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# Immunohistochemical localization of DARPP-32 in the brain and spinal cord of anuran amphibians and its relation with the catecholaminergic system

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ABSTRACT

The relationship between dopaminergic neuronal structures and dopaminoceptive structures in the amphibian brain and spinal cord are assessed by means of single and double immunohistochemical techniques with antibodies directed against DARPP-32 (a phosphoprotein related to the dopamine $D_1$-receptor) and tyrosine hydroxylase (TH) applied to the brain of the anurans *Rana perezi* and *Xenopus laevis*. The DARPP-32 antibody yielded a well-differentiated pattern of staining in the brain of these anurans. In general, areas that are densely innervated by TH-immunoreactive fibers such as the nucleus accumbens, striatum, amygdaloid complex, thalamus, optic tectum, torus semicircularis and spinal cord display a remarkable immunoreactivity for DARPP-32 in cell bodies and neuropil. Distinct cellular DARPP-32 immunoreactivity was also found in the septum, preoptic area, suprachiasmatic nucleus, tuberal hypothalamic region, habenula, retina, midbrain tegmentum, rhombencephalic reticular formation and solitary tract nucleus. Hodological data supported that striatal projection neurons were DARPP-32 immunoreactive. Double immunohistofluorescence staining revealed that catecholaminergic cells generally do not stain for DARPP-32, except for some cells in the ventral mesencephalic tegmentum of *Xenopus* and cells in the nucleus of the solitary tract of *Rana*. Several interspecies differences were noted for the DARPP-32 distribution in the brain of the two anurans, namely in the habenula, the thalamus and prethalamus, the cerebellum and octavolateral area and the structures with DARPP-32/TH colocalization. However, in general, the distribution of DARPP-32 in the brain of the anuran amphibians resembles in many aspects the pattern observed in amniotes, especially in reptiles.

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*Abbreviations:* ac, anterior commissure; Ac, caudal subnucleus of anterior nucleus of thalamus; Acc, nucleus accumbens; AOB, accessory olfactory bulb; Av, anteroventral tegmental mesencephalic nucleus; B, neuropil of Bellonci; BST, bed nucleus of the stria terminalis; C, central nucleus of thalamus; c, central field of spinal cord gray; Cb, cerebellum; cc, central canal; CeA, central amygdala; cHT, caudal hypothalamus; CT, caudal tuberal nucleus; d, dorsal field of spinal cord gray; DARPP, dopamine and cAMP-regulated phosphoprotein; DB, diagonal band of Broca; DCN, dorsal column nucleus; DF, dorsal funiculus; dh, dorsal horn of spinal cord; Dienc, diencephalon; DN, dorsal nucleus of the octavolateral area; Dp, dorsal pallium; gl, glomerular layer; GvN, ventral geniculate neuropil; GT, griseum tectale; Hb, habenula; Hd, dorsal habenular nucleus; igl, internal granular layer; III, oculomotor nucleus; INL, inner nuclear layer; Ip, interpeduncular nucleus; IPL, inner plexiform layer; Is, isthmic nucleus; IT, intermediate tuberal nucleus; IV, trochlear nucleus; IX-X, glossopharyngeal-vagal motor column; Jc, juxtacommissural nucleus; l, lateral field of spinal cord gray; LA, lateral amygdala; Lc, locus coeruleus; LDT, laterodorsal tegmental nucleus; LF, lateral funiculus; lfb, lateral forebrain bundle; lm, lateral motor field of spinal cord gray; Lp, lateral pallium; Lsd, lateral septum pars dorsalis; Lsv, lateral septum pars ventralis; LVN, lateral vestibular nucleus; Ma, mammillary nucleus; MeA, medial amygdala; Mes, mesencephalon; ml, mitral layer; MOB, main olfactory bulb; Mp, medial pallium; MPO, median preoptic nucleus; Ms, medial septum; MT, median tuberal nucleus; MVN, medial vestibular nucleus; Nsol, nucleus of the solitary tract; nlla, anterior lateral line nerve; nVII, facial nerve; nVIIIv, ventral octaval nerve; OB, olfactory bulb; oc, optic chiasm; ov, organum vasculosun laminae terminalis; ONL, outer nuclear layer; OT, optic tectum; ot, optic tract; PA, pallidum; p1–p3, diencephalic prosomeres 1–3; PB, parabrachial area; pc, posterior commissure; Pc, precommissural nucleus; Pd, posterodorsal tegmental nucleus; Pdi, posterodorsal tegmental nucleus, isthmic part; POa, preoptic region, caudal part; POb, preoptic region, rostral part; POC, commissural preoptic nucleus; PT, pretectum; PTh, prethalamus; PV, paraventricular nucleus; Pv, posteroventral tegmental nucleus; Pvi, posteroventral tegmental nucleus, isthmic part; r0, rhombomere 0 (isthmus); r1–r8, rhombomeres 1–8; Ra, raphe nucleus; RC, superficial retrochiasmatic nucleus; RF, reticular formation; Rh, rhombencephalon; rHT, rostral hypothalamus; Ri, inferior reticular nucleus; rLLN, rostral nucleus of the lateral line nerve; RM, retromammillary nucleus; Rm, median reticular nucleus; Rs, superior reticular nucleus; SC, suprachiasmatic nucleus; SCc, suprachiasmatic nucleus, caudal part; SCr, suprachiasmatic nucleus, rostral part; SCO, subcommissural nucleus; sgr, stratum granulare of the cerebellum; SM, superficial mammillary nucleus; smol, stratum moleculare of the cerebellum; sol, solitary tract; sP, stratum of Purkinje cells; spmc, spinal motor neurons; SPV, supraoptic paraventricular nucleus; Str, striatum; Tc, commissural nucleus of the torus semicircularis; Tel, telencephalon; Th, thalamus; Tl, laminar nucleus of the torus semicircularis; Tmg, magnocellular nucleus of the torus semicircularis; Tor, torus semicircularis; TP, posterior tubercle; Tp, principal nucleus of the torus semicircularis; v, ventricle; VF, ventral funiculus; vh, ventral horn of the spinal cord; VIa, accessory abducens nucleus; VIIIc, caudal octaval nucleus; VIIm, facial motor nucleus; vl, ventrolateral field of spinal cord gray; VL, ventrolateral prethalamic nucleus; vm, ventromedial field of spinal cord gray; VM, ventromedial prethalamic nucleus; Vm, trigeminal motor nucleus; Vp, ventral pallium; XII, hypoglossal nucleus.

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## 1. Introduction

Studies of dopamine signaling led to the discovery of the molecule DARPP-32 (an acronym for Dopamine and cAMP-Regulated PhospoProtein, with an apparent molecular weight of 32,000 Da), also known as PPP1R1B (Protein PhosPhatase 1, Regulatory (inhibitor) subunit 1B), which is a cytosolic protein that is phosphorylated by cAMP-dependent protein kinase in response to the activation of the $D_1$-dopamine receptor (Hemmings and Greengard, 1986; Greengard et al., 1998; Greengard, 2001) and is considered a marker for neurons that contain this receptor (Rajput et al., 2009). In addition, DARPP-32 plays a central role in the interactions among diverse complex signaling pathways that involve primarily (but not exclusively) dopamine and glutamate (Greengard, 2001; Fernandez et al., 2006).

DARPP-32 is highly concentrated in the neostriatum (the caudate and the putamen) and DARPP-32 immunoreactive (DARPP-32-ir) medium-sized spiny neurons are densely dopamine- and glutamate-innervated (Ouimet and Greengard, 1990). The stimulation of dopaminergic and glutamatergic receptors regulates the phosphorylation of DARPP-32, but with opposite effects. The stimulation of dopamine $D_1$-receptor enhances the phosphorylation of DARPP-32 (Walaas and Greengard, 1984) that acts as a potent protein phosphatase-1 inhibitor (Hemmings et al., 1984), whereas the activation of NMDA receptor promotes the elevation of intracellular calcium and induces dephosphorylation of DARPP-32 (Halpain et al., 1990) reducing its phosphatase-1 inhibitory activity. Therefore, DARPP-32 acts like a third messenger that integrates multiple signaling pathways in the brain (Greengard et al., 1998; Svenningsson et al., 2002; Andersson et al., 2005; Kuroiwa et al., 2008; Hara et al., 2010).

Previous immunohistochemical studies have analyzed the distribution of DARPP-32 in the brain of mammals (Gustafson and Greengard, 1990; Ouimet et al., 1984, 1992; Ouimet and Greengard, 1990; Anderson and Reiner, 1991; Barbas et al., 1993; Greengard et al., 1998; Wang et al., 2004; Ishikawa et al., 2007; Glausier et al., 2010), birds (Schnabel et al., 1997; Durstewitz et al., 1998; Reiner et al., 1998; Absil et al., 2001; Roberts et al., 2002; Bálint et al., 2004; Reiner et al., 2004; Metzger et al., 2006; Bálint and Csillag, 2007; Csillag et al., 2008) and reptiles (Smeets et al., 2001, 2003). Many of these studies were mainly focused on the basal ganglia and only a few works have analyzed the distribution of DARPP-32 in regions outside the telencephalic basal ganglia and their related mesencephalic structures (Ouimet et al., 1984, 1992; Schalling et al., 1990; Perez and Lewis, 1992; Smeets et al., 2001, 2003; Metzger et al., 2006). In these studies, DARPP-32-ir cells were primarily found in brain regions densely innervated by dopaminergic fibers, like the basal ganglia, where DARPP-32 containing cells are abundant. In addition, the DARPP-32 immunohistochemical techniques reveal the morphology of dopaminoceptive neurons to their full extent since this phosphoprotein is present in the soluble rather than the particulate subcellular fraction (Walaas et al., 1983; Walaas and Greengard, 1984) and somata, dendrites and axons are well labeled with DARPP-32 antibodies. Moreover, the immunohistochemical detection of DARPP-32 can be easily combined with other immunohistochemical staining in the study of relationships between the dopaminergic system and other neurotransmitter systems. Thus, in two previous studies, the relationships between the dopaminergic neuronal structures and dopaminoceptive cells were studied in the reptilian brain by combining the immunohistochemical detection of DARPP-32 and tyrosine hydroxylase (TH; Smeets et al., 2001, 2003). These double labeling experiments showed that, in general, the brain regions with dense TH-ir fibers also posses a high numbers of DARPP-32-ir cells bodies.

A phylogenetic survey of DARPP-32 immunoreactivity using radioimmunoassay in nervous tissue from nonmammalian species concluded that DARPP-32-like proteins were also present in dopaminoceptive brain regions of amniote vertebrates (birds and mammals), whereas none was identified in anamniotes (bony fishes and amphibians) (Hemmings and Greengard, 1986). However, in that study rostral portions of the frog and fish brain were mainly investigated. In previous studies in reptiles using a mouse anti-DARPP-32 serum it was demonstrated that very dense immunoreactive cells and fibers are located in the brainstem and spinal cord (Smeets et al., 2001, 2003). Using the same antibody, our preliminary results revealed specific patterns of distribution of DARPP-32-like immunoreactivity not only in the brain of amphibians but also of lungfishes (González and Northcutt, 2009). In the present study we have carried out a Western blotting analysis for the antibody used and demonstrated that it recognizes in amphibians a protein band similar to that of the rat sample. Therefore, the aim of the present analysis was to describe the presence and distribution pattern of DARPP-32-ir structures in the central nervous system of two anuran amphibians, *Rana perezi* and *Xenopus laevis,* using the same immunohistochemical techniques applied in other studies in amniotes. Considering the crucial phylogenetic position of amphibians as anamniote tetrapods, our study highlights primitive and derived traits in the DARPP-32 system. As in previous studies in other vertebrates, we have also analyzed the relationships between dopaminergic and dopaminoceptive cells and fibers by means of double DARPP-32 and TH immunohistochemistry. Because in amniotes most DARPP-32-ir cells in the striatum are projection neurons (Anderson and Reiner, 1991), we have conducted retrograde tracing experiments for labeling projection neuron in the frog striatum in combination with DARPP-32 immunohistochemistry to evaluate the situation in amphibians.

The two anuran amphibians used have been the core species in numerous cytoarchitectonic, chemoarchitectonic and hodologic studies, in particular with respect to catecholamines and basal ganglia organization (González and Smeets, 1991, 1994; Marín et al., 1997a,b, 1998a,b; Smeets and González, 2000) and offer, therefore, the opportunity to study the distribution pattern of DARPP-32 in a wide neuroanatomical perspective.

## 2. Materials and methods

For the present study, a total of 18 *Rana perezi* and 8 *Xenopus laevis* were used. The animals were obtained from the laboratory stocks of the Department of Cell Biology, University Complutense of Madrid and from commercial suppliers. The original research reported herein was performed under the animal care guidelines established by European Union (86/609/EEC) and the Spanish Royal Decree 223/1998.

All animals were deeply anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz; pH 7.3) and perfused transcardially with physiological saline followed by 150–200 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4). The eyes, brains and the spinal cord were removed and kept in the same fixative for 2–3 h. Subsequently, they were immersed in a solution of 30% sucrose in PB for 3–5 h at 4 °C until they sank, blocked in a solution of 20% gelatine with 30% sucrose in PB, and stored overnight in a solution of 4% formaldehyde and 30% sucrose in PB. The brains were cut on a freezing microtome at 40 μm thickness in the transverse or sagittal plane and sections were collected in cold PB.

### 2.1. DARPP-32 immunohistochemistry

The free-floating sections were rinsed twice in PB, treated with 1% $H_2O_2$ in PB for 15 min to reduce endogenous peroxidase activity, rinsed again three times in PB and processed by the peroxidase antiperoxidase (PAP) method (Sternberger, 1979). This included a first incubation of the sections in a primary serum of mouse anti-DARPP-32 (kindly donated by Dr. H.C. Hemmings Jr., The New York Hospital, Cornell Medical Center, NY, USA) diluted 1:10,000 in PB containing 0.5% Triton X-100 (PBS-T), 15% normal rabbit serum (NRS), and 2% bovine serum albumin (BSA), for 48 h at 4 °C. Subsequently, the sections were rinsed three times in PB for 10 min and incubated for 60 min at room temperature in rabbit anti-mouse serum (Chemicon, Temecula, CA; catalogue reference: AP160) diluted 1:50. After rising again three times for 10 min, the sections were incubated for 90 min in mouse PAP complex