

Absence of coexistence between NADPH-diaphorase and antidiuretic hormone in the hypothalamus of two galliforms: Japanese quail (*Coturnix japonica*) and chicken (*Gallus domesticus*)

F. Sánchez^{a,*}, J.R. Alonso^b, R. Arévalo^b, G. Brüning^c, G.C. Panzica^d

^aDepartment of Human Anatomy and Histology, Faculty of Medicine, University of Salamanca, Avda. Campo Charro s/n. 37007 Salamanca, Spain

^bDepartment of Cell Biology and Pathology, University of Salamanca, Salamanca, Spain

^cDepartment of Anatomy, Free University of Berlin, Berlin, Germany

^dDepartment of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Turin, Italy

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Abstract

Based on previous studies demonstrating coexistence of NADPH-diaphorase (ND) and vasopressin (VP) in the rat hypothalamus, ND histochemistry and vasotocin (VT) immunocytochemistry have been combined in order to study the distribution of both markers in the hypothalamus of the Japanese quail (*Coturnix japonica*) and chicken (*Gallus domesticus*). No coexistence was found, however, close anatomical relationships between ND-positive and VT-immunoreactive elements were observed in specific preoptic and hypothalamic locations. These findings indicate interspecies differences in the expression of ND and the antidiuretic hormone with functional implications in osmoregulation.

Keywords: Antidiuretic hormone; Interspecies differences; Magnocellular neurons; Nitric oxide; Osmoregulation

Nitric oxide (NO), a gas produced by different cell types, has been identified as a novel messenger molecule which carries out diverse signalling tasks in the brain [9]. NADPH-diaphorase (ND) is an enzymatic activity that reduces tetrazolium salts by consuming NADPH. ND histochemical technique has been widely used as a marker for NO synthesizing cells (see Ref. [1]). Over the last decade, based on data obtained from in situ hybridization, immunocytochemistry, and ND histochemistry, an important number of studies have been published showing the anatomical location of the NO-producing cells and processes throughout the central nervous system of vertebrates. In the mammalian hypothalamus, a widely distributed neuronal population express either ND or neuronal nitric oxide synthase (NOS). These neurons demonstrate characteristic topographical location and morphology [2,14,21,23]. In the avian brain, a few general studies have recently been published [5,6,17] reporting a specific and relatively com-

mon distribution pattern of ND/NOS activity in most brain areas of two galliform species (chicken and Japanese quail). There are several common features with the mammalian distribution pattern (i.e. a large population of ND-positive elements in the hypothalamic ventromedial region), but there are also important differences in the periventricular preoptic and supraoptic regions that were almost devoid of ND-positive elements in galliforms [5,17].

At present, it is well known that ND-hypothalamic cells express several neurochemical markers in rat including the antidiuretic hormone vasopressin (VP) [21] and angiotensin 1–7 [7]. These two coexistences, the plasticity of the hypothalamic neurons after osmotical manipulations [4,14,23] and the implications of NO in the control of the secretion of VP [19] indicate a direct involvement of NO in osmoregulation.

No information is at present available about a possible coexpression of ND and vasotocin (VT) (the avian antidiuretic hormone) in the avian hypothalamus. We have hence specifically investigated the putative expression of

* Corresponding author. Tel.: +34 23 241240; fax: +34 23 294559; e-mail: ssh@gugu.usal.es

VT by ND-positive neurons in the two galliform species that have previously been considered for the general distribution of ND-positive elements.

Four adult (2-month-old) Japanese quails (*Coturnix japonica*) and four adult (12-month-old) chickens (*Gallus domesticus*) were used in the present study. On their arrival in the laboratory the birds were isolated in individual cages, provided with food and water ad libitum, and exposed to 16 h of light each day until the sacrifice. Animals were transcardially perfused under deep anaesthesia (quails, Hypnodil®, Janssens Pharmaceutica, Belgium; 50 mg/kg body weight; chickens, 2 mg ketamine and 2 mg xylazine).

After rinsing the vascular system with 50–100 ml of 0.9% NaCl, animals were perfused with 200 ml of a fixative made of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Following this, brains were dissected out, placed in the same fixative for 12 h at 4°C, washed in PB for 1–2 days, then immersed for 48 h in a solution of 30% sucrose (v/v) in PB for cryoprotection. Thick cryostat sections (30 µm) were cut at the frontal plane and collected in cold (4°C) PB in three different series for detection of ND, VT, and both markers, respectively.

Visualization of ND-activity was carried out according to previous papers [5,17]. Briefly, free-floating sections were thoroughly washed in 0.1 M Tris–HCl buffer (TRIS; pH 8.0) at room temperature and incubated for 45 min at 37°C in a solution made up of 1.2 mM β-NADPH, 0.3 mM nitro blue tetrazolium and 0.3% Triton X-100 in TRIS. The course of the reaction was controlled under the microscope. When the histochemical reaction was concluded, the sections were rinsed in cold PB.

Sections treated for VT-immunocytochemistry were incubated in anti-VT primary antibody [20] diluted 1:8000 in PB for 48 h at 4°C. Thereafter, sections were processed according to the avidin-streptavidin technique. Following several rinses, sections were incubated for 5 min in 0.025% 3,3′ diaminobenzidine (DAB) and hydrogen peroxide in 0.1 M Tris–HCl buffer (pH 7.6).

The third series of sections was processed for the simultaneous demonstration of both markers. The histochemical procedure was applied first, followed by an overnight storage in PB at 4°C, and then by the immunocytochemical detection of VT as described above. In double-labelled sections, the blue coloured reaction of the ND histochemistry and the brown DAB reaction product of the immunoperoxidase were clearly distinguishable. At the end of the histochemical, immunocytochemical, or double staining procedures, the sections were briefly washed in PB, mounted on gelatin-coated slides, dried overnight at room temperature, dehydrated through graded alcohols and xylene, and coverslipped with Entellan.

Controls of the histochemical and immunocytochemical techniques were performed as previously described [5,17,20,21], no residual reaction was observed for both histo- and immunocytochemical procedures. The exact

location of positive-immunoreactive neurons was determined by means of phase contrast microscopy.

The distribution of both ND- and VT-positive cells has previously been described in detail in quail [17,22] as well as in chicken [5,20]. Observations in the present material were in complete agreement with those previous descriptions. The regions considered in this study were the anterior hypothalamic and dorsal diencephalic regions, where, according to previous studies, the entire population of neurons involved in the magnocellular neurosecretory pathway is located. The nomenclature applied to identify different VT-positive groups was based on topographical criteria and has been validated for the pigeon [3] and for other non-passerine birds including quail and chicken [15,20,22]. Briefly, three main populations of VT-immunoreactive (ir) neurons have been considered: (1) periventricular groups, clusters of neurons located periventricularly (P1, P2 and P3) (Fig. 1(1)); (2) lateral groups, clusters of neurons located close to the pial surface and optic chiasma (L1 and L2) (Fig. 1(2)); and (3) groups of neurons situated between the two previous ones (L3, L4 and L5) (Fig. 1(1,L4)).

Inspection of adjacent sections stained for ND and VT revealed that the two neurochemically identified populations were distributed in different territories. In particular, the number of densely stained ND-positive neurons was very low in all locations corresponding to the main groups of large VT-ir elements. This fact reduces obviously the potential degree of coexistence of both neuronal markers. Only a few scattered, large and intensely ND-stained neurons were observed in the region of L1–L3 groups (Fig. 1(5,6)). A large cluster of medium-sized heavily-stained ND neurons was located lateral to the medial preoptic nucleus and it extended caudally very close to the more lateral part of L4 group of VT elements (Fig. 1(1)). No ND staining was detected at the level of the median eminence.

Weakly ND-stained neurons were however present in several hypothalamic locations as the preoptic region, the periventricular region, and the anterior portion of the nucleus of the pallial commissure, which are also sites where large and small VT-ir elements are detected.

In double-stained material, the same distribution and morphology of positive elements was observed as in the single-stained sections. No coexistence of the two considered markers is found; however, very close relationships are detected. At the level of the nucleus of the pallial commissure (Fig. 1(3)), scattered VT-ir small and large neurons surround the wide cluster of small ND-positive elements. Frequently, VT-ir cell bodies are deeply intermingled with processes arising from ND-positive cells. Large intensely ND-stained cells were observed intermixed with the large VT-ir neurons of the L3 group (Fig. 1(5,6)). In this location, ND-positive cells have long processes that could be followed surrounding the VTergic elements.

In the periventricular hypothalamus, very small, weakly

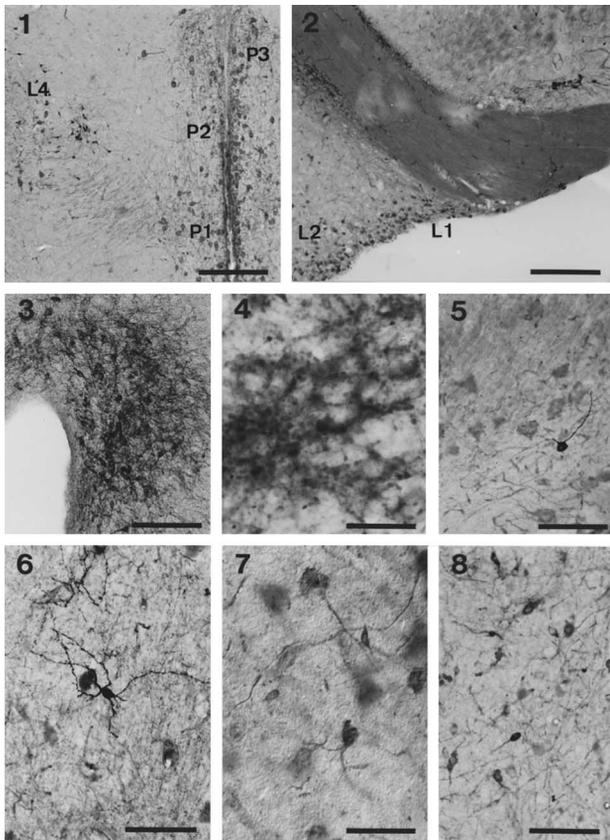


Fig. 1. The pictures are from Japanese quail or chicken sections sequentially double-stained for ND histochemical (blue colour) of VT immunocytochemical (brown colour) detection. Scale bar, (1,2) 50 μm ; (3,8) 20 μm ; (5–7) 10 μm ; (4) 5 μm . (1) Japanese quail, Low power enlargement of the hypothalamic periventricular region. P1–P3 and L4 indicate different subdivisions of the VTergic system in this bird. Note the absence of ND-positive cell bodies in the periventricular region and the presence of a cluster of ND-positive cells medially to the L4 group. $40\times$. (2) Chicken, Low power enlargement of the anterior preoptic region, showing the distribution of VTergic elements of the L1 and L2 groups. No ND-positive elements have been detected in this region, whereas a small group of them is located dorsally to the septo-mesencephalic tract. $40\times$. (3) Japanese quail, Small, weakly stained ND-positive elements in the nucleus of the pallid commissure. Note scattered small VT-ir cell bodies around and inside the nucleus, in close contact with ND-positive processes. $100\times$. (4) Japanese quail, VT-ir fibres surrounding the ND-positive cell bodies in the hypothalamic ventromedial nucleus. $400\times$. (5–6) ND-positive elements scattered among the VT-ir neurons in the L3 group of Japanese quail (5) and chicken (6) in the preoptic region. $200\times$. (7) Japanese quail, Small, weakly stained ND-positive elements intermixed with large VT-ir neurons in the periventricular region (the ventricle is on the right). $200\times$. (8) Chicken, Mixed population of small neurons ND- or VT-positive in the region connecting the P1–P3 groups (location top of the picture) and the more lateral L4 (bottom). $100\times$.

stained ND-cells were detected. They were frequently situated very close to the large VTergic elements of the P1–P3 groups (Fig. 1(7)). In the territory among the periventricular groups and the L4 group (which has been identified in previous studies as the avian homologue of the magnocellular portion of the rat paraventricular nucleus [20]), there were several small ND-positive or VT-ir neurons, but also

in this location no coexistence was found (Fig. 1(8)). The heavily stained ND-neurons located close to the L4 group (Fig. 1(1)) were also clearly situated more medially or more dorsally to the VT-ir elements, even if their processes reach the region occupied by VT-ir neurons. Lastly, at the level of the main group of ND-positive neurons in the hypothalamic region (the ventromedial nucleus), numerous VT-ir fibres were observed forming pericellular arrangements around the ND-positive cell bodies (Fig. 1(4)).

It has been clearly established over the last decade that NO plays a crucial role in the control of the secretion of hormones by the pancreas, anterior pituitary gland and hypothalamus. In the latter, NO is a key molecule in the regulation of the hypothalamic portal blood flow as well as in different neuroendocrine mechanisms [8]. There is also an important body of evidence suggesting that NO contributes variably to the regulation of the secretion of VP and renin [19], hormones implicated in the control of sodium and water balance. In rat, the role of NO in the osmoregulation is also reinforced by two facts: (1) the partial coexistence of ND and VP, and ND with angiotensin 1–7 found in the magnocellular hypothalamic neurons [7,21] and (2) the plastic changes observed following salt loading in the magnocellular neurons expressing NOS mRNA [14,23], NOS-immunoreactivity [23] and ND-activity [4].

On considering coexpression of NOS with other neuronal markers, at present a wide list of coexistences is available. Some of these coexistences seem to be species specific; for instance tyroxine hydroxylase and ND are largely coexisting in Japanese quail and lizard [11,18] whereas only a few neurons showed colocalization in rat [13], frog and urodeles [11]. It could be thus expected that for other neurochemical markers, in our case the antidiuretic hormone (VP/VT), similar interspecies differences might be also found. Moreover, previous studies suggest that the distribution and extent of the ND-activity in the vertebrate hypothalamus is highly variable, mainly at the level of the magnocellular region [5,12,16,17].

The present findings concerning simultaneous detection of ND and VT are the first conducted in non-mammalian species. Nevertheless, different analysis currently available in a few vertebrates of the distribution patterns of both markers considered individually indicate that this absence of coexistence might also occur in other species. In fact in *Pleurodeles* (amphibia urodele) the two markers are distributed in clearly different regions [10–12]. In the frog, there are ND elements in the magnocellular portion of the anterior hypothalamus [16], but the distribution does not match VT-elements. Moreover, both in avians and in amphibians no ND staining has been detected in the median eminence [5,12,16,17]. It strongly supports the hypothesis that neurons involved in the hypothalamic-neurohypophysial pathway do not produce NO by themselves in these classes of vertebrates.

Present results and the short summary of literature that

we have reported led to the conclusion that NO should have a minor role in the central control of osmoregulation in non-mammalian vertebrates. However, there are some considerations that should be born in mind before accepting this conclusion as definitive: at first, cells and fibres expressing ND-activity and thus, capable of releasing NO, are located in close anatomical relationship to specific VT-ir magnocellular elements. Since NO diffuses freely across membranes, it could be hypothesised that the effects of NO upon VT-ir neurons in birds are exerted transneuronally and not by direct synthesis in the magnocellular system. Secondly, the large majority of data concerning the involvement of NO in osmoregulation have been collected in the rat, so we need more experimental models also for mammals. Lastly, it has to be considered that as the NO system is highly plastic (at least in rat) it is possible that in birds, as well as in other non-mammalian vertebrates there is no or low expression of ND in vasotocinergic elements in normal conditions, and this expression could be increased by experimental manipulation of circulating salt levels. Thus, further studies should be addressed to check not only differences among other species but also possible modifications of the ND-expression in the magnocellular system of non-mammalian vertebrates after osmotic stimulation.

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