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Journal of Chemical Neuroanatomy 9 (1995) 27–40

Journal of Chemical Neuroanatomy

Ontogeny of vasotocinergic and mesotocinergic systems in the brain of the South African clawed frog *Xenopus laevis*

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Received 18 January 1995; revision received 10 May 1995; accepted 10 May 1995

Abstract

For a better understanding of the development of neurotransmitter systems and of their putative functional significance during ontogenesis, the development of the vasotocin (AVT) and mesotocin (MST) systems in the brain of *Xenopus laevis* was studied by means of immunohistochemical techniques. Weakly immunoreactive fibers were already present at late embryonic stage 38 in the caudoventral part of the telencephalon and in the ventral part of the diencephalon. The earliest immunodetectable AVT and MST immunoreactive cell bodies were found in the developing preoptic area at late embryonic stage 43. At the end of the embryonic period (stage 45), AVT immunoreactive fibers have reached the future medial amygdala, the midbrain tegmentum, the median eminence and the neural lobe of the pituitary. When compared with AVT immunoreactive fibers, the development of MST fibers shows some temporal delay. During the premetamorphosis (stages 45–52), AVT immunoreactive cell bodies appear in the medial part of the suprachiasmatic nucleus, the dorsal infundibular region, and the midbrain tegmentum, whereas fibers can now be traced to the nucleus accumbens, the septum and the medial amygdala in the forebrain, to the midbrain tegmentum, the reticular formation, the raphe nuclei, and the solitary tract nucleus in the brainstem, and to the spinal cord. Further maturation of the AVT system during prometamorphosis (stages 53–58) includes the appearance of immunoreactive cell bodies in the lateral part of the suprachiasmatic nucleus, the ventral preoptic area, and the dorsal infundibular region. By the end of the metamorphosis (stage 65), the maturation of the AVT/MST systems reaches an almost adult-like pattern. It should be noted that in amphibians, in contrast to mammals, the early appearance of the AVT/MST systems, including their extensive extrahypothalamic component, suggests that the two neuropeptidergic systems may play a significant role during development.

Keywords: *Xenopus laevis*; Brain; Vasotocin; Mesotocin

Abbreviations: Acc, nucleus accumbens; al, anterior lobe of the pituitary; Apl, amygdala, pars lateralis; Apm, amygdala, pars medialis; Cb, cerebellum; DB, diagonal band of Broca; Dp, dorsal pallium; em, eminentia mediana; H, ganglion habenulae; h-h, hypothalamo-hypophysial tract; Hyp, hypothalamus; III, nucleus nervi oculomotorii; Is, nucleus isthmi; Lp, lateral pallium; Ls, lateral septum; Mg, magnocellular preoptic nucleus; Mp, medial pallium; nl, neural lobe of the pituitary; on, optic nerve; POa, anterior preoptic area; Ri, nucleus reticularis inferior; SC, nucleus suprachiasmaticus; sol, solitary tract; Str, striatum; Tect, tectum mesencephali; tegm, tegmentum mesencephali; Thal, thalamus; Thal d, thalamus dorsalis; Thal v, thalamus ventralis; Tor, torus semicircularis; TP, tuberculum posterius; v, ventricle; VH, ventral hypothalamic nucleus; VM, ventromedial thalamic nucleus; Vm, nucleus motorius nervi trigemini.

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1. Introduction

The presence in the central nervous system of vasopressin/oxytocin (VP/OT) related peptides is a common feature not only of vertebrates (Acher, 1981; Acher and Chauvet, 1988; Sherwood and Parker, 1990), but also of invertebrates (Proux et al., 1987; Sherwood and Parker, 1990; Davis and Hildebrand, 1992; Reich, 1992). In mammals, VP and OT are well-characterized nonapeptides, which are present in magnocellular neurons of the hypothalamic supraoptic and paraventricular nuclei. Both peptides are synthesized in the hypothalamus as preprohormones and are processed

0891-0618/95/$09.50
SSDI 0891-0618(95)00063-D

during axonal transport for storage in, and release from, the neurohypophysis (Brownstein et al., 1980; Poulain and Wakerley, 1982; Mezey et al., 1987; Whitnall, 1993). Vasopressin promotes the reabsorption of water by the kidney and plays an important role in cardiovascular regulation in some species (Berecek and Swords, 1990), whereas OT is involved in parturition and lactation (e.g. Argiolas and Gessa, 1991; Kendrick et al., 1991). Moreover, during the past decade it has been shown that, apart from a strong involvement in the hypothalamo-pituitary axis, VP and OT containing fibers distribute widely throughout the brain and, therefore, may function not only as hormones, but also as neurotransmitters or neuromodulators (Buijs and Heerikhuize, 1982; Buijs, 1983; Argiolas and Gessa, 1991).

In nonmammalian vertebrates, the neuropeptides arginine vasotocin (AVT) and mesotocin (MST)/isotocin (IST) are present, which are considered equivalent to the mammalian VP and OT respectively. Studies of the distribution of these peptides in the brains of teleosts (Van den Dungen et al., 1982; Batten et al., 1990), reptiles (Stoll and Voorn, 1985; Thepen et al., 1987; Smeets et al., 1990), and birds (Kiss et al., 1987; Voorhuis and De Kloet, 1992) have revealed well-developed hypothalamo-hypophysial systems as well as extensive extrahypothalamic networks of immunoreactive fibers. Similar observations have been made in amphibians (Jokura and Urano, 1987; Boyd et al., 1992; González and Smeets, 1992a, 1992b), thus confirming the phylogenetic constancy of these peptidergic systems in vertebrates.

The widespread distribution of immunoreactive fibers in the CNS of amphibians strongly suggests that, as in mammals, AVT and MST act as neurotransmitters or neuromodulators. The variety of functions suggested by the distribution of the two neuropeptides throughout the brain is still the subject of investigation. Except for their involvement in reproductive behavior (e.g. Boyd, 1991, 1992; Penna et al., 1992; Moore et al., 1992), very little is known about the roles of AVT and MST in amphibian brains.

A way to get more insight into the putative roles of these peptides is by studying the development of these systems in relation to other developmental events, as has been done previously for catecholamines and neuropeptide Y (González et al., 1994a, 1994b; Tuinhof et al., 1994). Unfortunately, studies of the development of AVT in the brain of amphibians are limited both in number and in detail (Carr and Norris, 1990; Boyd, 1994), and they are totally lacking for MST. The aim of the present study was, therefore, to provide a detailed account of the development of AVT and MST systems in the brain of the South African clawed frog, *Xenopus laevis*. The choice of *Xenopus* was favored because of the possibility of hormone-induced breeding, the availability of an accurate timetable of development (Nieuwkoop and Faber, 1967), and the ease of maintenance of this species under laboratory conditions. An additional advantage is that neither the developing nor the adult brain of *Xenopus* contains much of the neuromelanin that obscures the immunoreactive cell bodies and fibers in other amphibians (González et al., 1994a). A preliminary report of this study has been presented at the 17th meeting of the European Society of Comparative Endocrinology in Córdoba, Spain (González et al., 1994c).

2. Methods

For this study, 70 *Xenopus laevis* tadpoles, ranging from developmental stage 38 through stage 65 and juveniles (after Nieuwkoop and Faber, 1967), were used. *Xenopus* larvae were obtained by Pregnyl (Organon, Oss, The Netherlands)-induced breeding and kept in tap water at 20–25°C throughout their development. The larvae were raised on nettle powder or canned spinach, whereas older larvae and young froglets were fed Tubifex. At appropriate times, embryos and tadpoles were anaesthetized in a 0.3% solution of buffered tricaine methanesulphonate (MS 222, Sandoz) and, subsequently perfused transcardially with saline followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). The brains were then removed and postfixed in the same fixative mixture for 1–2 h at room temperature. Animals that were too small to be perfused were fixed by immersion in the mixture just mentioned.

After fixation, the brains and eyes were immersed in a solution of 30% sucrose in 0.1 M phosphate buffer for 3–7 h, embedded in a solution of 15% gelatin with 30% sucrose, and stored for 5–7 h in a 4% formaldehyde solution at room temperature. The gelatin blocks were cut on a freezing microtome at 30 μm thickness in the transverse plane. The sections were collected in phosphate buffer and rinsed three times for 15 min in Tris-buffered saline (TBS). They were subsequently processed immunohistochemically according to the peroxidase–antiperoxidase (PAP) technique (Sternberger, 1979), using an arginine–vasotocin antiserum (provided by Dr. R.M. Buijs, Netherlands Institute for Brain Research, Amsterdam; diluted 1:5000) or an isotocin antiserum (donated by Dr. J.M. Guerné, Université de Strasbourg, France; diluted 1:2000). Working dilutions were used and tested for amphibian brains in previous studies (González and Smeets, 1992a, 1992b). All incubations were performed in TBS with 0.5% Triton X-100, pH 7.6. Since the primary antibodies were raised in rabbits, a second swine anti-rabbit (Nordic) serum was used, diluted 1:50, for 60 min. Subsequently, the sections were incubated in a rabbit PAP complex (Dakopatts, diluted 1:800) for 45–60 min and, after rinsing again three times in TBS, stained in 0.5 mg/ml 3,3-

diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in TBS for 10–20 min. In most cases, visualization was enhanced by adding 3.8% of a solution of 1% ammonium nickel sulfate to the DAB–H_2O_2 mixture. Finally, the sections were mounted on glass slides (mounting medium: 0.25% gelatin in Tris buffer, pH 7.6) and, after drying overnight, coverslipped. Some sections were counterstained with cresyl violet.

The specificity of the antisera was tested previously (Thepen et al., 1987). It was proved that the isotocin antiserum cross-reacts with MST and, to a much lesser extent, with AVT. The latter cross-reaction, however, was lowered to a non-immune serum level by adsorption of the antibody with AVT beads. Similarly, before adsorption, a cross-reaction of the AVT antiserum with MST was found. By adsorption with MST beads, the reaction for MST was reduced to the non-immune serum level. In the present study, we have used the antisera that were adsorbed to beads with the cross-reacting peptides. As a further control, we omitted the primary antisera from a few test sections in each experiment. No specific labelling of somata or fibers was found in these sections. The nomenclature in the present study is essentially the same as that used in our previous studies (González and Smeets, 1992b; González et al., 1994a).

3. Results

The development of the cell bodies and fibers immunoreactive for AVT or MST has been studied in the brain of embryos, larvae and froglets of *Xenopus laevis*, for which a detailed table of development is available (Nieuwkoop and Faber, 1967). The development of this species covers a period of about 60 days, systematically subdivided by Nieuwkoop and Faber into 66 stages and grouped into four successive sets of stages, as follows. (1) Late embryonic stages, extending over a rather long period of time which begins with the appearance of the operculum, an ectodermal evagination that covers the external gills. The gills slowly disappear at the end of the embryonic life and the larval period starts when they are totally resorbed. (2) Premetamorphic stages, during which the tadpole merely grows in size and small buds of the hindlimbs are formed on the lateral side of the body. (3) Prometamorphic stages, characterized by the progressive formation of the hindlimbs. This period ends when the length of the tail is at its maximun and the more drastic changes of the metamorphosis start. (4) Metamorphic climax, marking the period in which the transformation of the tailed larval form into the tailless, four-legged juvenile occurs.

In the following sections, we describe the development of AVT- and MST-immunoreactive (AVTi, MSTi, respectively) cell bodies and fibers, keeping these generalized sets of stages in mind. It should be noted that the pattern of AVT and MST immunostaining observed in animals, fixed at the same stage of development, was generally consistent. However, small variations were occasionally observed between animals taken at the same stage and treated identically. Such small variations have been observed before in developmental studies using immunohistochemical techniques (González et al., 1994a, 1994b; Tuinhof et al., 1994). They might be due either to variations in internal development between individuals that are staged exclusively on the basis of external morphological features or to small variations in the technical procedures.

3.1. Late embryonic stages

The first weakly AVTi and MSTi fibers in the brain of *Xenopus* were observed at embryonic stage 38. Weak-

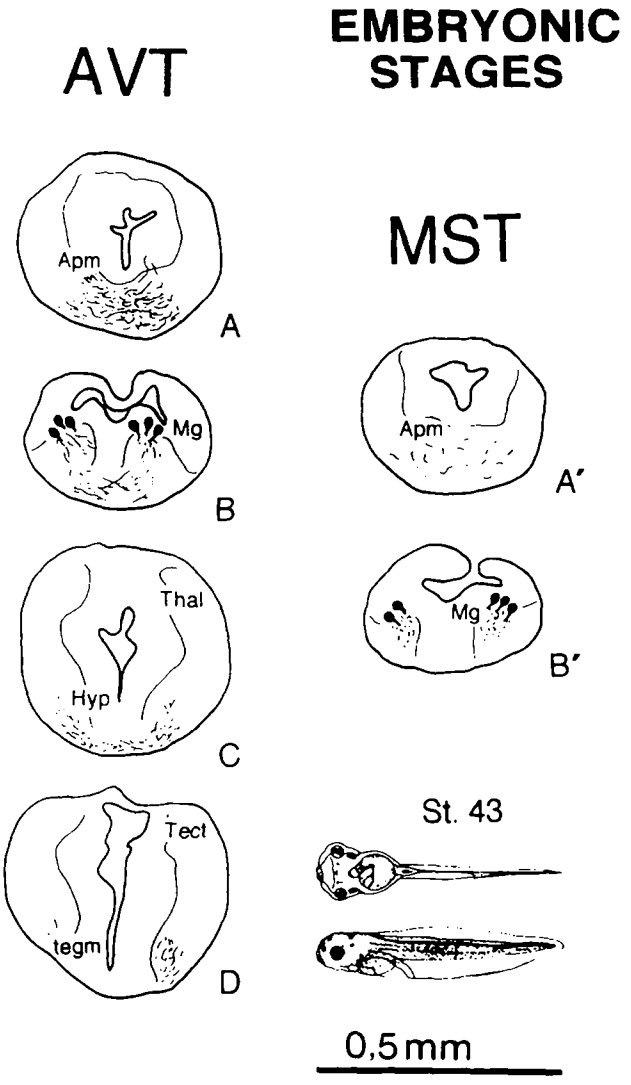

Fig. 1. Diagrams of transverse sections from rostral (A) to caudal (D) through the brain of a *Xenopus* tadpole at *stage 43*, showing the localization of AVTi cell bodies (large dots) and fibers (small dots, wavy lines). Sections 1A′ and 1B′, which are adjacent to sections 1A and 1B, show MSTi cell bodies and fibers.

ly immunoreactive fibers were found in the ventral aspect of both the caudal telencephalon and the diencephalon. The number of AVTi and MSTi fibers as well as the intensity of staining increase considerably during stages 41–43 when the animals are just prior to hatching and independent feeding. The first immunodetectable cell bodies appear in the caudal telencephalon at stage 43 (Figs. 1B, 1B′, 2a). The 5–8 cells on each side of the brain are round and possess short, thin processes that are directed ventrally. At this

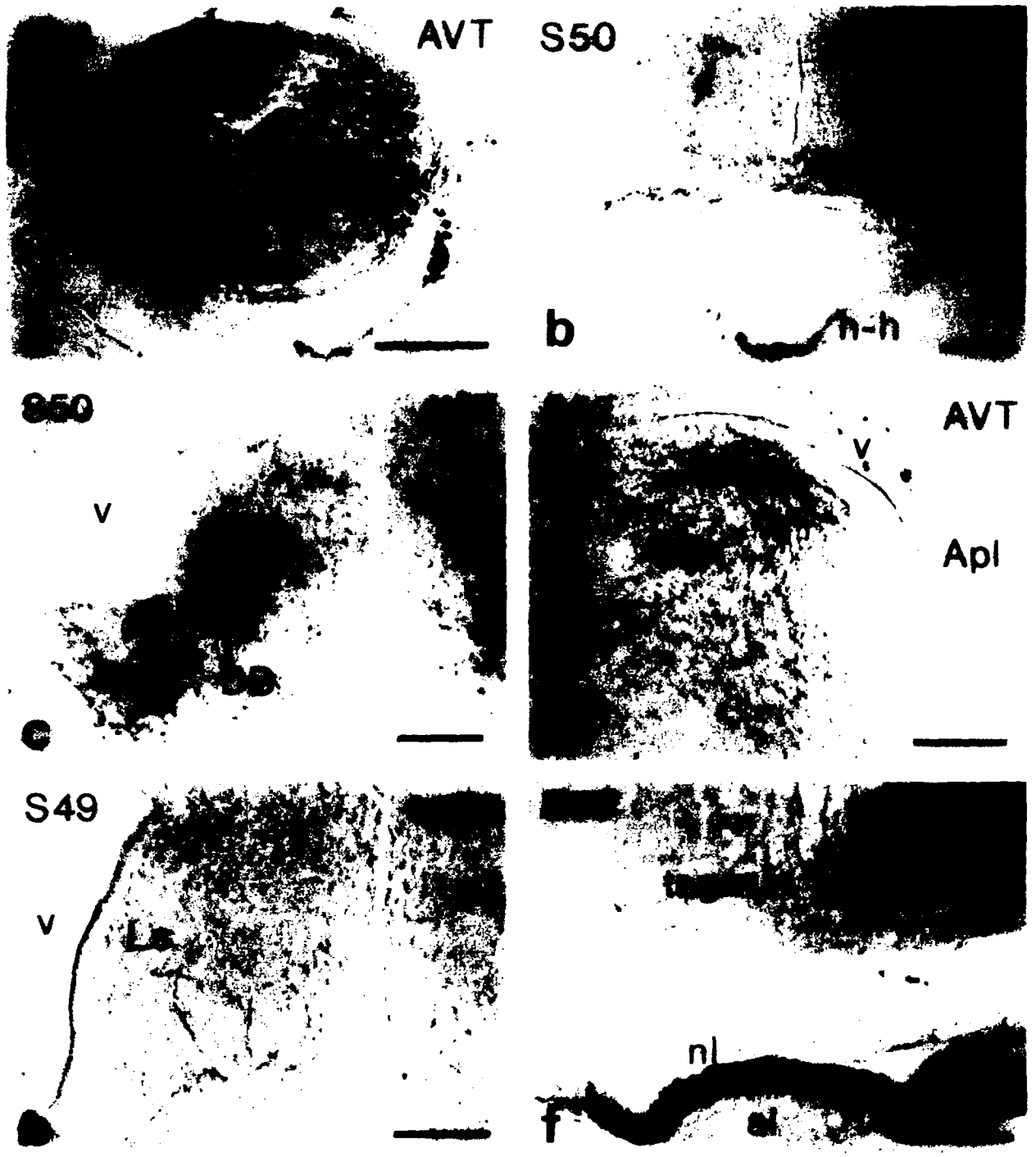

Fig. 2. Photomicrographs of transverse sections through the brain of *Xenopus laevis* tadpoles, showing the localization of AVTi cells and fibers in: (a) the diencephalon at *stage 43* (arrows point to immunoreactive cell bodies); (b) the mesencephalic tegmentum and infundibulum at *stage 50* (arrows point to AVTi cell bodies); (c) the rostroventromedial telencephalon at *stage 50*; and (d) the amygdala, pars medialis at *stage 52*. (e) and (f) show MSTi fibers in the rostro-ventromedial wall of the telencephalic hemisphere at *stage 49* and in the neural lobe of the pituitary at *stage 50*, respectively. Scale bars, 100 μm.

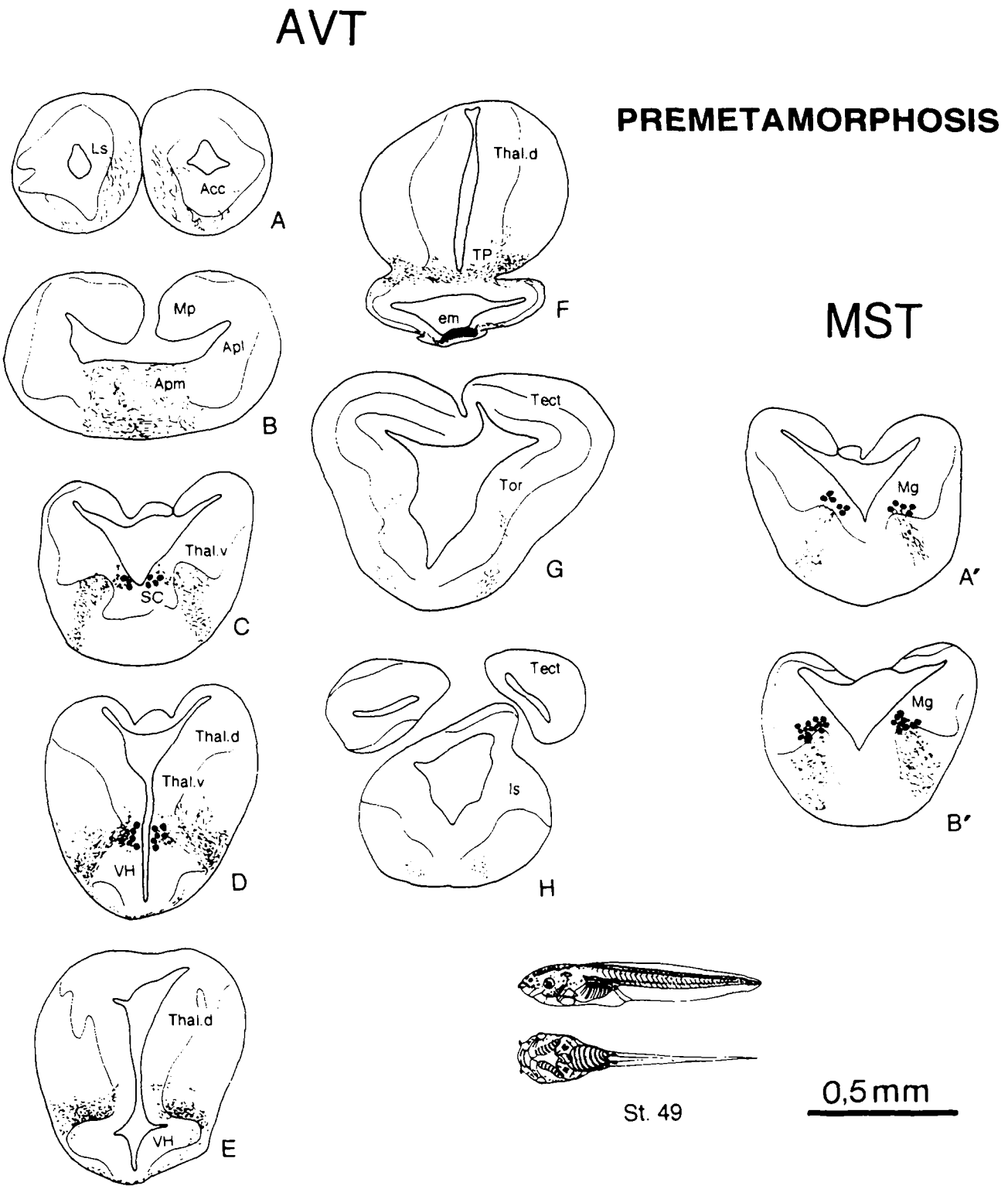

Fig. 3. Diagrams of transverse sections from rostral (A) to caudal (H) through the brain of a *Xenopus* tadpole at *stage 49*, showing the distribution of AVTi cell bodies (large dots) and fibers (small dots and wavy lines). Sections 3A′ and 3B′, which are adjacent to section 3C, illustrate the localization of the MSTi cell bodies at these levels.

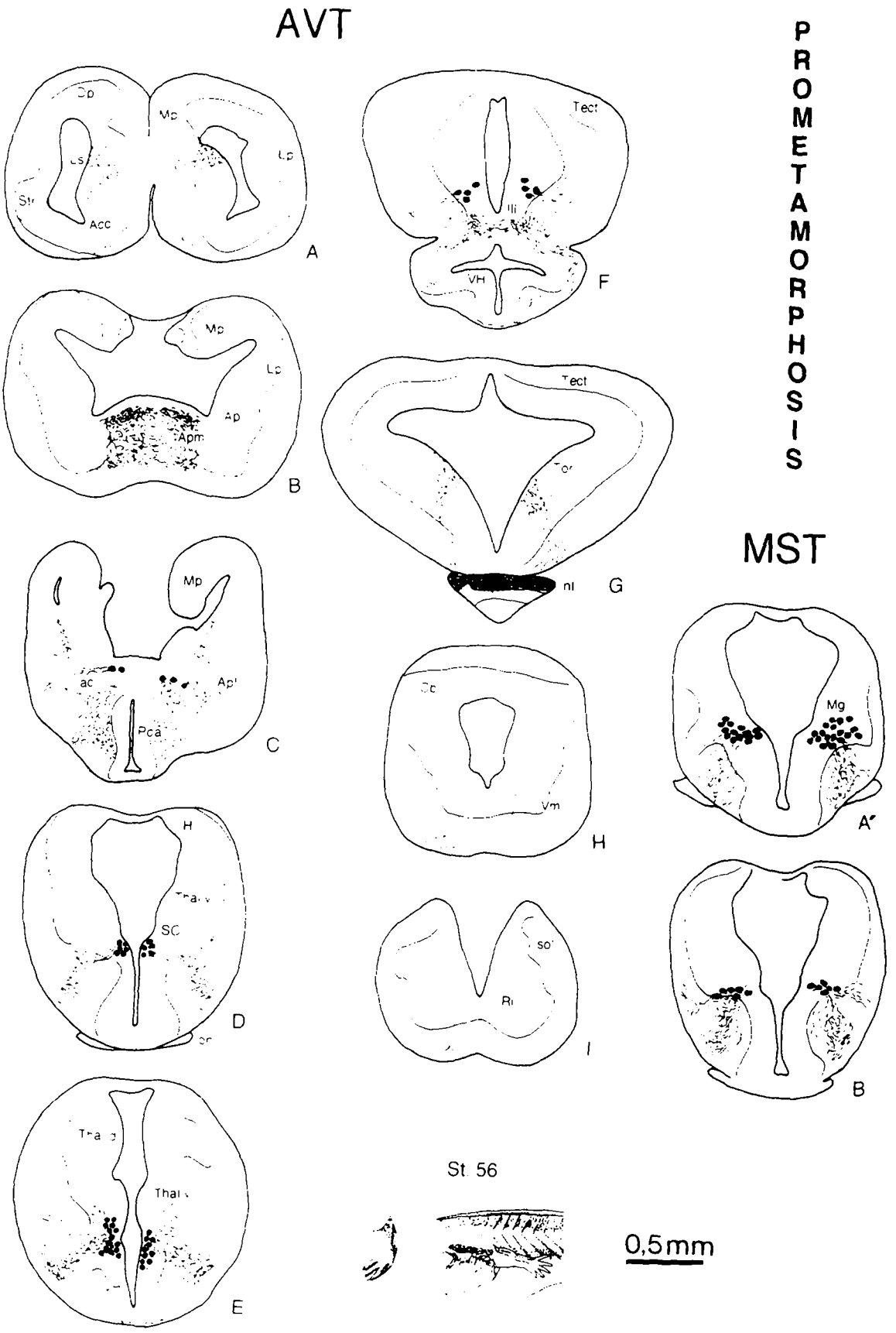

Fig. 4. Diagrams of transverse sections from rostral (A) to caudal (I) through the brain of a *Xenopus* tadpole at *stage 56*, showing the distribution of AVTi cell bodies (large dots) and fibers (small dots, wavy lines). Sections 4A′ and 4B′, which are adjacent to section 4D, illustrate the localization of the MSTi cell bodies at these levels.

stage, distinct immunoreactive fiber plexuses are present in the ventral telencephalon, i.e. the future amygdala pars medialis (Fig. 1A), and the ventral diencephalon (Fig. 1C). Also, the midbrain tegmentum contains AVTi fibers (Fig. 1D). From stage 45 onwards, immunoreactive fibers are found in the median eminence and the neural lobe of the pituitary.

During all these late embryonic stages (38–45), the location of AVTi and MSTi cell bodies is identical and also their morphology shows the same degree of development. However, a considerable difference is observed between the numbers of AVTi and MSTi fibers. The general impression is that, throughout the brain, the number of AVTi fibers exceeds that of MSTi fibers (compare Figs. 1A and 1B with Figs 1A′ and 1B′). The latter are almost exclusively confined to the ventral forebrain.

3.2. *Premetamorphic stages*

During the premetamorphosis, a rather complex development of AVTi and MSTi cells and fibers occurs in the hypothalamo-hypophysial system as well as in extrahypothalamic areas. At stage 49, weakly AVTi cell bodies are found close to the ventricle in the medial part of the suprachiasmatic nucleus (Fig. 3C) and, more caudally, in the dorsal infundibular region (Fig. 3D). Starting with stage 50, weakly AVTi cell bodies are present in the rostral part of the midbrain tegmentum (Fig. 2b) and in the caudal telencephalon intermingled with fibers of the anterior commissure. It should be emphasized that MSTi cell bodies were never observed in the suprachiasmatic nucleus, the dorsal infundibular region, the rostral midbrain tegmental area, or the anterior commissure (see Fig. 8). The distribution of MSTi cells appeared to be restricted to the area just rostral to the incipient optic chiasm, slightly dorsal and lateral to the AVTi perikarya (Figs. 3A′, 3B′).

The maturation of the AVTi/MSTi fiber systems also proceeds during the premetamorphic stages. Distinct plexuses of AVTi fibers are found in the ventromedial part of the telencephalic hemisphere (nucleus accumbens, septal region; Figs. 2c, 3A) and the medial amygdala (Figs. 2d, 3B). In the diencephalon, a conspicuous plexus of AVTi fibers is present in the ventrolateral hypothalamus (Fig. 3D), of which the majority courses caudoventrally and, via the internal zone of the median eminence (Figs. 2b, 3E, 3F), enters the neural lobe of the pituitary. Moreover, a dense plexus of AVTi fibers is already present in the posterior tubercle (Fig. 3F) and the ventral tegmentum of the mesencephalon where a number of fine fibers reach the developing torus semicircularis (Fig. 3G). A bilateral bundle of AVTi fibers can even be traced to rostral rhombencephalic levels occupying a ventromedial position and sending off fibers to the reticular formation and raphe nuclei (Fig. 3H). In sharp contrast to the AVTi fiber systems, the maturation of the MSTi fiber systems seems to stay behind. During premetamorphic stages, the extent of the distribution of MSTi fibers is still limited. Except for the medial amygdala, the ventrolateral hypothalamus (Figs. 3A′, 3B′), the median eminence and the neural lobe (Fig. 2f), where rather dense plexuses of MSTi fibers are found, only a few scattered fibers are present in the lateral septal region (Fig. 2e) and the lateral part of the midbrain tegmentum. Although few in number, MSTi fibers can be traced caudally to the nucleus of the solitary tract and even to the rostral spinal cord levels, where a plexus is found dorsolateral to the central canal.

3.3. *Prometamorphic stages*

During the prometamorphosis, the AVT/MST systems of the *Xenopus* larvae gain almost all the features of the corresponding systems in the adult brain (Fig. 4). The maturation of the AVTi cell groups during pro-

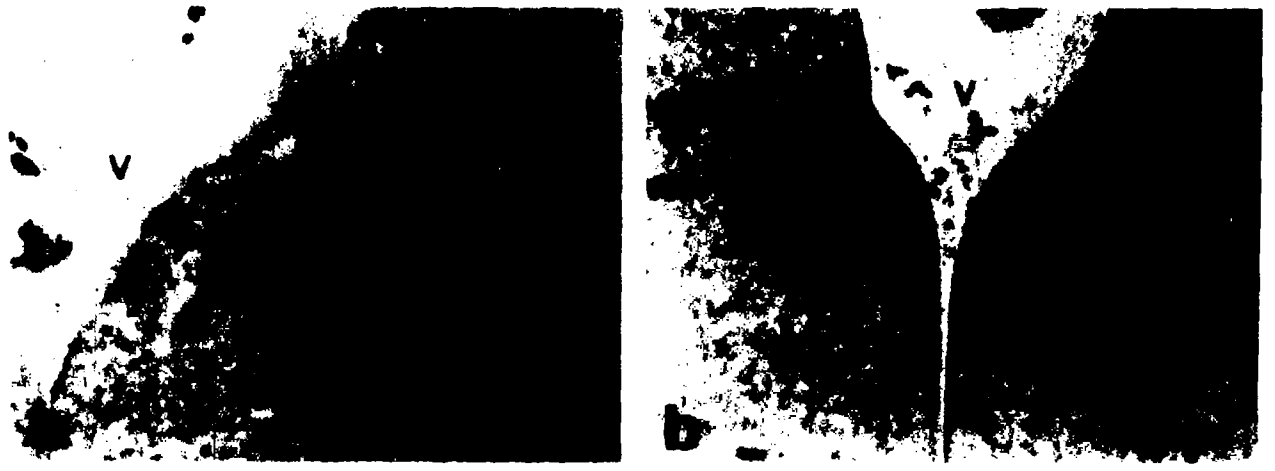

Fig. 5. Photomicrographs of transverse sections through the brain of *Xenopus laevis* during *prometamorphosis* (stages 55–56), showing MSTi cell bodies in the caudal (a) and rostral (b) parts of the magnocellular nucleus. Scale bars, 100 μm.

metamorphic stages is characterized by a substantial increase in number of cells in all areas previously described. Moreover, from stages 54–55 onwards, AVTi cells are found not only in the medial, but also in the lateral part of the suprachiasmatic nucleus (Fig. 4D). The last AVTi cell groups to appear are those in the ventral part of the preoptic area (Fig. 4C) and in the ventral infundibulum.

With respect to the distribution of MSTi cell bodies, a few cells are found in the lateral part of the suprachiasmatic nucleus at caudal preoptic area levels. The development of MSTi cell bodies in the magnocellular nucleus parallels that of AVTi cells in time but not in location (Figs. 4A′, 4B′). By mid-prometamorphic stages (54–56), the MSTi cells in the caudal part of the magnocellular nucleus (Fig. 5a) stain more intensely than those in the rostral part (Fig. 5b). The AVTi and MSTi cells in the magnocellular nucleus tend to show a gradient in development from dorsocaudal (stage 52) to rostroventral (stage 56).

A profuse innervation of the brain with AVTi fibers is achieved during prometamorphosis. The medial wall of the telencephalic hemispheres contains many immunoreactive fibers which occur predominantly in the nucleus accumbens, the lateral septal area, the diagonal band of Broca and the amygdala pars medialis (Figs. 4A, 4B). A few fibers are present in the olfactory bulb, the medial pallium, the dorsal striatum, and the amygdala pars lateralis. The most striking innervation is found in the the anterior preoptic area (Fig. 4C) and the lateral hypothalamus (Fig. 4E). In the midbrain, rather dense plexuses of AVTi fibers are observed at these stages in the tegmentum and the torus semicircularis (Figs. 4F, 4G). A considerably smaller number of fibers continues caudally into the rhombencephalic tegmentum (Figs. 4H, 4I), occupying a ventromedial position.

The overall distribution of MSTi fibers in the brains of *Xenopus* larvae during the prometamorphosis is less conspicuous than that of AVTi fibers. Nevertheless, an almost parallel distribution is found in the forebrain where the lateral septum, the amygdala pars medialis, the anterior preoptic area and the lateral hypothalamus are the most densely innervated regions (Figs. 4A′, 4B′). In contrast, the brainstem shows a predominance of MSTi fibers. They are abundantly present in the torus semicircularis, in an area just medial to the isthmic nucleus, the nucleus of the solitary tract, the rhombencephalic reticular formation and the spinal cord. Therefore, while AVTi fibers seem to be the major component in the forebrain, MSTi fibers likely play a major role in brainstem functioning during prometamorphosis. By the end of the prometamorphosis (stages 58–59), the distribution of AVT and MST immunoreactivities is essentially the same as in the previous stages, but some immunoreactive fibers now reach the dorsal thalamus and the mesencephalic tectum.

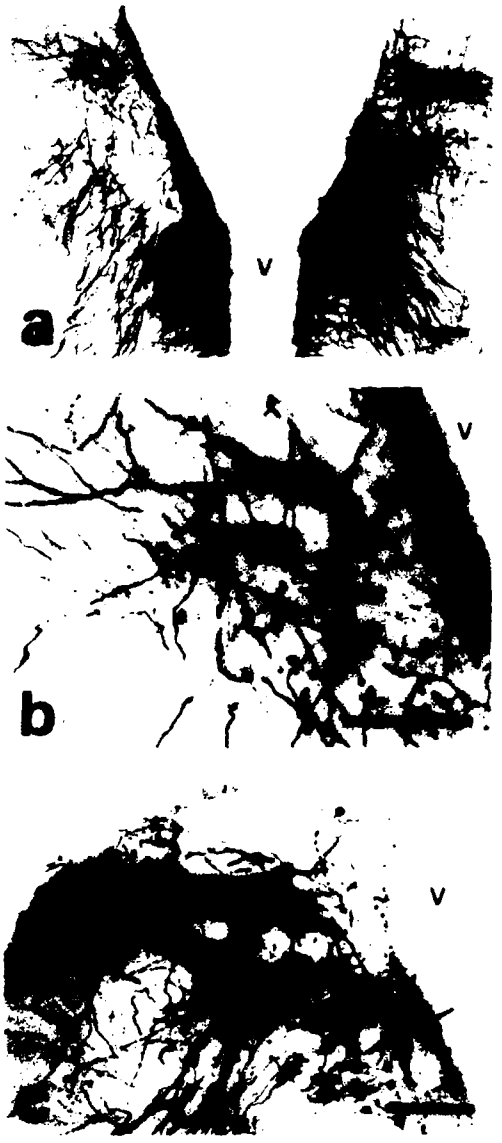

Fig. 6. Photomicrographs of transverse sections through the brain of *Xenopus laevis* at juvenile stages, showing AVTi cell bodies in the magnocellular nucleus. (a) shows the localization of the ventral and dorsal populations of immunoreactive cells within the nucleus; (b) shows, at a higher magnification, the morphology of the cells in the dorsal group in more detail; (c) shows AVTi cell bodies in the caudal part of the magnocellular nucleus where the parvocellular component is also observed (arrow). Scale bars, 100 μm.

3.4. Climax of the metamorphosis

During the metamorphosis from larvae to juvenile froglets, the maturation of the AVT/MST systems continues, reaching an adult-like pattern by stage 65. Regarding the distribution and morphology of the AVTi and MSTi cells, several features require comment. In the preoptic magnocellular nucleus, dorsal and ventral subdivisions, as seen in adult brains, are now recognized (Fig. 6a). The dorsal group consists of multipolar neurons which lie separated from the ventricle. These cells possess long processes that extend primarily in lateral

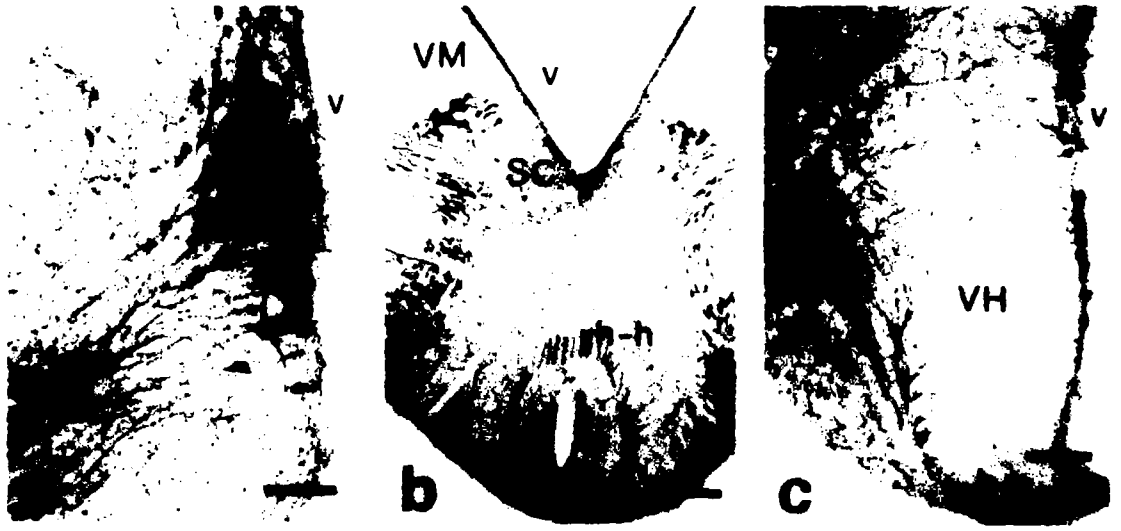

Fig. 7. Photomicrographs of transverse sections through the brain of *Xenopus laevis* at juvenile stages showing MSTi cell bodies in the anteroventral portion of the magnocellular nucleus (a), and in the caudal pole of the lateral suprachiasmatic nucleus (b). (c) shows AVTi cells and fibers at infundibular levels. Scale bars, 100 μm.

and dorsal directions, although smaller processes constitute a plexus that lines the ventricle (Fig. 6b). The ventral group is constituted by numerous cells that lie close to the ventricle and possess long varicose processes that are directed ventrolaterally (Fig. 6a). These cells are usually arranged in parallel laminae and frequently show club-like processes that extend to the ventricle. Caudally, a population of small cells, immunoreactive

Fig. 8. Diagrams of transverse sections through the brain of *Xenopus laevis* at juvenile stages. The upper row shows the distribution of AVTi cell bodies (large dots); the lower row presents the location of MSTi perikarya in adjacent sections. Arrows point to the sites where exclusively AVTi cells are present.

for both AVT or MST, is observed ventral to the magnocellular elements (Fig. 6c). Although their location is the same, CSF-contacting MSTi cells in the magnocellular nucleus are less numerous than AVTi cells (Fig. 7a).

In the posterior part of the suprachiasmatic region, both AVTi and MSTi cells occur in the lateral part of the suprachiasmatic nucleus (Fig. 7b), but only AVTi perikarya are found in its medial portion. Caudally, exclusively AVTi cells are observed close to the ventricle in the hypothalamic infundibular region (Fig. 7c). Thin processes of these cells course laterally into the lateral hypothalamus. In the brainstem the group of AVTi cells observed earlier in development is still represented by a few cells located dorsolateral to the oculomotor nucleus. A comparison of the distribution of AVTi and MSTi cells, as observed in adjacent transverse sections of the brain of juvenile froglets (Fig. 8), shows that there are several places where no codistribution occurs.

The distribution pattern of AVTi and MSTi fibers is also becoming adult-like at juvenile stages. When compared with that of previous stages, a clear maturation has taken place as characterized by the presence of a higher number of fibers and varicosities in every region. Among the last brain regions that become innervated are the ventral habenula, the lateral dorsal thalamus and the mesencephalic tectum, all of which contain primarily AVTi fibers. Also, the MSTi plexuses in the pretrigeminal region, the nucleus of the solitary tract and the spinal cord, already present at prometamorphic stages, are now much more distinct.

4. Discussion

4.1. Comparative aspects of development of AVT and MST systems in the brain of amphibians

From the above account, it is clear that the two neuropeptidergic systems develop early during embryonic stages. In the earliest stage studied, i.e. stage 38, weakly AVTi as well as MSTi fibers are already present in the ventral forebrain. However, immunoreactive cell bodies were not observed until stage 43. Although we did not find a temporal difference in the onset of the AVT and MST systems, an obvious difference was observed in the maturation of both systems, the MST system staying behind the AVT system. This was especially noted for the development of the fiber systems. Nevertheless, at the end of the metamorphosis both systems have gained an almost adult-like appearance.

An explanation for the observation that fibers stain at earlier embryonic stages than cell bodies might be that the content of AVT/MST within perikarya is below the detection level of the immunohistochemical technique used. This argument is supported by previous studies in amphibians (Kar and Naik, 1987) as well as mammals (Sinding et al., 1980; Whitnall et al., 1985) which show that cell bodies are immunoreactive to neurophysin at a considerably earlier stage of development. Since antibodies against neurophysin frequently recognize also the prohormones of AVT/MST in nonmammalians and VP/OT in mammals, it is most likely that there is a temporal delay between the first expression of the prohormone and the first expression of peptide in the brain of vertebrates. Despite these technical shortcomings, there are several features of the developing AVT/MST systems that can be commented on.

The development of both the AVT and MST systems is characterized by a steady increase in number of immunodetectable cell bodies and fibers, unlike, for instance, the ontogeny of the neuropeptide Y-containing cell bodies, which show a dramatical decrease in number during larval development (Tuinhof et al., 1994). However, it is also obvious that the distribution of MSTi cell bodies, both in the developing and the adult brain, is more restricted than that of AVTi cell bodies (González and Smeets, 1992b; present study). A remarkable finding of the present study is the predominance of MSTi fibers over AVTi fibers in the caudal brainstem, which is in contrast with the condition found in adults where AVTi fibers prevail in the corresponding region. Another impression gained from the present study is that the AVTi and MSTi cell groups show less overlap at late embryonic stages than in larval or juvenile stages. However, since information on the development of MSTi cell bodies and fibers in the brain of other amphibians is lacking, no general conclusions can be drawn.

Recently the development of the vasotocinergic system in the brain of the bullfrog, *Rana catesbeiana*, was studied by Boyd (1994). Unfortunately, the earliest stage studied was stage III (stages after Taylor and Kollros, 1946), which is already a premetamorphic stage. For clarity, stages 45/46 of *Xenopus* correspond to stage I of *Rana*, stages 52/53 to VI/VII, 58/59 to XVIII/XIX, and 65/66 to XXIV/XXV. It is therefore not surprising that Boyd found distinct groups of AVTi cells in the magnocellular preoptic nucleus, the suprachiasmatic nucleus, and the hypothalamic infundibular region at stage III, which corresponds to stages 49/50 of *Xenopus*. In fact, a similar distribution of AVTi cell bodies is found in *Xenopus*, where AVTi cells are immunodetectable in the magnocellular nucleus (starting at stage 43), and in the suprachiasmatic nucleus and dorsal infundibular region from stage 49 onwards. Slightly later (stage 50), AVTi cells were observed for the first time in the midbrain and the caudal telencephalon at the level of the anterior commissure. In *Rana*, AVTi cell bodies in the medial amygdala were detected somewhat later (stage VI), but a midbrain AVTi cell group seems to be lacking in both the developing and the adult brain. On the other hand, AVTi cell bodies were found, although not constantly, in the septal nucleus (starting at stage

VI) and the pretrigeminal region (from stage XX/XXI onwards) of *Rana* (Boyd, 1994), but never in *Xenopus* (present study). From studies in adults it was known that interspecies differences with respect to AVTi cell groups occur within the class of amphibians (Boyd et al., 1992; González and Smeets, 1992a, 1992b). Some of these differences may be due to the sensitivity of the immunohistochemical techniques, as suggested by the inconstant staining of the pretrigeminal cell group. The latter group was only observed in frogs killed in the fall (Boyd et al., 1992). Other differences most likely reflect real interspecies differences. For example, a remarkable finding of Boyd (1994) is that during development, AVTi perikarya in the amygdalar region of *Rana* have a medial position, whereas in adults the cells occur primarily in the pars lateralis of the amygdala. In this respect, it seems that in *Xenopus* (González and Smeets, 1992b) and *Pleurodeles* (González and Smeets, 1992a) the embryologically primitive condition has been retained, whereas in *Ranidae* a shift to a more lateral position within the amygdalar region has taken place (Boyd, 1994; Boyd et al., 1992; González and Smeets, 1992a).

The present study of the development of AVTi/MSTi cell bodies in *Xenopus* has revealed also some differentiation in the time of origin of cells within a group. In the suprachiasmatic nucleus, AVTi perikarya first appear in its medial portion at stage 49, whereas AVTi/MSTi cell bodies in the lateral part of the nucleus do not appear before stages 54/55. Also, in the hypothalamic infundibular region a time gradient was observed from dorsal to ventral. AVTi cells in the dorsal part appear at stage 49, whereas immunoreactive cells in the ventral infundibular region are observed for the first time at stages 54/55. Finally, the appearance of immunoreactive cells in the magnocellular nucleus shows a gradient from dorsocaudal to rostroventral.

4.2. Development of AVT/MST systems in relation to other developmental events

The present study is the third in a series that aims to correlate the ontogeny of neurotransmitter systems with developmental aspects of structure and function of the CNS of *Xenopus laevis*. It has become obvious that the use of a core species in such studies is a prerequisite. In previous studies, detailed information about the development of catecholamine (CA) systems, in particular the dopamine system, as well as of the neuropeptide Y (NPY) system has been provided (González et al., 1994a, 1994b; Tuinhof et al., 1994). The data on the development of AVT/MST systems of the present study can, therefore, be directly compared with those of the CA and NPY systems. Such a comparison is of particular interest, since mutual relationships between these neurotransmitter systems likely exist in several places, such as the preoptic area, the hypothalamus, the hypophysis, the midbrain tegmentum and the solitary tract region (compare González and Smeets, 1992, 1994; Tuinhof et al., 1994).

Of the transmitter systems studied so far, the NPY system seems to develop earlier than the CA and AVT/MST systems. The first, immunodetectable NPY cell bodies and fibers are seen at stages 33/34, whereas the adult-like pattern of distribution is already reached at the end of the premetamorphosis (Tuinhof et al., 1994). Although the first appearance of CA cell bodies (stage 38) precedes that of AVTi/MSTi perikarya (stage 43), the overall development of the CA systems and AVT/MST systems is very much alike, reaching an adult-like appearance at juvenile stages (González et al., 1994a; present study). Remarkably, despite the differences in development, the expression of NPY- and dopamine-immunoreactivity in the suprachiasmatic nucleus occurs at about the same time (around stage 40). This coincides with several other events related to background adaptation, suggesting that the latter nucleus plays a key role in this complex neuroendocrine mechanism (Tuinhof et al., 1994). It should be noted that dopamine and NPY coexist in the nerve terminals that make synaptic contacts with the melanotrope cells in the intermediate lobe of the hypophysis (de Rijk et al., 1990, 1992), whereas AVTi/MSTi hypothalamo-hypophysial fibers terminate exclusively in the neural lobe (González and Smeets, 1992b). Thus, there seems to be little interaction between AVT/MST systems and CA systems or NPY systems at the level of the pituitary. On the other hand, there is ample opportunity for these neurotransmitter systems to influence each other at preoptic and hypothalamic levels, as has been demonstrated for mammals (Buijs et al., 1984; Shioda and Nakai, 1992; Horie et al., 1993; Tillet, 1994).

Another area where catecholamine, AVT/MST and NPY neuronal structures are intimately linked together is the nucleus of the solitary tract. From previous studies (González et al., 1993; González and Smeets, 1994), it is known that the solitary tract nucleus contains dopaminergic, noradrenergic and putative adrenergic cell bodies. In addition, NPYi cell bodies have been demonstrated in the same area (Tuinhof et al., 1994). Furthermore, elaborate networks of CA-, NPY- and AVT/MST-immunoreactive fibers occur in this area, suggesting close relationships and modulatory effects of these neurotransmitter systems on each other. Such relationships have already been experimentally confirmed in rats (see e.g. Hermes et al., 1989; Shioda and Nakai, 1992; Shioda et al., 1992), but remain to be established for amphibians. From our studies it is clear that NPYi cell bodies appear first during development in the solitary tract nucleus of *Xenopus* (already at stage 40), whereas CA perikarya are not immunodetectable before stage 50. Remarkably, slightly before stage 50 the first AVTi/MSTi fibers are found within the solitary tract area. If in amphibians, as in mammals (Sawchenko and

Swanson, 1982a), the major portion of the ascending projection from the solitary tract nucleus to the hypothalamus is catecholaminergic, these AVT/MST fibers may play a role in the control of the relay of visceral information to the various forebrain regions.

Clearly magnocellular cells in the preoptic area were not seen till late larval stages in the present study. However, from studies on bullfrogs it is known that the antidiuretic action of AVT starts at premetamorphic stages (stages III to V, after Taylor and Kollros, 1946) and is well-established at prometamorphic and metamorphic climax stages (Alvarado and Johnson, 1966; Bentley and Greenwald, 1970). Rapid cell proliferation and cellular migration in the magnocellular part of the preoptic nucleus takes place around stages 58–60 in *Xenopus* tadpoles (Gorlick and Kelley, 1987). The present study has revealed clearly 'magnocellular' AVTi/MSTi cell bodies almost at the same time.

No sexual dimorphism in the AVT system of *Xenopus* tadpoles and froglets was found, which is in line with the data obtained by Boyd (1994) in developing bullfrogs. However, in contrast to *Xenopus*, the AVT system in the brains of adult *Rana catesbeiana* displays measurable dimorphic features in several regions, such as the lateral amygdala, the habenular nucleus and the suprachiasmatic nucleus (Boyd, 1992; Boyd and Moore, 1992). Thus, the differentiation into a male-like or female-like pattern of AVT immunoreactivity takes place during juvenile development or at adulthood.

4.3. Comparison with development in other vertebrates

In rats, the presence of vasopressin (VP) and its neurophysin in neuronal structures was not immunodetectable till fetal day 16, which is rather late considering the total prenatal development of 21 days (Buijs et al., 1980; Whitnall et al., 1985; Boer, 1987). The first immunoreactive cell bodies were observed in the supraoptic nucleus (fetal day 16), followed by those in the paraventricular nucleus (fetal day 17/18), and in the suprachiasmatic nucleus (from postnatal day 2 onwards). The earliest extrahypothalamic VP immunoreactive fibers were found at fetal day 20 in the central amygdala, whereas fibers in the lateral septum were not seen before postnatal day 10.

Oxytocin (OT) and its neurophysin were not detected immunohistochemically before postnatal day 4, suggesting that this peptide hormone does not play a significant role during the embryonic development. A similar delay in maturation of the VP and OT is also found in the appearance during development of the VP- and OT-binding sites in rats (Snijdewint et al., 1989). While VP binding sites were first detected at fetal day 20, OT binding sites were not seen until postnatal day 1.

The present study has revealed that also in *Xenopus* a temporal delay in development between the two systems seems to exist, at least with respect to the maturation of the fibers. However, it should be noted that in amphibians, in contrast to mammals, the mesotocinergic sytem may play a significant role during development.

Substantial differences in development and organization between mammals and amphibians may also exist in the hypothalamic cell groups. Studies in mammals have shown that the paraventricular nucleus consists of both magnocellular and parvocellular VP/OT elements (Swanson and Sawchenko, 1983; Dubois-Dauphin et al., 1989a, 1989b). Several studies have provided evidence that the parvocellular component is the source of the projections to the brainstem and spinal cord (Sawchenko and Swanson, 1982b; Cechetto and Saper, 1988), whereas the magnocellular component is the main source of projections to the hypophysis (Poulain and Wakerley, 1982). Magnocellular as well as parvocellular elements are also observed in the hypothalamus of amphibans, but a distinction between the two categories could not be made until late in development (Conway and Gainer, 1987; Boyd, 1994; present study). Nevertheless, extensive projections to the hypophysis and the brainstem and spinal cord are observed much earlier during development. These findings suggest that, if only the parvocellular component is the source of extrahypothalamic fibers, the future magnocellular elements grow in size during development. However, this hypothesis can only be confirmed by double labeling experiments combining tract tracing techniques with AVT/MST immunohistochemistry.

Acknowledgments

The authors are much indebted to Dr. R.M. Buijs and Dr. J.M. Guerné for donating the antisera, to the Department of Cellular Animal Physiology, University of Nijmegen, for providing the animals, and to Mr. D. de Jong for preparing the photomicrographs. The study was financially supported by Spanish Research Grant DGICYT PB93-0083 and NATO Collaborative Research Grant CRG 910970.

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