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Colocalization studies in the avian brain provide new insights on the roles of NO in higher vertebrates

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SUMMARY

Several studies have elucidated the distribution of nitric oxide (NO)-producing system in the vertebrate brain by the use of the histochemical reaction for NADPH-diaphorase (ND) or of the immunocytochemistry for neuronal NO synthase (nNOS). These investigations confirmed the existence of a general pattern of distribution among different species and comparable to that of the mammalian (rodent) brain. A large group of positive neurons is located in the basal ganglia, and a wide system is distributed in the mesencephalic and pontine tegmental regions. However, in mammals a group of neurons has been detected within the hypothalamic magnocellular nuclei. By contrast, these elements have not been found in other vertebrates (namely in birds) even under conditions of stimulation of the magnocellular system (salt-loading). Moreover, there are also differences in the presence and distribution of areas where ND coexists with other systems of neurotransmitters. In specific locations (substantia nigra, area ventralis of Tsai) of the mesopontine system of galliforms, a large amount of tyrosine-hydroxylase (TH)-positive neurons (about 40%) is also ND-positive, whereas in rodents only a very limited amount of TH-positive neurons show TH/ND positivity. Contrariwise, several studies in diverse mammalian species have demonstrated that co-existence of ND with cholineacetyltransferase (ChAT) is highly consistent within the mesopontine system; in birds the extent of this co-existence is reduced and variable according to the location, reaching its maximum in the area that surrounds the locus coeruleus. In conclusion, the general distribution of the ND system seems to be preserved in higher vertebrates. However, the functional role played by NO in important phenomena such as osmoregulation, the regulation of the catecholaminergic or cholinergic systems may vary greatly in mammals and birds. Based on these data and on several reports in other vertebrates (reptiles, amphibia), it is difficult to believe that NO may play a general role in the control of specific physiological activities in the vertebrate brain.

Key words: NADPH-diaphorase - vasotocin - vasopressin - tyrosine hydroxylase - cholinacetyltransferase

INTRODUCTION

Nitric oxide (NO) is an inorganic free radical gas (N=O), whose synthesis from L-arginine requires an enzyme known as NO synthase (NOS), that contributes to the formation of citrulline. Besides these substrates, NO synthesis also requires coenzymes, cofactors, the presence of calmodulin (Knowles and Moncada, 1994), as well as the co-operation of superoxide-dismutase (SOD, Schmidt et al., 1996). NO is believed to be a neuronal messenger (Vincent and Hope, 1992) whose action takes place by inducing an increase in soluble cyclic guanosine monophosphate in target cells (Miki et al., 1977). NOS isoforms have been purified and characterized from brain (Bredt and Snyder, 1990), macrophages (Stuehr et al., 1991), and

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endothelial cells (Mayer et al., 1989; Pollock et al., 1991). Molecular cloning and the study of immunological properties have suggested that there are at least three types of NOS (Bredt and Snyder, 1990).

Neuronal NOS (nNOS or NOS type I) is Ca2+/calmodulin-dependent (Knowles et al., 1989) and has been purified and cloned in the rat (Bredt et al., 1990, 1991). In the brain, the synthesis of NO requires the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. Therefore, NADPH-diaphorase (ND) histochemistry (a reaction based on NADPH as substrate and on the reduction of tetrazolium dyes as chromogen) allows the detection of specific populations of neurons that use NO as a neuronal messenger molecule. A series of early investigations (Hope et al., 1991; Dawson et al., 1991) established that the distribution of ND activity in the brain is a reliable marker of neuronal NOS activity in conventionally fixed tissue. Identification of neuronal systems involving NO as a neurotransmitter or neuromodulator by means of ND histochemistry or anti-nNOS immunocytochemistry has yielded similar and overlapping results in many investigations in both the central (Dawson et al., 1991; Brüning et al., 1994a) and peripheral nervous systems (Ward et al., 1992; Li et al., 1994). However, exceptions to this rule are the mammalian olfactory system (Kishimoto et al., 1993) and spinal cord (Vizzard et al., 1994).

NOS is localized in diverse cell types belonging to a variety of neuronal systems, suggesting a widespread role in neuromodulation for the free radical NO. It appears to be involved in a variety of physiological activities, such as long-term potentiation, neuroprotection, neural degeneration, and the regulation of peptidergic secretion (for reviews see: Dawson et al., 1992; Dawson and Snyder, 1994).

NOS (or its histochemical marker ND) also appears to be co-localized with other neurotransmitters or neuropeptides, including cholinacetyltransferase (ChAT), galanin (Pasqualotto and Vincent, 1991), neuropeptide Y and somatostatin (Vincent et al., 1983a, 1983c; Kowal et al., 1987), substance P (SP), bombesin/gastrin-releasing factors, corticotrophin-releasing factor (CRF), serotonin, calbindin 28K (for a review see Vincent, 1986), vasopressin and oxytocin (Calka and Block, 1993a; Miyagawa et al., 1994; Sánchez et al., 1994; Villar et al., 1994; Hatakeyama et al., 1996). Based on the literature, it appears that the ND system in the brain does not overlap completely with any other known neurotransmitter system.

Study of the distribution and characteristics of nNOS or ND-containing neurons (ND-neurons) has been performed mostly in mammalian species. More recently, several papers have described the presence and distribution of ND-neurons in the brain of other vertebrates such as fishes (Holmqvist et al., 1994; Östholm et al., 1994; Villani and Guarnieri, 1995a, 1995b; Moroz and Gillette, 1996), amphibia (Brüning and Mayer, 1996; Munoz et al., 1996), reptiles (Luebke et al., 1994; Brüning et al., 1994b; Bennis et al., 1996; Jiang and Terashima, 1996; Smeets et al., 1997) and birds (Brüning, 1993; Panzica et al., 1994, 1996a; Wallhäusser-Franke et al., 1995; Montagnese and Csillag, 1996; Sánchez et al., 1996; Cozzi et al., 1997; Panzica and Garzino, 1997; von Bartheld and Schober, 1997).

The general pattern of distribution of ND-neurons is similar in the mammalian, avian and reptilian brains, with some noteworthy variations. Thus, populations of positive neurons were observed in the basal ganglia and in the so-called mesopontine system, whereas neuronal populations located in the diencephalon or in higher telencephalic centers showed a high degree of variation in distribution, depending on the species investigated (for a discussion see Brüning, 1993; Panzica et al., 1994; Cozzi et al., 1997; Smeets et al., 1997).

In birds, a recent series of investigations, partly done in our laboratories, have elucidated that the differences are even more marked when considering the extent of neurotransmitters or neuropeptides co-localization. We will discuss here three examples; namely, the hypothalamic magnocellular system (Sánchez et al., 1996), the tyrosine hydroxylase (TH) system (Panzica et al., 1996a), and the ChAT mesopontine system (Panzica and Garzino, 1997).

NO AND THE HYPOTHALAMIC MAGNOCELLULAR SYSTEM

ND-hypothalamic cells express several neurochemical markers in the rat, including the antidiuretic hormone vasopressin (VP) (Calka and Block, 1993a; Sánchez et al., 1994; Hatakeyama et al., 1996) and angiotensin 1-7 (Calka and Block, 1993b). Several important evidences suggest that NO contributes to the regulation of the secretion of VP and renin, both implicated in the control of sodium and water balance (Calapai et al., 1992; Reid, 1994; Petrov et al., 1995; Wang and Morris, 1996; Rauch et al., 1997). In the rat, the putative direct role of NO in osmoregulation is also reinforced by two facts: 1) the aforementioned partial coexistence found in the magnocellular hypothalamic neurons of the rat for NOS and VP (Calka and Block, 1993a; Sánchez et al., 1994; Hatakeyama et al., 1996) and for angiotensin 1-7 (Calka and Block, 1993b) and 2) the plastic changes observed following salt loading in the magnocellular neurons expressing NOS mRNA (Kadowaki et al., 1994; Villar et al., 1994),

1994). In birds, the cell typology of ND-positive neurons and that of TH-immunopositive neurons are similar (von Bartheld and Bothwell, 1992; Bailhache and Balthazart, 1993; Brüning, 1993; Panzica et al., 1994); a possible indication of co-existence of the two systems. In mammals, the co-existence of ND with monoamines was suggested in early studies (Kauffman et al., 1974). However, it has recently been shown that only a few ND-positive neurons co-express TH in the rat brainstem, whereas a higher number of such neurons co-express serotonin (Johnson and Ma, 1993). Moreover, data from other species (cat: Mizukawa et al., 1989; human: Geula et al., 1993; Egberong-

Fig. 1 B

Fig. 1A-B– Rostro-caudal (A-E) distribution of VT- and ND-positive elements within the quail diencephalon. Drawings on the left are based on the nomenclature of the quail brain from Panzica et al., 1994. **AC**, nucleus accumbens; **AM**, nucleus anterior hypothalami; **CO**, optic chiasma; **DLAl**, nucleus dorso-lateralis thalami, pars lateralis; **DLAm**, nucleus dorso-lateralis thalami, pars medialis; **E**, ectostriatum; **FPL**, fasciculus prosencephalicus lateralis; **GLv**, nucleus geniculatus lateralis, pars ventralis; **IF**, infundibular region; **LA**, nucleus lateralis thalami; **ME**, median eminence; **nCPa**, nucleus of the pallial commissure; **nST**, nucleus of the stria terminalis; **PA**, paleostriatum augmentatum; **POA**, nucleus preopticus anterior; **POM**, nucleus preopticus medialis; **PVN**, nucleus paraventricularis; **PVO**, paraventricular organ; **ROT**, nucleus rotundus; **SCNm**, nucleus suprachiasmaticus pars medialis; **SL**, septum lateralis; **SM**, septum medialis; **Tn**, nucleus taeniae; **TSM**, tractum septo-mesencephalicum; **VLT**, nucleus ventrolateralis thalami; **VMN**, nucleus ventromedialis. VT-immunopositive cell groups are subdivided into periventricular (**P1-P3**), lateral (**L1-L4**), and dorsal (**DD1-DD2**) groups (Panzica et al., 1997).

be et al., 1994) indicate a general lack of co-localization of ND or NOS with monoaminergic neurons (mostly at the level of the SN). In amphibians, co-localization of ND or NOS with TH seems to be very limited or absent (González et al., 1996).

Recently, several studies have considered the problem of ND-TH co-localization in birds and reptiles. In the quail, we have observed that cells expressing these two neurochemical markers coexist throughout a large part of the SN

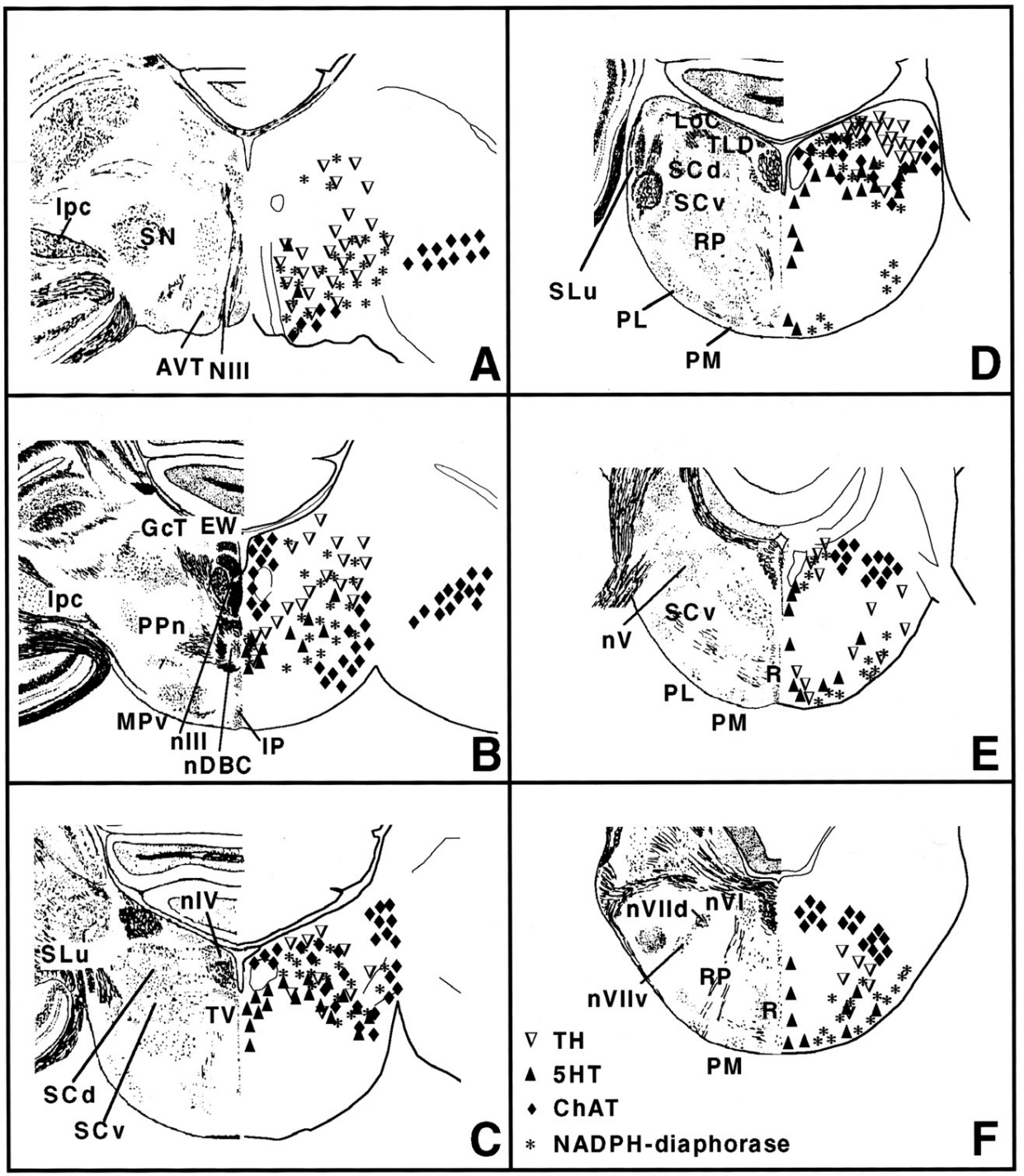

Fig. 2.– Semischematic drawings of coronal sections of the quail brainstem, arranged from the rostral (A) to the caudal levels (F). The location of nuclei identified in Nissl-stained sections is reported on the left side. Distribution of NADPH-diaphorase, tyrosine hydroxylase (TH), cholinacetyltransferase (ChAT), and serotonin (5HT) positive neurons is reported on the right side. The nomenclature is based on previous studies of the galliform brainstem (Guglielmone and Panzica, 1982; Panzica et al., 1996; Panzica and Garzino, 1997). **AVT**, area ventralis Tsai; **EW**, Nucleus of Edinger-Westphal; **GcT**, substantia grisea centralis; **IP**, nucleus interpeduncularis; **Ipc**, nucleus isthmi, pars parvicellularis ; **MPv**, nucleus mesencephalicus profundus ventralis; **nDBC**, nucleus decussationis brachiorum conjuntivorum; **nIII**, nucleus nervi oculomotori; **nIV**, nucleus nervi trochlearis ; **nV**, nucleus nervi trigemini; **nVI**, nucleus nervi abducentis; **nVIId**, nucleus nervi facialis, pars dorsalis; **nVIIv**, nucleus nervi facialis, pars ventralis; **PL**, nucleus pontis lateralis; **PM**, nucleus pontis medialis; **PPn**, nucleus tegmentalis pedunculo-pontinus; **R**, nuclei raphes; **RP**, nucleus reticularis pontis; **SCd**, nucleus subcoeruleus dorsalis; **SCv**, nucleus subcoeruleus ventralis; **SLu**, nucleus semilunaris; **SN**, substantia nigra; **TLD**, nucleus tegmentalis latero-dorsalis; **TV**, nucleus tegmentalis ventralis.

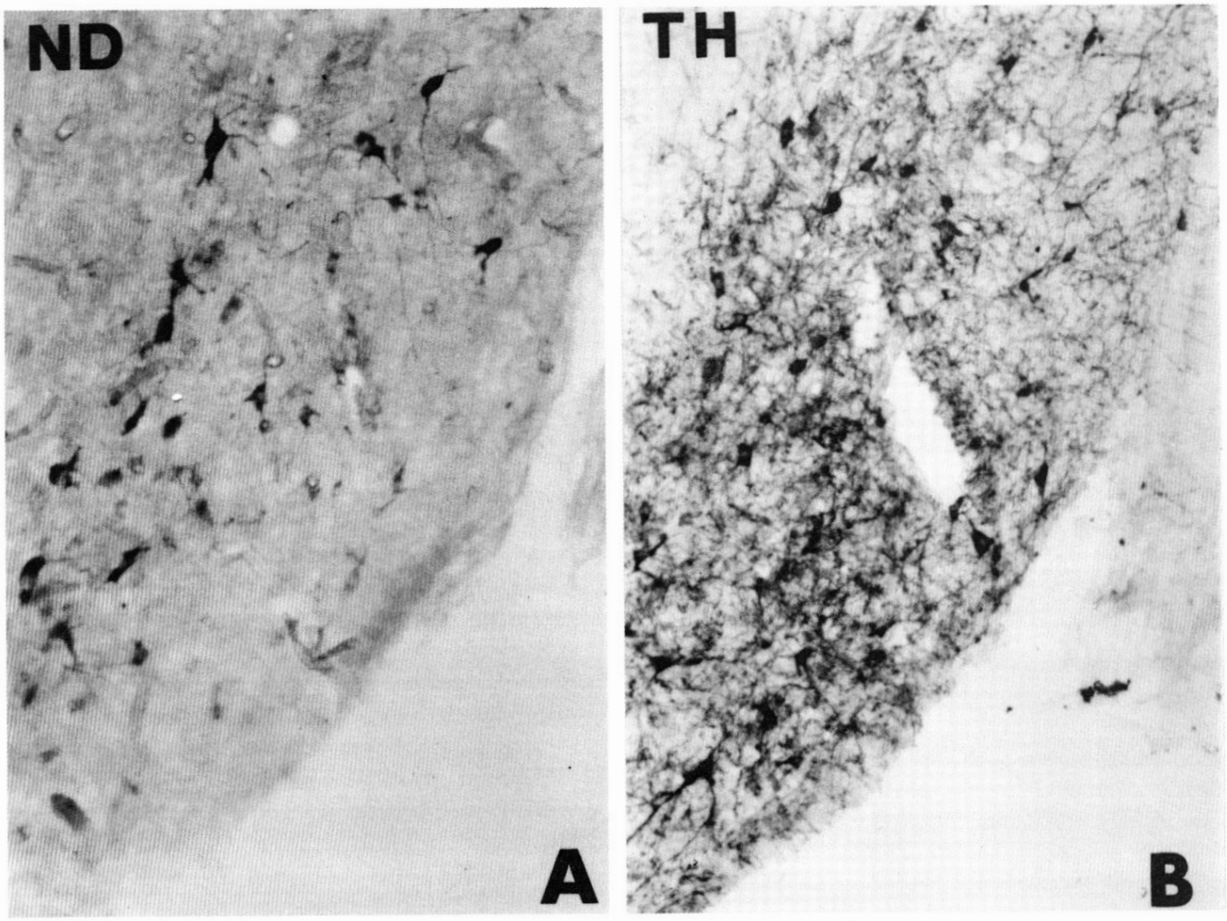

Fig. 3.– Comparison of TH-stained and ND-stained neurons in two adjacent sections corresponding to the area ventralis of Tsai (AVT). x190.

and of the AVT (Fig. 2-3). About 40% of the neurons in these two regions that contain immunoreactive TH also exhibit NADPH-diaphorase activity (Fig. 4A). This is not a general property of the quail catecholaminergic system: other dopaminergic groups, i.e. the nucleus tegmentalis pedunculo-pontinus (PPN), show a very limited degree of co-localization (only 17% of TH neurons are also ND-neurons and only 5% of ND-neurons are also TH-neurons). Moreover, in the nucleus tegmentalis laterodorsalis (TLD), which includes the avian LoC (the main noradrenergic group), there is a complete separation between these two neuronal populations (Fig. 2, 4A). Our results in the quail have been later confirmed in the adult chicken (von Bartheld and Schober, 1997) and similar findings have been reported also for the lizard *Gekko gekko* (Smeets et al., 1997).

The possible coexistence of ND (or NOS) with serotonin in birds can be excluded on the basis of the analysis of detailed mapping for serotonin (Cozzi et al., 1991) and ND in quail (Panzica et al., 1994). Double staining procedures confirmed that discrete regions (i.e., the ventral aspect of the TLD, Fig. 5) show several serotonin-positive fibers surrounding ND-neurons but no coexistence of the two systems (Panzica and Garzino, 1994).

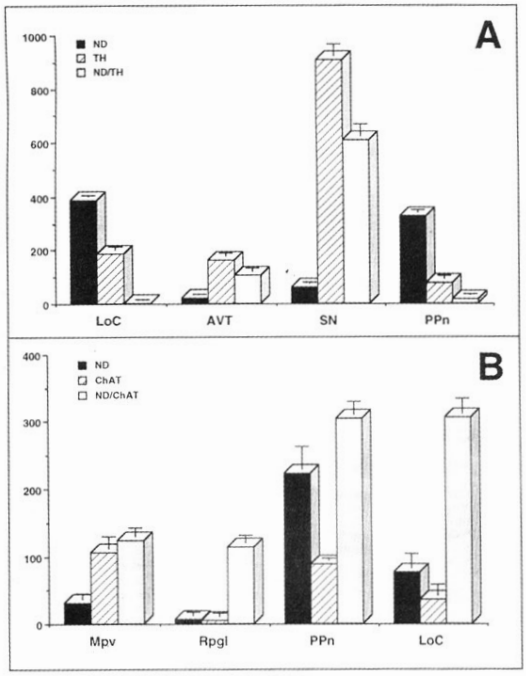

Fig. 4.– A: Histograms representing the number of TH-, ND-, or TH/ND-positive neurons observed in the quail mesopontine region (data from Panzica et al.1996a). **B:** Histograms representing the number of ChAT-, ND-, or ChAT/ND-positive neurons observed in the quail mesopontine region (data from Panzica and Garzino, 1997). **AVT**, area ventralis Tsai; **LoC**, locus coeruleus; **MPv**, nucleus mesencephalicus profundus ventralis; **PPn**, nucleus tegmentalis pedunculopontinus; **Rpgl**, nucleus reticularis paragigantocellularis; **SN**, substantia nigra.

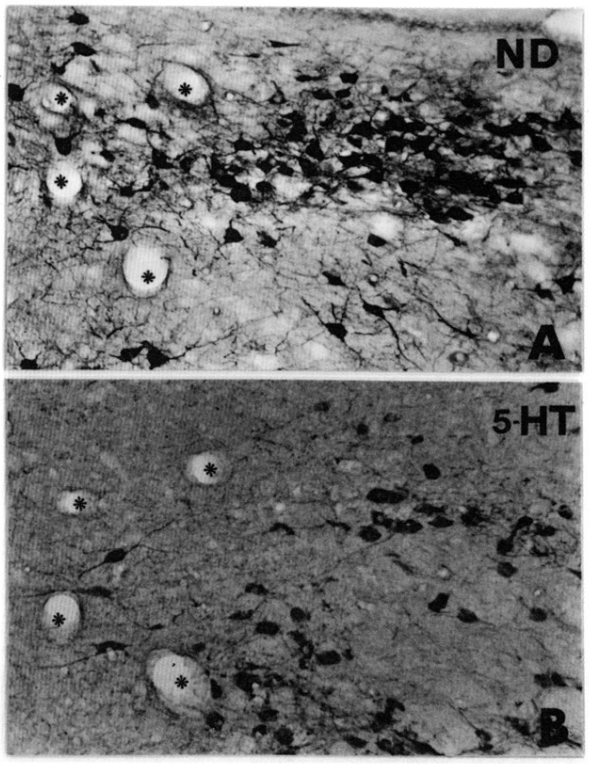

Fig. 5.– Comparison of serotonin and ND- stained neurons in two adjacent sections corresponding to the ventral border of the locus coeruleus. * indicates the same four blood vessels in both sections. x120.

NO AND THE CHOLINERGIC SYSTEM

Several studies in mammals (Vincent et al.,1983b, 1986; Reiner and Vincent, 1987) and reptiles (Luebke et al., 1994) suggest that ND or NOS could represent a specific marker of the cholinergic elements of the reticular mesopontine system. However, the aforementioned studies performed in birds (Panzica et al., 1996a) and reptiles (Smeets et al., 1997) demonstrated that ND positivity is widely co-localized with TH immunoreactivity, at least in part of this system. These data suggest a probable reduction in the extent of the co-localization of ND- and ChAT-positivity in the avian as well as in the reptilian mesopontine system in comparison to mammals.

ChAT-neurons have a wide distribution (Fig. 2) in the avian mesopontine region, (Sorenson et al., 1989; von Bartheld and Bothwell, 1992; Medina and Reiner, 1994; Panzica and Garzino, 1997). All motoneurons of the cranial motor nerves are immunopositive for ChAT, as well as groups of neurons in other nuclei [nucleus mesencephalicus profundus (MPv), PPN, nucleus isthmi parvicellularis (Ipc), nucleus semilunaris (SLu), TLD, nucleus reticularis paragigantocellularis (Rpgl)]. Interspecific differences have been also reported; for example ChAT immunoreactivity was not detected in the lateral lemnisci nucleus, in the AVT or in the trapezoid nucleus of the quail (Panzica and Garzino, 1997) whereas these regions do show positivity in pigeon (Medina and Reiner, 1994).

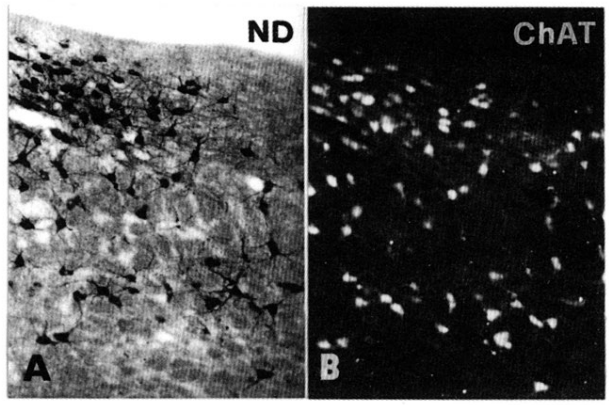

Fig. 6.– Comparison of ChAT- and ND-stained neurons in two adjacent sections corresponding to the medial aspect of the locus coeruleus (nucleus tegmentalis latero-dorsalis). x 90.

In the quail, comparison of adjacent sections stained for ND or for ChAT demonstrated (Fig. 6) the existence of extended regions with a comparable pattern of distribution for the two neurochemical markers (Panzica and Garzino, 1997). In particular, ND- and ChAT-neurons were both present in PPN, MPv, TLD, and Rpgl. However, in some regions only one marker was present (i.e. the AVT and SN, where ND elements were particularly abundant and ChAT-neurons almost undetectable) or the oculomotor nuclei (where only ChAT positivity was detected).

The percentage of co-localization of ND and ChAT detected in double stained sections varied greatly among these different nuclei (Fig. 4B). In the PPN of the quail, 77% of ChAT-neurons also showed ND positivity. The PPN is located caudally to the SN, a region in which about 40% of the TH-positive elements coexist with ND (Panzica et al., 1996a), and only a few scattered ChAT-neurons are observed. In the MPv, a cluster of ChAT+ neurons located ventrally in the isthmic region and clearly distinguishable from the PPN, the degree of co-localization of the two markers was of 54%. By contrast, the degree of co-localization is consistently higher in the TLD (89% of ChAT-positive elements), and especially in the Rpgl (96%).

These data demonstrate that ND and ChAT are co-localized in a large part of the mesopontine system of *Coturnix japonica*. They also confirm that, contrary to what has been demonstrated in rodents (Vincent et al., 1983b) and some reptiles (*Varanus exanthematicus*, Luebke et al., 1994), ND positivity cannot be considered as an unique marker of the mesopontine reticular cholinergic system in the vertebrate brain. In the quail, there are regions where ND is present and largely co-localized with TH (Panzica et al., 1996a) while ChAT is almost totally absent (AVT, SN). Moreo-

ver, in the regions where both markers are represented, the percentage of double stained neurons varies from 50 to 100%. Some of these regions show a complex array of neurochemical markers. In the TLD there are ND-, ND-/ChAT-, ChAT-, and TH-neurons segregated in discrete parts of the nucleus, where they probably identify subregions with different functional significance (so TH elements can be considered the equivalent of the mammalian locus coeruleus in birds. See Guglielmone and Panzica, 1982; von Bartheld et al., 1995; Panzica et al., 1996a).

Discussion

As briefly summarized in the Introduction, recent data collected in our laboratory and elsewhere concerning the distribution of ND/NOS-neurons have confirmed that the general pattern of distribution of this system is largely preserved in higher vertebrates. However, the examples reported demonstrate the existence of species- or interclass-specific variations in the extension and the degree of colocalization with other important neurochemical markers such as catecholamines (Panzica et al., 1996a; Smeets et al., 1997; von Bartheld and Schober, 1997), cholinacetyltransferase (Panzica and Garzino, 1997), or neurohypophyseal hormones (Sánchez et al., 1996). It must be stressed that these three examples refer to highly conservative important systems. The distribution and respective physiological roles of VT (or of vasopressin in mammals), TH, and ChAT are highly comparable among the different classes of higher vertebrates (for further references see Simon Oppermann et al., 1988; Ramieri and Panzica, 1989; Sorenson et al., 1989; Sánchez et al., 1991; Smeets, 1991; Medina and Reiner, 1994; Reiner et al., 1994; Grossmann et al., 1995; Cookson et al., 1996; Panzica et al., 1997).

Functional hypotheses about the general role of NO have sometimes been forwarded only on the basis of the presence (or absence) of ND or NOS positivity within specific systems in the central nervous system of mammals (and most of the data only refer to rodents). The data summarized in the present review suggest that the neurochemical criteria of coexistence developed on the basis of several investigations in the rodent brain are not general and cannot be automatically applied to other species under investigation. As mentioned before, some investigators may consider the distribution of ND positivity as a marker of the distribution of the mesopontine cholinergic system (i.e. see Luebke et al., 1994, in *Varanus exanthematicus*). By contrast, our data on ND/ChAT co-existence in the quail mesopontine system (Panzica and Garzino, 1997) suggest that no biunivocal correlation can be made.

The first, and probably the most important comment is that any morphological description of the distribution of ND or NOS positivity alone gives information only about the presence of the molecule (NOS immunocytochemistry) or its functional capability (NADPH-diaphorase reaction). However, synthesis of NO by the intervention of NOS is based on the availability of the aminoacid arginine: Therefore, detection of the substrate (Kharazia et al., 1997) should also be performed to demonstrate that the enzyme is active *in vivo*. A second point, also related to the mechanism by which NOS synthesizes NO, concerns the enzyme superoxide dismutase (SOD). The classical theory is that SOD can oxidize NO produced in excess, forming ONOO-. However, a recent study (Schmidt et al., 1996) has suggested that NOS does not form NO directly, but through a precursor (probably NO-) on which SOD will act to produce NO (see fig. 7 for a description of the cycle). Again, this implies that the detection of NOS or NADPH-diaphorase activity is not sufficient to unequivocally demonstrate the possibility of formation of the final product (NO) but that knowledge of the levels of SOD in the same system is also necessary.

A number of additional references points to other interesting hypotheses about the role of NO in the central nervous system of higher vertebrates. In the Japanese quail, neurons and fibers expressing ND-activity, and thus capable of releasing NO, are located in close relationship to specific VT-immunoreactive magnocellular elements (Sánchez et al., 1996). Since NO diffuses freely across membranes, it could be hypothesized that the effects of NO upon VT in birds are exerted transneuronally and not by direct synthesis in the magnocellular system. Moreover, it has been demonstrated in mammals that the MnPO is the region directly involved in central osmoception (Honda et al., 1990, 1992). This area is characterized by the presence of a large population of ND/NOS-neurons. Recently, the avian nucleus of the pallial commissure has been demonstrated to respond to acute osmotic challenges with the appearance of c-fos protein. This nucleus has therefore been considered the homologue of the mammalian MnPO (Sharp et al., 1995). It is a highly ND-positive structure and our unpublished results (Panzica) obtained in quail with an *in vitro* tracing technique (DiI, see Balthazart et al., 1994) suggest that this nucleus projects to the magnocellular system. Our hypothesis is that, in birds, the action of NO on osmoregulation could be mediated by the action of MnPO on the vasotocinergic system rather than through the vasotocin system itself.

Similar speculations can also be made about for the role of NO in the dopaminergic system.

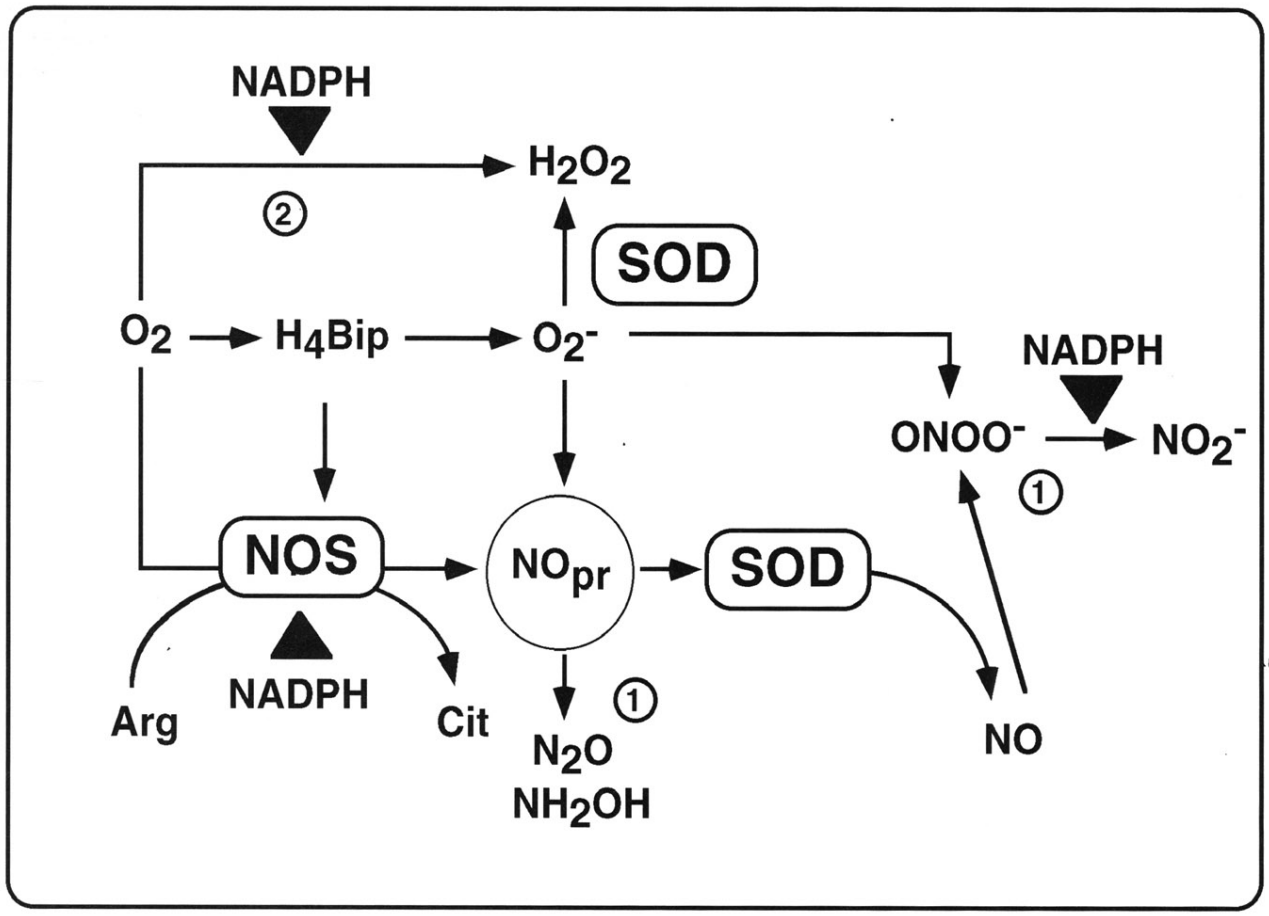

Fig. 7.– Diagram illustrating the putative role of superoxide-dismutase (SOD) and of nitric oxide synthase (NOS) in the production of NO (redrawn from Schmidt et al., 1996). According to this hypothesis, NOS consumes O_2, NADPH, and tetrahydrobiopterin (H_4Bip) to produce a NO precursor (NOpr), this latter is converted into NO only by the intervention of SOD. NADPH can also be consumed in parasitic reactions (2) or for the degradation of NO (1).

In mammals, the gonadal hormone testosterone up-regulates NOS activity in the medial preoptic area by increasing the number of positive neurons. The increased amount of NO produced by these cells may promote basal and copulation-induced dopamine release (for a review of these data, see Hull et al., 1997). In the quail, very few ND positive neurons have been observed in the medial preoptic region (Panzica et al., 1994). However, the detection of a high degree of co-existence of TH and ND activity in the AVT and SN of the quail (Panzica et al., 1996a) suggests that NO may play an important role in the regulation of the activity of the dopaminergic systems and, particularly, of the target areas of these centers. Dopamine is thought to be important in the regulation of the sexual behavior of the quail, possibly through the regulation of aromatase-producing cells (aromatase is the enzyme that locally converts testosterone into estradiol in the brain, see Balthazart and Ball, 1992). It has been hypothesized that dopaminergic afferents from the AVT (Balthazart et al., 1994; Balthazart and Absil, 1997) directly regulate the aromatase system in the medial preoptic nucleus of the quail (a sexually dimorphic nucleus that is believed to control male copulatory behavior in quail. See Panzica et al., 1996b). The presence of large numbers of cells co-expressing ND/TH within the quail AVT suggests a mechanism by which NO could play a direct role in the control of sexual behavior in this species.

These two last examples indicate that NO could have a similar role in different species, even though the disposition of the neural circuitries is highly different. The gaseous nature of the transmitter could account for the discrepancies in distribution. However, the anatomical findings reported here do not support the idea of a general role of NO in the vertebrate brain, suggesting by contrast different physiological roles in different species (or phyla).

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