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# Immunohistochemical analysis of Pax6 and Pax7 expression in the CNS of adult *Xenopus laevis*

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ABSTRACT

Pax6 and Pax7 are transcription factors essential for the development of the CNS. In addition, increasing data, mainly obtained in amniotes, support that they are expressed in subsets of neurons in the adult, likely playing a role in maintaining neuron type identity. In the present study we analyzed the detailed distribution of Pax6 and Pax7 cells in the adult CNS of *Xenopus laevis*. Immunohistochemistry with antibodies that are required for high-resolution analysis of Pax-expressing cells was conducted. A wide distribution of Pax6 and Pax7 cells throughout the CNS was detected, with distinct patterns that showed only slight overlapping. Only Pax6 was expressed in the telencephalon, including the olfactory bulbs, septum, striatum and amygdaloid complex. In the diencephalon, Pax6 and Pax7 were distinct in the alar and basal parts, respectively, of prosomere 3. Large numbers of Pax6 and Pax7 cells were distributed in the pretectal region (alar plate of prosomere 1) but only Pax6 cells extended into basal plate. Pax7 specifically labeled cells in the optic tectum, including the ventricular zone, and Pax6 cells were the only cells found in the tegmentum. Pax6 was found in most granule cells of the cerebellum and Pax7 expression was found only in the ventricular zone. In the rostral rhombomere 1, Pax7 labeling was detected in cells of the ventricular zone of the alar plate, but numerous migrated cells were located in the basal plate, including the griseum centrale and the interpeduncular nucleus. Pax6 cells also formed a column of scattered neurons in the reticular formation and were found in the octavolateral area. The rhombencephalic ventricular zone of the alar plate expressed Pax7. Dorsal Pax7 cells and ventral Pax6 cells were found along the spinal cord separated from the ventricle, which did not show immunoreactivity. Our results show that the expression of Pax6 and Pax7 is widely maintained in the adult brain of *Xenopus*, like in urodele amphibians and in contrast to the situation described in amniotes. Therefore, in amphibians these transcription factors seem to be needed to maintain specific entities of subpopulations of neurons in the adult CNS.



*Abbreviations:* A, anterior nucleus of the thalamus; ABB, alar-basal boundary; Acc, accumbens nucleus; al, anterior lobe of the hypophysis; aol, area octavolateralis; ap, alar plate; APt, anterior pretectal nucleus; Av, anteroventral tegmental nucleus; b, basal band of the mesencephalon; bp, basal plate; BSM, bed nucleus of the stria medullaris; BST, bed nucleus of the stria terminalis; CB, calbindin D-28k; Cb, cerebellum; cc, central canal; CeA, central amygdala; ChAT, choline acetyltransferase; chp, choroid plexus; CoP, commissural pretectum; d, dorsal band of the mesencephalon; dh, dorsal horn of the spinal cord; DMB, diencephalo-mesencephalic boundary; DMN, dorsal medullary nucleus; Dp, dorsal pallium; fr, fasciculus retroflexus; Gc, griseum centrale; gl, glomerular layer of the olfactory bulb; GT, griseum tectale; H, hypothalamus; Hd, dorsal habenular nucleus; igl, internal granular layer of the olfactory bulb; III, oculomotor nucleus; il, intermediate lobe of the hypophysis; Ip, interpeduncular nucleus; Ipn, interpeduncular neuropil; Is, isthmic nucleus; ISc, inner nucleus of the subcommissural organ; Isl1, islet 1; IV, trochlear nucleus; JcP, juxtacommissural pretectum; l, lateral band of the mesencephalon; LA, lateral amygdala; Lc, locus coeruleus; LDT, laterodorsal tegmental nucleus; Lp, lateral pallium; LR, laterorostral mesencephalic nucleus; m, medial band of the mesencephalon; Ma, mammillary region; MeA, medial amygdala; Mes, mesencephalon; MOB, main olfactory bulb; Mp, medial pallium; nl, neural lobe of the hypophysis; NOS, nitric oxide synthase; NPv, nucleus of the periventricular hypothalamic organ; Nsol, nucleus of the solitary tract; nIII, oculomor nerve; nV, trigeminal nerve; nVI, abducens nerve; on, olfactory nerve; OPT, olivary pretectal nucleus; OSc, outer nucleus of the subcommissural organ; OT, optic tectum; 0tp, orthopedia; p1–p3, prosomeres 1–3; pc, posterior commissure; PcP, precommissural pretectum; PcPC, parvocellular nucleus of the posterior commissure; Pdi, posterodorsal tegmental nucleus, isthmic part; pe, postolfactory eminence; PO, preoptic area; po, pineal organ; PPN, pedunculopontine tegmental nucleus; PrPt, principal pretectal nucleus; PTh, prethalamus; PThE, prethalamic eminence; r0, isthmus (rhombomere r0); r1–r8, rhombomeres 1–8; Ras, superior raphe nucleus; Rh, rhombencephalon; Ri, nucleus reticularis inferior; Rm, nucleus reticularis medius; Rs, nucleus reticularis superior; SC, suprachiasmatic nucleus; ScO, subcommissural organ; Sd, septum dorsalis; sgr, stratum granulare of the cerebellum; Sl, septum lateralis; Sm, septum medialis; sm, stria medullaris; smn, spinal motor neurons; smol, stratum moleculare of the cerebellum; sol, solitary tract; sP, stratum of Purkinje cells; SpL, nucleus spiriformis lateralis; Str, striatum; tc, tectal commissure; Teg, mesencephalic tegmentum; Tel, telencephalon; TH, tyrosine hydroxylase; Th, thalamus; TP, nucleus of the tuberculum posterior; Ts, torus semicircularis; Tub, tuberal hypothalamic region; v, ventricle; vh, ventral horn of the spinal cord; VIa, accessory abducens nucleus; VIm, main abducens nucleus; VIIm, facial motor nucleus; VL, ventrolateral nucleus of the prethalamus; VJc, ventral juxtacommissural nucleus; VM, ventromedial nucleus of the prethalamus; Vm, trigeminal motor nucleus; vz, ventricular zone.

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## 1. Introduction

Pax genes encode a set of transcription factors that are involved in a wide range of developmental processes in metazoans, acting as tissue-specific regulators of organogenesis (cell fate and patterning), cell proliferation and disease (Chalepakis et al., 1993; Noll, 1993; Stuart et al., 1994; Wehr and Gruss, 1996; Balczarek et al., 1997; Mansouri et al., 1999; Chi and Epstein, 2002; Haubst et al., 2004; Buckingham and Relaix, 2007; Lang et al., 2007; Blake et al., 2008; Wang et al., 2008). In particular, among the Pax genes, Pax6 and Pax7 are expressed in regionally restricted patterns in the developing brain, controlling neuronal proliferation, brain regionalization, cell differentiation and neuronal survival (Wehr and Gruss, 1996; Lang et al., 2007; Thompson et al., 2007; Osumi et al., 2008; Wang et al., 2008). Interestingly, they are highly conserved across vertebrates and the proteins encoded by these two genes are also conserved (Goulding et al., 1993; Matsuo et al., 1993; Epstein et al., 1994; Li et al., 1994, 1997; Kallur et al., 2008), and the deduced amino acid sequences show more than 85% overall identity across vertebrates (Callaerts et al., 1997; Hirsch and Harris, 1997; Seo et al., 1998). Pax6 and Pax7 also appear to be involved in maintaining pluripotency throughout adulthood, in subsets of cell populations characterized as stem/progenitor cells, as well as subpopulations of mature nerve cells within certain brain regions, with ability to respond to environmental signals (Chi and Epstein, 2002; Maekawa et al., 2005; Thomas et al., 2007; Thompson et al., 2007, 2008; Blake et al., 2008; Fedtsova et al., 2008; Osumi et al., 2008). Thus, Pax6 and Pax7 expressing neurons were reported in the adult brain of rodents. In general, Pax6 is expressed in retinal cells, telencephalon, diencephalon, ventral mesencephalon, cerebellum and pons/medulla (Stoykova and Gruss, 1994; Kohwi et al., 2005; Maekawa et al., 2005; Nacher et al., 2005; Stanescu et al., 2007; Duan et al., 2013), whereas Pax7 is expressed in the superior colliculus and in specific nuclei of the pons/medulla and thalamus (Stoykova and Gruss, 1994; Shin et al., 2003; Thomas et al., 2007; Thompson et al., 2007, 2008). In all these regions, Pax6 and/or Pax7 seem to be required for maintaining distinct neuronal identity (Ninkovic et al., 2010) and physiological functions in mature neurons (Stoykova and Gruss, 1994; Shin et al., 2003).

Research during development of the CNS in some representatives of all major vertebrate classes has shown that the Pax6 and Pax7 expression patterns are highly comparable across species (Stoykova and Gruss, 1994; Stuart et al., 1994; Kawakami et al., 1997; Murakami et al., 2001; Derobert et al., 2002; Haubst et al., 2004; Pritz and Ruan, 2009; Moreno and González, 2011; Duan et al., 2013). Moreover, detailed distribution maps of Pax6 and Pax7 expressing cell in the brain of amphibians have been recently obtained by means of immunohistochemical procedures (Bandín et al., 2013; Joven et al., 2013b). The use of these highly sensitive techniques also revealed that the expression of Pax6 and Pax7 is widely maintained in the brains of adult urodeles, in contrast to the situation observed in other tetrapods, and this discrepancy was proposed to be related to the pedomorphic features of urodele brains (Joven et al., 2013a). In contrast, a previous study based on in situ hybridization techniques, found that Pax6 expression continued in the brain of juvenile and reproductive adults *Xenopus* only in very restricted regions of the forebrain, including the dorsal portion of the septum and the prethalamus (Moreno et al., 2008a).

In the present study we have analyzed the distribution patterns of Pax6- and Pax7-immunoreactive cells (Pax6 and Pax7 cells, respectively) in the adult brain of the anuran *Xenopus laevis* using antibodies that have been proven to be highly sensitive to unravel these transcription factors (Hitchcock et al., 1996; Wullimann and Rink, 2001; González and Northcutt, 2009; Ferreiro-Galve et al., 2012; Bandín et al., 2013; Joven et al., 2013a,b; González et al., 2014). For the correct identification of the cell groups expressing Pax6 and/or Pax7, we used combined immunofluorescence to reveal simultaneously several transcription factors and neuronal markers, which in turn served as landmarks for brain regions, because their distribution is well established in the brain of *Xenopus*, as previously reported (González et al., 1993; Marín et al., 1997; López and González, 2002; González et al., 2002; Moreno et al., 2008b; Morona and González, 2008, 2009; Domínguez et al., 2011, 2013, 2014). These markers included the γ-aminobutyric acid (GABA), calbindin D-28k (CB), choline acetyltransferase (ChAT), nitric oxide synthase (NOS), serotonin (5-HT), tyrosine hydroxylase (TH), and the transcription factors Nkx2.1, Nkx2.2, Islet 1 (Isl1), and orthopedia (Otp). For comparisons across species, the currently adopted paradigm of brain segmentation based on spatially restricted gene expression patterns has proven extremely useful (Gilland and Baker, 1993; Marín and Puelles, 1995; Puelles et al., 1996; Fritzsch, 1998; Cambronero and Puelles, 2000; Díaz et al., 2000; Puelles and Rubenstein, 2003; Straka et al., 2006). Our results show a highly conserved pattern of Pax6 and Pax7 expression across vertebrates and demonstrate that the expression of these transcriptions factors is widely maintained in adult *Xenopus* brains, supporting their importance throughout life.

## 2. Materials and methods

### 2.1. Animals and tissue processing

Adult specimens of the anuran amphibian *X. laevis* ($n = 26$) were purchased from commercial suppliers (XenopusOne, Dexter, MI), and kept in tap water at 20–25 °C. The animals were treated according to the regulations and laws of the European Union (2010/63/EU) and Spain (Royal Decree 53/2013) for care and handling of animals in research, after approval from the University Complutense to conduct the experiments described.

The animals were anesthetized by immersion in a 0.4 mg/ml solution of tricaine methanesulfonate (MS222, Sigma Chemical Co., St. Louis, MO) and perfused transcardially with 100 ml 0.9% NaCl, followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4), or the fixative MEMFA (0.1 M MOPS [4-morpholinopropanesulfonic acid], 2 mM EGTA [ethylene glycol tetraacetic acid], 1 mM $MgSO_4$, 3.7% formaldehyde). The brain and spinal cord were dissected out and postfixed approximately 24 h in the same fixative solution at 4 °C. Subsequently, they were immersed in a solution of 30% sucrose in 0.1 M phosphate buffer (PB; pH 7.4) for 4–6 h at 4 °C until they sank. For sectioning on a freezing microtome (Thermo Scientific Microm HM 450) the tissue was embedded in a solution of 20% gelatin with 30% sucrose in PB, and stored overnight in formaldehyde diluted 1:10 in 30% sucrose in PB at 4 °C. Sections were obtained at 30–40 μm thickness in the transverse or sagittal plane, and collected in PB in four series of adjacent sections.

### 2.2. Immunohistochemistry

Immunohistofluorescence procedures were conducted for different primary antibodies, all of which were diluted in 5–10% normal goat or mouse serum (depending on the source of the primary antibody) in PB with 0.1% Triton X-100 (Sigma, St. Louis, MO) and 2% bovine serum albumin (BSA, Sigma). Different protocols were carried out on free-floating sections, with incubation in the primary antibodies for 72 h at 4 °C, or for 16–24 h at room temperature in the antigen retrieval pre-treated slides. The dilution of each primary antibody used is detailed in Table 1.

Single-staining protocols for the detection of Pax6 and Pax7 were carried out on the free-floating sections as follows: (1) incubation for 72 h at 4 °C in the dilution of each primary serum (see Table 1). (2) According to the species in which the primary antibody was raised, the second incubations were conducted with the appropriately labeled secondary antibody diluted 1:500 for 90 min at room temperature: Alexa 594-conjugated goat anti-rabbit (red fluorescence; Molecular Probes, Eugene, OR; catalog reference: A11037), Alexa 488-conjugated goat anti-mouse (green fluorescence; Molecular Probes; catalog reference: A21042).

For bright field immunohistochemistry, free-floating sections were rinsed twice in PB, treated with 1% $H_2O_2$ in PB for 20 min to reduce endogenous peroxidase activity, rinsed again three times in PB, incubated in the primary antibody dilution (mouse anti-Pax6 or mouse anti-Pax7) with 0.025% Triton in PB, revealed with biotinylated horse anti-mouse (1:100; Vector, Burlingame, CA; catalog reference: BA-2000), rinsed three times in PB, and visualized by the ABC-DAB kit method (Vector, SK4100).

To study the relative distribution of two proteins in the same sections, the two-step protocol for immunohistofluorescence was used, with cocktails of pairs of primary antibodies (always developed in different species), at the same dilutions specified in Table 1 and following the conditions detailed above for the