Pergamon

www.neuroscience-ibro.com

PII: S0306-4522(02)00326-3

Neuroscience Vol. 114, No. 3, pp. 567–575, 2002

0306-4522/02 $22.00+0.00

REGIONAL EXPRESSION OF THE HOMEOBOX GENE NKX2-1 DEFINES PALLIDAL AND INTERNEURONAL POPULATIONS IN THE BASAL GANGLIA OF AMPHIBIANS

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Abstract—The distribution of gene expression domains during development constitutes a novel tool for the identification of distinct brain regions. This is particularly useful in the brain of amphibians where cell migration is very limited and most neurons organize in a periventricular layer. Here we report the expression pattern of NKX2-1 protein in the developing *Xenopus* telencephalon. In mammals, the *Nkx2-1* gene is expressed in distinct subpallial regions such as the septum, the medial ganglionic eminence and preoptic region. The results of the present study demonstrate that the expression of NKX2-1 delineates the pallidal anlage and its derivatives in amphibians, as in mammals and birds. In addition, double-labeling immunohistochemistry and the combination of tracing experiments with NKX2-1 immunohistochemistry demonstrate that the amphibian striatum contains interneurons, which express NKX2-1 and produce, among other possible neurotransmitters, nitric oxide and acetylcholine. In sum, the results of the present study strengthen the notion that similar developmental programs exist during basal ganglia development in all tetrapods. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: NKX2-1, basal ganglia, interneuron, medial ganglionic eminence, lateral ganglionic eminence, striatum, telencephalon.

The identification of distinct nuclei within the telencephalon of amphibians is arduous since, even in the adult, cell migration is very limited and most cells concentrate in a periventricular position (Northcutt and Kicliter, 1980). Despite this caveat, experimental distribution studies of neurochemical markers and connectivity of chemically identified regions have recently led to propose a subdivision of the amphibian basal telencephalon (Marín et al., 1997a,b,c,d, 1998a). With those approaches, structures such as the dorsal and ventral pallidum, the bed nucleus of the stria terminalis and distinct amygdaloid divisions were tentatively delineated in the amphibian telencephalon (Marín et al., 1998b,c).

The relative distribution of gene expression domains during development constitutes a novel tool for the delineation of distinct regions in the brain of vertebrates (Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Smith-Fernandez et al., 1998; Puelles et al., 2000). Through this approach, for example, different progenitor zones and mantle domains have recently been identified in the developing avian and mammalian telencephalon (Smith-Fernandez et al., 1998; Puelles et al., 2000). Since the patterns of expression of such developmental genes are largely conserved (Smith-Fernandez et al., 1998; Puelles et al., 2000; see Williams and Holland, 1998 for discussion), they may be used with due precautions as an independent method to test the proposed hypothesis on the homology of different telencephalic subdivision in vertebrates.

Nkx2-1 is a member of the vertebrate *Nkx* family of homeobox genes (Price, 1993; Pera and Kessel, 1998; Qiu et al., 1997). During mouse and chicken brain ontogeny, *Nkx2-1* is expressed in progenitor cells of the subpallial telencephalon and hypothalamus (Pera and Kessel, 1998; Sussel et al., 1999), and its function is required for the proper development of these regions (Kimura et al., 1996; Pera and Kessel, 1998; Sussel et al., 1999; Marín et al., 2000). In the subpallium, *Nkx2-1* expression is found in the septum, medial ganglionic eminence (MGE) and preoptic region (Price, 1993; Sussel et al., 1999; Puelles et al., 2000). Interestingly, *Nkx2-1* expression is not restricted to progenitor cells, but it is also present in postmitotic neurons. For example, *Nkx2-1* is found in derivatives from the MGE, such as the dorsal and ventral pallidum (Sussel et al., 1999). In addition, *Nkx2-1* is also expressed in most striatal interneurons, which derive from the MGE and migrate tan-

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Abbreviations: BSA, bovine serum albumin; ChAT, choline acetyltransferase; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NOS, nitric oxide synthase; PB, phosphate buffer; PBS, phosphate-buffered saline; TRDA, Texas Red-conjugated dextran amine; TX, Triton X-100.

gentially to the developing striatum (Marín et al., 2000). Both cholinergic and GABAergic subpopulations of NKX2-1-expressing interneurons have been reported in the mammalian striatum (Marín et al., 2000).

Recently, it has been shown that *Xenopus* NKX2-1 protein is highly similar to other vertebrate NKX2-1 proteins, sharing 73% or 85% overall amino acid identity with mouse and chicken NKX2-1, respectively (Small et al., 2000). Moreover, by means of whole-mount *in situ* hybridization, it has been shown that *Xenopus Nkx2-1* is expressed in the developing ventral forebrain, as in birds and mammals (Hollemann and Pieler, 2000; Small et al., 2000). Nonetheless, the precise neuroanatomical distribution of NKX2-1 in the developing *Xenopus* forebrain has not been analyzed yet.

In the present study, we have analyzed the expression pattern of NKX2-1 protein in the developing *Xenopus* telencephalon by means of immunohistochemical methods. With this approach, we aimed to assess whether the subdivisions of the amphibian ventral telencephalon previously proposed on the basis of hodological and neurochemical criteria endured also developmental criteria, and in particular, if NKX2-1 expression in the basal telencephalon was readily comparable among tetrapods. The results of the present study demonstrate that expression of NKX2-1 delineates the pallidal anlage and its derivatives in amphibians, as in birds and mammals. In addition, expression of NKX2-1 in striatal neurons, along with tract-tracing experiments, provides the first evidence that the amphibian striatum contains interneurons and suggests that local circuit neurons follow similar developmental programs in all tetrapods.

EXPERIMENTAL PROCEDURES

For the present study, a total of 54 *Xenopus laevis* embryos and larvae, ranging from developmental stage 45 to the juvenile stage 65 (Nieuwkoop and Faber, 1967), were used (Table 1). Animals were obtained by Pregnyl-induced (Organon) breeding and maintained in tap water at 20°C throughout their development. At appropriate times, embryos and tadpoles were deeply anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz, Basel, Switzerland) in distilled water (pH 7.4) and then processed for immunohistochemistry. In the case of six juveniles, retrograde tracing experiments with Texas Red-conjugated dextran amines (TRDA) were combined with immunohistochemistry. The original research reported herein was performed under animal care guidelines established by the Spanish Royal Decree 223/1988.

Under anesthesia, the animals were perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and kept in the same fixative for 2–3 h. Animals that were too small to be perfused were, after anesthesia, fixed by immersion. After fixation, the brains were immersed in a solution of 30% sucrose in PB for 3–5 h at 4°C until they sank, embedded in a solution of 15% gelatin with 30% sucrose in PB, and then stored for 5 h in a 4% formaldehyde solution at 4°C. The gelatin blocks were cut on a freezing microtome at 40 μm in the frontal plane and collected in PB. The sections were subsequently processed immunohistochemically.

Immunohistochemistry single labeling

Free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (TX) in phosphate-buffered saline (PBS) for 1 h at room temperature, and subsequently incubated with the primary antisera for 48 h at 4°C in 2% normal serum and 0.3% TX in PB. The following antibodies were used: rabbit anti-NKX2-1 (Biopat Immunotechnologies, Caserta, Italy), diluted 1:500; goat anti-choline acetyltransferase (ChAT) (Chemicon, Hofheim, Germany), diluted 1:100; sheep anti-neuronal nitric oxide synthase (NOS) (K205 antibody, kindly donated by Dr. P.C. Emson), diluted 1:20 000. Sections were then incubated in biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA), diluted 1:100, and processed by the ABC histochemical method (Vector). The sections were then mounted (mounting medium: 0.25% gelatin) and, after drying overnight, coverslipped. Some sections were counterstained with Cresyl Violet to facilitate the analysis of the results. In each experiment, primary antiserum omission controls and normal serum controls were used to further confirm the specificity of the immunohistochemical labeling.

The NKX2-1 antibody used in this study, raised against rat NKX2-1, has been previously shown to present cross-reactivity with NKX2-1 proteins in amphibians, birds and mammals (Lazzaro et al., 1991; Ruiz i Altaba, 1998; Marín et al., 2000; L. Puelles, personal communication). The specificity of the antibody was further proven by the exclusive staining of the ventral forebrain areas that showed *Nkx2-1* mRNA expression in early *Xenopus* embryos (Hollemann and Pieler, 2000; Small et al., 2000). In addition, the antibody labeled developing thyroid and lung (data not shown), as shown in mammals (Lazzaro et al., 1991).

Immunohistochemistry double labeling

Free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% BSA and 0.4% TX in PBS for 1 h at room temperature, and subsequently incubated in a cocktail of primary antisera for 48 h at 4°C. The cocktail always includes a rabbit anti-NKX2-1 antiserum, diluted 1:500 in 2% normal serum and 0.4% TX in PB, and one of two antisera: goat anti-ChAT (diluted 1:100) or sheep anti-NOS (diluted 1:20 000). The sections were then rinsed in PB and incubated for 2 h in a cocktail of secondary antibodies diluted 1:100 in the same solution as the primary antisera. FITC-conjugated chicken anti-rabbit (Chemicon) and Rhodamine-conjugated donkey anti-sheep (Chemicon) were used for NKX2-1 and NOS double labeling, whereas FITC-conjugated chicken anti-rabbit and Rhodamine-conjugated donkey

Abbreviations used in the figures

ac	anterior commissure	MeA	medial amygdala
BST	bed nucleus of the stria terminalis	MGE	medial ganglionic eminence
CeA	central amygdala	Mp	medial pallium
DB	diagonal band of Broca	Ms	medial septum
DP	dorsal pallidum	Poa	preoptic area
Dp	dorsal pallium	Str	striatum
lfb	lateral forebrain bundle	v	ventricle
Lp	lateral pallium	VP	ventral pallidum
Ls	lateral septum	vz	ventricular zone

anti-goat (Chemicon) were used for NKX2-1 and ChAT double labeling. After rinsing three times in PB, the sections were mounted on glass slides and coverslipped with Vectashield (Vector).

Retrograde tracing combined with immunohistochemistry

In a series of experiments with six juveniles, the tracer 10-kDa TRDA (Molecular Probes, OR, USA), recrystallized from distilled water onto sharp tungsten needles, was applied unilaterally into the ventrolateral diencephalon, interrupting the lateral forebrain bundle. All surgical experiments were carried out following a ventral approach through the roof of the mouth and under anesthesia with a 0.3% solution of MS222. Survival times varied from 7 to 14 days. Following this period, the animals were deeply anesthetized and perfused transcardially with 50 ml saline followed by 100 ml fixative (4% paraformaldehyde in PB). The brains were removed, blocked in gelatin and cut in the frontal or sagittal plane at 40 µm thickness on a freezing microtome as described above. Subsequently, brain sections were processed for NOS, ChAT or NKX2-1 immunohistochemistry according to the indirect immunofluorescence method. In brief, they were first incubated for 48 h at 4°C with sheep anti-NOS (diluted 1:20 000), goat anti-ChAT (diluted 1:100) or rabbit anti-NKX2-1 (diluted 1:500), as described above. They were then incubated in the corresponding secondary antisera, FITC-conjugated rabbit anti-sheep (Vector), FITC-conjugated rabbit anti-goat (Vector) or FITC-conjugated chicken anti-rabbit (Chemicon). All secondary antisera were diluted 1:100 and the incubation was made at room temperature for 90 min. After rinsing in PB, the sections were then mounted on glass slides and coverslipped with Vectashield (Vector).

RESULTS

NKX2-1 immunoreactivity in the basal telencephalon

Nkx2-1 expression is first detectable in the *Xenopus* forebrain around stage 20 (Hollemann and Pieler, 2000; Small et al., 2000). At the end of the embryonic stages, NKX2-1-immunoreactive (NKX2-1+) cells were primarily confined to the ventromedial aspect of the developing telencephalic hemisphere (Fig. 1
). We have designated this region as the MGE, since it forms a noticeable protrusion into the lateral ventricle and occupies the same position as the MGE of birds and mammals (Smart, 1976; Puelles et al., 2000). NKX2-1+ cells were also found in the septal anlage at rostral telencephalic levels (data not shown), but were not present in more lateral regions of the basal telencephalon or in the pallial primordia (Fig. 1). At these stages, NKX2-1+ cells were primarily restricted to the ventricular zone, although NKX2-1 immunoreactivity was also found in some superficially migrated cells

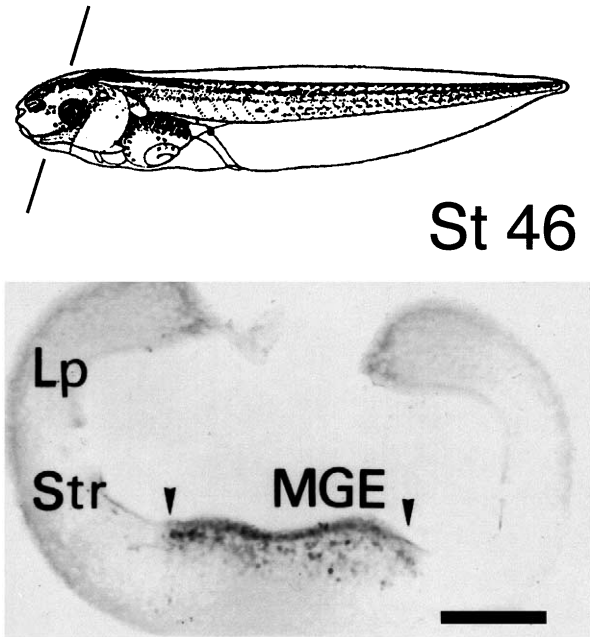

Fig. 1. Photomicrograph of a transverse section through the telencephalon of *Xenopus* at stage 46 illustrating the NKX2-1 immunoreactivity in the MGE (arrowheads). Scale bar = 100 µm.

(Fig. 1). In all cases, NKX2-1 immunoreactivity was always restricted to the cell nucleus.

As development continued, the number of postmitotic NKX2-1+ cells increased rapidly. By stage 50 (premetamorphic larva), NKX2-1+ cells were primarily restricted to several immature basal telencephalic nuclei. At rostral levels, strongly labeled NKX2-1+ cells migrated superficially and medially to constitute part of the ventral septum (Fig. 2a, b). Scattered NKX2-1+ cells were also found in the region of the developing striatum, despite the fact that the ventricular zone of this region never contains NKX2-1+ cells. More caudally, many NKX2-1+ cells migrated superficially from the MGE to form the bed nucleus of the stria terminalis (medially) and the dorsal pallidum (laterally) (Fig. 2c–f), two structures of the mature basal telencephalon of amphibians (Marín et al., 1998a). Noteworthy, the largest and strongest NKX2-1+ cell nuclei were located in the caudal pole of the dorsal pallidum (previously described as the entopeduncular nucleus), which is located in close relation with the growing lateral forebrain bundle (Fig. 2f). Scattered NKX2-1+ cells were also found in the developing amygdala. In the anterior preoptic area, NKX2-1+ cells were primarily located in the ventricular zone (Fig. 2f).

Table 1. Number of animals investigated at different stages of development with immunohistochemistry for NKX2-1

Developmental stages																		
Embryonic	Premetamorphic					Prometamorphic						Metamorphic climax						
45	46	47	50	51	52	53	54	55	56	57	58	59	61	63	64	65	juvenile	
6	4	3	4	3	2	1	2	4	2	3	2	2	2	3	2	3	6	number of animals

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

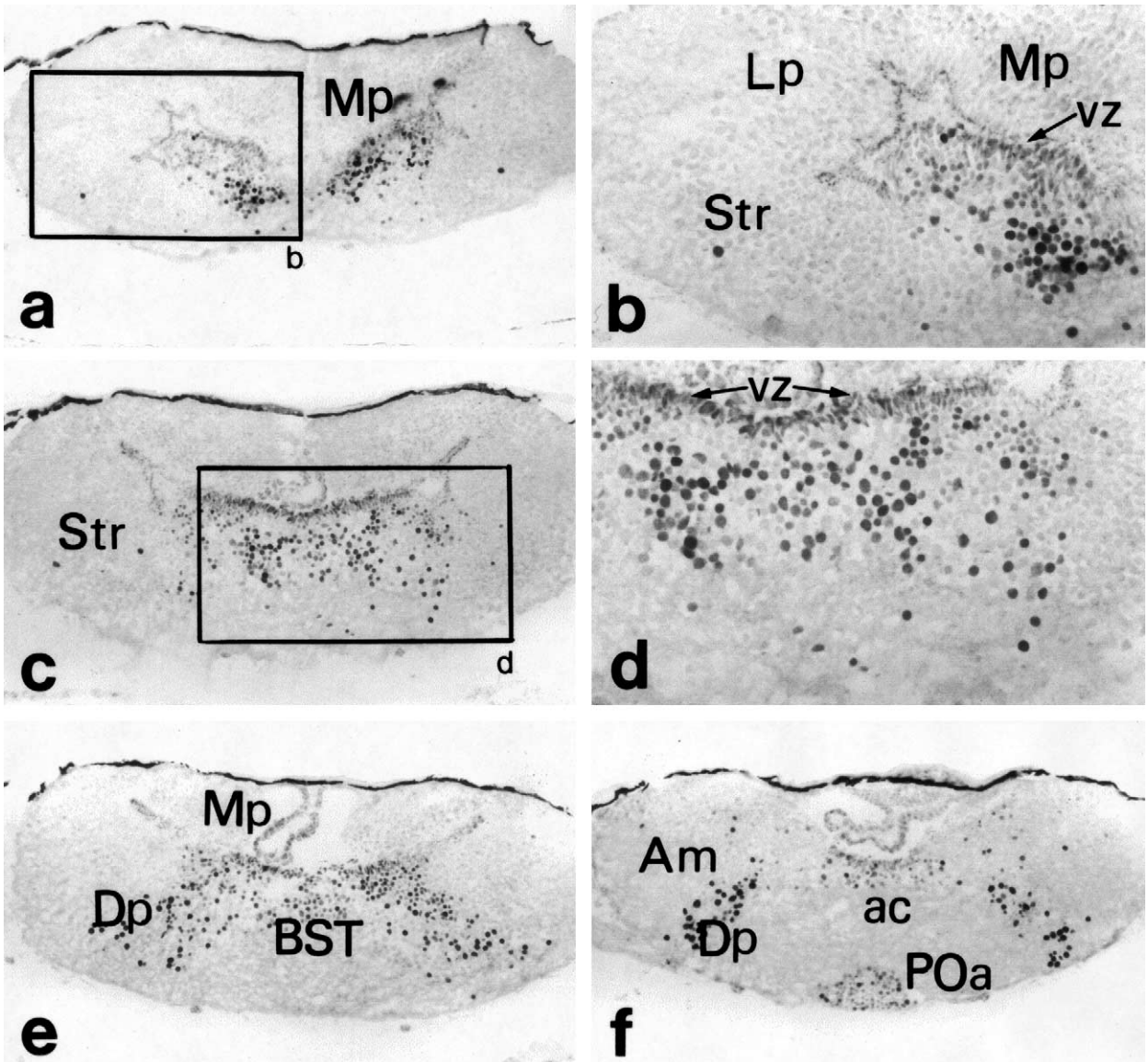

Fig. 2. Photomicrographs of transverse sections through the telencephalon of *Xenopus* at stage 50 taken from rostral (a) to caudal (f) levels. The localization of NKX2-1 immunoreactivity is shown in cell nuclei of the ventral telencephalon. Micrographs b and d are enlargements of the square areas in a and c, respectively. Scale bar = 100 µm (a, c, e, f) and 50 µm (b, d).

Throughout the metamorphosis, cells located in various basal telencephalic nuclei maintain expression of NKX2-1 (Fig. 3). Thus, postmitotic cells were found in both the medial and lateral divisions of the septum, as well as in the diagonal band (Fig. 3a–c). In addition, the ventricular zone of the lateral septum region contained NKX2-1+ cells, which may constitute the remains of the embryonic MGE. As in previous stages, a relatively large and disperse cell population of NKX2-1+ cells was present in the striatum and nucleus accumbens (Fig. 3a–c, f). Although some of these cells occupied rather dorsal positions within the striatum, no NKX2-1+ cells were ever found in pallial regions (Fig. 3a–e). In the caudal telencephalon, numerous NKX2-1-immunoreactive cells were present in the bed nucleus of the stria terminalis and dorsal pallidum (Fig. 3g). In addition, portions of the medial and central amygdala contained abundant labeled cells in juvenile frogs. The preoptic area also conserved NKX2-1 immunoreactivity through metamorphic stages.

In sum, NKX2-1+ cells are present in two main populations within the amphibian basal ganglia: (1) scattered cells in the striatum and nucleus accumbens; and (2) a large number of pallidal cells.

NKX2-1 is expressed in most nitrergic and cholinergic cells in the basal ganglia

The location of NKX2-1+ cells in the basal ganglia of juvenile frogs largely resembled the previously described distribution of nitrergic and cholinergic neurons in this region of the amphibian telencephalon (Muñoz et al., 1996; Marín et al., 1997d; López and González, 2002). To test whether nitrergic and cholinergic cells in the amphibian basal ganglia also express NKX2-1, we carried out double immunohistochemical double-labeling experi-

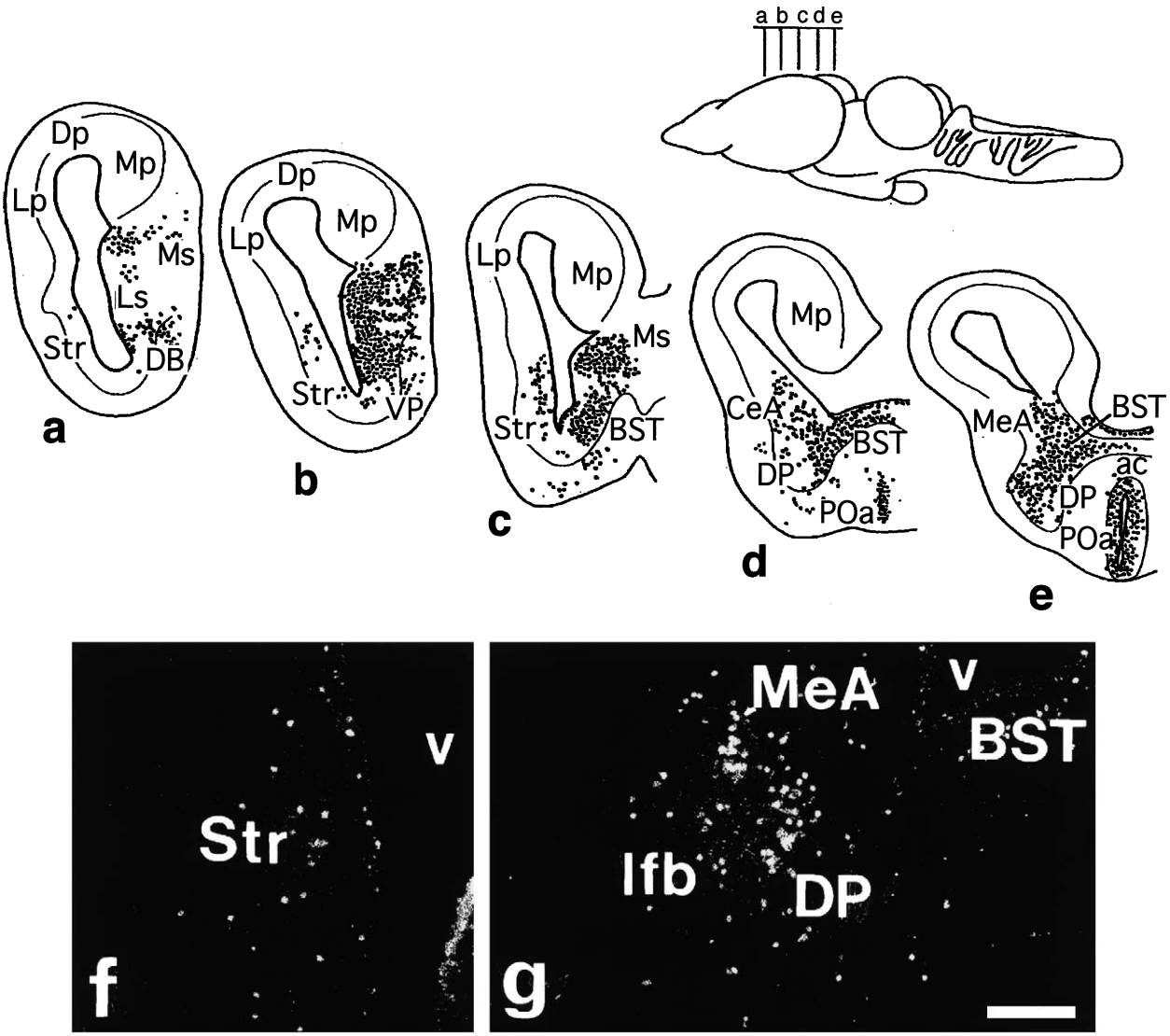

Fig. 3. Diagrams of transverse sections (a–e) through the forebrain of *Xenopus* at stage 65 (juvenile), at levels indicated in the schematic drawing of the brain. The localization of NKX2-1-immunoreactive cells is indicated by black dots. The photomicrographs show NKX2-1+ cells in the striatum (f) and in the caudal pallidal–amygdaloid region (g). Scale bar = 100 µm.

ments. Such experiments revealed that all striatal nitrergic neurons, as identified using antibodies against the synthetic enzyme NOS, were also labeled for NKX2-1 (Fig. 4a). Similarly, double-labeling experiments showed that all striatal cholinergic neurons, as identified by the presence of the synthetic enzyme ChAT, were also stained for NKX2-1 (data not shown). At caudal telencephalic levels, most nitrergic and cholinergic cells located in pallidal and amygdaloid regions of the basal telencephalon were also NKX2-1+ (Fig. 4b, c). Of note, a large number of NKX2-1+ cells in striatal and pallidal regions did not label with antibodies against NOS and ChAT, suggesting that NKX2-1 is also expressed in other neurochemical populations of basal ganglia.

NKX2-1 is expressed in nitrergic and cholinergic striatal interneurons

In the mammalian striatum, NKX2-1 is exclusively expressed in local circuit neurons, including nitrergic and cholinergic interneurons (Marín et al., 2000). Conversely, NKX2-1 is never found in striatal projection neurons (Marín et al., 2000). Since NKX2-1 is also expressed in NOS+ and ChAT+ cells in *Xenopus*, the amphibian striatum may contain a population of local circuit neurons homologous to that found in mammals. To confirm that NKX2-1+/NOS+ and NKX2-1+/ChAT+ neurons in the striatum of *Xenopus* are interneurons, we labeled all striatal projection neurons by injecting the retrograde tracer TRDA into the lateral forebrain bundle, the main efferent pathway of the striatum (see Marín et al., 1997c). Tract-tracing experiments in which TRDA was applied to the lateral forebrain bundle resulted in the massive labeling of striatal projection neurons. Experiments combining retrograde tracing with TRDA and immunohistochemistry against NOS or ChAT failed to double label cells in the striatum (Fig. 4d), demonstrating that NOS+ and ChAT+ cells in the

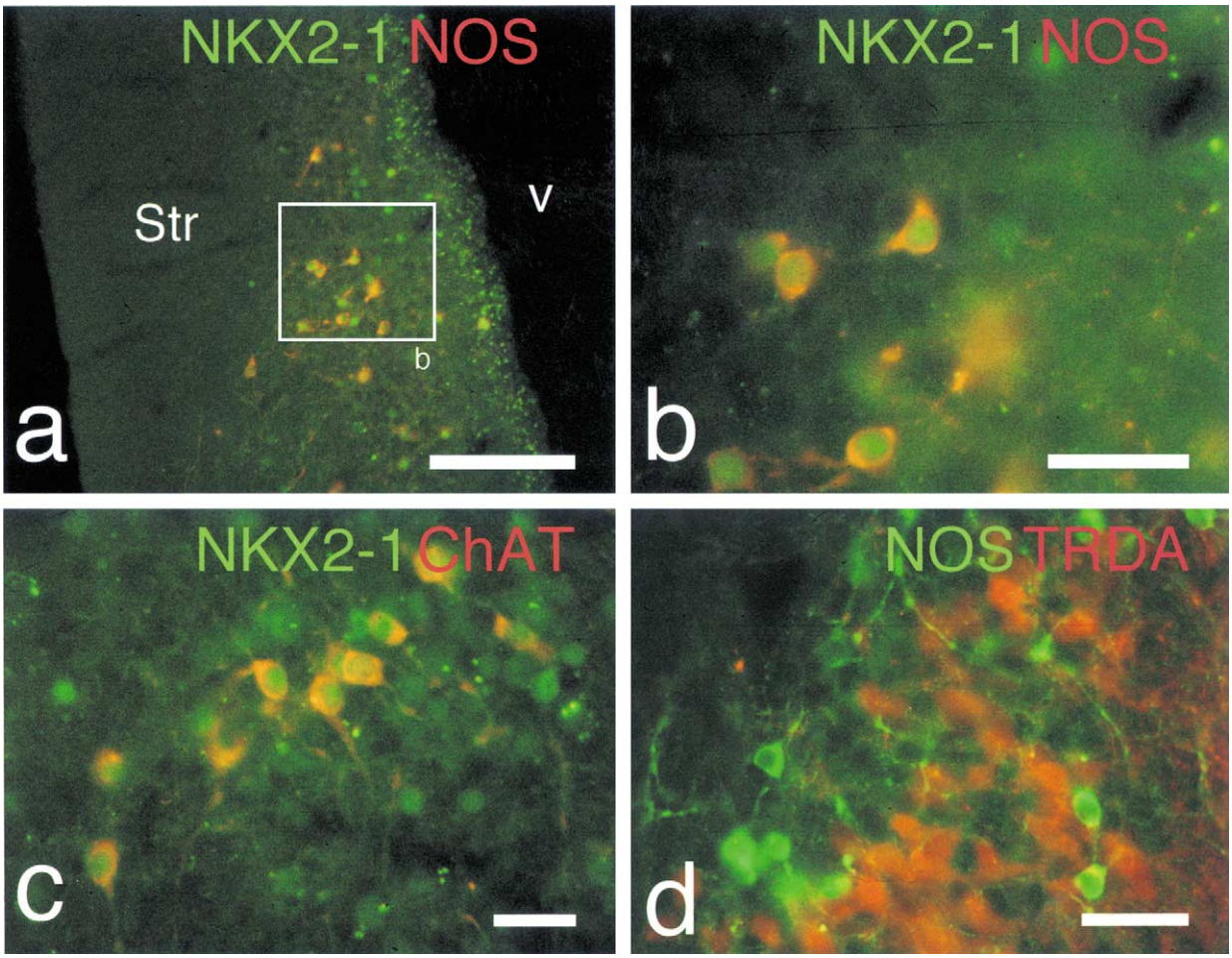

Fig. 4. Photomicrographs of double-labeled sections through the brain of *Xenopus* juveniles showing NKX2-1-immunoreactive cell nuclei (green in a–c) in comparison with NOS and ChAT staining of cell cytoplasms (orange-red). (a, b) Striatum at rostral levels, where all NOS-immunoreactive neurons are also NKX2-1+. (c) Caudal telencephalon, where the ChAT-immunoreactive cells of the pallidal–amygdaloid region are simultaneously NKX2-1+. (d) Micrograph of a section through the striatum showing the distinct populations of projection neurons (retrogradely labeled in red with TRDA) and NOS-immunoreactive cells (green). Scale bars = 100 µm (a) and 25 µm (b–d).

striatum are interneurons. Similar retrograde tracing experiments combined with NKX2-1 immunohistochemistry also revealed lack of NKX2-1 expression in striatal projection neurons (data not shown), reinforcing the notion that NKX2-1+/NOS+ and NKX2-1+/ChAT+ cells in the striatum of *Xenopus* are interneurons.

DISCUSSION

In the present study, we have extended previous analyses on the early expression of *Nkx2-1* in the amphibian embryonic forebrain (Hollemann and Pieler, 2000; Small et al., 2000) by examining the expression of NKX2-1 protein in the developing telencephalon of *Xenopus laevis*. NKX2-1 is expressed in a regionally restricted manner in the amphibian telencephalon, both in progenitor and postmitotic cells. Expression of NKX2-1 in progenitor cells was limited to the ventral septal region, the MGE and the preoptic area, but it was absent from the primordia of the striatum (lateral ganglionic eminence, LGE), dorsal region of the septal primordia and pallial ventricular zones. Expression of NKX2-1 in postmitotic cells was observed at all stages studied, including young adults. Labeled cells were found in the septum, pallidum and bed nucleus of the stria terminalis. In addition, scattered cells were also present in the striatum and amygdala. NKX2-1+ cells were never found in pallial regions. Insights from these findings are discussed below.

NKX2-1 defines distinct progenitor zones in the amphibian telencephalon

Previous studies have demonstrated that the nested expression of several transcription factors defines distinct progenitor domains in the telencephalon of birds and mammals (Smith-Fernandez et al., 1998; Puelles et al., 2000; Yun et al., 2001). For example, expression of *Dlx2* defines the entire subpallium, whereas expression of *Nkx2-1* in a subdomain of the *Dlx2*-expressing domain delineates the pallidal anlage, the MGE (Lazzaro et al., 1991; Bulfone et al., 1993; Price, 1993; Liu et al., 1997; Pera and Kessel, 1998; Sussel et al., 1999; Puelles et al.,

2000; Yun et al., 2001). In addition, *Nkx2-1* is also expressed in progenitor cells in the septum and preoptic area in both birds and mammals (Puelles et al., 2000; Yun et al., 2001).

In *Xenopus*, expression of *Dlx* genes delineates the entire subpallial telencephalon (Dirksen et al., 1993; Papalopulu and Kintner, 1993; Smith-Fernandez et al., 1998). As in mammals and birds, the present analysis of the expression of NKX2-1 demonstrates that the amphibian subpallium can be subdivided into *Dlx*+/*Nkx2-1*− (i.e. the striatal anlage, Deacon et al., 1994) and *Dlx*+/*Nkx2-1*+ regions. The latter domain comprises septal, MGE and preoptic progenitor domains. Of note, the MGE of amphibians also expresses the LIM homeodomain transcription factor *x-Lhx7* (Bachy et al., 2001), reinforcing the notion that the MGE of amphibians and mammals are homologue structures (Grigoriou et al., 1998). In conclusion, molecular analysis of the basal telencephalon in amphibians strongly suggests that the existence of separated progenitor zones for the striatal and pallidal components of the basal ganglia is a primitive feature in the tetrapod brain.

NKX2-1 expression delineates the amphibian pallidum

The present results on the distribution of NKX2-1 in postmitotic cell populations within the developing telencephalon in *Xenopus* provide further support to the notion that the amphibian basal ganglia contain pallidal structures (Marín et al., 1998a,b). Thus, as in birds and mammals, neurons in the pallidum of *Xenopus* are characterized by the expression of the homeobox transcription factor NKX2-1 (Sussel et al., 1999; Puelles et al., 2000; present results), suggesting that pallidal structures share a common developmental origin in tetrapods. Moreover, a primitive anlage for pallidal-like structures might also be present in fish (Smeets et al., 2000) where a similar pattern of gene expression has been observed in the ventral telencephalon of zebrafish (Rohr et al., 2001). However, the lamprey telencephalon lacks *Nkx2-1* expression during development (Ogasawara et al., 2001). Therefore, *Nkx2-1* expression in the ventral telencephalon might be a shared feature in all gnathostome vertebrates in contrast with the situation in agnathostomes.

In addition to the pallidum, the basal telencephalon of *Xenopus* contains other neuronal populations that express NKX2-1 and are readily comparable to their mammalian counterparts. For example, cholinergic neurons in the nucleus of the diagonal band and basal forebrain complex express NKX2-1 in both mammals and amphibians (Marín et al., 2000; present study). Moreover, the telencephalic cholinergic cell groups of *Xenopus* do not send projections outside the telencephalon (Neary, 1990; Scalia et al., 1991; Marín et al., 1997c; present study), and are responsible for the cholinergic innervation of the pallium, as in mammals (Marín et al., 1997d; Woolf, 1991). Finally, NKX2-1+ neurons are also found in the diagonal band region and bed nucleus of the stria terminalis in *Xenopus*, as it occurs in their homologue counterparts in birds and mammals (Sussel et al., 1999; Puelles et al., 2000). In sum, expression of NKX2-1 in pallidal and septal postmitotic neurons is a highly conserved feature of telencephalic organization in the brain of tetrapod vertebrates.

The amphibian striatum contains projection neurons and interneurons

The striatum of amniotes possesses projection neurons and local circuit neurons, whose axons do not leave the striatum (interneurons). Interneurons comprise only about 5–10% of striatal neurons in the mouse, and belong to two main categories: (1) cholinergic interneurons, and (2) GABAergic interneurons. The second group can be further divided into distinct subpopulations attending to the simultaneous expression of GABA with calcium binding proteins (calbindin, parvalbumin, calretinin), neuropeptides (somatostatin, neuropeptide Y) or NOS (Kawaguchi et al., 1995; Figueredo-Cardenas et al., 1996). The presence of most of these types of striatal interneurons is a conserved feature in the brain of amniotes (Reiner et al., 1998).

In amphibians, most striatal cells are projection neurons with long descending axons in the lateral forebrain bundle (Lázár and Kozicz, 1990; Marín et al., 1997c). Moreover, calbindin-, parvalbumin-, calretinin-, somatostatin- and neuropeptide Y-immunoreactive cells seem to be lacking from the amphibian striatum (Danger et al., 1985; Cailliez et al., 1987; Petkó and Orosz, 1996; Vallarino et al., 1998; Necchi et al., 1999), leading to the suggestion that striatal interneurons are a unique feature of the amniote basal ganglia (Reiner et al., 1998). In contrast to this hypothesis, two lines of evidence presented here suggest that the amphibian striatum contains local circuit neurons. First, both cholinergic and nitrergic neurons have been identified in the striatum of anurans (Brüning and Mayer, 1996; Muñoz et al., 1996; Marín et al., 1997d, 1998a), and their axons do not project out of the striatum. Second, striatal cholinergic and nitrergic neurons express NKX2-1 in amphibians. The latter finding strongly supports the notion that these cell populations are local circuit neurons, because NKX2-1 expression in the mammalian striatum is exclusively restricted to interneurons (Marín et al., 2000). Moreover, the amphibian striatum may contain additional types of striatal interneurons, since the population of striatal NKX2-1+ cells is larger than that of cholinergic and nitrergic cells together. In sum, the existence of interneurons in the striatum appears to be a conserved characteristic of the basal ganglia of tetrapods.

Striatal interneurons in amphibians are most likely derived from a progenitor zone different than the striatal primordia (LGE), because progenitor cells in this region never express NKX2-1 during development. This is in agreement with the development of striatal interneurons in mammals, which derive from the MGE and migrate tangentially to the striatal mantle (Marín et al., 2000). Similarly, the striatal anlage in birds does not express *Nkx2-1* in its ventricular zone (LGE) and a similar tangential migration of *Nkx2-1*-expressing cells seems to

occur from the pallidal primordia to the striatal mantle (Puelles et al., 2000).

Genetic studies in mammals have shown that development of a large fraction of cortical interneurons depends on NKX2-1 function (Sussel et al., 1999; Pleasure et al., 2000), and experimental embryological analysis has demonstrated that a large population of cortical interneurons derives from the MGE (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Anderson et al., 2001). However, NKX2-1+ cells are absent from pallial regions in amphibians, bird and mammals (Sussel et al., 1999; Marín et al., 2000; Puelles et al., 2000; present study), suggesting that cortical interneurons derived from the MGE down-regulate the expression of *Nkx2-1* during their tangential migration to the cortex. Although the present study does not provide evidence supporting the existence of interneurons in the amphibian pallium, the similarity in the expression pattern of NKX2-1 in the ventral telencephalon of amphibians, birds and mammals, as well as the postulated existence of tangential migrations of interneurons from the MGE to the striatum in amphibians, makes it tempting to hypothesize that a migration of interneurons from the MGE to the pallium might be also a primitive feature of the tetrapod brain. In line with this idea, the amphibian pallium contains cells that express the homeobox gene *Dlx2* (Brox et al., 2000), whose expression in the mammalian cortex is exclusively restricted to interneurons (Stühmer et al., 2002). Future experiments should provide a definitive answer to this question.

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(*Accepted* 6 *June* 2002)