

# Basal Ganglia Organization in Amphibians: Development of Striatal and Nucleus Accumbens Connections With Emphasis on the Catecholaminergic Inputs

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## ABSTRACT

To broaden our insight into the organization of the basal ganglia of amphibians, the development of the connections of the striatum and the nucleus accumbens was studied by means of tract-tracing techniques based on the transport of biotinylated dextran amines. In a number of experiments, these techniques were combined with tyrosine hydroxylase immunohistochemistry to identify the sources of catecholaminergic inputs to the striatum and the nucleus accumbens. Already at *late embryonic* stages, the basal telencephalon receives inputs from cells located in the amygdala, the thalamus, the suprachiasmatic nucleus, the raphe nucleus, and the rhombencephalic reticular formation. At these stages, the rostral part of the posterior tubercle seems to be the only source of the dopaminergic input to the basal telencephalon. During *premetamorphosis*, not only a differentiation between connections of the striatum and the nucleus accumbens could be made, but new sources of inputs were also detected in the mesencephalic and isthmus tegmentum, the parabrachial nucleus, and the nucleus of the solitary tract. Double-labeling experiments revealed that, at these stages, in addition to the posterior tubercle, cells within the mesencephalic tegmentum, the locus coeruleus, and the solitary tract nucleus contribute to the catecholaminergic innervation of the basal forebrain. During *prometamorphic* stages, a gradual increase occurs in the number of cells that project to the basal telencephalon. At the beginning of the *metamorphic climax*, the organization of the basal ganglia afferents largely resembles the pattern observed in juveniles and adults. Remarkably, during larval stages, the cells that contribute to the dopaminergic innervation of the basal forebrain show a rostrocaudal gradient in time of appearance. Moreover, the dopaminergic fibers reach the striatum earlier than the nucleus accumbens, and they precede markedly the development of the efferent connections of both brain structures. These developmental aspects are easily correlated with the situation in amniotes; therefore, the notion that amphibians share an essentially similar pattern of basal ganglia organization with other tetrapods is further strengthened. *J. Comp. Neurol.* 383:349-369, 1997. © 1997 Wiley-Liss, Inc.

**Indexing terms:** striatum; substantia nigra; nucleus of the solitary tract; locus coeruleus; evolution

In part of a research program that aims to answer the question to what extent the basal ganglia organization of amphibians is comparable with that of amniotes, the afferent and efferent connections of the striatum and the nucleus accumbens have been studied in the brains of adult anurans and urodeles (Marín et al., 1995, 1996, 1997a-c). Significant differences in connections between

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the nucleus accumbens and the striatum were demonstrated, suggesting the presence of functional subdivisions. Moreover, the results of those studies strongly support the notion that a common pattern of catecholaminergic inputs to the basal forebrain exists among tetrapod vertebrates.

A comparison of the development of basal ganglia structures of amphibians with that of amniotes would certainly contribute to answering the question whether these structures are basically similarly organized among vertebrates. A notable feature of the embryonic development of the basal ganglia of amniotes is the early appearance of dopaminergic fibers in the developing striatum (Olson and Seiger, 1972; Specht et al., 1981; Voorn et al., 1988; Medina et al., 1994a,b). It has been hypothesized that the early presence of dopaminergic fibers in the striatum indicates an important role in organizing the development of this brain region and directly influences the maturation of striatal neurons (Tennyson et al., 1973; Specht et al., 1981; Voorn et al., 1988; Medina et al., 1994a,b). Moreover, the catecholaminergic innervation of the striatum precedes the formation of the long descending projections to the midbrain tegmentum (Fishell and van der Kooy, 1987).

Recently, the ontogeny of the catecholamine systems in amphibians has been studied immunohistochemically by means of antibodies against tyrosine hydroxylase (TH) and dopamine (González et al., 1994a,b, 1995). These studies revealed that, as in amniotes, dopaminergic fibers reach the developing striatum and nucleus accumbens at early stages of development. However, the origin and progressive organization of this innervation during the embryonic and larval stages remained unknown, because data about the formation of connections of the basal ganglia of amphibians were not available. A major reason for the absence of such information was the lack of sensitive tracers that could be injected in vivo at restricted sites in the developing basal telencephalon of amphibians. With the development of an in vitro approach in which the tracer biotinylated dextran amine (BDA) is applied to

restricted brain areas, this problem has been largely overcome (Luksch et al., 1996; Muñoz et al., 1996).

The aims of the present study were 1) to establish the temporal sequence of appearance of striatal and accumbal connections in amphibians, with emphasis on the catecholaminergic inputs; and 2) to compare the development of basal ganglia connections of amphibians with that reported for amniotes in order to gain more insight into the evolution of the basal ganglia of vertebrates. To reach those goals, the South African clawed toad, *Xenopus laevis*, has been selected as the core species because of the availability of an accurate time table of development (Nieuwkoop and Faber, 1967) and the existence of recent hodological data of basal ganglia connections in adult brains of this species (Marín et al., 1997a–c). Low-weight (3 kD) BDA has been used to selectively study the connections of the developing striatum and nucleus accumbens. BDA, depending on the method of application, is both an anterograde and a retrograde tracer (Fritsch, 1993; Muñoz et al., 1996; Marín et al., 1997a–c).

## MATERIALS AND METHODS

For the present study, a total of 88 *Xenopus laevis* embryos and larvae ranging from developmental stage 45 to developmental stage 65 (Nieuwkoop and Faber, 1967) were used (Table 1). The original research reported herein was performed under guidelines established by the Spanish Royal Decree 223/1988. Animals were obtained by Pregnyl-induced (Organon) breeding and were 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 methanesulphonate (MS222; Sandoz, Basel, Switzerland) in distilled water, pH 7.4, and then processed for tracing experiments under in vitro conditions, as previously described (Luksch et al., 1996). Briefly, under anesthesia, the animals were cooled to a body temperature of 4°C and were perfused transcardially with iced Ringer's solution (75 mM NaCl, 25

### Abbreviations

A	anterior thalamic nucleus	Ms	medial septum
Acc	nucleus accumbens	ob	olfactory bulb
Ad	anterodorsal tegmental nucleus	oc	optic chiasm
aob	accessory olfactory bulb	P	posterior thalamic nucleus
Apl	amygdala, pars lateralis	Pb	parabrachial nucleus
Apm	amygdala, pars medialis	pc	posterior commissure
Av	anteroventral tegmental nucleus	Pd	posterodorsal tegmental nucleus
C	central thalamic nucleus	POa	preoptic area
Cb	cerebellum	Pv	posteroventral tegmental nucleus
Dp	dorsal pallidum	Ra	raphe nucleus
Ea	anterior entopeduncular nucleus	Ri	nucleus reticularis inferior
gl	glomerular layer of the olfactory bulb	Rm	nucleus reticularis medius
gr	granular layer of the olfactory bulb	Rs	nucleus reticularis superior
Hab	habenula	SC	suprachiasmatic nucleus
Hyp	hypothalamus	SIR	superficial isthmic reticular nucleus
III	nucleus nervi oculomotorii	sol	solitary tract
Ip	interpeduncular nucleus	Str	striatum
La	lateral thalamic nucleus, anterior division	tect	tectum
Lc	locus coeruleus	tegm	tegmentum
lfb	lateral forebrain bundle	Thal	thalamus
Lp	lateral pallidum	Tor	torus semicircularis
Lpv	lateral thalamic nucleus, posteroventral division	TP	posterior tubercle
Ls	lateral septum	v	ventricle
m	medial tegmental region	VH	ventral hypothalamus
mfb	medial forebrain bundle	Vm	nucleus motorius nervi trigemini
ml	mitral cell layer of the olfactory bulb	VM	ventromedial thalamic nucleus
Mp	medial pallidum	vt	ventral thalamus

TABLE 1. Number of Animals Investigated at Different Stages of Development With Tracer Application in the Striatum (Str) or the Nucleus Accumbens (Acc)<sup>1</sup>

Embryonic		Developmental stages															n	
		Premetamorphic					Prometamorphic					Metamorphic climax						
45		46	47	50	51	52	53	54	55	56	57	58	59	61	63	64	65	
6	Str	4	3	4	3	2	1	2	4	2	3	2	2	2	3	2	3	42
	Acc	3	2	3	2	3	2	2	3	4	2	4	1	3	1	2	3	40

<sup>1</sup>Staging of the embryos and larvae according to Nieuwkoop and Faber (1967).

mM NaHCO<sub>3</sub>, 2 mM CaCl<sub>2</sub>, 2 mM KCl, 0.5 mM MgCl<sub>2</sub>, 11 mM glucose; Merck, Darmstadt, Germany), which was oxygenated with carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) to a pH of 7.3 (Straka and Dieringer, 1993). Subsequently, the brain and spinal cord were rapidly isolated, and, after removal of the dura mater and the choroid plexuses, they were transferred to fresh iced Ringer's solution.

Applications of 3 kD BDA (D-7135; Molecular Probes, Eugene, OR) were made unilaterally within the ventrolateral (striatal) and ventromedial (nucleus accumbens) subregions of the basal telencephalon. The tracer was applied by impaling the selected regions with a very sharp tungsten needle. On the tip of the needle, BDA was recrystallized from a saturated solution in distilled water. The brains were maintained for 15–24 hours at 15°C in continuously oxygenated Ringer's solution. They were then fixed for 3–5 hours in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, blocked in a solution of 15% gelatin and 30% sucrose in PB, and stored for 5 hours at 4°C in a solution containing 4% formaldehyde and 30% sucrose in PB. Sections were cut on a freezing microtome at 30–40 µm thickness in the frontal or the sagittal plane and were collected in cold PB.

In a first series of experiments, BDA was visualized with an avidin biotin complex (Vectastain ABC standard kit; Vector Laboratories, Burlingame, CA) and peroxidase activity with 3,3'-diaminobenzidine (DAB)-nickel as chromogen (for details, see Marín et al., 1997a). The sections were mounted on glass slides (mounting medium: 0.2% gelatin in Tris buffer, pH 7.6) and dried overnight. After ethanol dehydration and xylene cleaning, they were coverslipped with Entellan (Merck). Some sections were counterstained with cresyl violet. In a second set of experiments, visualization of BDA was combined with indirect immunofluorescence for TH. Briefly, brain sections were first incubated for 48 hours at 4°C with a mouse anti-TH antibody (Incstar, Stillwater, MN) diluted 1:1,000. They were then incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse-immunoglobulin (IgG) complex (Incstar) diluted 1:150 for 90 minutes. BDA was visualized by incubation with a Texas red-conjugated streptavidin complex (Vector Laboratories; diluted 1:200) together with the secondary antibody. The sections were then mounted on glass slides and coverslipped with Vectashield (Vector Laboratories). Alternating the appropriate filter combinations in a Zeiss fluorescence microscope allowed the identification of BDA retrogradely labeled cells and TH-immunoreactive (THir) cells.

The distribution of labeled cells and fibers in the brains of *Xenopus* embryos and larvae was charted in representative transverse sections by means of a camera lucida or a computer-aided X-Y plotting system (MD-2 digitizer and software; Minnesota Datametrics). The nomenclature used in this study is largely the same as that used in our previous studies of amphibians (González et al., 1994a,b).

## RESULTS

In the present study, BDA was applied to the developing striatum and nucleus accumbens in *Xenopus* embryos and larvae. The developmental sequence of characteristic stages in the embryonic and larval periods of *Xenopus* has been summarized in Figure 1. For each stage, the pattern of labeling was consistent among animals treated identically. The different methods for visualizing BDA, as reported previously for adult amphibians (Marín et al., 1997c), gave essentially the same results. In the following sections, attention is paid to the development of the afferent connections of the striatum and the nucleus accumbens and, in particular, to their catecholaminergic inputs. Furthermore, because BDA is also anterogradely transported, the development of the main efferent projections of the striatum and the nucleus accumbens was also studied.

### Development of the afferent connections

**Late embryonic stages.** The earliest tadpoles of *Xenopus* that were analyzed corresponded to developmental stages 44–45, which are the last stages of the embryonic period. At these stages, the hemispheric evaginations leading to the formation of the lateral ventricles have not taken place yet, and subregions within the subpallium are still not recognizable. Nevertheless, applications of BDA restricted to the telencephalic hemisphere revealed several projections already at these stages (Fig. 2). Numerous retrogradely labeled neurons were found bilaterally in the medial amygdala in the caudal part of the telencephalon (Fig. 2B). In the rostral diencephalon, labeled cells were seen in the suprachiasmatic nucleus, mainly in its ventrolateral part (Fig. 2C). More caudally, scattered cells were observed in the hypothalamus (Fig. 2D,E,a). In addition, numerous labeled cells were found throughout the rostro-caudal extent of the thalamus and the posterior tubercle (Fig. 2D,E,a). Already at stage 45, three brainstem cell groups were found to project to the telencephalon. The latter groups included strongly labeled cells in the parabrachial region and the raphe at caudal isthmus levels (Fig. 2F,b) and a few weakly labeled cells in the reticular formation at more caudal brainstem levels (Fig. 2G).

**Larval stages: Striatum.** At larval stages 46–47, three distinct regions were identified within the telencephalic hemisphere, i.e., a rostral area bulbaris and lateral and medial hemispheric walls. Starting from these stages, tracer applications could be restricted to the ventrolateral (striatum) or the rostral ventromedial (nucleus accumbens) regions of the telencephalic hemisphere.

**Premetamorphic stages.** The development of the striatal afferent projections during premetamorphic stages is characterized not only by a progressive maturation of existing connections but also by the appearance of new cell groups projecting to the striatum (Fig. 3). Already at stage 47, numerous cell groups became retrogradely labeled

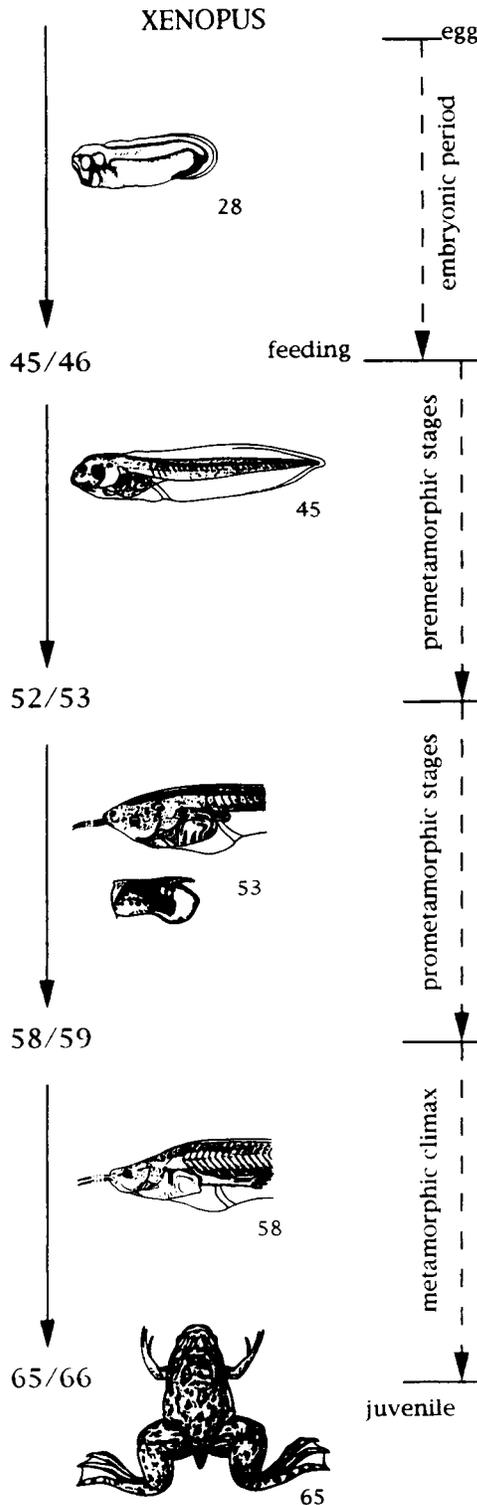


Fig. 1. Developmental stages of *Xenopus laevis* from egg to juvenile after Nieuwkoop and Faber (1967). Numbers on the left indicate developmental stages. Drawings represent *Xenopus* at selected developmental stages. Lower part of drawing at stage 53 is an enlargement of the hindlimb.

after tracer applications to the ventrolateral telencephalic wall. Labeled cells were found in the olfactory bulb, medial pallium, amygdala, suprachiasmatic nucleus, dorsal thalamus, posterior tubercle, parabrachial region, raphe, and reticular formation (Fig. 4a). At stage 50, retrogradely labeled cells in the suprachiasmatic nucleus after BDA application in the developing striatum were still restricted to its lateral aspect (Figs. 3D, 4b). At about the same time, two distinct populations of labeled cells that corresponded to the anterior division of the lateral thalamic nucleus and the central thalamic nucleus were recognized in the dorsal thalamus at intermediate levels (Fig. 3E, 4c). In addition, starting at stages 50–51, labeled cells were found in the ventral thalamus just dorsal to the lateral forebrain bundle (Figs. 3D, 4b) and in the nucleus of the solitary tract (Figs. 3J). At the end of premetamorphosis, a notable increase in the number of cells projecting to the striatum occurred in the dorsal thalamus (Fig. 4d).

**Prometamorphic stages.** During the prometamorphosis, the distribution of cell bodies that projected to the striatum showed a clear resemblance to the pattern observed in adults (Fig. 5; see also Marín et al., 1997a). In the caudal telencephalon, numerous retrogradely labeled cells constituted a rather compact group in the anterior entopeduncular nucleus, whereas a few cells occurred in the preoptic area (Fig. 5C,D). In the hypothalamus, labeled neurons were mainly restricted to the rostral aspect of the ventral hypothalamic nucleus (Fig. 5F). In the dorsal thalamus, cells in the anterior division of the lateral thalamic nucleus were now clearly segregated from those of the central thalamic nucleus (Figs. 5E–G, 6a). Finally, from stage 54, tracer applications in the striatum consistently labeled cells in the rostral aspect of the mesencephalic tegmentum (Fig. 5I).

**Metamorphic climax.** This period of development is characterized by a general maturation of the brain and a steady increase in the number of cells projecting to the striatum (Fig. 7). For example, the number of cells in the caudal extent of the ventral striatum and in the anterior entopeduncular nucleus that projected to rostral striatal territories became considerably larger (Figs. 6b, 7C,D). Similarly, the number of labeled cells located in the caudal aspect of the central thalamic nucleus and in the posterior tubercle region increased substantially during these stages (Figs. 6c, 7H,I). Finally, during the metamorphic climax, cells in the posteroventral division of the lateral thalamic nucleus became labeled for the first time after BDA application to the striatum (Fig. 7H).

#### Larval stages: Nucleus accumbens

**Premetamorphic stages.** Restricted tracer applications to the ventromedial telencephalic wall of larvae at late premetamorphic stages revealed the existence of several well-developed afferent projections to the nucleus accumbens (Fig. 8). Labeled cells were found in the olfactory bulb, medial amygdala, preoptic area, the rostral aspect of the suprachiasmatic nucleus, anterior thalamic nucleus, ventromedial thalamic nucleus, posterior tubercle, parabrachial nucleus, raphe, reticular formation, and nucleus of the solitary tract (Figs. 8, 9a,b). Labeling was bilateral in the amygdala, suprachiasmatic nucleus, anterior thalamic nucleus, and posterior tubercle, although the ipsilateral projection was always predominant (Fig. 8C,D,G).

**Prometamorphic stages.** In the prometamorphic period, the distribution of cells that projected to the nucleus accumbens was largely comparable with that observed in

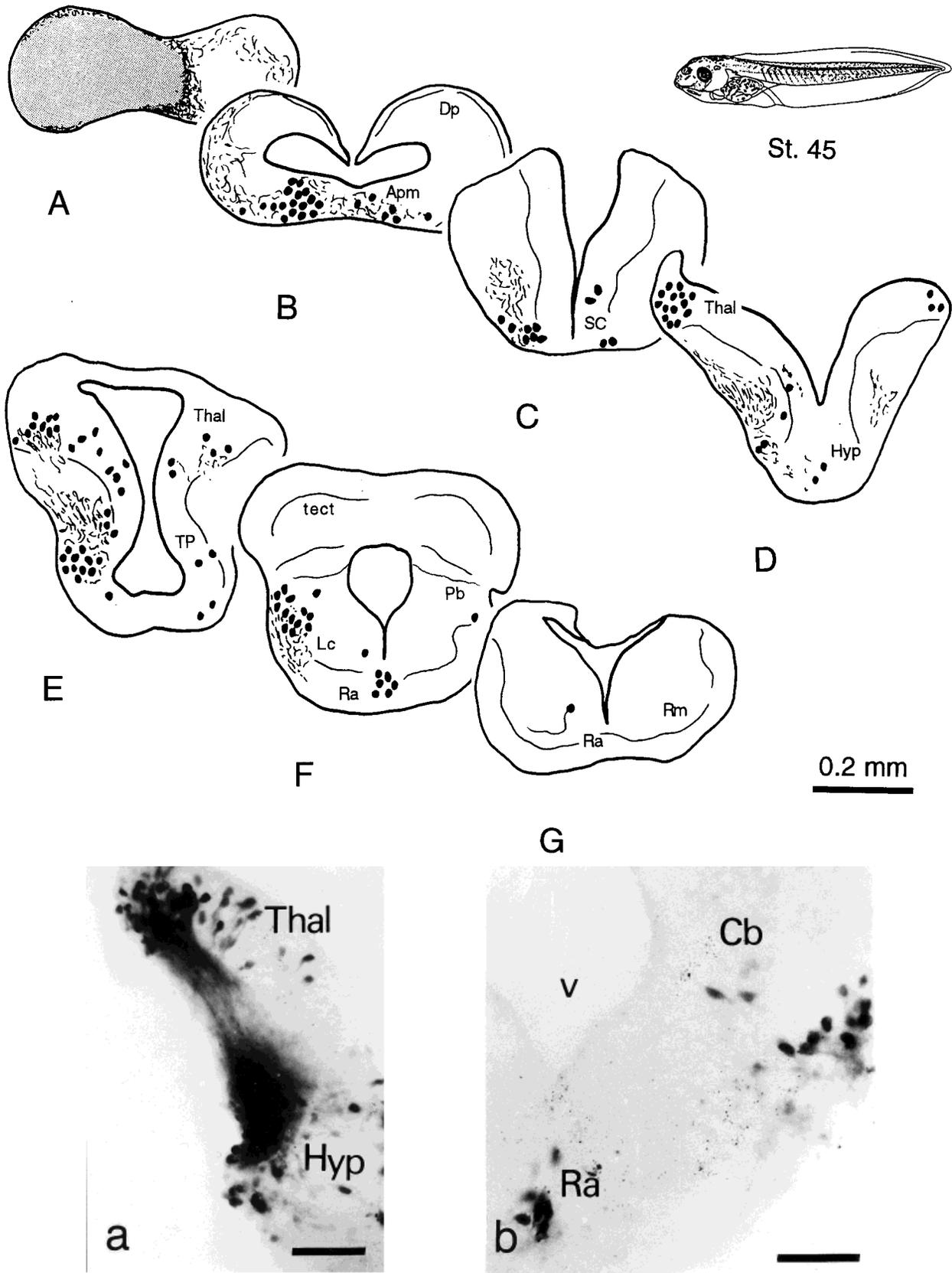


Fig. 2. A-G: Charting of labeling observed after biotinylated dextran amine (BDA) application to the developing hemisphere (shaded area in A) at embryonic stage 45. Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. Photomicrographs (a,b) illustrate labeled cells in the thalamus (Thal) and the hypothalamus (Hyp; comparable to level D of the

scheme) in a and the raphe (Ra), the locus coeruleus, and the parabrachial region (comparable to level F of the scheme) in b. In a, dorsal is up, and medial is to the right; whereas, in b, dorsal is up, and medial is to the left. For other abbreviations, see list. Scale bars = 50  $\mu$ m in a and b.

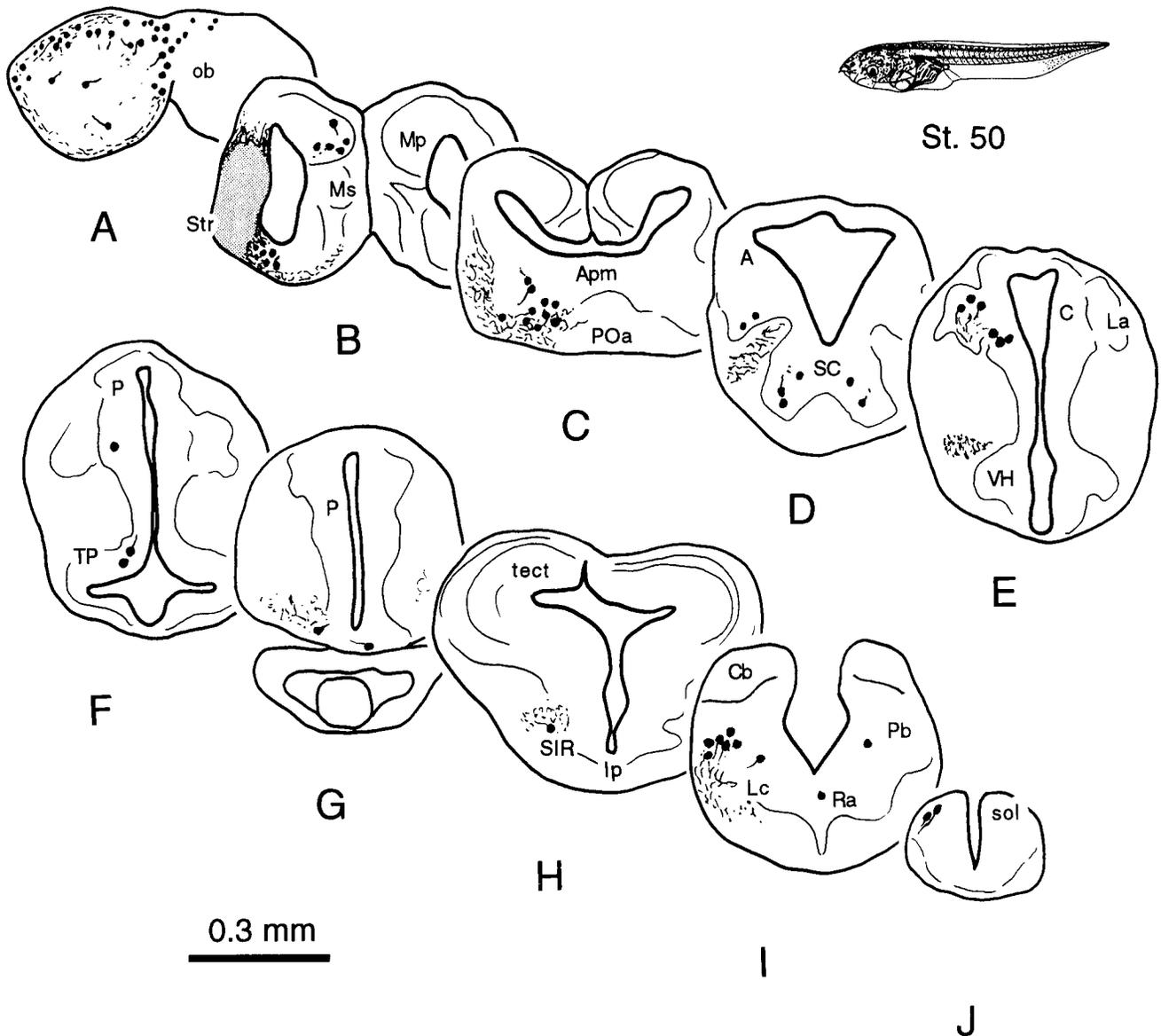


Fig. 3. A–J: Charting of labeling in the brain of *Xenopus* at stage 50 after BDA application to the striatum (shaded area in B). Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. For abbreviations, see list.

adults (Fig. 10; see also Marín et al., 1997a). Additional retrogradely labeled cells occurred in the caudal aspect of the suprachiasmatic nucleus (Fig. 10E) as the prometamorphosis proceeded. Moreover, cells in the posterior tubercle region reached a high degree of organization (Fig. 9c). Starting at stages 52–53, labeled cells were found in the anteroventral tegmental nucleus (Fig. 10I). At isthmic levels, only a few cells were present in the parabrachial region, whereas the number of neurons in the adjacent reticular formation increased (Fig. 10J). Another notable feature of this period was the rise in the number of labeled cells in the raphe, which remained restricted mainly to the upper rhombencephalon (Fig. 9d). At the end of the prometamorphic period, the main afferent projections to the nucleus accumbens arose from the medial amygdala,

the anterior thalamic nucleus, and the posterior tubercle-mesencephalic tegmentum continuum (Figs. 9e,f, 10C–H).

**Metamorphic climax.** Already at early metamorphic climax stages, the afferent connections to the nucleus accumbens were highly organized (Fig. 11). Major events that take place during these stages concern the maturation of inputs from the ventral hypothalamus, posterior tubercle, and midbrain tegmentum (Fig. 11F–J).

### Development of the catecholaminergic innervation

**Late embryonic stages.** Unilateral applications of BDA to the telencephalic hemisphere of *Xenopus* embryos at stage 45 revealed retrogradely labeled cells in the

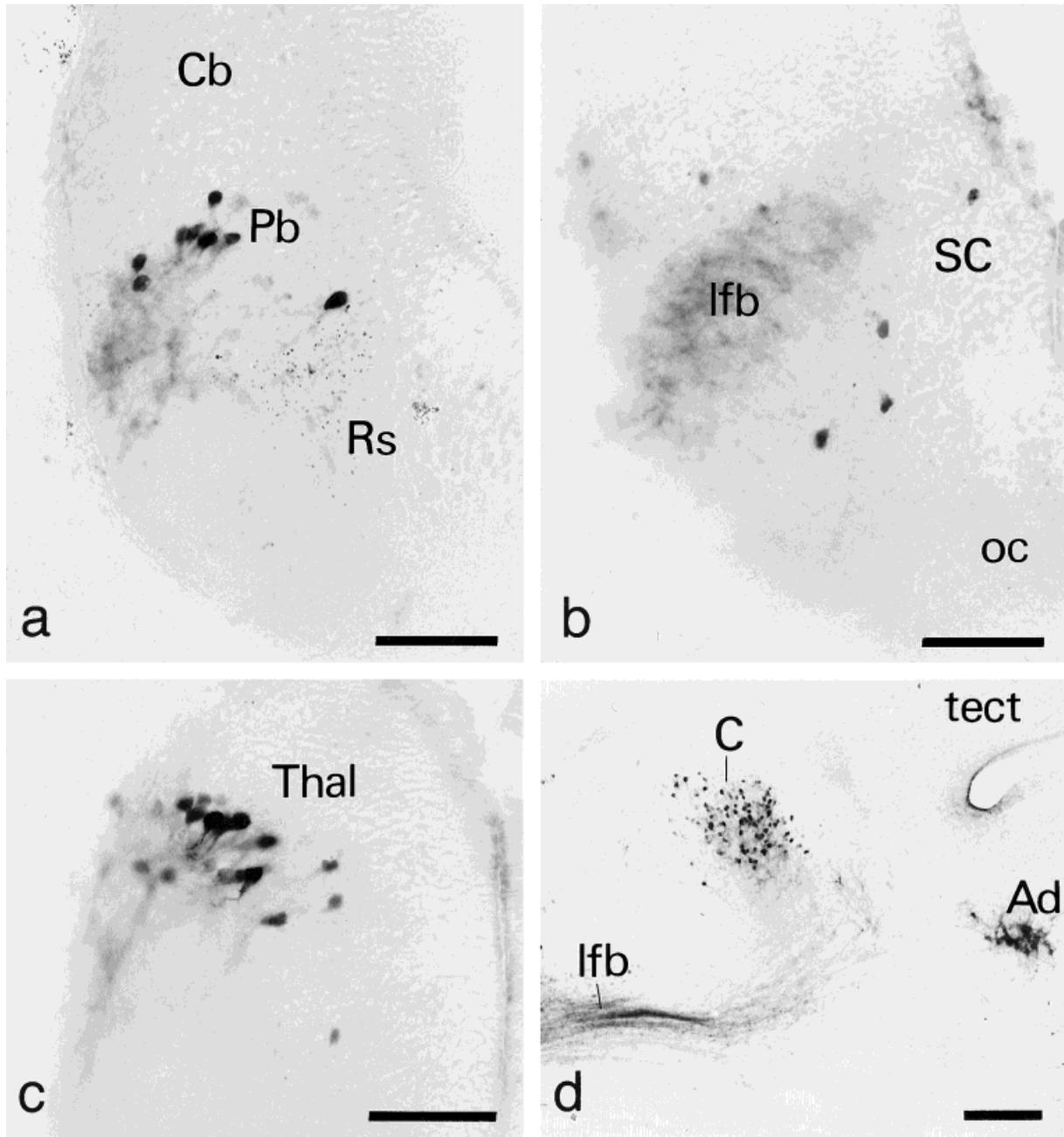


Fig. 4. Photomicrographs of transverse (a–c) or sagittal sections (d) illustrating the labeling after BDA application in the striatum. **a:** Retrogradely labeled cells in the parabrachial region at stage 47. **b:** Labeled cells in the developing suprachiasmatic nucleus and dorsal to the lateral forebrain bundle at stage 51. **c:** Retrogradely labeled cells

in the dorsal thalamus at stage 51 **d:** Retrogradely labeled cells in the central thalamic nucleus and anterogradely labeled fibers in the anterodorsal tegmental nucleus. In a–c, dorsal is up, and medial is to the right; whereas, in d, dorsal is up, and rostral is to the left. For abbreviations, see list. Scale bars = 50  $\mu$ m.

rostral portion of the ipsilateral posterior tubercle (Fig. 2). At this stage, the dopaminergic cells of the posterior tubercle are located close to the surface of the brain, dorsal to the infundibulum (Fig. 12A; see also González et al., 1994a,b). The combination of retrograde tracing and TH immunohistochemistry revealed that, already at stage 45, a number of these dopaminergic cells project to the telencephalon (Fig. 12A,B). In contrast, no double-labeled cells

are found in the locus coeruleus after BDA application in the telencephalon at the end of the embryonic period, although, as early as stage 39, weakly THir neurons are observed in that nucleus (González et al., 1994a,b).

**Larval stages: Striatum.** Tracer applications restricted to the ventrolateral aspect of the telencephalic hemisphere, in combination with TH immunohistochemistry, revealed double-labeled cells at intermediate levels of

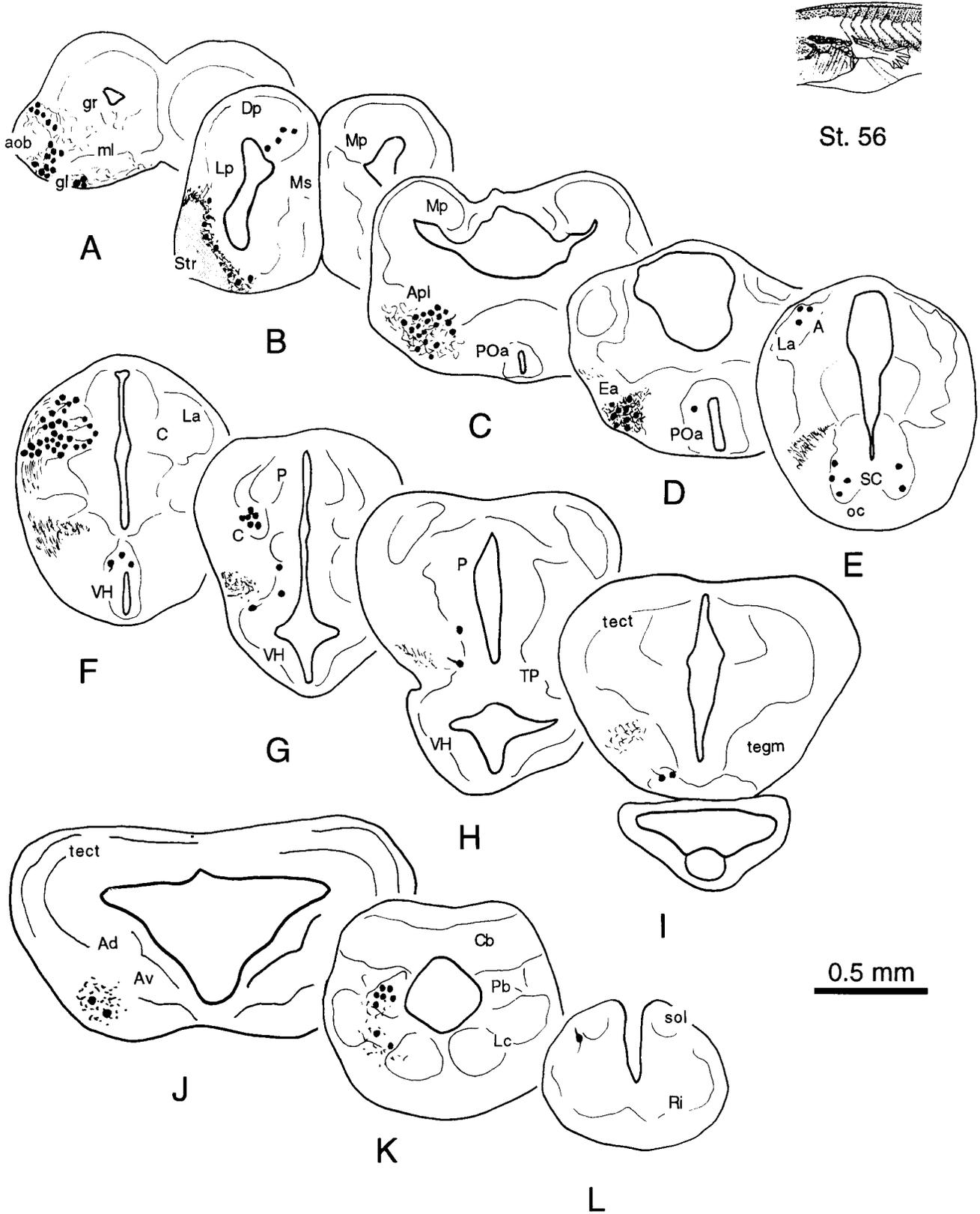


Fig. 5. A-L: Charting of labeling in the brain of *Xenopus* at stage 56 after BDA application to the striatum (shaded area in B). Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. For abbreviations, see list.

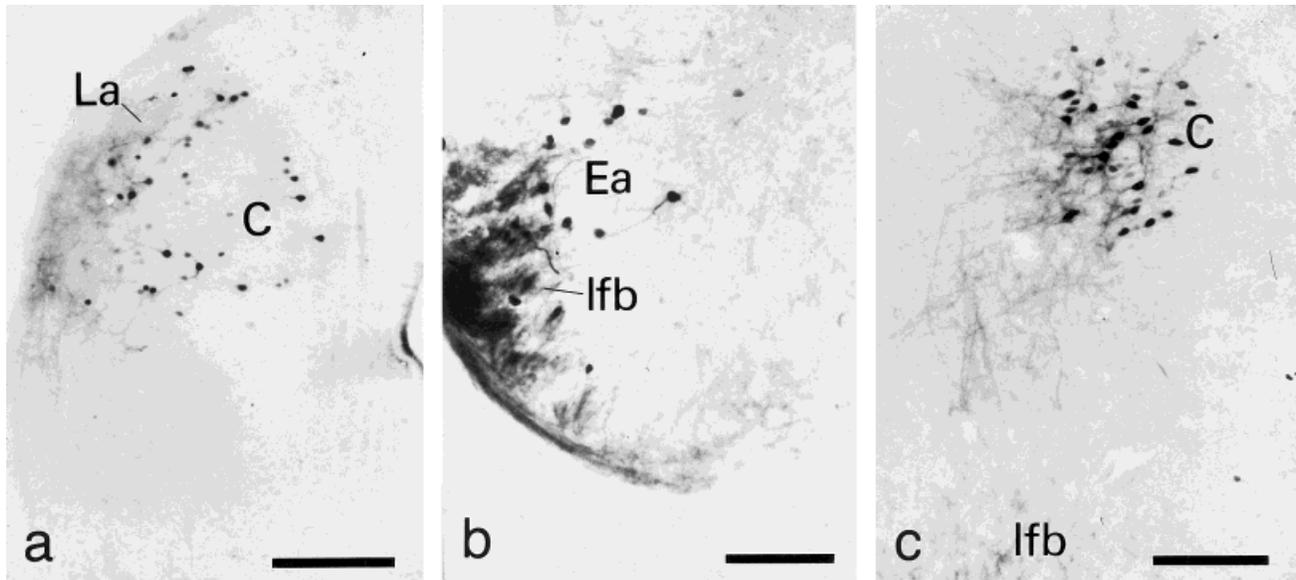


Fig. 6. Photomicrographs of transverse sections through the brain of *Xenopus* tadpoles illustrating retrogradely labeled cells after BDA application in the striatum. **a:** Dorsal thalamus at stage 56. **b:** Anterior entopeduncular nucleus at stage 58. **c:** Caudal pole of the central thalamic nucleus at stage 58. In all photomicrographs, dorsal is up, and medial is to the right. For abbreviations, see list. Scale bars = 100  $\mu$ m.

the posterior tubercle at *premetamorphic* stages 46–47 (Fig. 12C,D). At about the same time, a few double-labeled cells were also found in the locus coeruleus. The number of double-labeled cells in the posterior tubercle increased strikingly at stage 50, and the cell group extended farther caudally to the border between the diencephalon and the mesencephalon (Fig. 3G). In addition, a distinct cell population of striatal afferent cells that did not contain dopaminergic neurons was located in the dorsomedial aspect of the posterior tubercle, mainly at rostral levels (Fig. 3F). From stages 50–51, a few weakly THir cells in the nucleus of the solitary tract were found to project to the ventrolateral aspect of the telencephalic hemisphere.

During the *prometamorphic* period, the previously described catecholaminergic cell groups mature primarily by increasing their number of cells (Fig. 12E,F). In addition, beginning at stage 55, double-labeled cells are found in the rostral aspect of the mesencephalic tegmentum. During the *metamorphic climax*, a basic pattern of catecholaminergic innervation is observed that is already similar to that found in the adult brain (Marín et al., 1997c).

**Larval stages: Nucleus accumbens.** From *premetamorphic* stages 46–47, a population of dopaminergic neurons in the posterior tubercle already projects to the immature nucleus accumbens. Starting with stage 47, dopaminergic neurons in the caudal posterior tubercle extend to the diencephalic-mesencephalic transition area, where numerous cells are found to project to the nucleus accumbens (Fig. 12G,H). Slightly later (stage 48), large nondopaminergic cells that project to the nucleus accumbens are located in the rostral aspect of the posterior tubercle, where a few double-labeled cells are also present (Fig. 13A,B). At about the same time, double-labeled cells are found in the locus coeruleus after tracer application into the nucleus accumbens (Fig. 13C,D). At stages 50–51,

double-labeling experiments show that most of the cells in the solitary tract nucleus that were labeled after application of BDA to the nucleus accumbens are also THir. At the end of the *premetamorphic* period, considerably denser catecholaminergic plexuses are found in the nucleus accumbens (see also González et al., 1994a), which is directly related to the increase in number of double-labeled cells found in the posterior tubercle region.

During the *prometamorphic* stages, dopaminergic cells are found in the mesencephalic tegmentum. From stages 57–58, most of the double-labeled cells found after tracer applications in the nucleus accumbens are located in the caudal aspect of the posterior tubercle and, predominantly, in the midbrain tegmentum (Fig. 13E,F). Finally, the development of the catecholaminergic innervation of the nucleus accumbens during the *metamorphic climax* is characterized by an increase in the number of catecholaminergic cells projecting to this nucleus. However, no significant changes in the organization of these afferent systems are observed.

### Development of the efferent connections

Previous studies have revealed significant differences in afferent and efferent connections between the nucleus accumbens and the striatum in adult amphibians (Marín et al., 1997a–c). From those studies, it became clear that dextran amines can be preferentially transported retrogradely or anterogradely, depending on the method of application and the size of the injection. Therefore, because BDA is also distributed anterogradely, data on the development of the main basal ganglia efferent connections could also be studied.

**Striatum.** The major efferent systems of the striatum, as observed in adult brains, are formed by ipsilateral descending projections to the anterior entopeduncular

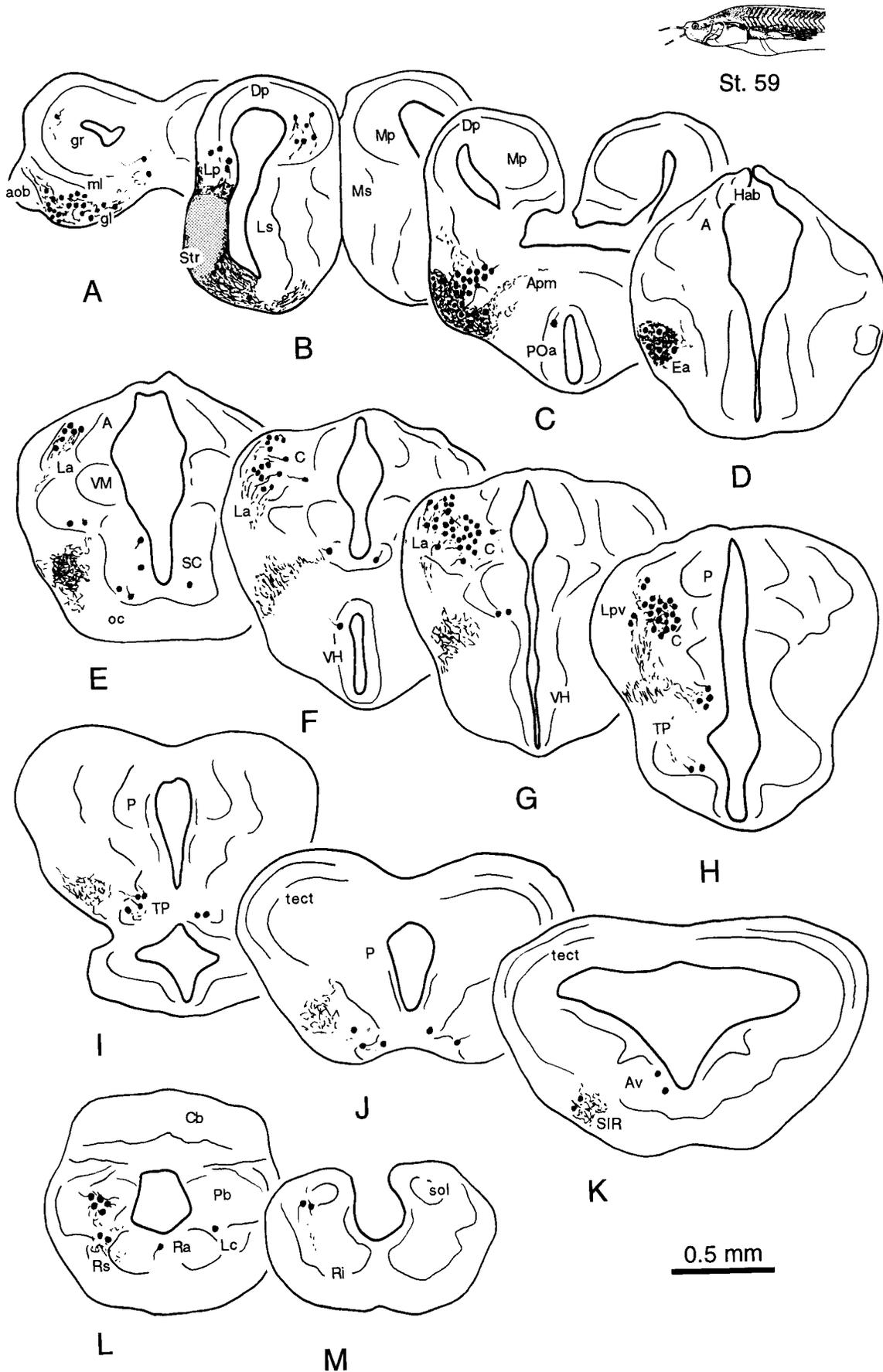


Fig. 7. A-M: Charting of labeling in the brain of *Xenopus* at stage 59 after BDA application to the striatum (shaded area in B). Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. For abbreviations, see list.

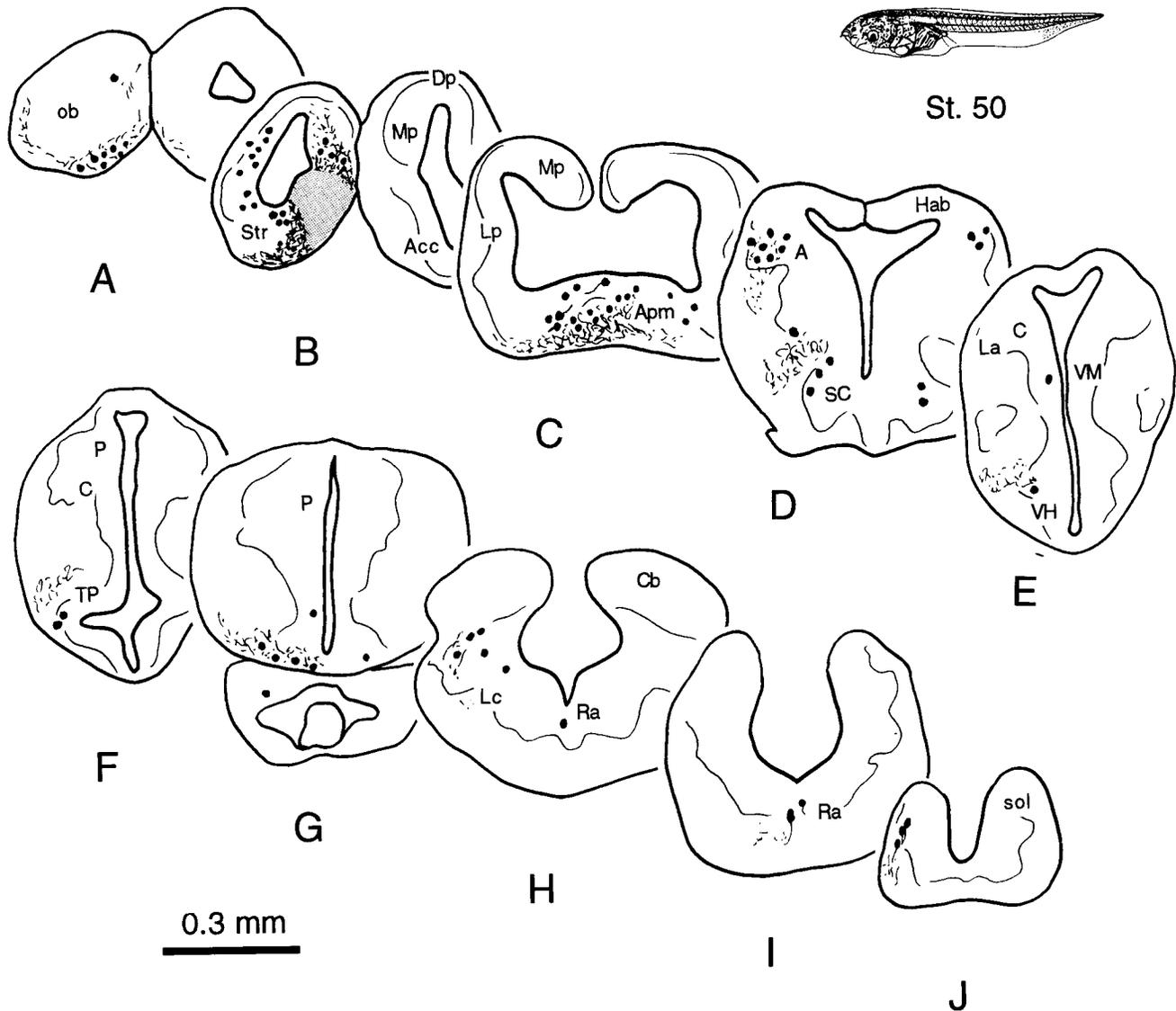


Fig. 8. A–J: Charting of labeling in the brain of *Xenopus* at stage 50 after BDA application to the nucleus accumbens (shaded area in B). Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. For abbreviations, see list.

nucleus, the pretectum, and the mesencephalic and isthmic reticular formation (Marín et al., 1997b). During the *premetamorphic* stages, striatal efferent projections are very restricted. Only minor projections reach the immature caudal portion of the ventral striatum and its caudal continuation, i.e., the anterior entopeduncular nucleus. In addition, no anterogradely labeled fibers are present in the pretectum (Fig. 14A), and only minor projections reach the rostral mesencephalic tegmentum (Fig. 15A). The striatal efferent systems, as the *prometamorphosis* proceeds, reach a high degree of organization. A prominent bundle of labeled fibers projects to the caudal extent of the ventral striatum-anterior entopeduncular continuum. Furthermore, starting at stage 53, anterogradely labeled fibers are found in the pretectum (Fig. 14B,C). Considerably denser plexuses extend caudally in the mesencephalic tegmentum

(Fig. 15B,C) as the *prometamorphic* period continues. The development of the efferent projections of the striatum during the *metamorphic climax* is characterized by an increase in the number of fibers that reach the pretectum and, remarkably, the mesencephalic and isthmic reticular formation (Figs. 14D,E, 15D–G).

**Nucleus accumbens.** In adult amphibians, the nucleus accumbens projects mainly to the preoptic area, the ventral hypothalamus, and the posterior tubercle-medial tegmental region continuum (Marín et al., 1997b). Starting at *premetamorphic* stages 49–50, efferent fibers are recognized in the anterior aspect of the preoptic area and the ventral hypothalamus. An increase in the number of efferent fibers from the nucleus accumbens is observed as the larval development proceeds. From *premetamorphic* stage 55, numerous anterogradely labeled fibers are found

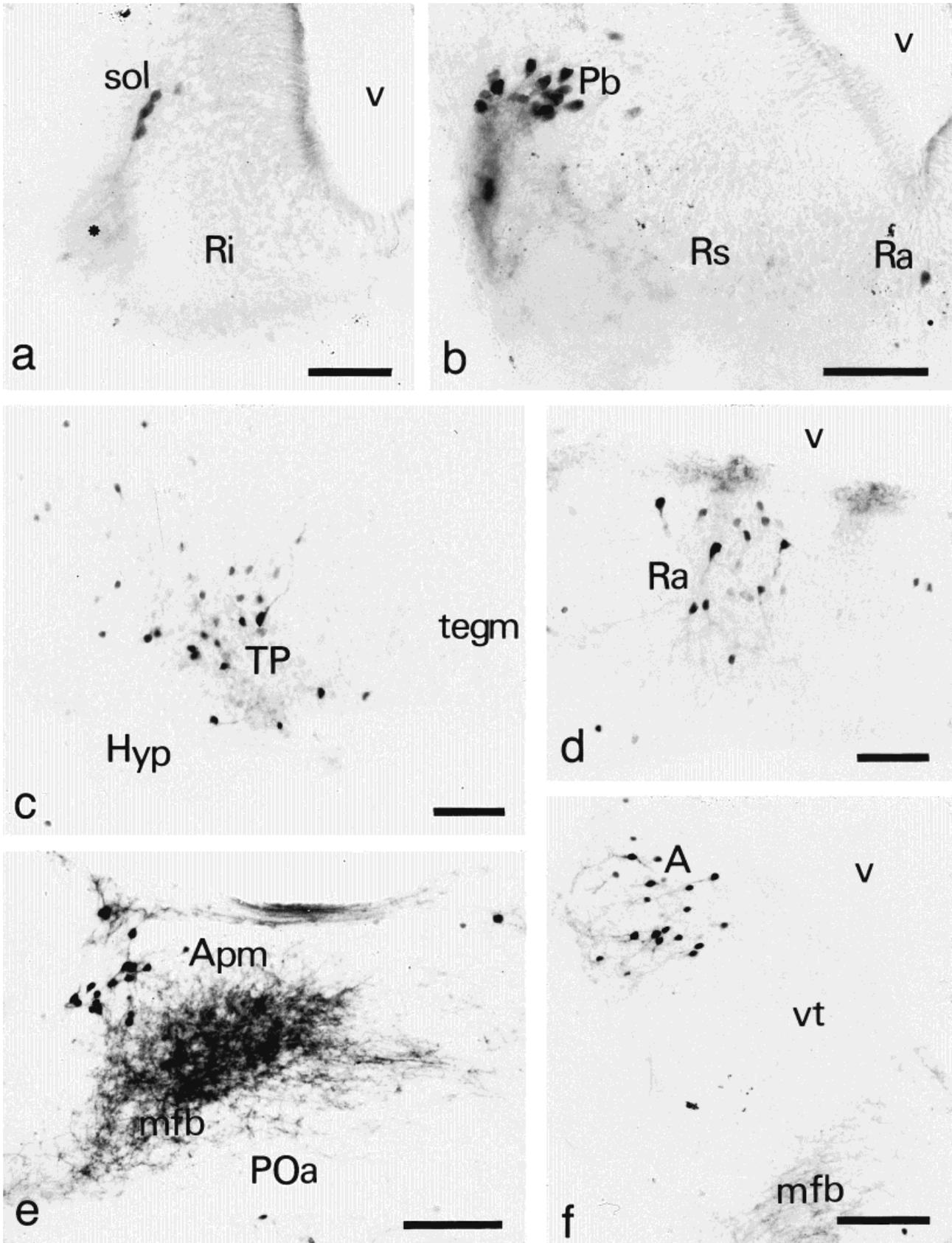


Fig. 9. Photomicrographs of transverse (a,b,e,f) or sagittal (c,d) sections through the brain of *Xenopus* tadpoles after tracer application into the nucleus accumbens illustrating labeled cells in the nucleus of the solitary tract at stage 50–51 (a), in the raphe and parabrachial regions at stage 51 (b), in the posterior tubercle region at stage 52–53 (c), in the rostral raphe at stage 52–53 (d), in the medial amygdala

above the medial forebrain bundle at stage 55 (e), and in the anterior thalamic nucleus at stage 55 (f). In a, b, e, and f, dorsal is up, and medial is to the right; whereas, in c and d, dorsal is up, and rostral is to the left. For abbreviations, see list. Scale bars = 50  $\mu$ m in a–d, 100  $\mu$ m in e, f.

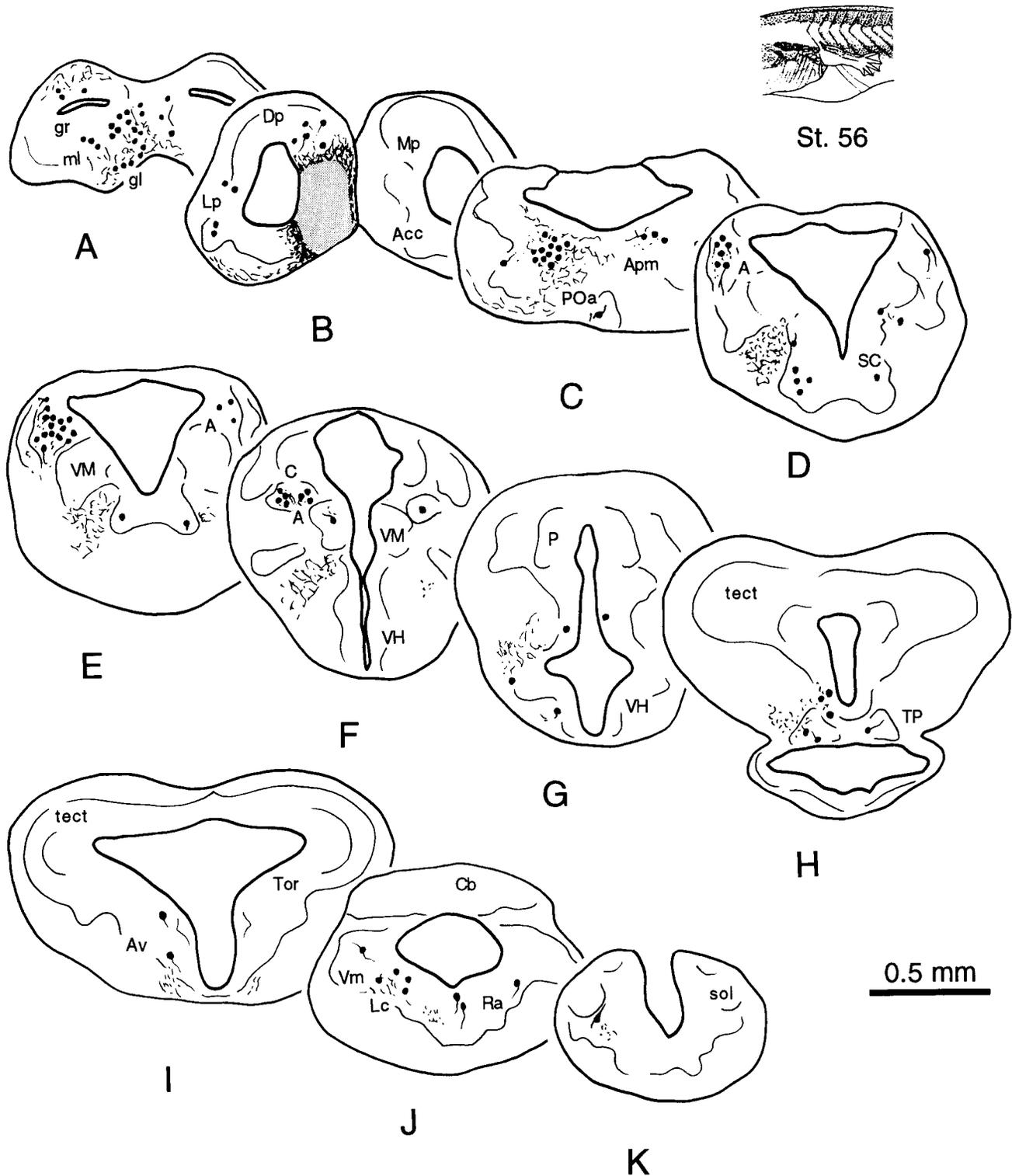


Fig. 10. A-K: Charting of labeling in the brain of *Xenopus* at stage 56 after BDA application to the nucleus accumbens (shaded area in B). Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. For abbreviations, see list.

in the posterior tubercle, and, at the end of the prometamorphosis, the pattern of innervation observed in the ventral hypothalamus is comparable with that found in adult

brains. Finally, during the *metamorphic climax*, no major changes in the development of the main efferent systems of the nucleus accumbens are observed.

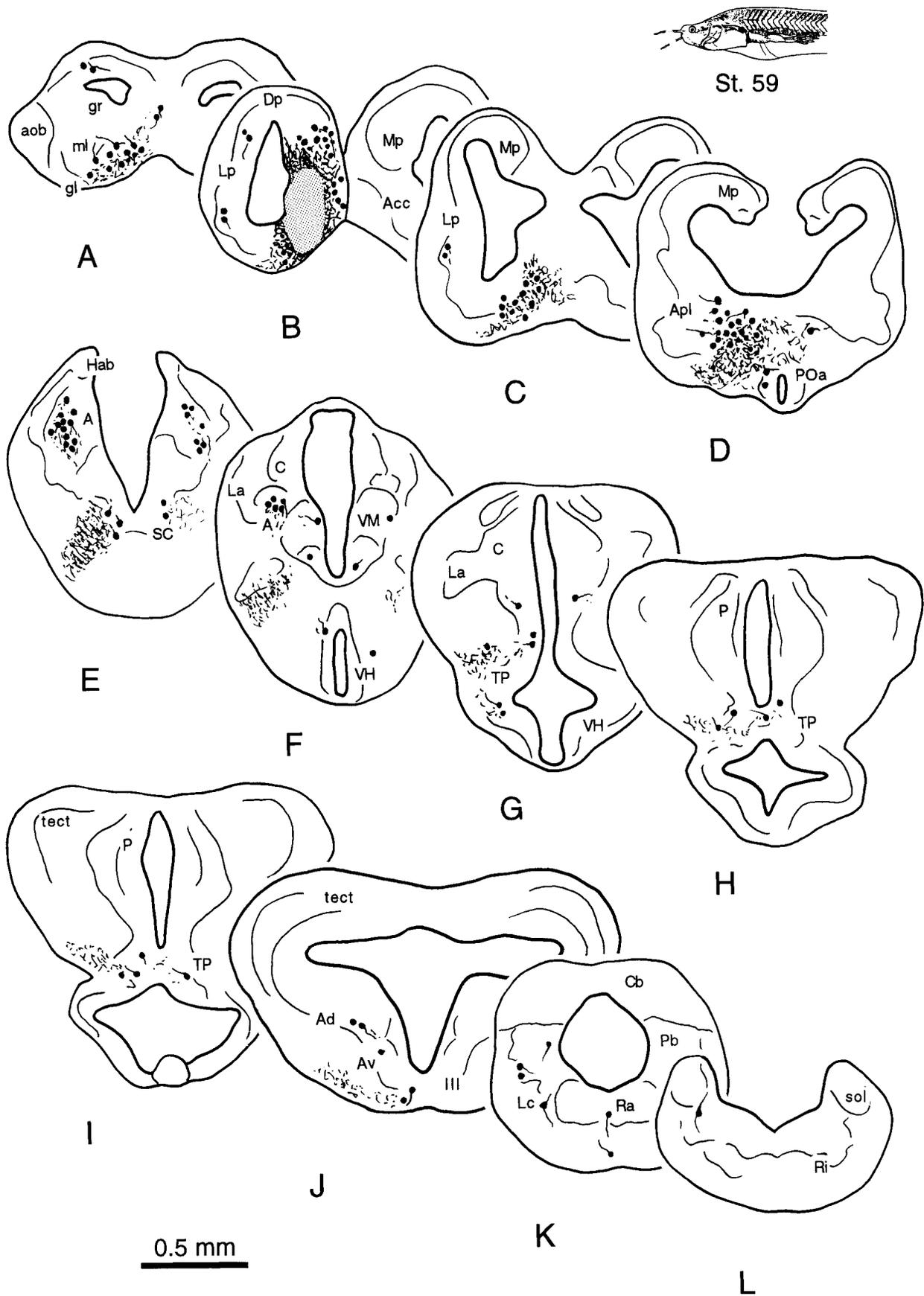


Fig. 11. A-L: Charting of labeling in the brain of *Xenopus* at stage 59 after BDA application to the nucleus accumbens (shaded area in B). Dashes and small dots represent labeled fibers, whereas large dots indicate retrogradely labeled cell bodies. For abbreviations, see list.

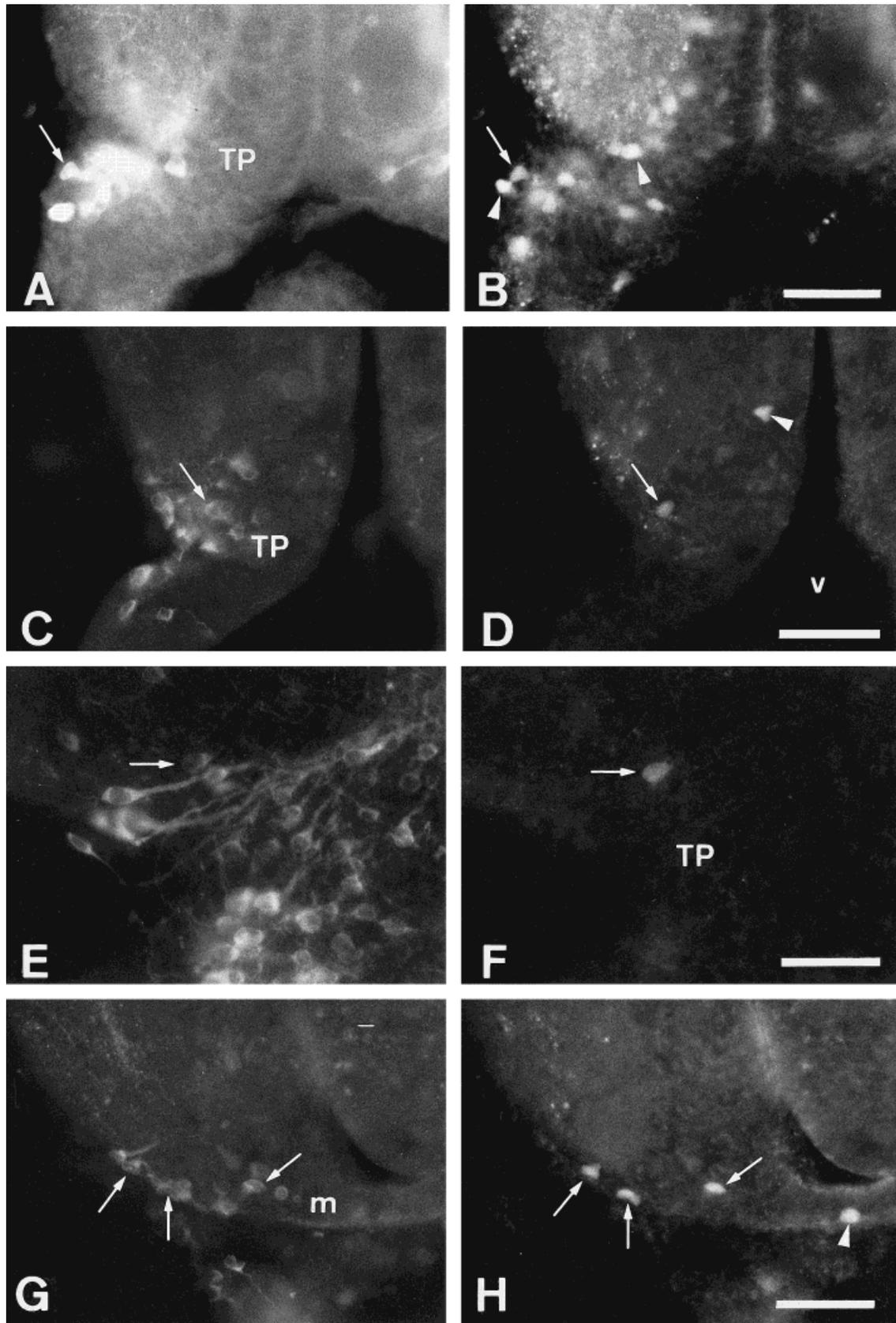


Fig. 12. Photomicrographs illustrating tyrosine hydroxylase-immunoreactive (THir) cells (A,C,E,G) and retrogradely labeled cells (B,D,F,H) in transverse sections through the brain of *Xenopus* tadpoles. **A,B:** Corresponding photomicrographs of the same section at the level of the posterior tubercle after BDA application to the telencephalic hemisphere at stage 45. **C,D:** Illustrations of the same section through the rostral aspect of the posterior tubercle in a experiment with BDA application in the striatum at stage 48. **E,F:**

Photomicrographs of the same section at the level of the caudal posterior tubercle after BDA application in the striatum at stage 56. In both photomicrographs, medial is to the right. **G,H:** Representations of the same section through the rostral midbrain tegmentum at stage 47. Arrows in all photomicrographs indicate double-labeled cells, whereas arrowheads indicate retrogradely labeled cells that are not THir. For abbreviations, see list. Scale bars = 50  $\mu$ m.

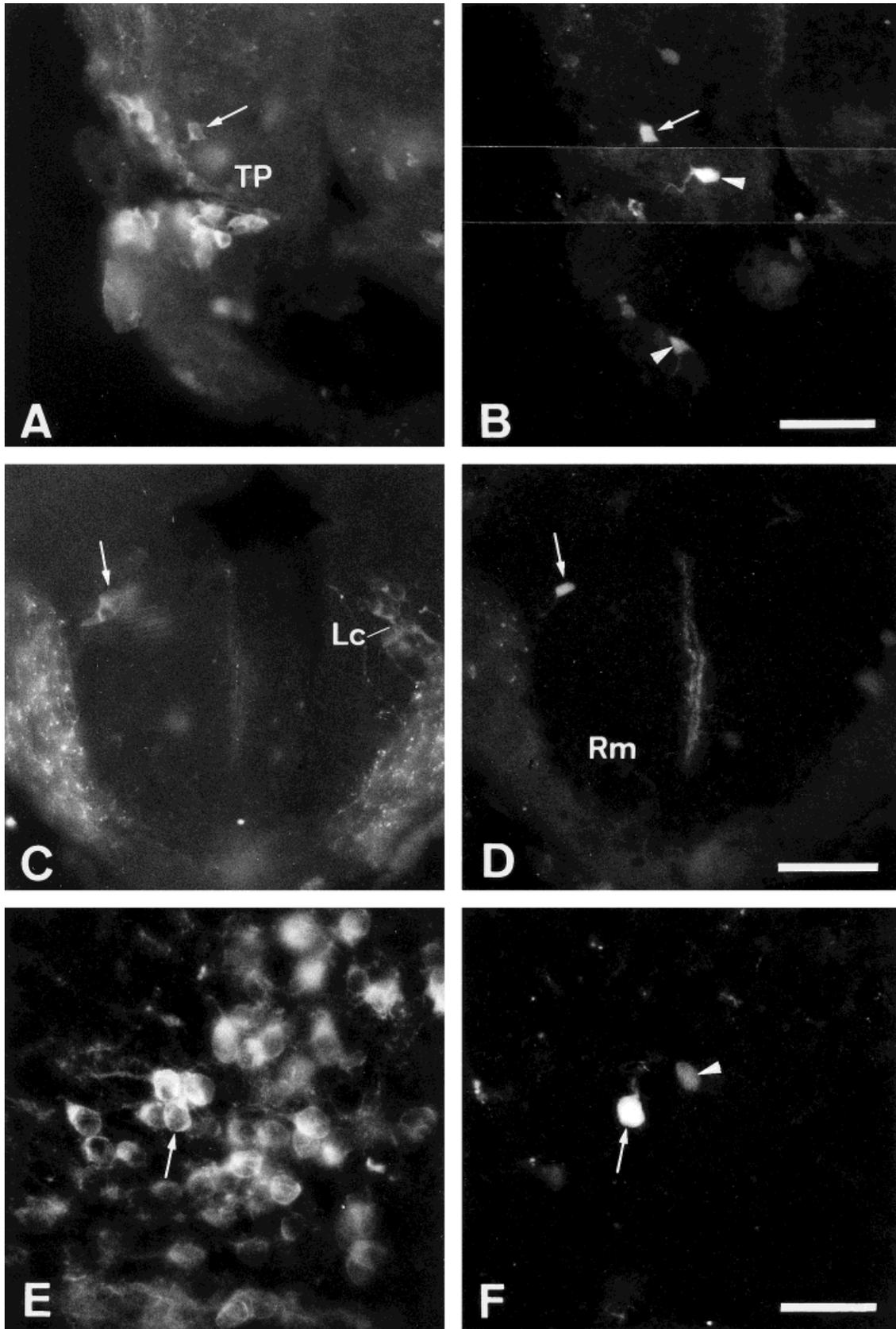


Fig. 13. Photomicrographs illustrating THir cells (A,C,E) and retrogradely labeled cells (B,D,F) in transverse sections through the brain of *Xenopus* tadpoles after BDA applications into the nucleus accumbens. **A,B:** Corresponding photomicrographs of the same section through the rostral aspect of the posterior tubercle at stage 48. **C,D:** The same section at the level of the locus coeruleus at stage 48. **E,F:**

Illustrations of the same section through the midbrain tegmentum at stage 57. In both photomicrographs, medial is to the right. Arrows in all photomicrographs indicate double-labeled cells, whereas arrowheads indicate retrogradely labeled cells that are not THir. For abbreviations, see list. Scale bar = 50  $\mu$ m.

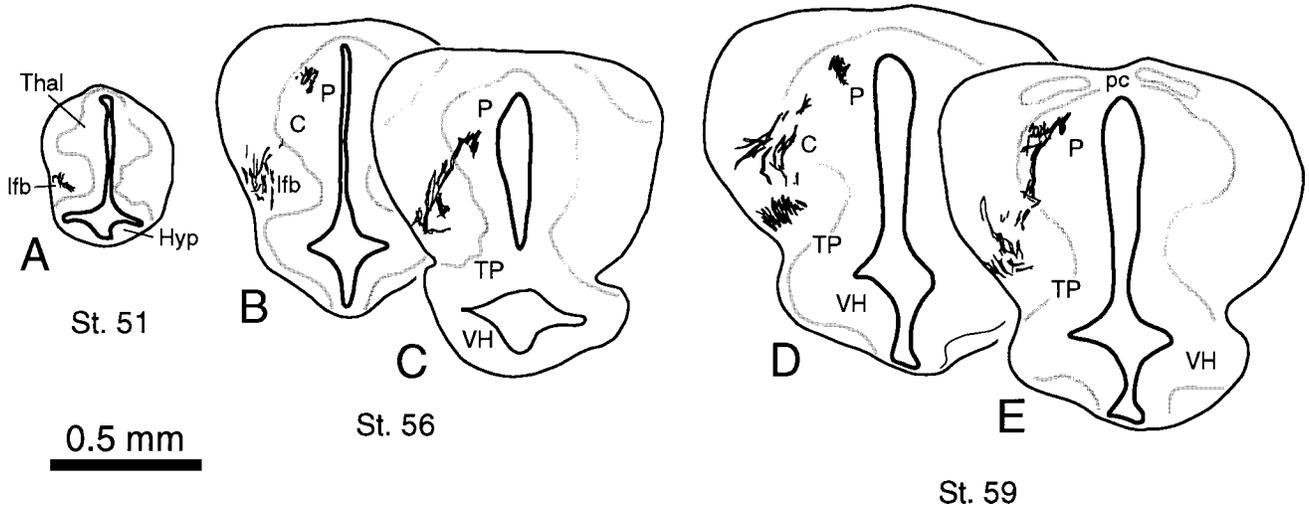


Fig. 14. A-E: Schematic drawings of transverse sections through the brain of *Xenopus* tadpoles at levels of the pretectal region. The progressive innervation of the pretectal areas from the striatum is illustrated for stages 51, 56, and 59. Note that B and D are rostral to C and E, respectively. For abbreviations, see list.

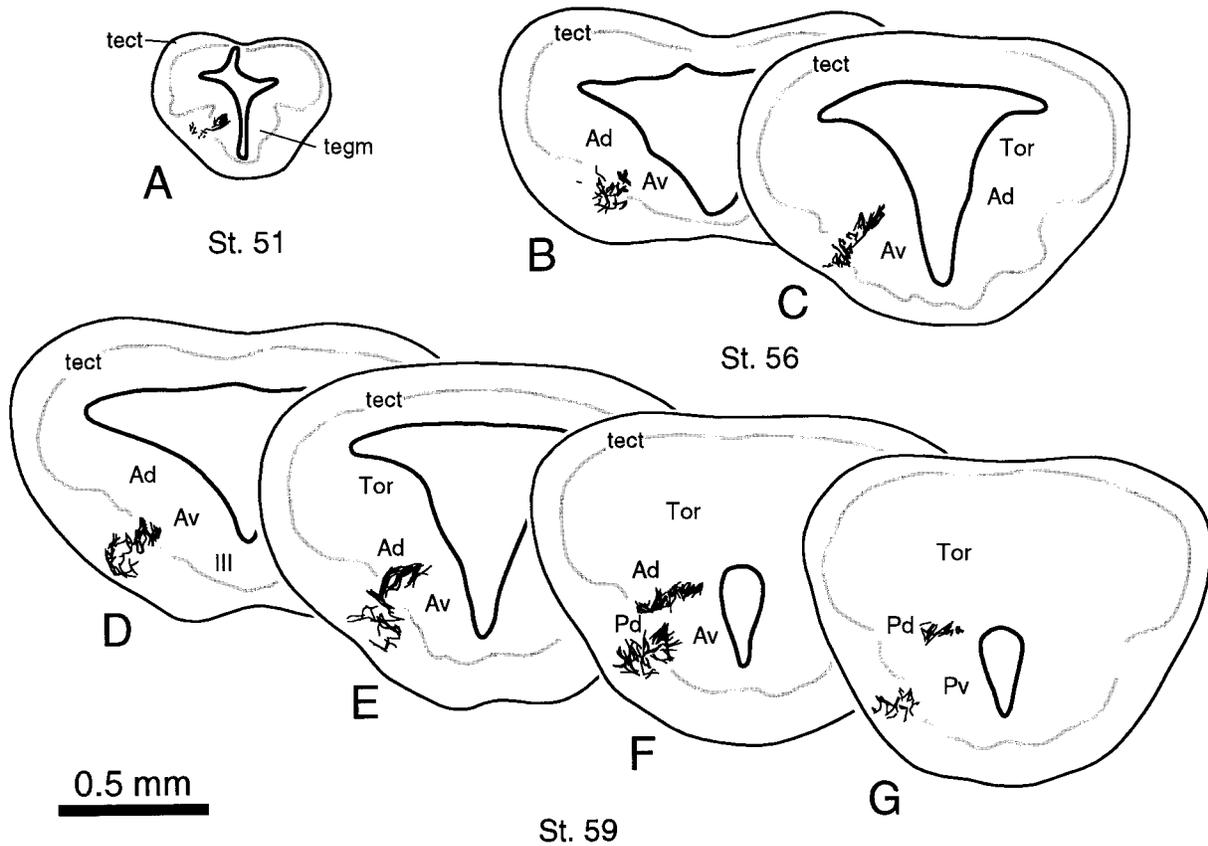


Fig. 15. A-G: Schematic drawings of transverse sections through the brain of *Xenopus* tadpoles illustrating the progressive innervation of the mesencephalic and isthmic tegmental areas from the striatum at stages 51, 56, and 59. Note that B is rostral to C, and D-G are ordered from rostral to caudal. For abbreviations, see list.

## DISCUSSION

In the present study, the developing connections of the basal ganglia have been investigated in amphibians. It should be noted that the afferent connections have been revealed by means of retrograde tracing, and, in some cases, minor spread of the tracers into bordering areas could not be avoided. In those cases, the verification of afferent striatal and/or nucleus accumbens projections from brainstem regions awaits confirmation by means of anterograde tracing.

### General features of the development of striatal and accumbal connections in *Xenopus*

From the present account, it is clear that, already at the beginning of the larval period, distinct sets of projections characterize the ventrolateral and ventromedial regions of the hemispheres. In that perspective, the present study provides further evidence for an anatomical and functional subdivision of the amphibian basal telencephalon into a striatum (caudatoputamen in mammals) and a nucleus accumbens, as proposed previously (González and Smeets, 1991; Marín et al., 1997a–c).

The present study demonstrates that the inputs to the striatum and the nucleus accumbens develop before the end of the embryonic period and show an almost adult appearance at late prometamorphic stages. Moreover, the ingrowth of afferent fibers into the striatum slightly precedes that of the afferent connections to the nucleus accumbens, which is in agreement with the overall development of the hemispheres (Clairambault, 1976). Accordingly, the first catecholaminergic fibers innervating the telencephalon are found in caudal and lateral regions (amygdala and striatum) followed by rostral and medial territories (nucleus accumbens and septum; González et al., 1994a,b; present study).

Compared with the afferent connections, the main efferent connections of the striatum and the nucleus accumbens develop much later. Their development starts at early prometamorphic stages, and the adult-like pattern of efferents is recognized only at metamorphic climax stages. However, as in the case of the afferent connections, the projections of the striatum develop slightly earlier than those of the nucleus accumbens.

### Temporal correlation between the development of striatal and accumbal connections and other developmental events in the central nervous system of anurans

**Diencephalic afferents.** The majority of the early developing inputs to the basal telencephalon of *Xenopus*, as shown by the present study, arises from the diencephalon. Previous studies by Clairambault (1976) and Tay and Straznicky (1982) have shown that the development of the anuran diencephalon starts in its caudoventrolateral portion and then proceeds in a rostradorsomedial direction. Accordingly, the cells of the posterior tubercle region are generated early during development, from stages 20 onward (Tay and Straznicky, 1982). Therefore, it was not surprising that a projection from the posterior tubercle region to the basal telencephalon could already be recognized before the end of the embryonic period (stages 44–45) in the present study. Because cells within the posterior tubercle become immunoreactive for TH and

dopamine as early as stage 39, and the basal telencephalon contains dopaminergic fibers already at stage 41 (González et al., 1994a,b), it seems likely that the first dopaminergic projections from the posterior tubercle reach the telencephalon at that early stage.

Another remarkable finding of the present study is the early development of thalamic projections to the basal telencephalon. A study on the development of somatosensory systems of *Xenopus* (Muñoz et al., 1993) revealed that projections from the dorsal root ganglion cells to the dorsal column nucleus (DCN) develop slightly earlier than those of the DCN to the diencephalon, which can be recognized at stage 51. The projection from the DCN to the thalamus reaches primarily the ventral thalamus, although some fibers terminate in dorsal thalamic regions (Neary and Wilczynski, 1977; Muñoz et al., 1995). The present study provides evidence that a projection from the ventral thalamus to the striatum is present at stages 50–51, approximately at the same time when fibers from the DCN reach the diencephalon (Muñoz et al., 1993). On the other hand, the present study also reveals that projections from the dorsal thalamus to the striatum are established apparently before the first fibers originating from the DCN arrive at the mesodiencephalic border.

The suprachiasmatic nucleus constitutes another source of early developing inputs to the basal telencephalon. Cells located in the lateral aspect of the nucleus are generated early during the development of the diencephalon according to Tay and Straznicky (1982), which is in agreement with the results of the present study. In addition, the present study has demonstrated that the telencephalic projections arising from cells in the rostral part of the suprachiasmatic nucleus develop remarkably earlier than those originating from its caudal part, thus supporting the notion that the anuran suprachiasmatic nucleus may be comprised of at least two distinct components (Puelles et al., 1996).

**Brainstem afferents.** At late embryonic stages, two major sources of inputs to the basal forebrain are noted, i.e., the raphe nucleus and the parabrachial nucleus. The early appearance of projections from the raphe nucleus to the basal telencephalon, as in the case of dopamine, suggests that serotonin plays an important role in the development of the striatum. On the basis of Nissl-stained material, only one raphe nucleus could be recognized in anurans (Nikundiwe and Nieuwenhuys, 1983). However, an immunohistochemical study of the development of the serotonergic system in *Xenopus* by van Mier et al. (1986) revealed the existence of two nuclei, i.e., a nucleus raphes superior extending between the level of the octaval cranial nerve and the midbrain and a nucleus raphes inferior located between the level of the octaval cranial nerve and the obex. The serotonergic input to the spinal cord originates primarily from the inferior raphe nucleus, whereas the superior raphe nucleus gives rise to predominantly ascending projections (van Mier et al., 1986; Tan and Miletic, 1990). The early appearance of telencephalic projection neurons within the superior raphe nucleus has been established by the results of the present study. However, the study by van Mier et al. (1986) indicates that these projections may exist much earlier, because serotonergic ascending projections were already observed at stage 32, reaching the basal telencephalon at stages 35–36 (see Fig. 1 in van Mier et al., 1986).

Apart from the raphe nucleus, the parabrachial nucleus is among the first brainstem cell populations innervating the basal telencephalon. In adult amphibians, the projections from the parabrachial nucleus terminate primarily in the caudal division of the striatum (Marín et al., 1997a), which, on the basis of its connections, has been compared with the mammalian central nucleus of the amygdala (Bruce and Neary, 1995; Marín et al., 1997a,b). The parabrachial nucleus also projects to the caudal aspect of the ventromedial telencephalic wall, but it projects only sparsely to the nucleus accumbens proper. The observed massive input from the parabrachial nucleus into the basal telencephalon during the embryonic period is in line with the development of the telencephalic hemispheres, which follows a caudorostral gradient (Clairambault, 1976). The high numbers of retrogradely labeled cells at late embryonic stages may be explained by the involvement in the injection site of relatively large portions of the caudal striatum and the amygdala. The size and differentiation of the telencephalon increase as the larval period proceeds. This enables more restricted applications of tracers to either the striatum or the nucleus accumbens, which, in turn, lead to lower numbers of labeled cells within the parabrachial nucleus.

**Efferent connections.** Striatal projections to the pretectum as well as to the mesencephalic and isthmic reticular formation are considered to have an indirect, modulatory effect on the optic tectum of anurans (Wilczynski and Northcutt, 1983; Marín et al., 1997b), which plays a key role in the control of visually triggered orienting movements (for review, see Ewert, 1987). The present study has demonstrated that the development of striatal projections to the mesencephalic and isthmic reticular formation follows a rostrocaudal gradient, in parallel to the general development of the brainstem (Straznicki and Gaze, 1972; Lewis and Straznicki, 1979). Striatal fibers reach the mesencephalic tegmentum during the premetamorphic period, but the adult-like pattern is not reached until the end of the prometamorphosis. Striatal projections to the pretectum develop slightly later than striatotelmental projections, reaching a high degree of organization also at the end of the prometamorphic period. Remarkably, Debski and Constantine-Paton (1993) found that the projection from the mesencephalic and isthmic tegmentum to the optic tectum of *Rana pipiens* precedes the formation of pretectotectal connections, but both afferent connections develop during the premetamorphic period, apparently prior to the arrival of the striatal fibers to the brainstem tegmentum and pretectum (present study). Taken together, the available data suggest that the neuronal circuitry underlying striatal modulation of visuomotor behavior is already established at the end of the prometamorphosis. Accordingly, behavioral tests have demonstrated that, at the end of the metamorphosis, the neuronal mechanisms underlying visually oriented behavior in anurans are functional (Ewert, 1987).

### Comparative aspects of the development of the catecholaminergic innervation of the basal forebrain

In contrast to data from adult brains, results from studies of developmental aspects of striatal connections in vertebrates are scarce. Most of these studies have been carried out in neonatal animals that already possess fairly mature striatal connections and, thus, do not provide

substantial information about the ontogeny of these pathways (Morris et al., 1977, 1979; Jacobowitz et al., 1980). However, developmental studies in opossums and rats, animals that are born with rather immature striatal connections, suggest that projections from the thalamus, midbrain dopaminergic groups, and raphe are among the first to reach the striatal anlage (Martin et al., 1989; Iñiguez et al., 1990), similar to what occurs in amphibians (present study). On the other hand, the development of the dopaminergic projections to the striatum of amniotes has been studied immunohistochemically in detail (Specht et al., 1981; Voorn et al., 1988; Medina et al., 1994a,b), enabling a comparison with the development of such pathways in amphibians.

In adult amphibians, the dopaminergic cells in the posterior tubercle and the adjacent medial tegmental region constitute a continuous field along the rostrocaudal axis, extending from the retromammillary region (after Puelles et al., 1996) to the level of the exit of the oculomotor nerve (González and Smeets, 1991, 1994). The generation of these dopaminergic cells starts in the lateral aspect of the posterior tubercle region at the end of the embryonic period and continues in caudomedial direction by rapidly increasing the number of cells (González et al., 1994a,b). Thus, at the beginning of the prometamorphic period, the organization of the dopaminergic cells in the caudal diencephalon and mesencephalon largely resembles that observed in adult amphibians. This continuum of cells forms a V shape along the rostrocaudal axis, with the tip of the V pointing caudally within the mesencephalic medial tegmental region (González and Smeets, 1994; González et al., 1994a).

Comparison of the dopaminergic cell populations in adult amphibians with those of amniotes has commonly led to the conclusion that important differences exist in basal ganglia organization. Moreover, because the dopaminergic cell groups in the ventral tegmental area (A10), the substantia nigra (A9), and the retrorubral area (A8) have been classically considered to originate within the ventral mesencephalon of mammals (Specht et al., 1981), reptiles, and birds (Parent et al., 1986), fundamental differences between amphibians and amniotes appear to exist already during the development of the midbrain dopaminergic cell groups. However, recent reviews by Puelles and Medina (1994) and Smeets and Reiner (1994) have shown that a different conclusion is reached when a segmental approach is applied to the localization of the catecholaminergic cell groups in the brains of vertebrates. Comparison of the dopaminergic cell groups located in the diencephalic and mesencephalic basal plate suggests close topographic similarities among tetrapods (Medina et al., 1994a,b; Puelles and Medina, 1994; Marín et al., 1997c). In that perspective, the A8–A10 complex of amniotes stretches across several segments and is constituted by distinct diencephalic (retromammillary, posterior tubercle, and basal synencephalic), mesencephalic, and isthmic cell groups (Medina et al., 1994a,b; Puelles and Medina, 1994). Similarly, the dopaminergic cell field corresponding to the A10 complex is almost completely present in amphibians, although they seem to lack the isthmic portion of the A10 complex and a laterally migrated substantia nigra and retrorubral area (Marín et al., 1997c).

Misunderstanding of the boundary between the diencephalon and the mesencephalon has also contributed to the confusion about the site of origin of A8–A10 dopaminergic

gic cell groups during development. Thus, as it was recently established by Puelles and Medina (1994) in their elegant study of the segmental development of catecholaminergic neurons in chicks, there is evidence that the development of the dopaminergic cells in the diencephalic and mesencephalic basal plate is roughly similar in tetrapods. Reassessment of the actual limits of the mesencephalon during development suggests that a precocity of the dopaminergic cell groups in the diencephalic prosomeres, compared with those in the mesencephalon, is a common feature of amphibians, birds, and mammals (Voorn et al., 1988; González et al., 1994a,b; Puelles and Medina, 1994). Furthermore, the expression of the TH gene starts rostral to the cephalic flexure and extends afterward into the caudal mesencephalon (Burgunder and Young, 1990).

The present study has provided further evidence for the existence of a common pattern of the development of the dopaminergic innervation of the basal forebrain among tetrapods. First, as in the rat (Voorn et al., 1988), the first cells projecting to the prosencephalon are located in the rostromedial portions of the future A9–A10 complex. Second, early generated dopaminergic cells appear to migrate to their final position after their axons have invaded the target areas in both amphibians and amniotes (Tennyson et al., 1973; Voorn et al., 1988; Medina et al., 1994a,b; Marin et al., 1997c). Third, in all tetrapods studied, the dopaminergic innervation of the dorsal striatum precedes that of the nucleus accumbens (Voorn et al., 1988; González et al., 1994a,b; Medina et al., 1994a,b). Fourth, the arrival of dopaminergic fibers in the striatum precedes the formation of long, descending projections arising from the striatum (Fishell and van der Kooy, 1987; present study). Finally, at the metamorphic climax, the organization of the amphibian dopaminergic cell groups strikingly resembles that of such groups at the beginning of the last week in the rat embryonic development. These groups, as in amphibians, form a V shape along the rostrocaudal axis (see Fig. 38 in Voorn et al., 1988). During the last week of embryonic development, the rat dopaminergic cell groups expand caudally and laterally to occupy the entire lateral zone of the substantia nigra (Voorn et al., 1988). Taken together, all of these data strengthen the notion that the amphibian equivalent of the A9–A10 groups of amniotes represents an early stage in the evolution of these groups, which, most probably, have developed further by increasing their number of neurons and expanding caudally and laterally (Marín et al., 1997c).

### Functional considerations

The present study has shown that the early development of catecholaminergic projections to the basal telencephalon appears to be a common feature not only for amniotes but probably for all tetrapod vertebrates. Numerous studies have suggested that dopamine signaling could play a role in establishing synaptic connections (Lankford et al., 1987, 1988; Rodrigues and Dowling, 1990; Lauder, 1993). It has also been suggested that the arrival of dopaminergic fibers in the striatum may influence the maturation of the striatal neurons (Tennyson et al., 1973; Specht et al., 1981; Voorn et al., 1988; Medina et al., 1994a,b). Nevertheless, depletion of dopamine does not produce substantial cell death of striatal neurons and does not prevent the fundamental organization of the striatum (Lança et al., 1986; Zhou and Palmiter, 1995). On the other hand, an intact connection between the developing substan-

tia nigra and the striatum is crucial for the normal expression of substance P, dynorphin, and opiate receptors in the striatum (van der Kooy and Fishell, 1992; Zhou and Palmiter, 1995). Although dopamine appears to be critically involved in the expression of some striatal markers, a number of experiments have suggested that another factor released by dopaminergic neurons may be implicated in the induction of some striatal markers (Moon, 1984; van der Kooy, 1984). Furthermore, the early appearance of serotonergic cells in the superior raphe nucleus and their projections to the basal forebrain indicate that serotonin may also play a key role in the developing striatum (Whitaker-Azmitia et al., 1995). In that perspective, anurans like the clawed toad, *Xenopus laevis*, offer a unique opportunity to study ontogenic aspects of neuronal connections, because their embryonic development occurs over a prolonged period of time, during which the animal is accessible for experimental studies. Experimental lesions on the developing mesostriatal system in amphibians would certainly contribute to understanding the role of this pathway in the development of striatal function in vertebrates.

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