

# Pattern of Calbindin-D28k and Calretinin Immunoreactivity in the Brain of *Xenopus laevis* During Embryonic and Larval Development

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## ABSTRACT

The present study represents a detailed spatiotemporal analysis of the localization of calbindin-D28k (CB) and calretinin (CR) immunoreactive structures in the brain of *Xenopus laevis* throughout development, conducted with the aim to correlate the onset of the immunoreactivity with the development of compartmentalization of distinct subdivisions recently identified in the brain of adult amphibians and primarily highlighted when analyzed within a segmental paradigm. CR and CB are expressed early in the brain and showed a progressively increasing expression throughout development, although transient expression in some neuronal subpopulations was also noted. Common and distinct characteristics in *Xenopus*, as compared with reported features during development in the brain of mammals,

were observed. The development of specific regions in the forebrain such as the olfactory bulbs, the components of the basal ganglia and the amygdaloid complex, the alar and basal hypothalamic regions, and the distinct diencephalic neuromeres could be analyzed on the basis of the distinct expression of CB and CR in subregions. Similarly, the compartments of the mesencephalon and the main rhombencephalic regions, including the cerebellum, were differently highlighted by their specific content in CB and CR throughout development. Our results show the usefulness of the analysis of the distribution of these proteins as a tool in neuroanatomy to interpret developmental aspects of many brain regions. *J. Comp. Neurol.* 521:79–108, 2013.

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**INDEXING TERMS:** calcium-binding proteins; telencephalon; thalamus; brainstem; evolution; amphibians

The use of calcium-binding proteins (CBPs) as neuroanatomical markers of distinct neuronal groups in the brain has been demonstrated a useful tool for the identification of otherwise unobservable specific groups of neurons, nuclear boundaries, brain subdivisions, fiber tracts, and neuropils. Among the numerous CBPs in the nervous system, calbindin-D28k (CB) and calretinin (CR) are of particular interest because they are localized within practically nonoverlapping cell groups, and this segregated pattern allows recognition subgroups within nuclei that represent distinct neuronal subcircuits, which are not cytoarchitectonically separated (for review, see Andressen et al., 1993). Thus, analysis of the distribution of CB- and CR-immunoreactive (CBir and CRir, respectively) cells and fibers has been widely conducted in the brain of many vertebrates to assist in the identification of regions and nuclei across species because their staining, in many cases, facilitates topological recognition of homologous structures. Most studies have been carried out in the

brain of adult mammalian species (e.g., García-Segura et al., 1984; Fournet et al., 1986; Jones and Hendry, 1989; Celio, 1990; Jacobowitz and Winsky, 1991; Résibois and Rogers, 1992; Rogers and Résibois, 1992; Winsky et al., 1992; Andressen et al., 1993; Arai et al., 1994; De Biasi et al., 1994; Molinari et al., 1994; Gutiérrez et al., 1995) but because these proteins are highly conserved throughout vertebrate evolution, studies were also accomplished in representatives of most vertebrate classes (Lunam, 1989; Rodríguez-Moldes et al., 1990; Pombal et al., 1999, 2002; Pritz, 1999; Dávila et al.,

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2000; Díaz-Regueira and Anadón, 2000; Castro et al., 2006; Huesa et al., 2006; Morona et al., 2006a,b; Yan et al., 2010).

The precise localization of CB and CR, two members of the EF-hand family of CBPs that act as buffers for calcium concentration in different systems (Bainbridge et al., 1992; Heizmann and Braun, 1992; Polans et al., 1996; Schäfer and Heizmann, 1996; Vecellio et al., 2000; Blatow et al., 2003; Cheron et al., 2004), has been functionally related to developmental events in the central nervous system that require precise regulation of calcium,

such as synaptic transmission and plasticity (Constantine-Paton et al., 1990; Singer, 1995; Schwaller et al., 2002; Blatow et al., 2003), determination of the spine morphology (Vecellio et al., 2000), gene expression (Spitzer et al., 1993; Spitzer, 1994), and the maintenance of cytoskeletal elements during neuronal migration events (Enderlin et al., 1987; Kater et al., 1988; al-Mohanna et al., 1992; Puelles et al., 1992; Porteros et al., 1997; Abbot and Jacobowitz, 1999; Guglielmino and Covertti, 2000; Ulfhig, 2002). However, each protein seems to play specific roles in different sets of neurons reflecting the

#### Abbreviations

A	Anterior thalamic nucleus	p1-p3	Prosomeres 1-3
Ac	Anterior thalamic nucleus (caudal part)	PA	Pallidum
ac	Anterior commissure	Pa	Pallium
Acc	Nucleus accumbens	Pb	Parabrachial area
Ad	Anterodorsal tegmental nucleus	Pc	Precommissural pretectal nucleus
AN	Anterior nucleus of the octavolateral area	Pd	Posterodorsal tegmental nucleus
AOB	Accessory olfactory bulb	Pdi	Posterodorsal tegmental nucleus, isthmic part
AOL	Area octavolateralis	PO	Preoptic area
Ar	Anterior thalamic nucleus (rostral part)	PThE	Prethalamie eminence
Av	Anteroventral tegmental nucleus	PV	Paraventricular nucleus
b	Basal band of the mesencephalon	Pv	Posteroventral tegmental nucleus
BST	Bed nucleus of stria terminalis	Pvi	Posteroventral tegmental nucleus, isthmic part
C	Central thalamic nucleus	Ra	Raphe nucleus
Cb	Cerebellum	r0	Isthmus (rhombomere r0)
CeA	Central amygdala	r1-8	Rhombomeres 1-8
CoP	Commissural pretectal nucleus	RC	Retrochiasmatic nucleus
d	Dorsal band of the mesencephalon	Rh	Rhombencephalon
DCN	Dorsal column nucleus	Ri	Nucleus reticularis inferior
DF	Dorsal funiculus	Rm	Nucleus reticularis medius
DMN	Dorsal medullary nucleus	RM	Retromammillary nucleus
Dp	Dorsal pallium	Rs	Nucleus reticularis superior
EC	Epichiasmatic nucleus	SC	Suprachiasmatic nucleus
fr	Fasciculus retroflexus	SCc	Caudal suprachiasmatic nucleus
Gc	Griseum centrale	SCR	Rostral suprachiasmatic nucleus
GT	Griseum tectale	Sd	Septum dorsalis
GTs	Griseum tectale superficiale	Se	Septum
Hb	Habenula	Sld	Dorsolateral septal nucleus
Hd	Dorsal habenula	Slv	Ventrolateral septal nucleus
Hv	Ventral habenula	SM	Superficial mammillary nucleus
Hy	Hypothalamus	Sm	Septum medialis
III	Oculomotor nucleus	sm	Stria medullaris
IV	Trochlear nucleus	spc	Spinal cord
IN	Intermediate nucleus of the octavolateral area	SPV	Supraoptoparaventricular area
Ip	Interpeduncular nucleus	St	Striatum
Is	Isthmic nucleus	SubP	Suballium
JcP	Juxtacommisural pretectal nucleus	Sv	Septum ventralis
I	Lateral band of the mesencephalon	Tel	Telencephalon
LC	Laterocaudal mesencephalic nucleus	Tht	Thalamotelencephalic tract
LDT	Laterodorsal tegmental nucleus	TI	Laminar nucleus of the torus semicircularis
Ifb	Lateral forebrain bundle	Tmg	Magnocellular nucleus of the torus semicircularis
LGE	Lateral ganglionic eminence	TP	Posterior tubercle
Lp	Lateral pallium	Tp	Principal nucleus of the torus semicircularis
Lpv	Lateral posteroventral nucleus	Ts	Torus semicircularis
LR	Laterorostral mesencephalic nucleus	Tub	Tuberous hypothalamic region
m	Medial band of the mesencephalon	v	Ventricle
Ma	Mammillary area	Vd	Descending trigeminal nucleus/tract
MeA	Medial amygdala	VIII	Octaval area
Mes	Mesencephalon	VIIIm	Facial motor nucleus
MesV	Mesencephalic trigeminal cells	VL	Ventrolateral thalamic nucleus
MGE	Medial ganglionic eminence	VM	Ventromedial thalamic nucleus
MOB	Main olfactory bulb	Vm	Motor trigeminal nucleus
MN	Spinal motoneurons	X	Vagal motor nucleus
Mp	Medial pallium	XI-spm	Nucleus of the accessory nerve and spinal cord motoneurons
MPO	Medial preoptic nucleus	XII	Hypoglossal nucleus
MVN	Medial vestibular nucleus	Vp	Ventral pallium
Nsol	Nucleus of the solitary tract	Vpr	Principal sensory trigeminal nucleus
OB	Olfactory bulb	Zli	Zona limitans intrathalamica
oc	Optic chiasm	ZI	Zona incerta
on	Optic nerve	Zip	Periventricular nucleus of the zona incerta
OT	Optic tectum		
ot	Optic tract		

functional and evolutionary dissimilarities in CB and CR sequence (Parmentier, 1990) that allow them to adapt to different targets (Schwaller et al., 2002; Palczewska et al., 2003; Schmidt et al., 2005, 2007).

Therefore, the early expression and the dynamics of the distribution of these proteins have been used as markers for developmental aspects of certain neuronal subpopulations in diverse studies in the brains of mammals (Baimbridge and Miller, 1982; Liu et al., 1988; Séquier et al., 1991; Liu and Graybiel, 1992; Résibois and Rogers, 1992; Soriano et al., 1994; Fonseca et al., 1995; Yan et al., 1995, 1996; Jiang et al., 1997; Meyer and Wahle, 1999; Dávila et al., 2005). It was demonstrated that CR and, to a lesser extent, CB are expressed early in the central nervous system (Andressen et al., 1993) and show either a progressively increasing expression from the fetal stage to maturity (Enderlin et al., 1987; Yew et al., 1997; Kwong et al., 2000) or a transient expression in neuronal subpopulations at certain developmental stages (Iacopino and Christakos, 1990; Puelles et al., 1992; Villa et al., 1994; Yan et al., 1995; Lohmann and Friauf, 1996; Jiang and Swann, 1997; Frassoni et al., 1998; Shetty and Turner, 1998; Setzer and Ulfhake, 1999; Abraham et al., 2009; Masliukov et al., 2012). Only a few studies have addressed the spatiotemporal sequence of appearance of CB and/or CR expression in the developing brain of nonmammalian vertebrates, and the observed patterns of distribution have been correlated with developmental aspects of brain maturation (Enderlin et al., 1987; Braun et al., 1988, 1991; Bastianelli and Pochet, 1993; Guglielmone and Corvetti, 2000; Castro et al., 2003; Guglielmotti et al., 2004).

Among vertebrates, amphibians are a class of anamniotes that marks a crucial point in evolution, the transition from aquatic to terrestrial lifestyles. Species of amphibians have generally been chosen in comparative studies of brain organization in vertebrates, and it is assumed that fundamental traits common to the brain of tetrapods can be found in amphibians (Herrick, 1948; Kicliter and Ebbesson, 1976; Fritzsch and Himstedt, 1980; Northcutt and Kicliter, 1980). The extensive distribution of CBir and CRir cells and fibers in the brains of adult amphibians has been demonstrated for several species (Necchi et al., 1999; Milán and Puelles 2000; Brox et al., 2004; Morona and González, 2008, 2009; Morona et al., 2011b). These studies proved the extreme usefulness of the analysis of the localization of these proteins to recognize most brain regions that constitute clear divisions in amniotes and that in anamniotes, particularly in amphibians, are not distinguishable due to the reduced cell migration from the ventricular lining that crowds most

neurons in a dense central gray layer in which nuclei and distinct brain structures can be recognized, at best, as local condensations, in particular during development (ten Donkelaar, 1998a,b).

In many developmental neurobiology studies the anuran *Xenopus laevis* has been employed as a model organism, mainly due to the convenience of “hormone induced” breeding that makes embryos available for experimentation at all times of the year and are easy to maintain under laboratory conditions. In addition, an accurate timetable of development exists (Nieuwkoop and Faber, 1967) that is widely used, allowing comparative analysis of developmental processes. The present study is a detailed spatiotemporal analysis of the localization of CBir and CRir structures in the brain of *X. laevis* throughout development, conducted with the aim to correlate the ontogeny of the immunoreactivity with the development of compartmentalization of many brain subdivisions recently identified in the brain of amphibians, mainly using the segmental approach based on modern neuromeric models of the forebrain (Puelles et al., 1996; Puelles and Rubenstein, 2003), midbrain (Díaz et al., 2000), and hindbrain (Gilland and Baker, 1993; Marín and Puelles, 1995; Fritzsch, 1998; Cambronero and Puelles, 2000; Straka et al., 2006). In order to identify some of these subdivisions better, we have used as reference the codistribution/colocalization of CB and CR with other markers, the distribution of which we have previously studied in the developing *Xenopus* brain (González et al., 1994; López and González, 2002; López et al., 2002). In particular, double immunohistochemistry for the simultaneous detection of CB and CR with tyrosine hydroxylase (TH), nitric oxide synthase (NOS), and choline acetyltransferase (ChAT) has been systematically performed.

## MATERIALS AND METHODS

### Animals and tissue processing

For the present study, a total of 132 *X. laevis* embryos and larvae were used. They were staged according to Nieuwkoop and Faber (1967) and sorted into early embryos (30–40), late embryos (41–45), premetamorphic larvae (46–53), prometamorphic larvae (54–59), metamorphic larvae (60–66), and juveniles (Table 1). The animals were obtained from the laboratory stock of the Department of Cell Biology, University Complutense of Madrid. *Xenopus* tadpoles were obtained by breeding induced by chorionic gonadotropin (Pregnyn; Organon, West Orange, NJ) and kept in tap water at 20–25°C. Young larvae were raised on Mikropan Growth Food (Sera, Hensbers, Germany), and older larvae and juveniles were fed with liver meat. The original research reported herein was performed under guidelines established by

**TABLE 1.**  
Number of animals investigated at different stages of developments

Developmental stages																					
Embryonic				Premetamorphic					Prometamorphic					Metamorphic climax							
23-30	30-39	40-45	46	47	48	50	51	52	53	54	55	56	57	58	59	60	62	63	65	66	n
20	23	35	2	6	3	6	3	3	5	6	2	4	0	4	2	3	0	3	0	2	132

Staging of the embryos and larvae according to Nieuwkoop and Faber, 1967.

the regulations and laws of the European Union (86/609/EEC) and Spain (Royal Decree 223/1998) for care and handling of animals in research.

At appropriate times, embryos and larvae were anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, pH 7.4; Sigma Chemical, St. Louis, MO). The late larvae and juveniles were perfused transcardially with 0.9% sodium chloride, followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). The embryos and premetamorphic larvae were fixed by immersion for 20 hours at 4°C in the same fixative solution. Subsequently, they were immersed in a solution of 30% sucrose in PB for 5 hours at 4°C until they sank, embedded in a solution of 20% gelatin with 30% sucrose in PB, and then immersed in a 3.7% formaldehyde solution at 4°C for 8–10 hours. The gelatin blocks were cut on a freezing microtome at 20–40 µm in the transverse, horizontal, or sagittal plane, and sections were collected in cold PB.

### Immunohistochemistry

Sections were rinsed in PB, treated with 1% H<sub>2</sub>O<sub>2</sub> in PB for 15 minutes to reduce endogenous peroxidase activity, rinsed again in PB, and processed by the peroxidase anti-peroxidase (PAP) method (Sternberger, 1979). This process included a first incubation of the sections in a mouse anti-calbindin-D28k (CB) or rabbit anti-calretinin (CR) serum (both by Swant; Bellinzona, Switzerland; cat. nos. 300 and 7699/4, respectively), diluted 1:1,000 in PB containing 0.5% Triton X-100 (PBT), for 48–72 hours at 4°C. Subsequently, they were rinsed in PB for 10 minutes and incubated in the second antibody goat antimouse (diluted 1:50 in PBT; Dako, Glostrup, Denmark) or swine antirabbit (diluted 1:50 in PBT; Dako), for 90 minutes at room temperature. After rinsing, the sections were incubated for 90 minutes in either mouse or rabbit PAP complex (diluted 1:500 in PBT; Dako) and rinsed three times in PB. Finally, the sections were stained in 0.5 mg/ml 3,3'-diaminobenzidine (DAB; Sigma) intensified with nickel (Adams et al., 1981) with 0.01% H<sub>2</sub>O<sub>2</sub> in PB for 10–20 minutes. Some series of sections were stained according to the glucose oxidase method (Shu et al., 1988), which enhances the staining of fibers and terminals. The sections were then mounted on glass slides from a solution of 0.25% gelatin in 0.05 M Tris-HCl buffer (TB, pH

7.6) and, after dehydration, the slides were coverslipped with Entellan (Merck, Darmstadt, Germany).

### Double immunohistofluorescence

To study the codistribution/colocalization of CB and CR (or either of these proteins with NOS, ChAT, and TH), different two-step protocols for immunohistofluorescence were used with antibody cocktails as follows.

#### CB/CR

1) Incubation for 72 hours at 4°C in a mixture of primary mouse anti-CB and rabbit anti-CR antibodies (both diluted 1:1,000 in PBS-T); 2) second incubation for 90 minutes at room temperature in a mixture of Alexa 488-conjugated goat antimouse (green fluorescence; diluted 1:300 in PBS-T; Molecular Probes, Eugene, OR) and Alexa 594-conjugated goat antirabbit (red fluorescence; diluted 1:500 in PBS-T; Molecular Probes).

#### CB/NOS

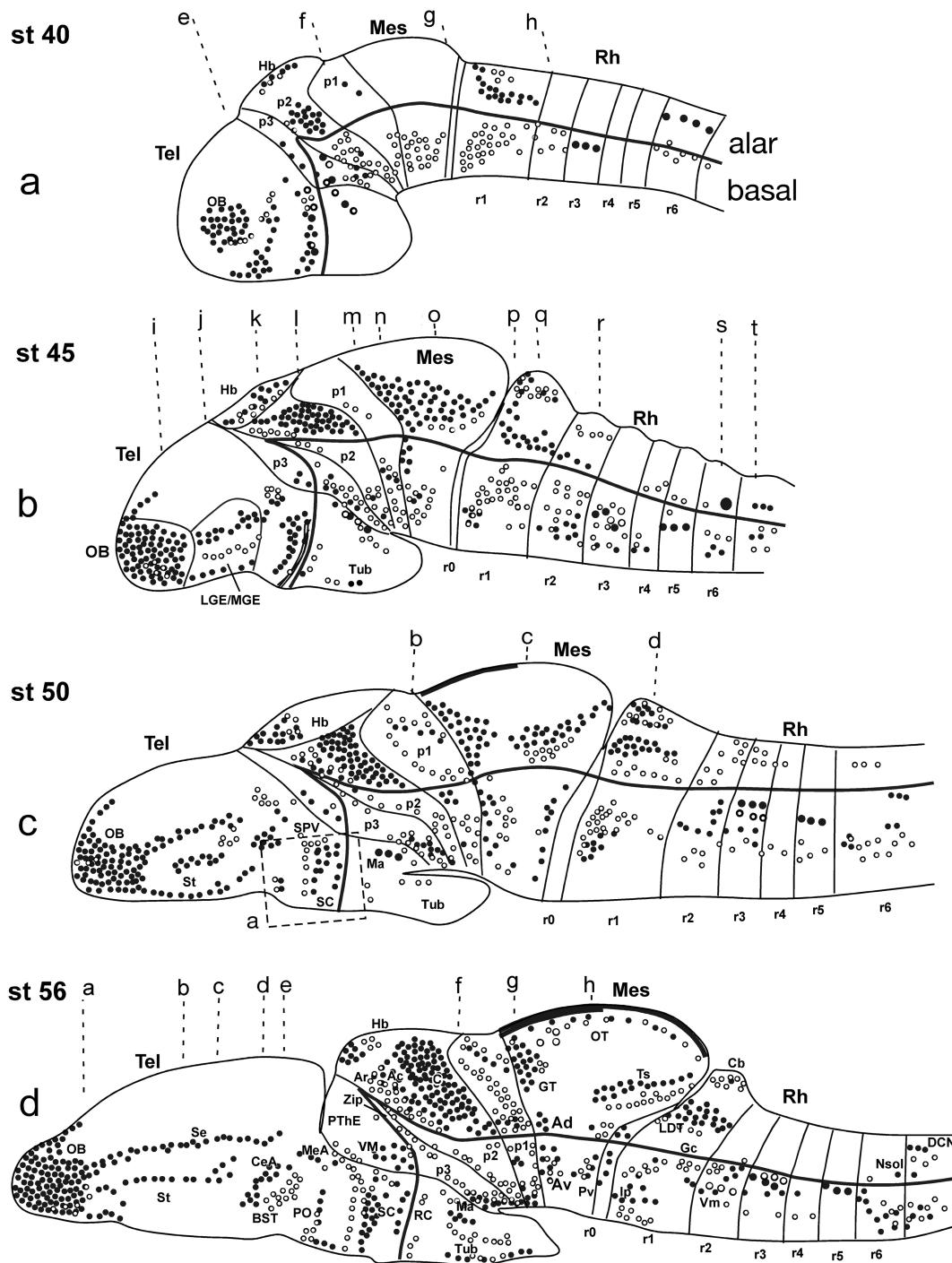
1) Incubation for 72 hours at 4°C in a mixture of primary mouse anti-CB (diluted 1:1,000 in PBS-T) and sheep anti-NOS antibodies (diluted 1:20,000 in PBS-T; K205 antibody, donated by Dr. Emson, Babraham Institute; Cambridge, UK); 2) second incubation for 90 minutes at room temperature in a mixture of rhodamine-conjugated rabbit antimouse (red fluorescence; diluted 1:300 in PBS-T, Chemicon, Temecula, CA) and FITC-conjugated rabbit antisheep (green fluorescence; diluted 1:500 in PBS-T; Chemicon).

#### CR/NOS

1) Incubation for 72 hours at 4°C in a mixture of primary rabbit anti-CR (diluted 1:1,000 in PBS-T) and sheep anti-NOS antibodies (diluted 1:20,000 in PBS-T); 2) second incubation for 90 minutes at room temperature in a mixture of FITC-conjugated chicken antirabbit (green fluorescence; diluted 1:100 in PBS-T, Chemicon) and rhodamine-conjugated donkey antisheep (red fluorescence; diluted 1:100 in PBS-T; Chemicon). Rinsing, mounting, and coverslipping were accomplished as described above.

#### CB/TH

1) Incubation for 72 hours at 4°C in a mixture of primary mouse anti-CB (diluted 1:1,000 in PBS-T) and rabbit



**Figure 1.** Schematic representation of the general distribution of calbindin (empty dots) and calretinin (filled dots) immunoreactive cells in a sagittal views of the brain at representative early embryonic (a) late embryonic (b) premetamorphic (c) and prometamorphic (d) stages. In all schemes dorsal is oriented upward and rostral to the left. The main subdivisions with regard to the neuromeric models are indicated. In each scheme the dashed lines represent the levels of the sections shown in the microphotographs in subsequent figures. Levels indicated in a and b correspond to photographs in Figure 2, levels indicated in c correspond to photographs in Figure 3, and levels indicated in d correspond to photographs in Figure 4. For abbreviations, see list.

anti-TH (diluted 1:100; cat. no. AB152, Chemicon) antibodies; 2) second incubation for 90 minutes at room temperature in a mixture of Alexa 488-conjugated goat antimouse

(green fluorescence; diluted 1:300 in PBS-T; Molecular Probes) and Alexa 594-conjugated goat antirabbit (red fluorescence; diluted 1:500 in PBS-T; Molecular Probes).

**TABLE 2.**  
Primary Antibodies used in This Study

Name	Immunogen	Commercial supplier	MW (kDa)	Dilution
CR	Recombinant human calretinin	Polyclonal rabbit anti-CR; Swant, Bellinzona, Switzerland; catalog No. 7699/4	29	1:1,000
CB	calbindin D-28k purified from chicken gut	Monoclonal mouse anti-CB; Swant, Bellinzona, Switzerland; catalog No. 300	28	1:1,000
TH	TH purified from rat PC12 cells	Monoclonal mouse anti-TH ImmunoStar, Hudson, WI; catalogue reference 22941	62	1:1,000
TH	TH Protein purified from rat pheochromocytoma	Polyclonal Rabbit-anti-TH Chemicon International, Inc, USA. Code number: AB152	62	1:1000
ChAT	Human placental choline acetyltransferase	Polyclonal goat anti-ChAT Chemicon; catalogue reference: AB144P	68	1:100
NOS	Recombinant rat NOS	Polyclonal sheep anti-NOS K205 antibody Dr. P.C. Emson, The Babraham Institute	155	1:20,000

### CR/TH

1) Incubation for 72 hours at 4°C in a mixture of primary rabbit anti-CR (diluted 1:1,000 in PBS-T) and mouse anti-TH (Immunostar, formerly Incstar, Hudson, WI; cat. no. P22941) antibodies (diluted 1:100 in PBS-T); 2) second incubation for 90 minutes at room temperature in a mixture of Alexa 488-conjugated goat antimouse (green fluorescence; diluted 1:300 in PBS-T; Molecular Probes) and Alexa 594-conjugated goat antirabbit (red fluorescence; diluted 1:500 in PBS-T; Molecular Probes).

### CB/ChAT

1) Incubation for 72 hours at 4°C in a mixture of primary mouse anti-CB (diluted 1:1,000 in PBS-T) and goat anti-ChAT antibodies (diluted 1:100 in PBS-T; cat. no. AB1582, Chemicon); 2) second incubation for 90 minutes at room temperature in a mixture of rhodamine-conjugated rabbit antimouse (red fluorescence; diluted 1:100 in PBS-T) and FITC-conjugated rabbit antigoat (green fluorescence; diluted 1:100 in PBS-T, Vector Laboratories, Burlingame, CA).

### CR/ChAT

1) Incubation for 72 hours at 4°C in a mixture of primary rabbit anti-CR (diluted 1:1,000 in PBS-T) and goat anti-ChAT antibodies (diluted 1:100 in PBS-T); 2) second incubation for 90 minutes at room temperature in a mixture of FITC-conjugated chicken antirabbit (green fluorescence; diluted 1:100 in PBS-T) and rhodamine-conjugated donkey antisheep (red fluorescence; diluted 1:100 in PBS-T).

In all double immunofluorescence experiments, after rinsing the sections were mounted on glass slides and coverslipped with Vectashield (Vector Laboratories).

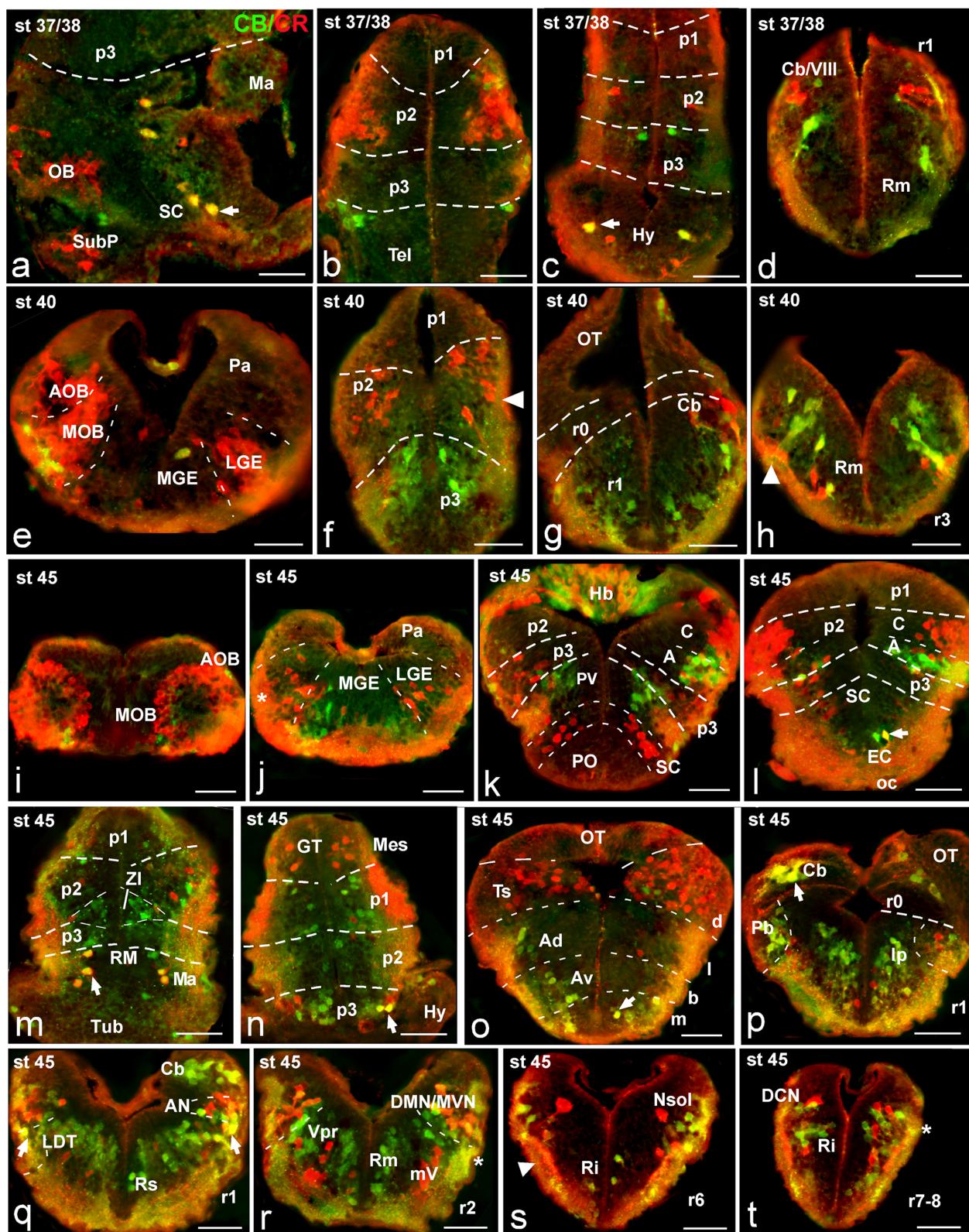
### Controls and specificity of the antibodies

All the antibodies had been previously tested in the brain of *X. laevis* and the patterns of staining showed the

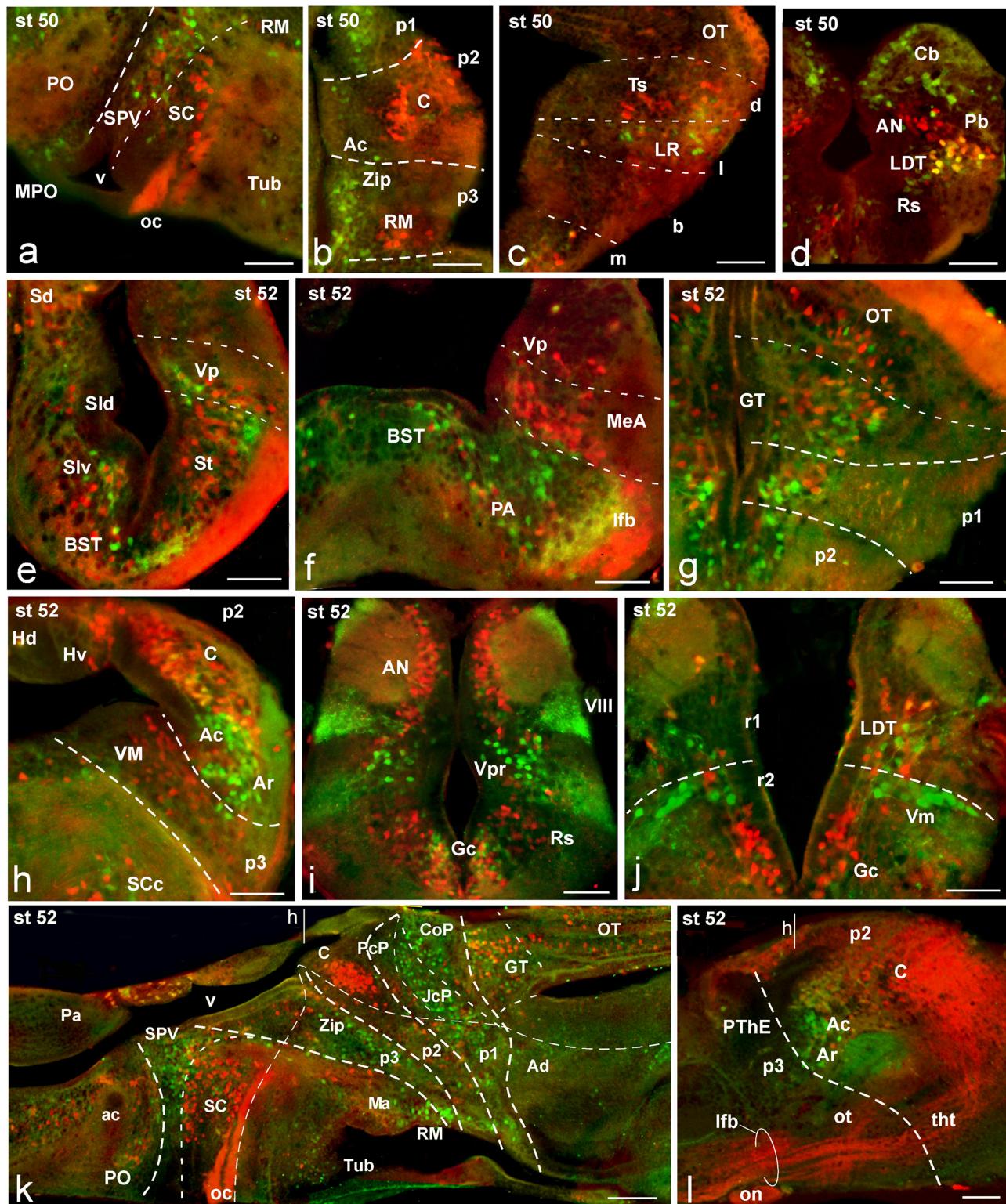
same distribution as that observed in the present study (González et al., 1994; López and González, 2002; López et al., 2003, Morona and González, 2008, 2009). Controls for the immunohistochemical experiments included: 1) western blots of brain extract of *Xenopus* showed that each of the antibodies used consistently labeled single bands that corresponded well to the expected molecular weights and, with small species variations, were similar to the bands labeled in the lane of rat brain extract (see fig. 1 in Morona and González, 2008, 2009; Moreno et al., 2012); 2) incubation of some selected sections with pre-immune mouse serum (1:1,000 for TH and CB), rabbit serum (1:1,000 for CR), or goat serum (1:1,000 for NOS and ChAT) instead of the primary antibody; 3) controls in which either the primary or the secondary antibody was omitted; and 4) preadsorption of the primary antibodies with synthetic peptides (Morona et al., 2006b, 2007b). In all these negative controls the immunostaining was eliminated. The characteristics of the used antibodies are shown in Table 2 and further specifications can be found in our preceding similar studies on the brain of *Xenopus* (Morona and González, 2008, 2009; Moreno et al., 2012).

### Evaluation and presentation of the results

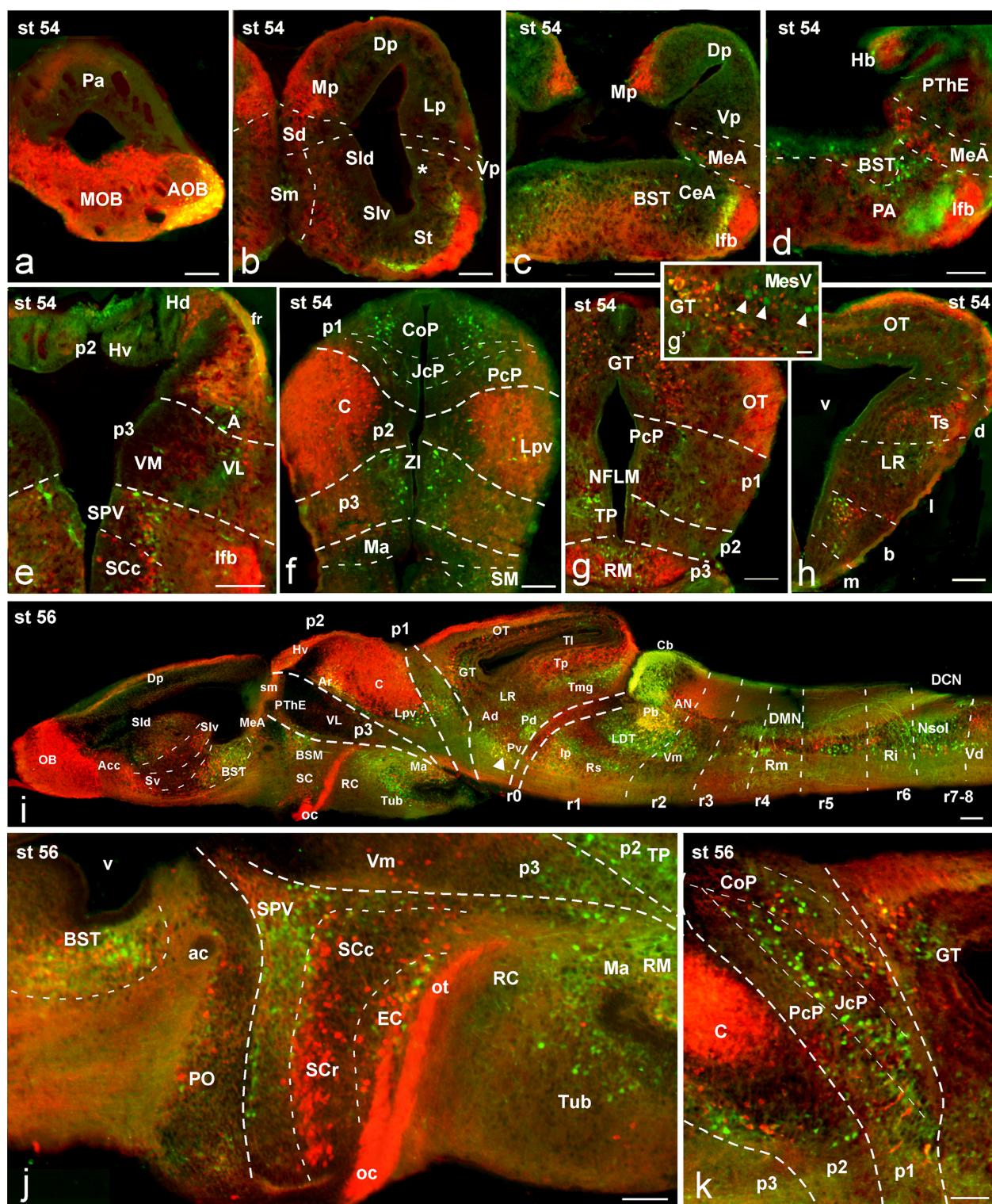
The localization of CBir and CRir cell bodies and fibers was studied in both the single-labeled sections and the double-labeled sections. Their relative localization was framed within the newly defined territories in the telencephalon of amphibians and attending to the neuromeric organization of the brain (Marín et al., 1998; Moreno and González, 2006, 2007b; Morona and González, 2008, 2009; Domínguez et al., 2010; Moreno et al., 2012). For a ready interpretation of the developing brain in *Xenopus* and an easier comparison with other species we used the same nomenclature as in our previous studies in the adult brain (Morona and González, 2008, 2009). The general distribution of CBir and CRir cells in the brain was charted at selected embryonic and larval stages in schemes



**Figure 2.** Photomicrographs of double-labeled CB/CR sagittal (a, dorsal is upwards and rostral to the left) and transverse (b–t) sections through representative levels of the brain of *Xenopus* at embryonic stages (indicated on each photomicrograph). The levels of transverse sections are indicated in Figure 1a,b. In all photographs green is CB and red is CR, whereas yellow represents double-labeled cells. Dashed lines mark the approximate boundary between neuromeres and telencephalic subdivisions. Arrowheads in f point to the lateral CRir fiber tract in p2, in h to the lateral CRir fiber tract in the basal plate, and in q to double-labeled cells in the LDT. The asterisks in r and t mark the CBir fibers in the lateral rhombencephalon. For abbreviations, see list. A magenta-green version of this figure is provided as Supporting Information Figure 1. Scale bars = 50  $\mu$ m in a–h; 100  $\mu$ m in i–t.



**Figure 3.** Photomicrographs of double-labeled CB/CR sagittal (a,k,l; dorsal is upwards and rostral to the left) and transverse (b–j) sections through representative levels of the brain of *Xenopus* at premetamorphic stages (indicated on each photomicrograph). The levels of transverse sections for stage 50, and the area shown in a, are indicated in Figure 1c. In all photographs green is CB and red is CR, whereas yellow represents double-labeled cells. Dashed lines represent the approximate boundary between telencephalic (e,f) or diencephalic (a,b,h,k,l) subdivisions, the mesencephalic longitudinal bands (c,g,k), and rhombencephalic regions (d,i,j). For abbreviations, see list. A magenta-green version of this figure is provided as Supporting Information Figure 2. Scales bar = 100 µm in a–j; 200 µm in k.



**Figure 4.** Photomicrographs of double-labeled CB/CR transverse (a-h) and sagittal (i-k; dorsal is upwards and rostral to the left) sections through representative levels of the brain of *Xenopus* at prometamorphic stages (indicated on each photomicrograph). The levels of transverse sections are indicated in Figure 1d. In all photographs green is CB and red is CR, whereas yellow represents double-labeled cells. Dashed lines represent the approximate boundary between telencephalic (a-d,i), diencephalic (e-g,i-k), mesencephalic (g,h), and rhombencephalic (i) subdivisions. i: Low magnification image of a lateral sagittal section showing the relationship between CB and CR labeled structures throughout the brain of a prometamorphic larva. j,k: Details of sagittal sections through the hypothalamic (j) and pretectal (k) region showing the relative localization of CB- and CR-labeled structures in each subdivision. Inset in g' shows trigeminal mesencephalic neurons (arrowheads) in the rostral optic tectum. For abbreviations, see list. A magenta-green version of this figure is provided as Supporting Information Figure 3. Scale bars = 100  $\mu$ m in a-h,k; 200  $\mu$ m in i,j.

obtained from a series of sagittal sections in which the main subdivisions were outlined attending to the neuromeric models (Fig. 1). Single- and double-labeled sections

were analyzed with an Olympus BX51 microscope equipped for fluorescence with appropriate filter combinations. For the illustrations, selected double-labeled

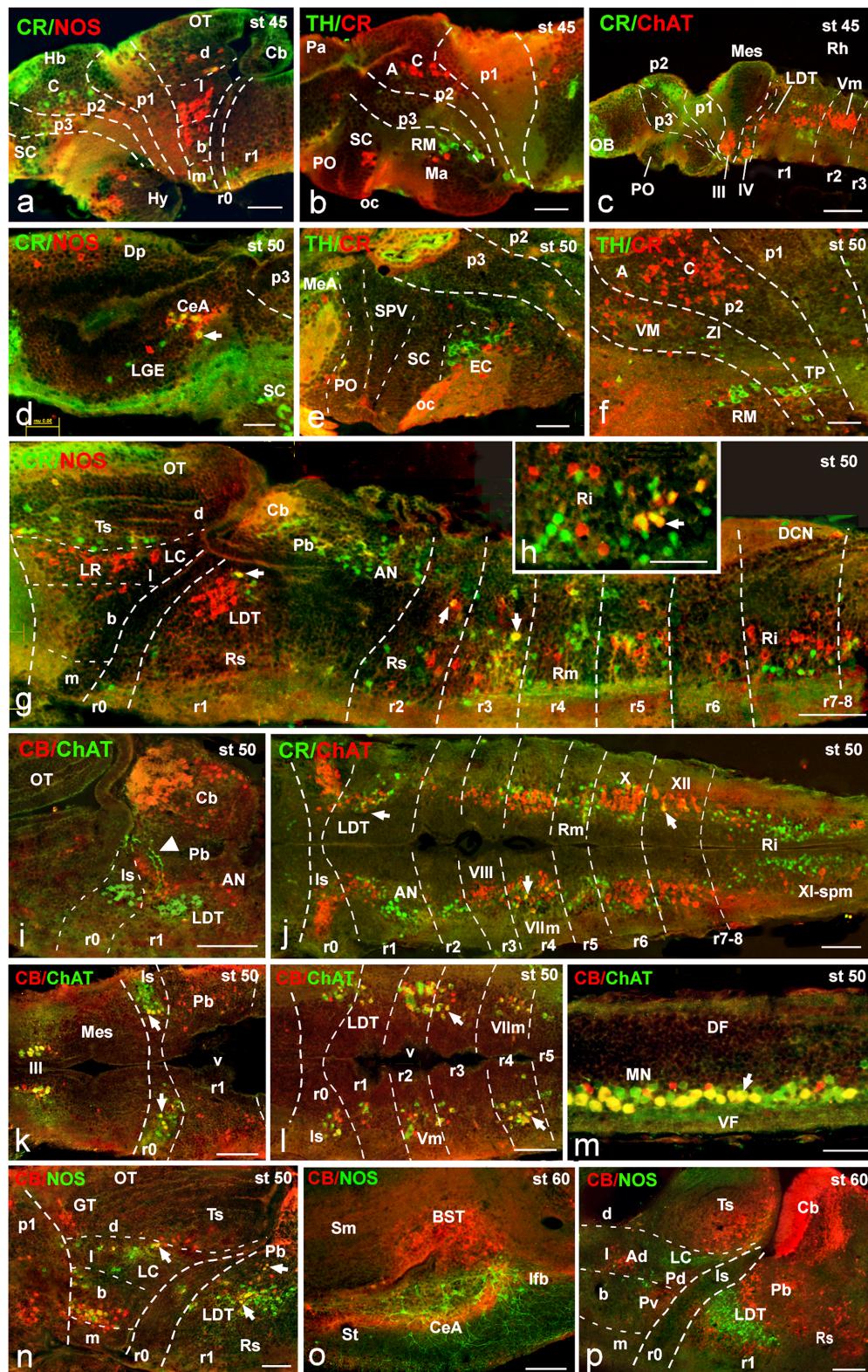


Figure 5

sections for CB/CR were photographed (Figs. 2–4). In addition, sections stained with double immunohistochemistry combining either CB or CR with NOS, TH, and ChAT were also photographed (Fig. 5). In all cases, photographs were obtained using a digital camera (Olympus DP70). Contrast and brightness were adjusted with Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) and photographs were mounted on plates using Canvas 11 (ACS Systems International, Santa Clara, CA).

## RESULTS

The staining patterns obtained for CBir and CRir cells and fibers in the developing central nervous system of *X. laevis* were studied in animals whose ages varied from early embryonic stages to the metamorphic climax (Table 3). For each stage, the pattern of labeling was consistent among animals treated identically. The long larval period, which starts with independent feeding, is generally subdivided into three stages (Gona et al., 1982; González et al., 1994): 1) premetamorphic stages; 2) prometamorphic stages; and 3) metamorphic climax. In the following sections we describe the development of the immunoreactive structures taking into account these periods of development. To clarify the description, the progressively or transiently appearing immunoreactive cell groups in the developing brain of *X. laevis* are summarized in Table 4.

### Early embryonic stages (34–40)

The first immunoreactive elements were detected by stage 34 (about 44 hours postfertilization). CBir cells were located in the hindbrain at mid-rhombencephalic levels within the reticular formation and in the developing octavolateral area, and labeled longitudinal fibers were observed through the brainstem and diencephalon up to the caudal telencephalon. In turn, at this early stage CRir

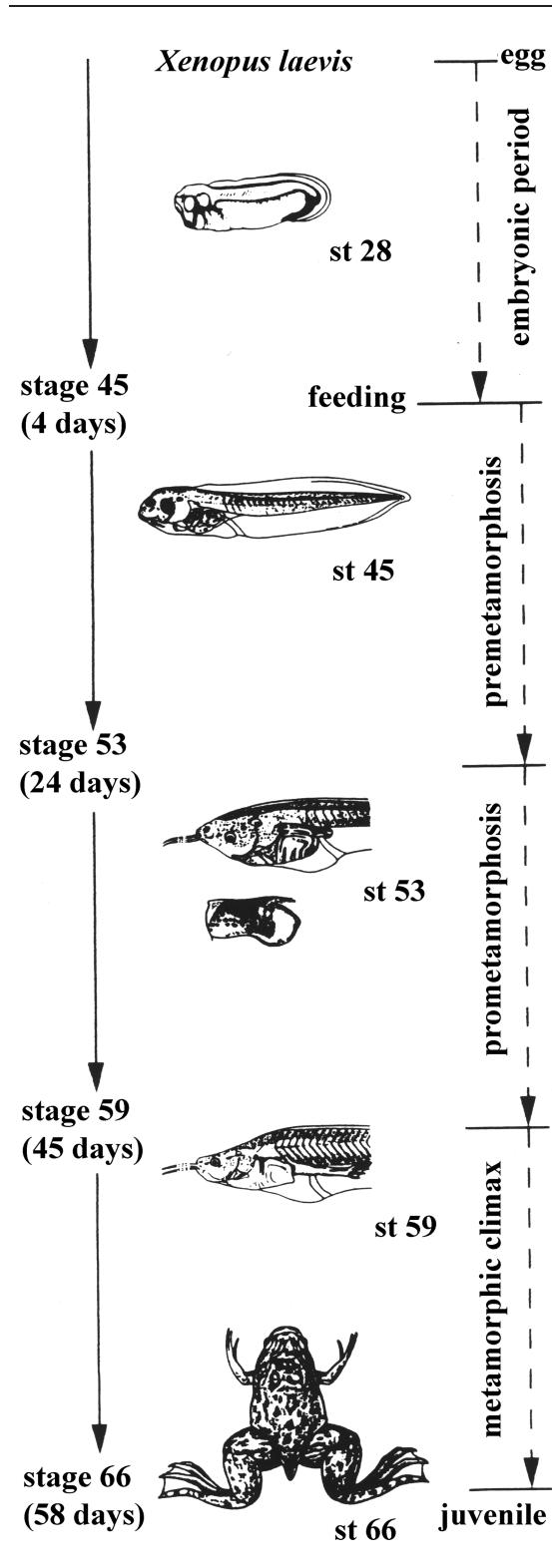
cells were primarily located in rostral positions, namely, in the prospective olfactory bulbs and subpallial regions. Shortly after, at the beginning of hatching (stages 36–38; 2 days and 2–5 hours), conspicuous CBir and CRir cells were scattered in the developing hypothalamus, with many of them being double-labeled along the early suprachiasmatic region (Fig. 2a,c). They soon extended more caudally into mammillary and retromammillary regions. Dorsally, in the lateral margin of the alar part of prosomere 2 (p2) the developing thalamus possessed numerous CRir cells and only a few CBir cells. Along prosomere 3 (p3) only fibers were observed and the lack of immunoreactive cells distinguished the prethalamus (alar p3) from the intensely labeled thalamus (Fig. 2b). Only a few CBir cells were seen at these stages in the basal part of p3 (Fig. 2c). In the mesencephalic tegmentum, CBir cells appeared progressively and, in the rhombencephalon, the reticular CBir cells were larger with long dendritic trees, whereas CRir and, to a lesser extent, CBir cells occupied alar regions in the rostral rhombencephalon, before the cerebellar anlage was observed (Fig. 2d).

The end of the early embryonic stages is marked when the mouth of the embryo breaks through (stage 40; 2 days and 18 hours) and the brain, at this stage, showed large numbers of CBir and CRir cells (Figs. 1a, 2e–h). In the telencephalon, intense CRir cells were abundant in the developing olfactory bulbs and, within the subpallium, in the lateral ganglionic eminence (LGE) extending caudally in the preoptic area (Fig. 2e). Only a few CBir cells were observed in the lateral margin of the olfactory bulbs and in the medial ganglionic eminence (MGE; Fig. 2e). The cells previously labeled in the hypothalamus (CBir and CRir) were not detectable at these stages and CBir cells were more abundant within tegmental regions through the diencephalon, mesencephalon, and rostral rhombencephalon, with the exception of the isthmic

**Figure 5.** Photomicrographs of double-labeled sagittal (a–i; dorsal is upwards and rostral to the left) and horizontal (j; rostral is to the left) sections through the brain of *Xenopus* at different developmental stages showing the distribution of CB or CR immunoreactive cells with nitric oxide synthase (NOS), tyrosine hydroxylase (TH), or choline acetyltransferase (ChAT). The markers shown in each panel are indicated in appropriate color code, and the developmental stage is indicated in the upper right corner. a–c: Relative localization of nitrorenergic (a) catecholaminergic (b) and cholinergic (c) populations compared to CR at stage 45. d: Colocalization of CR in the large nitrorenergic cells of the central amygdala (double-labeled cells in yellow). e: Distribution of CR in relation to the catecholaminergic cells in the preoptic and suprachiasmatic regions. f: Segmental distribution of the alar CRir cell groups in relation to the basal catecholaminergic cell groups, g: CRir and NOSir cells in the distinct dorsal and lateral bands in the mesencephalon and along the rhombencephalon. h: High magnification of double CR/NOS-labeled cells in the inferior reticular nucleus. i: CBir cells in the alar region of r0 and r1 in relation to the cholinergic nuclei. Arrowhead points to the fibers of the trochlear nucleus in the basal plate (not shown). j: Horizontal section showing the relative position and the segmental arrangement of CR groups in relation to the ChATir motor and nonmotor cholinergic groups in the rhombencephalon. k–m: Relative localization CBir cells in relation to the oculomotor, isthmic, rhombencephalic, and spinal cholinergic cell groups. Double-labeled cells always appear in yellow. n–p: CB and NOS-stained structures in a premetamorphic larva in the mesencephalon and rostral rhombencephalon (n) and in prometamorphic larvae in the caudal telencephalon (o) and mesencephalon and rostral rhombencephalon (p). Double-labeled CB/NOSir cells (in yellow) in the basal band of the mesencephalon and in the LDT at stage 50 were not observed at stage 60. For abbreviations, see list. Arrows point to double-labeled cells. A magenta-green version of this figure is provided as Supporting Information Figure 4. Scale bar = 50  $\mu$ m in a,b,d–f,h,k–p; 100  $\mu$ m in c,g,i,j.

TABLE 3.

Developmental stages of *Xenopus laevis* from egg to juvenile, summarized after Nieuwkoop and Faber (1967)



region (Figs. 1a, 2f,g). Within the thalamus, a rostral zone contained CBir and CRir cells (the prospective caudal part of the anterior nucleus), whereas only CRir cells

were found more caudally (Figs. 1a, 2f). From these stages the optic fibers were labeled for CR at the lateral margin of the diencephalon (arrowhead in Fig. 2f). Also remarkable were the progressively more abundant CRir cells located in the alar part of rhombomere 1 (r1) and, more caudally, in the reticular formation, where they often also contained CB (Fig. 2h).

#### Late embryonic stages (41–45)

The late embryonic stages, when the tadpoles began to swim constantly—not only in response to tactile stimuli as in previous stages—span for less than 2 days and end when the larval stages start with independent feeding. During these stages the brain grows considerably and its regionalization begins to be more evident, allowing the localization of labeled cells more accurately (Figs. 1b, 2i–t).

In the telencephalon, the number of CBir cells increased significantly (Fig. 1b) and were located from the olfactory bulbs through the subpallium in both the LGE and MGE, including the regions of the bed nucleus of the stria terminalis (BST) and the pallidum (Fig. 2j). In turn, CRir cells were particularly abundant in the main and accessory bulbs, which could be distinguished for the first time in these stages (Fig. 2i). Caudally, CRir cells were located mainly in the derivatives of the LGE (striatum and central amygdala) where a heavily labeled neuropil was observed in the lateral part (asterisk in Fig. 2j). In the medial pallium both CBir and CRir cells were noted at these stages, with CBir cells becoming the first to be detected (Table 4).

Different regions of the hypothalamus showed CBir and CRir cells whose precise localization, in many cases, was better evaluated by double labeling with NOS and TH (Figs. 2k–m, 5a,b). Thus, CRir cells were primarily found in the external division of the suprachiasmatic region, in close relation to the CRir fibers in the optic chiasm (Fig. 2k,l). A few CBir cells were also found close to the midline in the epichiasmatic nucleus that often were double-labeled for CR (Fig. 2l). A few CBir and CRir cells appeared also in the paraventricular nucleus (just caudal to the suprachiasmatic region). Beneath the optic chiasm in the tuberal hypothalamus, scattered CBir cells were detected, whereas more caudally in the mammillary and retromammillary regions cells containing both CB and CR were intermingled or doubly labeled (Fig. 2m).

At these stages, CRir cells were distinguished in alar (Figs. 2k,l, 5a) and basal domains within the diencephalon (Fig. 2m,n). In the dorsal part of p2, the habenula was now conspicuously immunoreactive and CRir cells tended to occupy the medial regions, while CBir cells and fibers were located more laterally, although there was some overlap and double-labeled cells were also observed (Fig.

2k). In addition, CBir fibers were seen in these stages in the fasciculus retroflexus. In the thalamus, the number of CRir and CBir cells increased and their distribution allowed the distinction of two portions: an anterior part with abundant CBir cells and a posterior part filled with CRir cells (Fig. 2k,l). Only a few cells were labeled in the alar region of p1 (pretectal region) that mainly contained CB (Figs. 1b, 2m,n). Additional CBir cells were located in the basal plate, from p3 to the mesencephalon, with a distribution that at rostral levels (retromammillary and posterior tubercle regions) was similar to that of the TH-containing cells (Fig. 5f).

By stage 45, the number of CRir cells in the dorsal mesencephalon increased significantly, mainly in the griseum tectale (rostrally) and in the torus semicircularis (caudally), whereas fewer cells were labeled in the tegmentum within the early anterodorsal and anteroventral tegmental nuclei (Fig. 2o). CBir cells in the mesencephalon prevailed in the tegmentum, first rostrally in the territory of the anterodorsal and anteroventral tegmental nuclei and then caudally in the posterodorsal and posteroventral tegmental nuclei. The pattern of distribution of the cell populations labeled for CB and CR, together with those observed at these stages labeled for NOS and ChAT, allowed the first indication of the organization of the mesencephalon into four longitudinal bands (dorsal, lateral, basal, and medial) (Fig. 2o). The boundary with the isthmic tegmentum was marked by the lack of CBir elements, as observed in the combination with ChAT that highlighted this region by the presence of the trochlear nucleus (Fig. 5c). In contrast, r1 showed large cell populations containing CB, CR, or both. Thus, in the alar domain, many large cells were double-labeled in the growing cerebellum (Fig. 2p) and smaller cells in this location spread more caudally (Fig. 2q), including the parabrachial area (Fig. 2p). Also at this level, the rostral extent of the octavolateral area showed single- and double-labeled cells (Fig. 2q) that continued caudally in the dorsal medullary and medial vestibular nuclei (Fig. 2r). CBir and double-labeled cells were also identified in the region of the laterodorsal tegmental nucleus (LDT, arrowhead in Fig. 2q), already well defined at these early stages by its ChAT immunoreactivity (Fig. 5c). Caudally in the alar plate of the rhombencephalon, double-labeled cells were observed in the nucleus of the solitary tract, whereas only CRir cells were detected close to the obex in the region of the dorsal column nucleus (Fig. 2s,t). In the rhombencephalic tegmentum, rostrally, abundant CBir cells were located in the interpeduncular nucleus (Fig. 2p) and small CBir and CRir cells occupied the central gray in r2, coincident with the rostral pole of the trigeminal motor nucleus, identified by the ChAT staining (Fig. 5c). Trigeminal motoneurons were labeled for CR at these stages more than

for CB, which labeled more abundantly the main sensory nucleus of the trigeminus and reticular cells (Fig. 2r). The latter cells were more scattered in the caudal rhombencephalon where isolated large CRir cells were located more dorsally only in these stages because they were no longer observed once the larval period started (Fig. 2s).

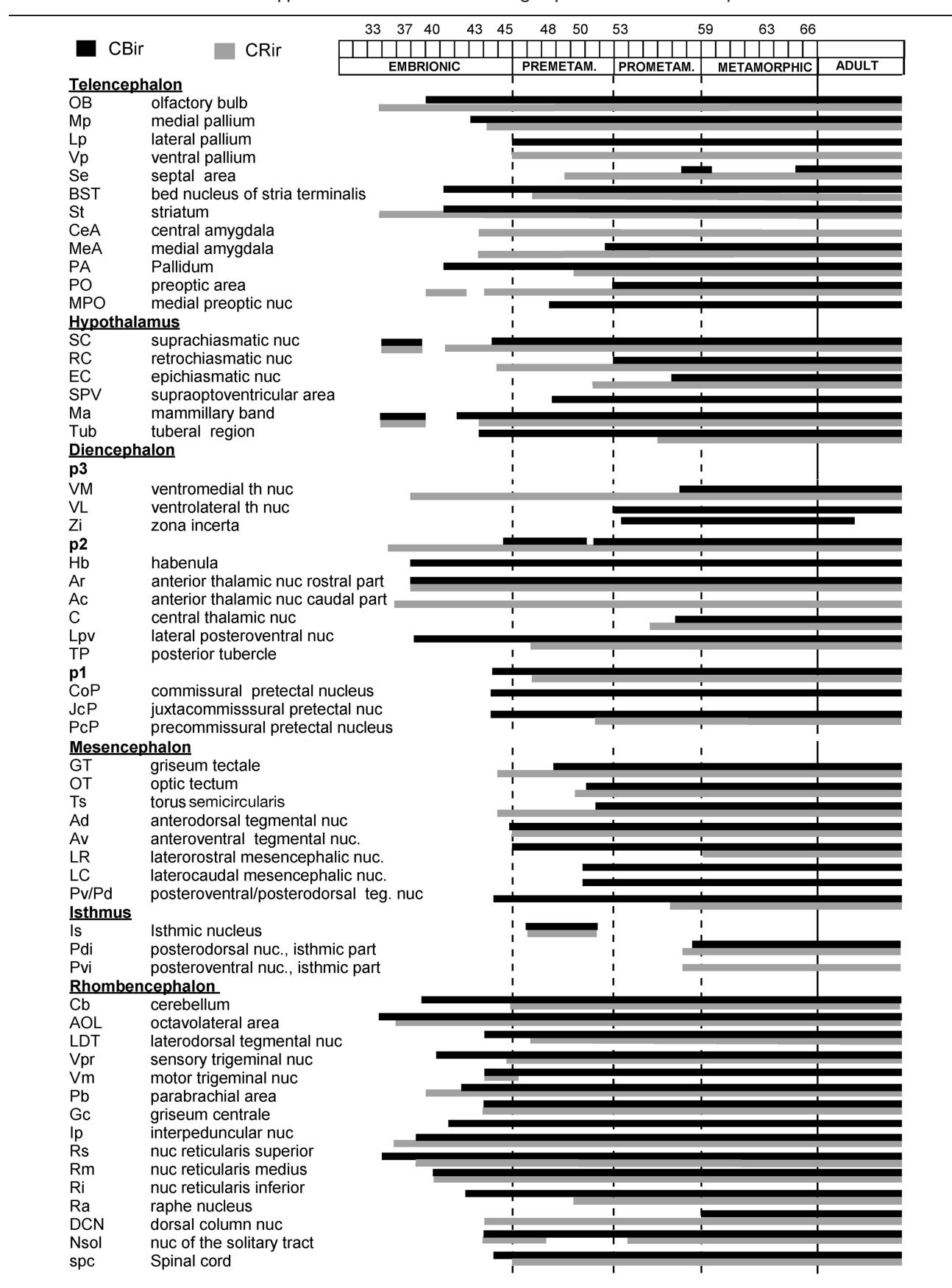
### Premetamorphic stages (46–53)

The premetamorphosis represents the start of the long larval period with active feeding. The free-swimming premetamorphic larvae accomplish a progressive growth for a period of ~20 days (stages 45–53). During this period, the hindlimb buds develop on the side of the body but digit formation is not initiated. At these stages, many of the CBir and CRir cell populations became gradually more segregated, the intensity of the immunoreaction increased, and new cell groups were distinctly identified. Also, the morphology of the labeled cells matured showing larger cell processes, and fibers tracts were clearly observed (Figs. 1c, 3, 5d–n).

In the telencephalon, labeled pallial cells were scarce. Most CRir cells were observed close to the palliosubpallial boundary (Figs. 1c, 3e). Medially these cells extended into the dorsal septum, whereas laterally they occupied the ventral pallial region (Fig. 3e). A few CBir cells were noted in the ventral lateral pallium and CRir cells in the dorsal pallium, both in caudal telencephalic levels. In the subpallium, CBir cells were more abundant in the dorsal and ventral lateral septum, and intermingled CBir and CRir cells were observed in the region of the BST (Fig. 3e). In the lateral part of the subpallium, the labeled structures allowed a better distinction of the striatal and amygdaloid components (Fig. 3e,f). CBir and CRir cells formed separated populations in the striatum, medially to the increasing neuropil labeled ventrolaterally (Fig. 3e). Caudally, the central and medial amygdala, the pallidum, and the BST could be distinguished on the basis of distinct patterns of labeling. The central amygdala, located just caudal to the striatum, was better identified by its NOSir cells, which in some cases colocalized CR (Fig. 5d). The medial amygdala contained CRir cells and was distinct from the pallidum and the BST that contained a higher number of CBir cells and were localized more ventromedially (Fig. 3f). In the caudal subpallium, the preoptic area was discernible by the scarcity of labeled cells, although the ventral medial preoptic nucleus showed a small population of weakly CBir cells at the beginning of the premetamorphosis that increased during this period and added a small number of CRir cells (Fig. 3a,k).

The alar subdivisions of the hypothalamus contained distinct populations of labeled cells that allowed their identification from these stages. The most dorsally located supraoptoparaventricular region (SPV) was

TABLE 4.

Timetable of appearance of CBir and CRir cell groups in the brain of *Xenopus laevis*

progressively highlighted by its content in CBir cells in contrast to the adjacent paraventricular and suprachiasmatic regions, where CRir cells prevailed (Fig. 3a,k), and the combination with TH particularly defined the epichiasmatic cell population (Fig. 5e). The boundary between the alar and basal hypothalamic regions could be assessed by the CRir fibers of the optic chiasm, and in the basal hypothalamus CBir cells were numerous in the tuberal regions, whereas both CBir and CRir cells codistributed in the caudal mammillary region (Fig. 3k).

In the diencephalon, the increasing population of CRir cell in the thalamus allowed the distinction between the anterior and central nuclei, and only the first showed CBir cells in its rostral subdivision (Figs. 1c, 3l). Intensely CRir fibers from the thalamic nuclei coursed in the lateral forebrain bundle and reached the striatal neuropil (Fig. 4l). Within the pretectal region, CBir cells were abundant and began to be segregated mainly into the juxtacommissural and precommissural nuclei, while the commissural and precommissural nuclei also contained CRir cells (Figs. 1c, 3b). The basal diencephalon contained CRir cell populations located rostrally in the retromammillary and posterior tubercle regions (Figs. 1c, 3b, 5f), whereas CBir cells were widely distributed along the diencephalic tegmentum (Figs. 1c, 3b,g,k).

The optic tectum grows enormously during premetamorphosis. In the organization of its layers, a mix of CBir and CRir cells was progressively discerned in the periventricular cell layers that were more abundant in the rostral griseum tectale (Fig. 3g,k), and a strongly CRir superficial fiber zone was formed from lateral to medial regions by the optic projection (Fig. 3c,g). In the torus semicircularis, as in earlier stages, CRir were abundant during premetamorphosis and CBir cells started to be observed laterally in this region by stage 50 (Fig. 3c). Also in the lateral mesencephalic band CBir cells were seen in the laterocaudal and laterorostral nuclei, which were also outlined by its cells labeled for NOS (Fig. 5g). Similarly, slightly more medially but in the basal band, CBir cells were numerous and some colocalized NOS (Fig. 5n). In contrast, CRir cells prevailed in the basal band (Fig. 3c) in which some motoneurons of the oculomotor nucleus were seen to contain CB (Fig. 5k). In the alar region of the isthmus (r0), a transient expression of CB and CR was observed during premetamorphosis, in cells located among the cholinergic cells, identified by the ChAT staining (Fig. 5i-k). These cells were no longer observed from stage 52 (see Table 4).

The developing cerebellum showed CRir cells, mainly in the lateral aspect of the cerebellar plate, whereas CBir cells were more abundant and extended medially. Caudally, both CBir and CRir cells extended in the dorsal alar plate of r1-3 (Figs. 3d, 5g,i). In particular the LDT, located in the alar part of r1 and identified by ChAT and NOS im-

munoreactivity (Fig. 5g,i,j,l,n), contained numerous CBir and CRir cells and double-labeled cells were observed from the beginning of the premetamorphosis (Fig. 3d,i). Also in the dorsal alar plate along the rhombencephalon, a segregation started in the distribution of labeled cells in the octavolateral area, which preferably contained CRir cells in the dorsal (lateral line) nuclei and CBir cells in the ventral (octaval) nuclei (Fig. 3i).

Along the brainstem and in the spinal cord, the presence of CB and/or CR in motor neurons was remarkably high during these stages (Figs. 3j, 5j-m). Also throughout the rhombencephalon, the reticular formation increased its complexity, as observed by the nuclear organization of CBir and CRir cells; better observed in combination with ChAT and NOS staining (Fig. 5g,j,n). Many cells were distributed along the superior median and inferior reticular nuclei and, in some cases, the colocalization with NOS and ChAT was already observed, as in the adult (Fig. 5g,h,j).

### Prometamorphic stages (54–59)

This larval period is marked by the development of the hindlimbs and ends just before the drastic metamorphic changes that transform the tadpole into a froglet. The prometamorphic stages (stages 54–59, ~21 days) were characterized by the progressive maturation of the cell groups, and the pattern of fiber labeling was more conspicuous than in previous stages. Thus, numerous fiber tracts and terminal-like neuropils were distinctly stained. During prometamorphosis the segregation of most cell groups is similar to the adult and the main landmarks and subregions are already established (Fig. 4).

In the telencephalon, the prevalence of CR immunoreactivity was evident and particularly noticeable in the olfactory bulbs, the striatal region, and the medial part of the medial pallium and septum (Figs. 1d, 4a-c). The CRir cells in the medial pallium were particularly abundant at caudal levels and sent their processes toward the dorsal pallium (Fig. 4b,i), forming a tract conserved up to the adult. Within the striatum, a segregated pattern of labeled fibers was already noted, in which the CBir fibers occupied more medial (internal) positions than those intensely CRir located in the ventrolateral neuropil (Fig. 4b) or, more caudally, in the lateral forebrain bundle (Figs. 4e,d, 5o). Also during prometamorphosis, in the region of the nucleus accumbens, which showed CRir cells from previous stages, a small number of CBir cells appeared. The group of CRir cells located near the palliosubpallial border (asterisk in Fig. 4b) became more apparent, and continued caudally in the medial amygdala (Fig. 4c,d), which contrasted with the central amygdala that mainly contained CBir cells. Medially in the caudal telencephalon, just rostral to the anterior commissure, the region of the

BST showed complementary patterns of CBir and CRir cells in which CBir cells were located more dorsally (Fig. 4c,d,i). The number of CBir cells in the BST increased notably along the prometamorphosis and its distinction from the amygdaloid regions was visualized by means of double labeling with NOS, particularly in the region of the central amygdala where no double-labeled cells were found, like in the adult (Fig. 5o).

In these stages, a marked pattern of bands served to delineate the preoptic and alar hypothalamic regions (Fig. 4j). Thus, scarce CBir and CRir cells were now observed in the preoptic area, whereas the amount of CBir cells in the SPV and of CRir in the suprachiasmatic region increased strikingly (Fig. 4j). In the latter region, CBir cells intermingled with CRir cells in the epichiasmatic nucleus. The number of CBir cells beneath the optic chiasm, in the retrochiasmatic and tuberal regions, progressively increased during prometamorphic stages and only a few CRir cells were detected in the tuberal hypothalamus and, in particular, in the mammillary region (Fig. 4i,j).

The three diencephalic prosomeres were clearly delineated by their CBir and CRir cell populations (Fig. 4e,f,i,k). Within the alar p3, no labeling was found in the prethalamic eminence (dorsal part of p3) and weakly CRir and CBir cells were located in the ventromedial and ventrolateral nuclei, respectively (Fig. 4e), and later in the prometamorphosis CBir cells were located ventrally in the nucleus of the zona incerta (Fig. 4f). In the basal portion of p3, the retromammillary region, both CBir and CRir cells were observed, mainly segregated into medial and lateral populations, respectively (Fig. 4g), and by the end of the prometamorphosis, the population of CBir cells was no longer observed. The alar p2 developed greatly during these stages and the pattern observed for the distribution of CR and CB was similar to that of the adult, with prominent CRir cell populations in the ventral habenula and in the thalamic anterior, central and lateral nuclei (Figs. 1d, 4f,i,k) and a population of CBir cells in the rostral division of the anterior nucleus (Fig. 4e,j). Numerous CBir cells were also distributed in the basal portion of p2, including the posterior tubercle (Fig. 4g,k). In the alar part of p1, the number of labeled cells was remarkably higher than in previous stages. CBir cells were distributed in the three rostrocaudal divisions of the pretectum (Fig. 4f,k) and CRir cells occupied just a rostral band within the commissural domain and a ventral subdivision of the precommissural domain (Fig. 4k).

Within the mesencephalon, the mixed population of CBir and CRir cells in the griseum tectale was progressively segregated into scarce CBir cells located medially and CRir cells placed more laterally (Fig. 4g,i,k). The most striking event observed in the midbrain at these stages was the lamination of the tectum and the massive arrival

of CRir fibers of the optic tract to the superficial laminae, with a lateromedial and rostrocaudal gradient (Fig. 4h,i). Among the mixed population of CBir and CRir cells, some large mesencephalic trigeminal cells were CBir in the rostral tectum (Figs. 1d, 4g'). The torus semicircularis significantly enlarged and began to make a protrusion into the mesencephalic ventricle. The complementary pattern of CB and CR expression observed in the adult could be distinguished in the prometamorphic larvae in which CBir cells and fibers were located in the caudal and lateral regions, while CRir elements occupied more rostral and dorsal regions (Figs. 4i, 5p). The groups of the lateral, basal, and medial bands of the mesencephalic tegmentum also reached a parcellation similar to the one observed in the adult (Figs. 4h,i, 5p) and by the end of the prometamorphosis, CRir cells were observed in the isthmic tegmentum, i.e., in the isthmic portion of the posterodorsal and posteroventral nuclei. Of note, during prometamorphosis (from stage 53) TH-positive cells were first observed in the medial band of the mesencephalic tegmentum and many of these cells contained CB.

In the rhombencephalon, the pattern of distribution of labeled cell groups and fibers showed the same features observed in the adult. However, the intensity of the staining was higher and the CBir and CRir cells were more numerous in many regions, showing overlapped distributions, with abundant double-labeled cells that were not observed in subsequent stages. Remarkably intense was the expression of CB in the cerebellum, and in the LDT and parabrachial region, whereas CRir cells were more abundant in the rostral octavolateral area (anterior nucleus) and parabrachial nucleus (Fig. 4i). A new feature observed from stage 57 in the rhombencephalon was the identification of CRir cells in the nucleus of the solitary tract.

## Metamorphic climax (59–66)

The metamorphic climax is characterized by the transformation of the tadpole into a juvenile, with the resorption of the tail and change in locomotor behavior, now depending mainly on the limbs. This period is fast (~10–12 days), and the adult-like pattern of staining was fully achieved at the end of the metamorphosis.

Only small differences were noted as compared to previous stages. In general, the intensity of the immunoreaction decreased through metamorphosis and some cells were identified more precisely in the now considerably larger brain. As a peculiarity, the CBir cell populations labeled transiently in the septum in previous stages were observed again from stage 64, and were maintained through metamorphosis. In particular, CRir cells were seen in regions of the isthmic tegmentum and CRir cells in the dorsal column nucleus. The rest of the pattern of

distribution was basically identical to the one described for the prometamorphic stages. However, as mentioned above, CB and CR expression in motor neurons was frequently observed in pre- and prometamorphic stages but at the end of the metamorphosis only a few cells were simultaneously labeled for ChAT and either CB or CR, and the number continued to decline with age, and only a small number of motor neurons contained CB or CR in animals beyond metamorphosis.

## DISCUSSION

The present study has demonstrated the usefulness of the analysis of the onset of CBir and CRir structures, in many cases in combination with other ancillary markers, to highlight the accurate extent of many regions in the developing *Xenopus* brain not observed on the basis of cytoarchitecture alone, and that were similarly identified in the adult brain (Morona and González, 2008, 2009). In previous studies, some of these regions were tentatively identified using immunohistochemistry to reveal diverse neurotransmitter systems throughout development (Roberts et al., 1987; González et al., 1994; López and González, 2002; López et al., 2002) and, more precisely, data on the regionalization of the developing brain have been obtained by the analysis of gene expression patterns (Bachy et al., 2001; González et al., 2002a,b; Moreno and González, 2007; Moreno et al., 2004, 2008a; Domínguez et al., 2010, 2011). Here we provide a comprehensive description of the onset and distribution of CBir and CRir elements in *Xenopus*. Both proteins are present in cell populations that, from early stages, can be easily framed within the proposed boundaries of the current segmental synthetic models (Díaz et al., 2001; Puelles and Rubenstein, 2003), which helps the identification and delineation of many brain regions and allows the observation of morphogenetic changes during development. In the following sections, the main features of the onset of CB and CR in the brain of *Xenopus* are discussed in relation to data available in other species, with respect to distinct brain areas from rostral to caudal levels.

### Telencephalon

#### *Olfactory bulbs*

A main feature observed in *Xenopus* was the early onset of CRir cells in the olfactory bulbs and the presence of a minor population of CBir cells. In line with these results, previous studies in teleosts described an early appearance of CR in the olfactory bulb that helped in the identification of granule (Porter et al., 1997) and mitral cells (Castro et al., 2008). In contrast, developmental studies in mammals revealed the onset of immunoreactiv-

ity for CB and CR only in late embryonic stages that increased until birth but decreased postnatally (Batista-Brito et al., 2008).

### *Pallium*

CB and CR proteins have been useful in amniotes to distinguish specific neuronal subpopulations and axonal networks in pallial regions (Dávila et al., 1997, 1999; Grateron et al., 2003; Suárez et al., 2006) and CRir was used for distinguishing pallial territories in the developing brain of the trout *Salmo trutta fario* (Castro et al., 2003) and the shark *Scyliorhinus canicula* (Rodríguez-Moldes, 2009). Within the pallium of anurans, the four divisions characterized in amniotes were also identified (Brox et al., 2003, 2004; Moreno et al., 2004) and, in the adult, showed distinct patterns of distribution for CB and CR (Morona and González, 2008).

The medial pallium, which is considered the counterpart of the hippocampus in mammals (Neary, 1990; Northcutt and Ronan, 1992; González and López, 2002; Westhoff and Roth, 2002), showed a noteworthy dynamic pattern of CRir in the developing *Xenopus* that resembles the transient CRir expression observed in similar regions of the developing rat hippocampus (Jiang and Swann, 1997).

Numerous studies reported a dynamic pattern of immunoreactivity for CB and CR in the mammalian neocortex. They consistently showed an early CR expression in bands in the Cajal–Retzius cells and in fibers of the marginal zone, and in neurons in the subplate (Fonseca et al., 1995). Subsequently, striking numbers of interneurons in different cortical layers express CB/CR in prenatal stages. This pattern of spatiotemporal distribution was related to the laminar formation and synaptic organization of the neocortex. Comparatively, the neocortex (or isocortex) is not present in anurans, although its forerunner might be located in the dorsal pallium (Aboitiz and Montiel, 2007). In addition, the anuran pallium possesses little neuronal migration outside the internal ventricular zone and laminar organization is not formed, what might be related to the scarce CBir/CRir cells observed during development in *Xenopus*.

The early onset of CB in the lateral pallium and CR in the ventral pallium and striatum of *Xenopus* is comparable to the patterns reported in mammals. More precisely, in the corticostriatal junction of the developing rat brain CB is expressed in the region that matches with the genetically defined ventral pallium (Puelles et al., 2000), whereas CR is found in the lateral subdivision of the lateral ganglionic eminence (Reblet et al., 2005).

A band of CRir and CBir fibers is formed from the medial to the dorsal and lateral pallial regions that in the adult was related with the thalamic projection from the anterior nucleus (Morona and González, 2008). In this

study we have seen that CRir fiber are detected earlier in young larvae, whereas CBir fibers were observed just at the metamorphic climax. This pattern coincides with the maturation observed in the thalamus and, in particular, in the anterior nucleus where the cells expressing CR precede those containing CB.

### **Subpallium**

CRir cells appear early in subpallial regions that later originate the striatal and pallidal areas and part of the amygdaloid complex. The dorsal septal subdivision possesses abundant CRir cells, close to the medial pallial CRir neurons early in development, and later in the ventral portions close to the striatum. The latter can be related with the observation that the dorsal part of the septum, just adjacent to the medial pallium, is of pallial origin in anurans (Bachy et al., 2001, 2002), whereas striatal-derived regions of the septum seem to arise from the subpallial CRir cell populations.

The distinction between striatal and pallidal regions in the anuran subpallium followed in the present study relies on hodological and immunohistochemical results obtained, among other anurans, in *Xenopus* (Marín et al., 1997a–c, 1998a,b). Of note, the precise localization of the pallidum has been alternatively proposed in the caudal striatum on the basis of connectivity (Endepols et al., 2004). However, studies during development and in the adult *Xenopus* have supported the localization of the pallidum in the region of Nkx2.1 and Lhx7 expression considered in this study (González et al., 2002a,b; Moreno et al., 2004, 2008a). The results in *Xenopus* suggest different spatiotemporal involvement of CR and CR in the development of the striatopallidal region. Thus, CRir cells were numerous in the LGE (prospective striatum) from very early (stages 37/38), whereas CBir cells were present at stage 40 in the MGE (prospective pallidal region) and later in the striatal areas at stage 47, which prefigure the pattern observed in the adult (Brox et al., 2002; Morona and González, 2008). The early presence of CBir cells in the MGE and not in the LGE, and their scattered distribution observed later in striatal regions, agree with the genetic data about the pallidal origin of some striatal neurons in anurans (Bachy et al., 2001; Moreno et al., 2004, 2008c), in line with the situation described in mammals (Marín et al., 2000; Anderson and Graybiel, 2001; Ulfhake, 2001). In our study most of the striatal CRir cells seem to arise from rostral telencephalic levels, in contrast to mammals, where immature striatal interneurons expressing CR derive almost exclusively from progenitors found in the caudal ganglionic eminence (CGE) (Xu et al., 2004; Butt et al., 2005; Flames et al., 2007). Noteworthy, in another anamniote, the brown trout (teleost), an early population of CB/CR-like immunoreactive cells was

reported in the dorsal and ventral telencephalic nucleus of the ventral telencephalon (Castro et al., 2003) that correspond to the striatal and pallidal areas of amniote vertebrates, respectively (Mueller et al., 2008).

### **Amygdaloid complex**

Each calcium-binding protein shows a distinct spatio-temporal expression pattern of development in the *Xenopus* amygdaloid complex, as has been also reported for the mammalian amygdaloid complex (Legaz et al., 2005; Dávila et al., 2008; Bupesh et al., 2011a,b).

In the present study we followed the terminology for the amygdaloid components proposed for *Xenopus* in our previous works, which considered the lateral, medial, and central amygdaloid subdivisions and the bed nucleus of the stria terminalis (see Moreno and González 2006, 2007b; Moreno et al., 2012). Similar regions were distinctly described in other studies, although different terminology was adopted and, for example, the lateral amygdala was referred to as the striatopallial transition area, and the bed nucleus of the stria terminalis (or part of it) as the central amygdala (for review, see Laberge et al., 2006).

The lateral amygdala of amphibians is located in the ventral pallium and contains CBir cells from early stages embryos (stage 41) whose number decreases in the prometamorphic period to the end of the metamorphosis, and then increases again. Some differences were found in the mouse, in which early CBir cells were also observed in the incipient pallial amygdala (embryonic day [E]13.5) followed by an increase in CBir in late embryonic stages, but this was a transient CB expression in migratory neuroblasts and developing neurons (Legaz et al., 2005). The increase in the CBir population seems to have a foreign origin, mainly in the anterior entopeduncular area / ganglionic eminences of the subpallium (Puelles and Rubenstein, 2003; Medina et al., 2004; Tole et al., 2005). Some subpallial contribution was reported to this area from cells originated in the MGE also in *Xenopus*, although it is not known if the cells possessed CB (Moreno et al., 2008b).

The medial amygdala in anurans is a complex structure, considered a main subpallial derivative that possesses important intermingled neuron populations with a different embryonic origin, expressing both pallial and subpallial markers (Brox et al., 2003, 2004; Moreno et al., 2004; Moreno and González, 2006, 2007a; Abellán and Medina, 2009; Bupesh et al., 2011a). In mice, from intermediate embryonic stages this structure shows numerous CRir fibers and a band of CRir or CBir cells in the superficial region of the medial amygdala. Based on these CBPs and other neurochemical markers, a pallial origin for the superficial band was suggested, and

subpallial contribution to the dorsal and ventral nuclei of the posterior medial amygdala (Legaz et al., 2005). In *Xenopus* we observed a gradual and rather late increase in CBir cells that precedes that observed in the lateral amygdala. Thus, based on the studies about the developmental origin of this region in amphibians (Moreno and González, 2006, 2007a) and the data in mammals (Marín and Rubenstein, 2002; Legaz et al., 2005), it is possible that the CBir cells in the MeA of *Xenopus* arise in the MGE. Differently, following the spatiotemporal development of the population of CRir cells that occupies the medial amygdala from early stages, they seem to originate in the LGE.

The central amygdala in anurans, as considered here, is a striatal component (Marín et al., 1998a; Moreno and González, 2005). This region has been alternatively compared to the pallidum of anurans (Endepols et al., 2004), although gene expression patterns do not support this comparison (Brox et al., 2003, 2004; Moreno et al., 2004). The central amygdala shows a pattern of CRir largely similar to the striatum. The NOS staining delineates this structure at late embryonic stages (López and González, 2002), and based just on CR the clear distinction of this structure from the LGE was reached at similar stages. The combination of NOS and CR immunohisto-fluorescence revealed double-labeled cells at stage 50, which were not found later in adults (Morona and González, 2008).

## Hypothalamus

The main hypothalamic subdivisions currently recognized in amniotes, attending to the segmental models (Morales-Delgado et al., 2011; Puelles et al., 2012a,b), were identified in the adult brain of amphibians on the basis of CB and CR immunohistochemistry (Milán and Puelles, 2000; Morona and González, 2008). In previous immunohistochemical studies during development of the brain in *Xenopus*, hypothalamic subdivisions were roughly described that were hardly recognized before stage 56 (López and González, 2002; López et al., 2002; Moreno et al., 2008a).

Many studies in mammals revealed that the hypothalamic areas contain distinct CBir and CRir cell groups and their analysis throughout ontogeny served to understand hypothalamic development (Lephart et al., 1997; Ikeda and Allen, 2003; Edelmann et al., 2007). The distinct spatiotemporal distribution of CBir and CRir cells in the hypothalamus along development of *Xenopus* allows the progressive identification of its main regions. Thus, the topologically dorsal (alar) hypothalamic regions, i.e., the SPV and the suprachiasmatic region, can be distinguished by their content in CBir and CRir cells, respectively, from premetamorphic stages. It should be noted

that an early transient immunoreactivity for CB and CR was found in large cells located in a region above the optic chiasm. Of note, in mammals a transient expression of CB in a cluster of cells in the suprachiasmatic nucleus was reported, and it was related to the arrival of the retino-hypothalamic tract (Ikeda and Allen, 2003; Antle et al., 2005). In *Xenopus*, the suprachiasmatic nucleus is innervated by retinal fibers (Tuihof et al., 1994) that are CRir (Morona et al., 2007b; Morona and González, 2008) and the presence of the double-labeled cells in this nucleus precedes the arrival of the retinal projection. Also, CBir cells in the suprachiasmatic nucleus are scarce at early stages, increase in premetamorphic stages, and then decrease in prometamorphic. Similarly, in mammals CBir cells decrease in the postnatal period when the retinohypothalamic input matures (Ikeda and Allen, 2003).

The basal (topologically ventral) regions of the hypothalamus include the tuberal region rostrally and the mammillary band caudally (Morales-Delgado et al., 2011). The progressive detection of CBir cells in the tuberal region highlights the development of this part of the hypothalamus. The region of the mammillary band, which includes the mammillary nucleus and the retromammillary region, is not clearly defined in early developmental stages (Moreno et al., 2004, 2008a; Domínguez et al., 2010). This region possesses an early transitory population of double CB/CR-labeled cells that extends in the basal plate of the diencephalon. At stage 45 these double-labeled cells remain in the rostral part of this mammillary band, whereas in the basal plate of p3 just CBir labeled cells are present. This pattern of distribution helps the identification of otherwise unclear boundaries (Domínguez et al., 2010).

## Diencephalon

The neuromeric subdivision of the adult diencephalon in anurans were established on the basis of CB and CR immunohistochemistry (Puelles et al., 1996; Milán and Puelles, 2000; Morona and González, 2008). Many studies also employed immunohistochemistry for both proteins to study the development of different diencephalic regions, especially the thalamus (Puelles et al., 1992; Frassoni et al., 1998; Abbot and Jacobowitz, 1999; González G. et al., 2002; Contreras-Rodríguez et al., 2003).

A remarkable feature of the alar region of p3 in the developing mouse diencephalon is a transient structure currently termed the prethalamic eminence, which is no longer distinguishable at P0 and expresses CR between E10 and E17 (Abbot and Jacobowitz, 1999; González G. et al., 2002; Puelles et al., 2012a,b). The thalamic eminence, however, is well developed in adult amphibians (Neary and Northcutt, 1983; Krug et al., 1993) but is

virtually devoid of CB or CR in the adult (Morona and González, 2008) as well as during development (present results). In other regions of the early developing prethalamus in *Xenopus*, a CRir cell population was observed located laterally, which then is progressively displaced medially and restricted to the rostral half of p3. At the same time a CBir population appeared preferentially in lateral positions and close to the zona limitans intrathalamic (Domínguez et al., 2010). This disposition closely resembles the situation described for mammals (Puelles and Rubenstein, 2003). These populations lately give rise to the CRir ventromedial/ventrolateral nucleus and the CBir zona incerta and part of the ventrolateral nucleus, as described in the adult (Milán and Puelles, 2000; Morona and González, 2008).

Within the thalamus, the temporal sequences of emergence for CB and CR are comparable in mammals and *Xenopus*. From early stages (37/38), CBir and CRir cells are located in the alar p2, although CR is more abundant than CB, similar to the situation found in the developing mouse (Enderlin et al., 1987; Puelles et al., 1992; Frassoni et al., 1998). In the mouse, the earliest CBir and CRir thalamic neurons (E13–E15) were both radially and non-radially oriented in the region of reticular thalamic migration, and most of them maintain their immunoreactivity throughout postnatal life (Puelles et al., 1992; Frassoni et al., 1998; Contreras-Rodríguez et al., 2003; Ortino et al., 2003). Their position and morphology led to the proposal that such cells are immature neurons en route to their final position, suggesting a possible role for both proteins in migration (Frassoni et al., 1998) and some aspects of neuronal differentiation (Jiang and Swann, 1997). In agreement with this assumption, CB and CR were expressed early in *Xenopus* in thalamic neurons located laterally and later these populations occupied more rostral and caudal regions, suggesting that they might migrate.

In mammals, CR has been described as a good thalamic marker during development from early stages (Frassoni et al., 1998; Abbot and Jacobowitz, 1999; González G. et al., 2002), in concordance with the present results in *Xenopus*. However, in the diencephalon of the shark *Scyliorhinus canicula* CR is found in thalamic cells only at late embryonic stages (Rodríguez-Moldes, 2009). More species of different vertebrate groups need to be analyzed before concluding a comparable pattern among tetrapods versus a different one in nontetrapods.

In the dorsal part of the alar region in p2, CBir and CRir cells intermingle in the habenula of *X. laevis* at early stages and transient double-labeled cells appear from stages 41 to 45, when CBir fibers also course in the fasciculus retroflexus. Also, transient expression of CR was described in the trout habenulo-interpeduncular system

during development (Porteros et al., 1998). Of note, marked variations in CR expression and habenular asymmetry were described in *Rana esculenta* (Guiglielmo et al., 2004), in contrast to the symmetrical expression present in *Xenopus*, both during development and in the adult (Morona and González, 2008).

The pretectal region has recently been reinterpreted in *X. laevis* based on combined patterns of gene expression (Morona et al., 2011a) and three anteroposterior histogenetic domains have been differentiated, with some dorsoventral subdivisions in each domain, in line with the pretectal genoarchitecture reported in chicken and mouse (Ferran et al., 2007, 2009). This tripartite model of the pretectal region is highlighted by the pattern of distribution of CBir and CRir cells in adult amphibians (Milán and Puelles, 2000; Morona and González, 2008), and during development the distinct distribution is reached only close to the metamorphic climax. Few data are available on the developmental expression of CB and CR in the pretectum in other vertebrates, but there seems to be a general situation in which the expression in different nuclei is reached only late in development (De Castro et al., 1998; Okoyama and Moriizumi, 2001). As proposed in the cat, the late development of the CBir pretectal cell groups may be related to the slow maturation of the pretectal motion processing system and the corticopretectal projections (Wahle and Reimann, 1997).

## Mesencephalon

CB and CR immunohistochemistry has been useful in delineating the amphibian midbrain topologic compartments, identified previously in reptiles (Díaz et al., 2000; Morona and González, 2008). They also proved to be good markers to follow the cytoarchitectonic changes and define different subdivisions of the avian torus semicircularis (Puelles et al., 1994), but developmental studies on CBPs in the midbrain are scarce, which precludes a detailed comparative analysis.

In *Xenopus* CRir is conspicuous at stages 43–45 in the dorsal band delineating the diencephalo-mesencephalic boundary (DMB) and the griseum tectale as it has been identified in reptiles and birds (Puelles et al., 1996; Díaz et al., 2000; García-Calero et al., 2002) and in a representative similar structure in advanced teleosts (Díaz-Regueira and Anadón, 2000; García-Crespo and Vecino, 2004). There are no data about CB or CR in its counterpart in mammals, the nucleus pretectalis posterior. An early onset in CBir cells has been observed in the anterior pretectal area (Enderlin et al., 1987) but its equivalence with the griseum tectale is not clear. CRir cells occupy also the toral region early, at the time of the appearance of the external organs of the lateral line and the differentiation of the ear and eye vesicles (Nieuwkoop and Faber,

1956). Later, the CRir cells progressively occupy the optic tectum and subdivisions of the anterodorsal and anteroventral tegmental nuclei, whereas CBir cells have a later onset.

### **Optic tectum**

CBir was used to follow developmental changes in the optic tectum of birds (Manns and Güntürkün., 2005) or its counterpart, the superior colliculus, in mammals (Dreher et al., 1996; Mize et al., 1996). CBir cells appear early in the rostralateral part of the superior colliculus and later spread widely except for the medial portion (Enderlin et al., 1987; Dreher et al., 1996). A maximal expression is reached when retinal ganglion cells establish the adult lamination and extrinsic afferents grow into the tectum (Mize et al., 1996). The pattern of morphology observed in the tectal CBir and CRir cells during development suggest that they are interneurons (Wu and Cline, 2003). A peculiarity observed in the tectum of *Xenopus* is the presence of CBir mesencephalic trigeminal cells, whose number increases until metamorphosis and then decreases, although they are scarcely persistent in the adult (Morona and González, 2009).

In zebrafish the early appearance of CR in retinorecipient tectal layers was described, with an almost identical pattern to that found during the embryonic period of *Xenopus* (García-Crespo and Vecino, 2004). It was used as a marker of the development of the retinal ganglion cell projection, being related to neurite elongation in development and axon regeneration (García-Crespo and Vecino, 2004).

### **Torus semicircularis**

The toral region of *Xenopus* constitutes the caudal part of the dorsal mesencephalic band (Díaz et al., 2000; Morona and González, 2009) and is a center of multisensory interaction (Zittlau et al., 1985). It receives general auditory input arising directly from the dorsal medullary nucleus to the laminar nucleus, whereas the principal and magnocellular nuclei receive lateral line input from the medulla (Lowe, 1986; Edwards and Kelley, 2001). During development, in this region a band of CRir cells is first detected and predominate until late premetamorphic stages. Subsequently, a mixed population of CBir and CRir cells appears in late premetamorphic stages that are progressively limited to peripheral regions, with a prevalence of CRir in dorsal parts and CBir in ventral parts. Notably, CR predominates at the time of the highest functional activity of the lateral line system in the tadpoles, coinciding with the higher levels of CR in the lateral line nucleus of the medulla. Accordingly, CR seems to be mainly related to the lateral line system input from early stages, whereas CB appears as the auditory input devel-

ops, and this relationship is maintained to the adult (Morona and González, 2009). CB and CR were described in cells of the inferior colliculus (homologous to the torus semicircularis) of several mammalian species during development (Friauf, 1994; Lohmann and Friauf, 1996; Zettel et al., 1997; Henkel et al., 2005). Both CB and CR showed a transient expression in this region with a peak of immunoreactivity in the perinatal period (Friauf, 1994; Lohmann and Friauf, 1996) with a subsequent decrease that is more evident in the central parts (Henkel et al., 2005). In all studies the adult-like pattern is achieved postnatally or after hatching, coinciding with the maturing of the circuitry once hearing begins.

### **Tegmental region**

The longitudinal subdivisions of the mesencephalon were evident from stage 45 in *Xenopus*. The basal tegmentum includes the lateral, basal, and the medial bands, and houses some nuclei related to motor behavior. In the lateral band, the combination of CB and NOS immunoreactivity allowed distinguishing the laterocephal and laterorostral nuclei (Morona and González, 2009). These cells show a distinct pattern of development and, while NOSir cells appear first located in the anterodorsal and anteroventral tegmental nuclei, CBir cells appear gradually in the lateral band. Progressively, NOSir cells occupy more dorsal positions, extending caudally until their final distribution in the lateral band. Double-labeled cells were first observed at stage 50 in the lateral band, after the final settlement of the nitrergic population.

In the basal band of *Xenopus*, CBir cells are present from embryonic stages and numerous CB/CR double-labeled cells were found at stages 56–58. This mesencephalic band houses the red nucleus that possesses CBir and CRir cells that project contralaterally to the spinal cord (Morona et al., 2005, 2006b). In the rat, CBir was not conspicuous in the basal band until day E21 and numerous double-labeled cells were found in the prenatal and perinatal period in the red nucleus (Enderlin, 1987). Also, in humans, outstanding CBir was demonstrated in cells of the magnocellular part of the red nucleus in the fetal and perinatal brain, but these cells were only weakly reactive in the adult (Ulfig, 2001, 2002). Also in the basal band, a significant population of oculomotor neurons contains CB during the larval period, but the number of double-labeled cells for ChAT and CB in this nucleus decreases markedly after metamorphosis and only a small number persists in the adult (Morona and González, 2009).

The floor plate and its derivatives have attracted much more attention and, in particular, the development of the CBir and CRir cells in the dopaminergic substantia nigra and the ventral tegmental area (SN and VTA) has been

analyzed in mammals (Alfahel-Kakunda and Silverman, 1997; Verney et al., 2001a,b). In the rat, CR appeared in the rostral half of the SN at E18 and distributed thereafter throughout both the VTA and SN, whereas CB developed later, especially in the caudal SN (Alfahel-Kakunda and Silverman, 1997). Only a minority of the CBir cells represented dopaminergic neurons and their appearance was related to the later developing (matrix) CBir compartment of the striatum (Liu and Graybiel, 1992). Comparatively in *Xenopus*, double-labeled cells for TH and CB are present in the mesencephalic tegmentum only after prometamorphosis, in a group of cells that was demonstrated to project to the striatum (Marín et al., 2007c,d). The terminal field of this projection forms a band close to the periventricular cell layer that matches the immunoreactive fiber labeling observed for TH and CB immunoreactivity (Morona and González, 2008).

### Rhombencephalon

During development, the first CBir and CRir neurons appear at early embryonic stages in the rhombencephalon and diencephalon, as has been also reported for the chicken (Guglielmone and Corvetti, 2000) and rat (Enderlin, 1987). Rhombencephalic cells appeared in the reticular formation close to the otic vesicle, where first differentiating neurons were described in *Xenopus* (Hartenstein, 1993). In chick, CRir neuroblasts appear to populate sites of the brainstem according to a spatiotemporal pattern of distribution similar to reticular THir and serotonergic neurons. However, in *Xenopus* CBir and CRir reticular neurons appear later than the serotonergic neurons and earlier than the THir cells (González et al., 1994; present results), coinciding with the serotoninergic innervation of the spinal cord and the appearance of collateral branches in the brainstem (van Mier et al., 1986). This timing overlaps with the beginning of coordinated movements and the switch on of the sensory pathways (Roberts et al., 1983, 1986). Thus, the location of CBir and CRir cells reflects that of the first neurons that form circuitries for the incipient motor patterns described for all anamniote larvae (van Mier and Donkelaar, 1984; van Mier et al., 1985). The results suggest a functional role of the CBPs in the early motor circuits.

The cerebellar anlage at the marginal alar plate of r1 early expresses CB and CR that later was identified in Purkinje neurons from premetamorphic stages (Uray et al., 1998; Uray and Gona, 1999; Morona and González, 2009). Purkinje somata were simultaneously labeled for CB and CR throughout premetamorphic stages and during the first and second wave of generation of these cells in the prometamorphic period, as they move to their final position and extend their neurites. However, in *Xenopus* CR is only transiently expressed in Purkinje cells, whereas

CB expression remains and is a reliable marker of all Purkinje cells in the adult (Morona and González, 2009). Many studies revealed an early CB expression in Purkinje and other cerebellar cells in diverse vertebrates (Enderlin et al., 1987; Hamon et al., 1991; Yew et al., 1997; Milosevic and Zecevic 1998) with some fluctuations in the levels of immunoreactivity (Enderlin et al., 1987; Yew et al., 1997; Kwong et al., 2000). In several mammals and in the chicken, the levels of CB in Purkinje cells increase early after birth (as in the premetamorphic to metamorphic stages in *Xenopus*) and then decrease (Enderlin et al., 1987; Iacopino et al., 1990; Bastianelli and Pochet, 1993). Consequently, CB was related to cellular maturation in the cerebellum (Iacopino et al., 1990; Milosevic and Zecevic, 1998) and with important roles in physiology and synaptic plasticity, with a special relevance in the dendritic spine morphology of Purkinje cells (Schiffman et al., 1999; Swaller et al., 2002). In contrast, in *Xenopus* the intensity of the CB immunoreaction increases in pre-metamorphic to metamorphic stages but strong reactivity is maintained in the adult.

Interestingly, CR was found in Purkinje cells of adult specimens of the genus *Rana* (Uray and Gona, 1999; Necchi et al., 1999; Morona and González, 2009), but it remains only in some fibers in the cerebellar molecular layer in *Xenopus*, as reported in rodents (Enderlin et al., 1987). A transient expression of CR in Purkinje cells was also observed in the chick cerebellum (Bastianelli and Pochet, 1993; Bastianelli, 2003). Among mammals, in primates some subpopulations of Purkinje cells are CRir (Fortin et al., 1998), whereas in humans CR appears only in the cerebellar cortex (Kwong et al., 2000). In contrast, in juvenile and adult sharks, CR appears in the molecular layer in stellate cells (Rodríguez-Moldes et al., 2008) as in adult teleosts (Díaz-Regueira and Anadón, 2000; Meek et al., 2008).

From early developmental stages, many CBir and CRir neurons distribute in the octavolateral column of the rhombencephalic alar plate. Mainly segregated populations were observed in which CR is expressed in the dorsally located cells related to the lateral line and CB is mainly located in ventrally located cells in the octaval nuclei. This segregation is achieved during the larval period and maintained through the adult (Morona and González, 2009). Noticeably, CR reaches a peak of intensity during the maximal development of the lateral line system in the tadpole (Russell, 1976). In other frogs with a predominantly terrestrial life as adults, the lateral line system is only present at larval stages, disappearing at metamorphosis. In these frogs CR is found in the dorsal (acoustic) branch of the eighth nerve and in their terminal fields in what we have considered the dorsal nucleus that would include the sacular nucleus (Necchi et al., 1999;

Morona and González, 2009). In birds the available data for CR (Parks et al., 1997; Kubke et al., 1999; Guiglielmino and Corvetti, 2000) showed that the avian auditory nuclei present CRir cells early in development but the cell number decreases until a week after hatching (Kubke et al., 1999). The presence of both CB and CR in octaval brainstem nuclei at very early stages in development has been reported in mammals (Lohmann and Friauf, 1996; Henkel and Brunso-Bechtold, 1998) and, in particular, their spatiotemporal appearance in vestibular afferents and nuclei led to suggest the involvement of these proteins in successive phases of synaptic remodeling of the vestibular input and their cerebellar control (Puyal et al., 2002).

Many large cells located in the motor and reticular nuclei of the rhombencephalon of *Xenopus* also exhibit a prominent CBir and only a few of these cells also contain CR. The numbers of CBir and CRir motoneurons are progressively higher along the larval life, being most abundant by the beginning of the metamorphic climax, at the time of maximal development of the tail (Will, 1986), with a steady decrease until adulthood. The segmental analysis and the double labeling for ChAT corroborate that CB is present in trigeminal, facial, and vagal motoneurons, whereas CR is only found close to the facial nucleus, likely in the accessory abducens nucleus (Marín et al., 1997e), like in the adult and in line with results in teleosts (Díaz-Regueira and Anadón, 2000; Castro et al., 2006).

## CONCLUSION

The present results showed that CB and CR are expressed in the brain of *Xenopus laevis* from early stages of embryogenesis, in temporally and spatially distinct patterns that implicate them in developmental processes. The expression of both proteins also showed to be dynamic during late stages of development that is likely related to the fine regulation of some subsets of neurons at these stages to reach the definite location and function in adults. Comparing our results with the localization found in adults (Morona and González, 2008, 2009) it is evident that, in general, the labeled structures in the developing brain retain their ability to express these proteins in the adult brain. However, transient expression has also been observed that in some cases suggested a relationship with the migration of the immature labeled cells, as inferred by the analysis of the spatiotemporal sequence of immunoreactivity.

Neurons containing CBPs have been primarily associated with the  $\gamma$ -aminobutyric acid (GABA)ergic phenotype (Gabriel et al., 1998; Beham et al., 2002), but some studies revealed colocalization in a wide range of neurochemical types (Geula et al., 1993; Isaacs and Jacobowitz,

1994) and it is therefore difficult to phenotypically characterize the cell groups containing CB and/or CR. Thus, in the present study and in previous works in amphibians (Morona and González, 2008, 2009) the presence of CB and CR in cholinergic, catecholaminergic, and nitrergic cell subpopulations supported the notion that these CBPs are not related to any neurochemical type.

The comparison of our results with previous data on the development of CBir and CRir brain structures has shown extensive variability across vertebrate classes. The consistent paradigm considered for comparing brain regions based on transversal and longitudinal brain subdivisions has served to clarify the accurate position of many labeled cell groups, in many cases with the help of combined labeling for other regional markers. The identification of distinct cell populations by means of immunohistochemistry is particularly useful in amphibians, primarily during development.

In spite of their variability, the immunocytochemically identified neuron populations compared across species have been demonstrated to be a useful auxiliary tool that gives important clues as to the homology of brain nuclei (Nieuwenhuys, 1998). Thus, in the present study the immunohistochemical detection of CB and CR has served to discern cell populations during development and helped to demonstrate neuronal heterogeneity. Of note, the straightforward comparison based only on the presence of CBPs should not be made, due to the great variability observed in well-established homologous regions in the brain of different vertebrates (see above). However, in cases of unclear topological relations, this immunohistochemical approach has been valuable to confirm homology, in particular when the demonstration of CBPs is combined with the presence of other neuronal markers (Morona and González, 2008, 2009). Moreover, the pattern of CB and CR expression during development and its comparison with that observed in the adult has provided new clues for the precise allocation and characterization of many neuronal populations.

Finally, it should be pointed out that although many neuronal systems in diverse species express these proteins during development, the exact function has not been elucidated. In the case of *Xenopus* during development, the present results support diverse roles for these proteins. For example, they are possibly involved in migration and differentiation of thalamic neurons, in maturation of cerebellar cells, and successive phases of synaptic remodeling of the octavolateral area. In addition, given that they are localized in neurons before synaptogenesis, functions not related to neurotransmission are suggested. Furthermore, CBPs may subserve  $\text{Ca}^{2+}$ -independent functions, as has been described for CR (Gander et al., 1996). Therefore, we sustain the usefulness of the

study of the differential distribution of CBPs in neuronal population as a tool in neuroanatomy, although we are far from understanding the functional significance of a particular CBP in specific groups of developing neurons.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ROLE OF AUTHORS

Both authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis, and contributed equally to the elaboration of the study and to the research that led to preparation of the article.

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