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ORIGINAL ARTICLE

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Developmental changes of NADPH-diaphorase positive structures in the isthmic nuclei of the chick

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Abstract Nicotine adenine dinucleotide phosphate-diaphorase staining was used to study nitric oxide synthase activity and distribution in the midbrain visual structures of white leghorn chick embryos and post-hatched chickens. Enzyme staining first appeared in the isthmic region at the tenth embryonic day (E10) in the neuropil of the nucleus isthmi, pars parvocellularis. At E11 faint enzyme positivity appeared also in the nucl. isthmi pars magnocellularis, the nucl. semilunaris and the nucl. isthmo-opticus. The staining intensity of the isthmic nuclei dramatically increased between the 12.5th and the 13th days of incubation. The nucl. isthmi, pars parvocellularis showed the strongest enzyme reaction throughout embryonic life. A day before hatching all the isthmic nuclei were heavily stained, however, nicotine adenine dinucleotide phosphate-diaphorase-positive cells occurred exclusively in the nucleus isthmo-opticus. In the tectum opticum, intensely stained cells occupied the stratum fibrosum et griseum superficiale. The layer containing the projection neurons to the isthmo-optic nucleus was unstained. In the isthmic region, the intensity of staining surpassed that of the tectum and reached its maximum at E17 and then slowly decreased till the end of the experimental period (120 days post-hatched). The tractus isthmo-opticus showed nicotine adenine dinucleotide phosphate-diaphorase activity throughout the investigated period of life of the chicken, but the tractus tectoisthmo-opticus was unstained. Our results suggest that in the isthmic nuclei, nicotine adenine dinucleotide phosphate-diaphorase-positive neurons occur only in the isthmo-optic nucleus and optic tectum. The other positively stained structures are the fibers and terminals of tectal cells. In most brain areas nicotine adenine dinucleotide phosphate-diaphorase indicates nitric oxide synthase that produces nitric oxide. The transient appearance of this molecule is probably necessary for neuronal differentiation or the establishment of synaptic connections in the isthmic nuclei, and these developmental changes are under the control of the optic tectum.

Key words Tectum opticum · Isthmic nuclei · Neuronal differentiation · Nitric oxide

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Introduction

In the last few years, some experimental data have suggested that nitric oxide (NO), among other functions, might have some role in neuronal development and synaptic plasticity (Edelman and Gally 1992; Wu et al. 1994; Cudeiro and Rivadulla 1999; Ernst et al. 1999). It was shown that the synthesizing enzyme of NO, neuronal nitric oxide synthase (nNOS) was transiently expressed in the cortical plate neurons of rat embryos (Bredt and Snyder 1994). In the ferret, NO that is produced by nNOS mediates activity-dependent changes in the formation of eye-specific laminae in the lateral geniculate nucleus (Cramer and Sur 1999). The development of ocular dominance columns in the monkey`s visual cortex could be correlated with nNOS activation (Aoki et al. 1993); even so the formation of ocular dominance columns seems to occur independently of NOS activity (Ruthazer et al. 1996). In chick embryos, peak expression of nNOS was observed in the optic tectum between embryonic day 12 (E12) and E15 (Williams et al. 1994), while in the adult chicken tectum, only a few cells were NADPH-diaphorase (NADPH-d, a marker for NOS)-positive (Brüning 1993; Montagnese and Csillag 1996). Since peak expression of NOS in tectal cells coincided with the refinement of initial synaptic connections, it was suggested that NO might be an important factor in this process (Williams et al. 1994). It was also shown that NO might be involved in the initiation of cell death

during development (Posada and Clarke 1999). Most NADPH-d-positive cells occurred in those layers of the embryonic chick tectum where the neurons that project to the nucleus isthmi, pars parvocellularis were located (Woodson et al. 1991). This structure, however, was not mentioned among the NOS-containing brain areas.

Another structure of the chick visual system where NOS-positive neurons occur in the adult chick is the isthmo-optic nucleus (Brüning 1993). This nucleus receives tectal afferents (see for references Hunt and Brecha 1984; Woodson et al. 1991), and projects to the contralateral retina. The isthmo-retinal terminals may use NO as transmitter (Morgan et al. 1994).

We supposed that NO might have an important role not only in retinotectal pattern formation, but also in development of the isthmic nuclei that receive the axons of NADPH-d-positive tectal cells. We investigated in chick embryos whether NOS was expressed in the neurons of the isthmic nuclei, and whether NOS-positive tectal axons terminated in these nuclei. The transient expression of NADPH-d in the developing isthmic complex was reported in a congress abstract by Dermon et al. (1998).

Materials and methods

Incubation and handling of animals

Forty-four white leghorn chick embryos and nine chickens hatched in our laboratory were used. The fertilized eggs (purchased from the Beijing Agricultural University) were incubated in an egg incubator at 37–38°C, with 60% humidity. They were placed in cardboard egg trays with the air sacs upward and were rotated four times a day (8 and 12 am, 6 and 10 pm). The day when the eggs were put into the incubator was marked as embryonic day 0 (E0), the next day E1, and the last E20. The chicks were hatched at the 21st day, which was labelled as H0. The experiments were carried out in five series. The details are given in Table 1.

In the first series, two batches of eggs were incubated with a 7-day difference in the beginning of incubation. The youngest embryo we studied was killed at E6. The brain of this embryo was processed simultaneously together with the brains of three E13 embryos. Next day the brain of one E7 embryo was processed together with three E14 embryos. Beginning with E9, two brains were processed together from the two age groups until we reached the E12 and E19 embryo pairs. By this we covered the embryonic time from E6 to E19. Our aim with this series of investigations was to record any possible great difference of NADPH-d-staining pattern in embryos widely separated in time of their embryonic development. Two E20 embryos and two H0 chicks were processed separately, since their possible pairs (E13 and E14) have already been stained. The brains of one 7-day-old, one 14-day-old, one 21-day-old and one 4-month-old post-hatched chicken were also processed and labelled as H7, H14, H21 and H120. By this series, we covered developmental stages from E6 to H0 with 1-day gap, H0 to H21 with 1-week gap and added the 4-month-old chicken.

Table 1 Groups of animals investigated [*S* sagittal sections, *H* horizontal sections, ( ) brains were processed together]

Groups	Age of embryos
I	(E6+E13), (E7+E14), (E8+E15), (E9+E16), (E10+E17), (E11+18), (E12+E19), E20, H0, (H7+H14+H21), H120
II	(E11+E14+E17+E20)
III	(E11+E11,5+E12+E12,5+E13)
IV^{S}	(E13+E17+E19+H7), H120
V^{H}	(E15+E19), H120

In the second series of investigation, four brains were processed and stained simultaneously in the combination of E11-E14-E17-E20. We reduced the gap between developmental stages expressed by the days of embryonic life to study possible minor differences in NADPH-d staining during embryonic development. By these parallel experiments, we expected to get preparations processed in exactly the same way, thus allowing comparison of younger and older animals for a precise description of differences in staining of isthmic nuclei.

In the third series, we investigated embryos between E11 and E13 using a time gap of 12 h. Since we have seen a sudden increase in the intensity of NADPH-d staining of the isthmic nuclei between E11 and E13 in our previous series, a smaller time scale was necessary to describe this critical period. The animals in this group were labelled as E11, E11.5, E12, E12.5, and E13. All brains in the first, the second and the third series were sectioned in the coronal plane, because in this plane two, three or all the isthmic nuclei could be located in the same single section, together with the optic tectum. Thus, the visual structures could be compared in the same section.

In the fourth series, the brains of five animals (E13, E17, E19, H7 and H120) were cut in the sagittal plane. Our aim was to locate the tractus isthmo-opticus (TIO) carrying efferent fibers to the retina.

In the fifth series, the brains of three animals (E15, E19 and H120) were cut in the horizontal plane, the most favorable one to locate the tractus tectoisthmo-opticus (TTIO).

All experiments were carried out according to the “Principles of laboratory animal care (NIH publication No 86-23, revised 1985). The animals were anaesthetized with diethylether before being killed. The E6 and E7 embryos were decapitated, the brains removed and placed into cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (PB). Embryos older than E7 and post-hatched chickens were perfused through the heart with saline (0.9% NaCl, 4°C) followed by 150 ml of fixative for 10–15 min, and then the brains were removed. The caudal mesencephalon together with the most rostral part of the pons was separated and placed in the same fixative for additional 3–4 h postfixation. For cryoprotection the brains were transferred into a 25% solution of sucrose overnight at 4°C. Sixty-μm serial frozen sections were cut on a cryostat microtome at –15°C and collected in PB in tissue culture wells 1 cm in diameter. Sections containing the nucleus isthmo-opticus and a few sections rostrally and caudally to it were transferred into 0.5 ml PB in other wells (2 or 3 sections of the younger together with one of the older embryos to the same well).

NADPH-d histochemistry

The incubation solution was prepared as follows: Triton X-100 (FARCO), 6 μl/ml, Nitro blue tetrazolium (Sigma N6876), 0.4 mg/ml, nicotine adenine dinucleotide phosphate tetrasodium salt reduced form (Sigma N1630, or sometimes Biomol 15156) 2 mg/ml, dissolved in PB. This solution (0.5 ml) was added to each of the wells containing the sections in 0.5 ml phosphate buffer. Using this technique we reached the optimum final concentration of chemicals, and the incubation started almost at the same time in each well. The sections were incubated at 37°C in darkness. In a pilot study using an E14 embryo we determined that the optimum incubation time was 40 min. This incubation time was used throughout the series of investigations to make the results comparable. Histochemical reaction was stopped by filling the wells with PB in the same order as the NADPH-containing solution was added. Then the sections were immediately transferred into PB, rinsed 3×5 min and finally were mounted on gelatine coated slides. They were air-dried overnight and then were placed in 100% ethanol, cleared in xylene and sealed in a synthetic medi-

um. Microphotographs were taken by a Nikon FXA microscope using Agfapan APX 25 film.

Results

The isthmic complex of the chick that receives primary or secondary visual information consists of four nuclei. Their locations have been described in several papers and atlases, and therefore we will not give an account in detail about them. In the following description we will use the nomenclature applied by Kuenzel and Masson (1988) for the brain of the 2-week-old chick. The largest among the isthmic nuclei is the nucleus isthmi, pars parvocellularis (Ipc). Next in size is the nucleus isthmi, pars magnocellularis (Imc) that is located between the optic tectum and the Ipc. The two smaller nuclei are the nucleus isthmo-opticus (IO) in the dorsolateral part of the mesencephalic tegmentum and the nucleus semilunaris (Slu). The rostral half of the Slu locates close to the ventrolateral surface of the tegmentum, and the caudal half close to the lateral surface. When cell migration is finished and the nuclei are formed, their locations are the same during embryonic development as in the two-week-old chick (Clarke 1982).

Developmental changes in the NADPH-d activity in the optic tectum were described in detail by Williams et al. (1994). Our finding was essentially the same, and therefore NADPH-d staining in the tectum will be mentioned only in relation with developmental changes of NADPH-d activity in the isthmic nuclei.

We distinguished three periods with characteristic patterns in the development of NADPH-d activity: periods I, II, III.

Period I

This spans the embryonic days before E10. In this period the cells that migrated towards the surface of the tectum showed already NADPH-d staining at day E8 in the rostral and at E9 in the caudal tectum, and occupied a layer corresponding later to the stratum fibrosum et griseum superficiale. We will designate these cells, without mentioning their exact locations, migrated or superficial cells in the following descriptions. This period corresponds to the generation and migration of the cells forming the isthmic nuclei (Clarke 1982; Puelles and Martinez-de-la-Torre 1987) that were unstained.

Period II

This comprises the embryonic days between E10 and H0. We divided this period into four sub-periods based on developmental changes of the isthmic nuclei during embryonic life.

Sub-period 1

This includes E10 and E11. During these two days an obvious enzymatic activity develops in the migrated tectal cells and the isthmic region begins to show NADPH-d staining (Fig. 1A,B). The first signs of weak diffuse staining in the isthmic complex appeared in the Ipc of the E10 embryo. By E11 the intensity of staining in the Ipc slightly increased, and a faint staining also appeared in the the IO (Fig. 1A), and also in some sections in the Imc (not shown). Under higher magnification, very faintly stained neurons could be identified within the IO. A weak diffuse background staining also indicated NADPH-d activity (Fig. 1C).

Sub-period 2

This covers developmental stages from E12 to E15 and is characterized by a continuous increase in the intensity of NADPH-d staining in the optic tectum and the isthmic nuclei. There was some increase in the intensity of staining in the Imc, Ipc and IO between E11.5 and E12, and a sudden increase occurred between E12.5 and E13. The unstained mesencephalic root of the trigeminal nerve divided the stained IO into a dorsolateral larger and a ventromedial smaller part (Fig. 1D). This separation could be observed already in the E11 embryo (Fig. 1C). The ventromedial part of the nucleus was less intensely stained than the dorsolateral part. This pattern was clearly seen in the E13 embryos, in which all visual structures, including the migrated cells of the tectum, were intensely stained (Fig. 1D–F). This period corresponds to the time of normal cell death in the IO according to Clarke (1985, 1992). In the stratum album centrale (SAC) of the tectum, fascicles of NADPH-d-positive axons were oriented radially around the periventricular lay-

Fig. 1A–F Development of NADPH-diaphorase-reactive structures in the optic tectum and the isthmic complex in chick embryos. All photographs show coronal sections. Dorsal is up. **A** Moderate enzyme positivity in the migrated cells (*open arrows*) in the tectum opticum (*Tec*) of an E11 embryo. *Arrowheads* point to the faintly stained nucleus isthmi, pars parvocellularis (*aq* aqueductus cerebri, *nIV* nucleus nervi trochlearis). The framed area shown enlarged in **C** contains the nucleus isthmo-opticus. *Bar* 500 μm and applies also to **E**. **B** NADPH-diaphorase-positive migrated neurons in the optic tectum of an E11 chick embryo. *Bar* 50 μm and applies also to **F**. **C** The nucleus istho-opticus (IO) of the E11 embryo shown framed in **A**. Note very faint staining and the unstained root of the mesencephalic trigeminal nucleus (*arrows*). *Bar* 125 μm. **D** The nucleus isthmo-opticus of an E13 chick embryo. Note the enzyme-positive cells (*dark spots*) embedded in the diffusely stained lighter background. The unstained root of the mesencephalic trigeminal nucleus (*arrows*) divides the structure into a ventromedial (lighter) and a dorsolateral (darker) part. *Bar* 100 μm. **E** The tectum opticum and the isthmic complex of an E13 embryo (*Imc* nucl. isthmi, pars magnocellularis; *IO* nucl. isthmo-opticus; *Ipc* nucl. isthmi, pars parvocellularis. *Open arrows* point to the migrated tectal cells and the two *small arrows* indicate the root of the mesencephalic trigeminal nucleus. The *black circle* labels the ventriculus tecti mesencephali. **F** Migrated tectal cells in the E13 embryo ▶

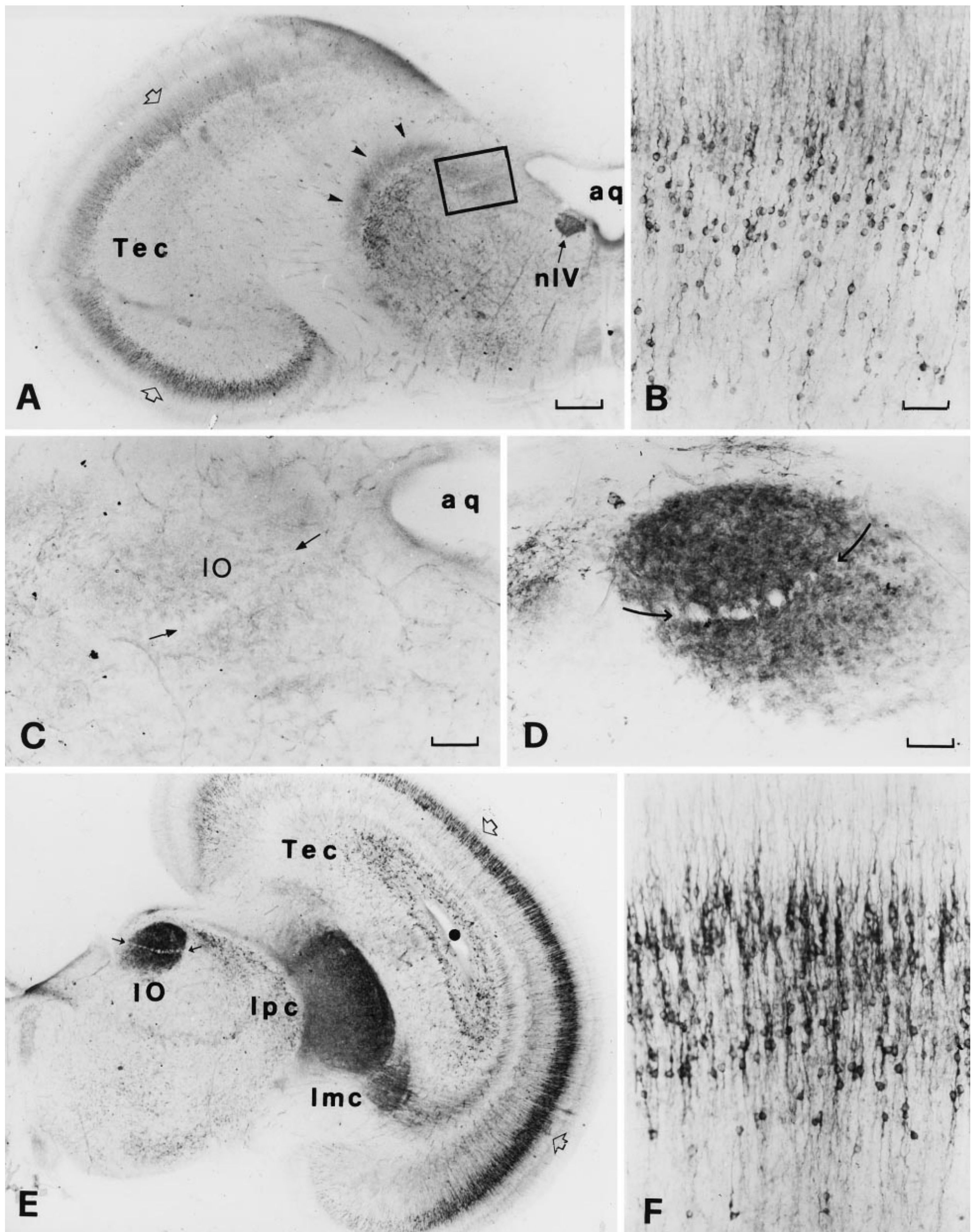


Fig. 1A–F

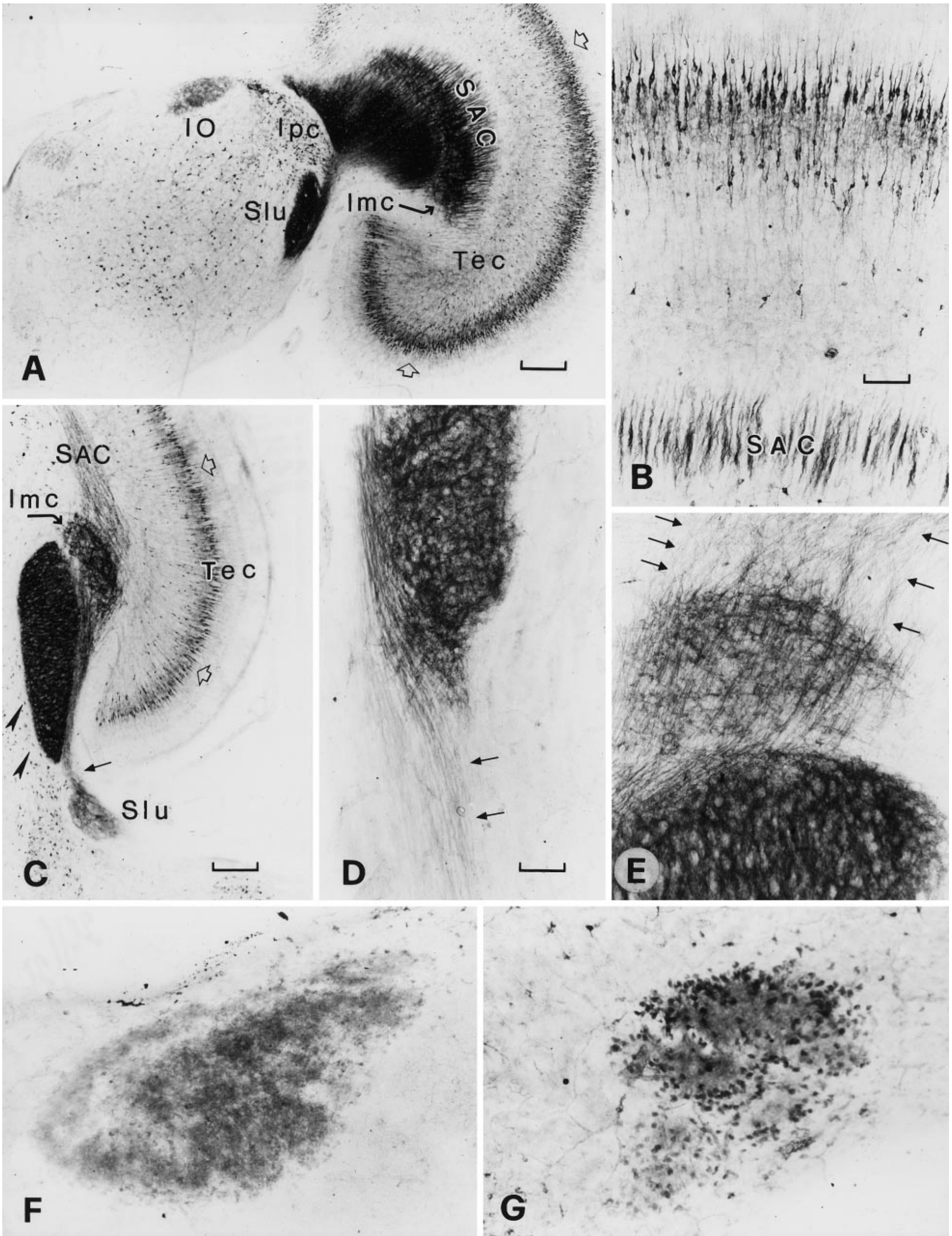


Fig. 2A–G

er and the Imc, at the level of the caudal tectum. Here the NADPH-d-positive fibers penetrated the Imc and entered the Ipc. The axon fascicles showed the same orientation as the migrated tectal cells and their axons. However, only a short segment of these axons was stained and an unstained area separated them from the fascicles piercing the SAC. These axon fascicles could be stained throughout the embryonic life (Fig. 2A,B,E). Individual terminal branches of axons could not be distinguished in any of the termination areas.

In the Imc, a loose network of probably fiber fascicles and the penetrating axons gave a reticulated appearance. In the Ipc, the intensity of staining was higher than in the Imc. In the IO, the intensity and appearance of staining was similar to that in the Ipc (Fig. 1E). Weakly stained perikarya were masked by the diffusely stained neuropil; however, they could be identified under high power magnification. In E15 embryos, unlike in early stages of development, the IO was not stained evenly. The appearance of weakly stained spots and folded stained strips might indicate the beginning of lamination within the nucleus. The Slu was faintly stained in the E13 embryos, and the intensity of its staining reached that of the IO only in the E15 embryos.

Sub-period 3

This covers the embryonic days E16–E18. It is characterized by changes in the intensity of NADPH-d staining in the tectum and isthmic nuclei. During this period, the intensity of staining in the migrated tectal cell layers was gradually decreasing, primarily caused by the redistribution of the NADPH-d-positive cells that formed two well-defined layers instead of one, and more scattered cells were stained. The origins of the most intensely stained axons of fusiform cells were also detected. They showed the typical “shepherd`s crook” shape by turning towards the deep tectal layers. These and other faintly stained, loosely arranged thin axons were identified between the NADPH-d-positive superficial tectal cell layers and the SAC, where they converged to form densely packed fascicles (Fig. 2A,B). In the deeper sublayers of the stratum fibrosum et griseum superficiale cell morphology was more variable. Some fusiform cells were oriented horizontally, while others had triangular or multipolar perikarya with short dendrites.

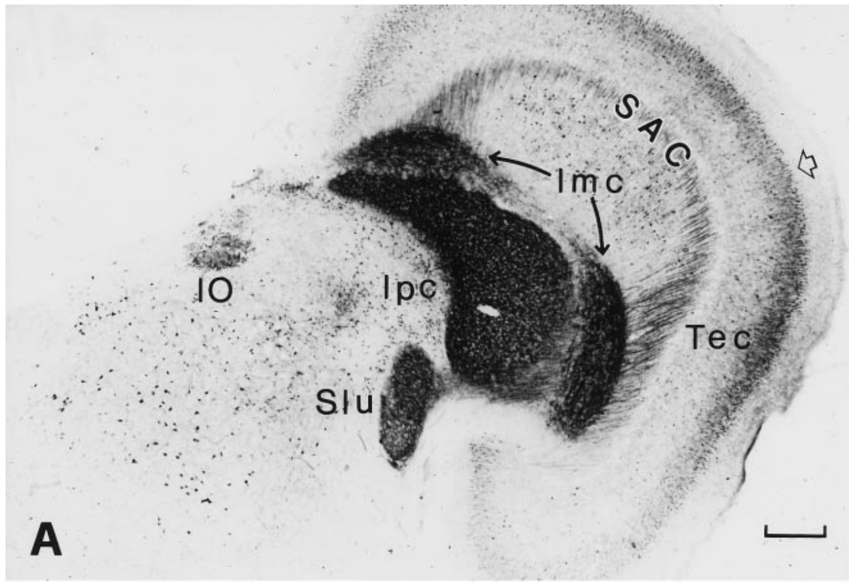
The most intense NADPH-d staining in the Ipc was seen in the E16 and E17 embryos during development. The reticulated appearance of the Imc and the Ipc was more obvious than in the E13 embryos. The numerous unstained spots among the dark-blue axon fascicles probably indicated unstained cells (Fig. 2C,E). The Slu also showed a similar staining pattern (Fig. 2D). By the end of this sub-period, the intensity of staining decreased somewhat in the Ipc and increased in the Imc, and both remained intense. In the IO, the intensity of diffuse staining slightly decreased between E15 and E16 and reached a moderate level in the E16 embryos (Fig. 2A). Meantime, the differentiation of the IO continued. The faint NADPH-d-positive neurons formed convoluted laminae; cell processes, however, were not stained (Fig. 2F). A well-stained NADPH-d-positive axon bundle that traversed the Imc and bypassed the ventral aspect of the caudal Ipc could be followed to the lateral aspect of the Slu (Fig. 2C). Most axons entered the diffusely stained Slu; some of them, however, proceeded for a short distance in the ventral direction. They ended abruptly without forming terminal branches in this particular section (Fig. 2D). The intensity of staining in the Slu was slowly increasing, and the axon fascicles in the SAC were intensely stained also in this sub-period (Fig. 2B).

Sub-period 4

This comprises the E19 and E20 embryonic stages and the freshly hatched chicks labelled as H0. The bilaminar arrangement of the migrated tectal cells was apparent, but their number and staining intensity were seemingly reduced in the E20 embryos (Fig. 3B) when compared to the tectum of an E13 embryo (Fig. 1F). The proportion of triangular and multipolar cells apparently increased. The

◀ **Fig. 2A–G** NADPH-diaphorase-positive structures in the optic tectum and the isthmic complex of chick embryos. All photographs, except **C**, show coronal sections in which dorsal is up. **A** E16 embryo (*SAC* stratum album centrale; *Slu* nucl. semilunaris). *Open arrows* point to the migrated tectal cell layer. *Bar* 500 μm. **B** Migrated cells in the tectum opticum of an E16 embryo. Note intensely stained axon fascicles in the stratum album centrale (*SAC*). *Bar* 50 μm. **C** Horizontal section through the isthmic complex of an E17 embryo. The most intensely stained structure is the nucl. isthmi, pars parvocellularis (*arrowheads*). *Arrow* points to tectal fibers terminating in the nucl. semilunaris. Rostral is up. *Bar* 300 μm. **D** The ventral half of the nucl. semilunaris densely packed with enzyme-positive fibre terminals. *Arrows* point to stained fibers passing the nucleus. Lateral is to the left. *Bar* 100 μm and applies also to **E**–**G**. **E** Enzyme-positive tectal fibers (*arrows*) penetrating the isthmic nucleus, pars magnocellularis and entering the pars parvocellularis in the lower one-third of the picture. White spots in the black area are unstained cells. E16 embryo. **F** The diffusely stained nucleus isthmo-opticus of an E17 embryo. At higher magnification, the darker spots are identified as faintly stained cells. **G** Intensely stained cells in the nucleus isthmo-opticus of an E20 embryo

Fig. 3 A NADPH-diaphorase-positive structures in the tectum opticum and the isthmic complex of a chick embryo 1 day before hatching (E20). All photographs show coronal sections. *Open arrow* points to migrated tectal cells. Dorsal is up. *Bar* 500 μm and applies also to **C**. **B** The migrated tectal cells form two layers in the E20 embryo. *Bar* 50 μm and applies also to **D**. **C** NADPH-diaphorase-positive structures in the isthmic region of a seven-day-old chick (H7). The *bracket* embraces the migrated tectal cell layer in which the number of stained cells and the intensity of staining is strongly reduced compared to the preceding stages. Among the isthmic nuclei the IO is the most intensely stained structure. Note intensely stained cells in the nucl. pontis lateralis (*PL*). **D** Enzyme-positive migrated cells in the tectum opticum of an H21 chick. The number of stained cells is obviously reduced when compared to the E20 embryo. **E** The nucleus isthmo-opticus of an H7 chick. *Arrow* shows the unstained root of the mesencephalic trigeminal nucleus (see also in **F**). *Bar* 100 μm. **F** The nucleus isthmo-opticus of a 4-month-old chick. *Bar* 125 μm ▶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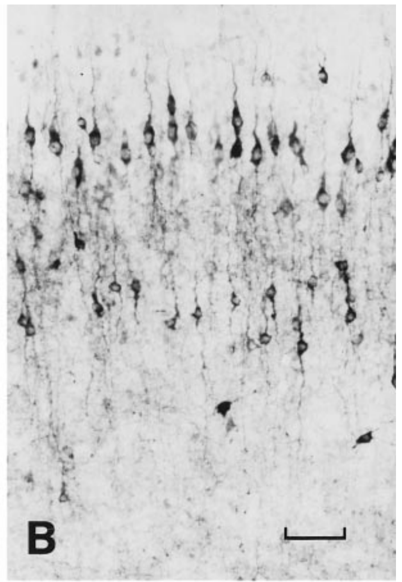
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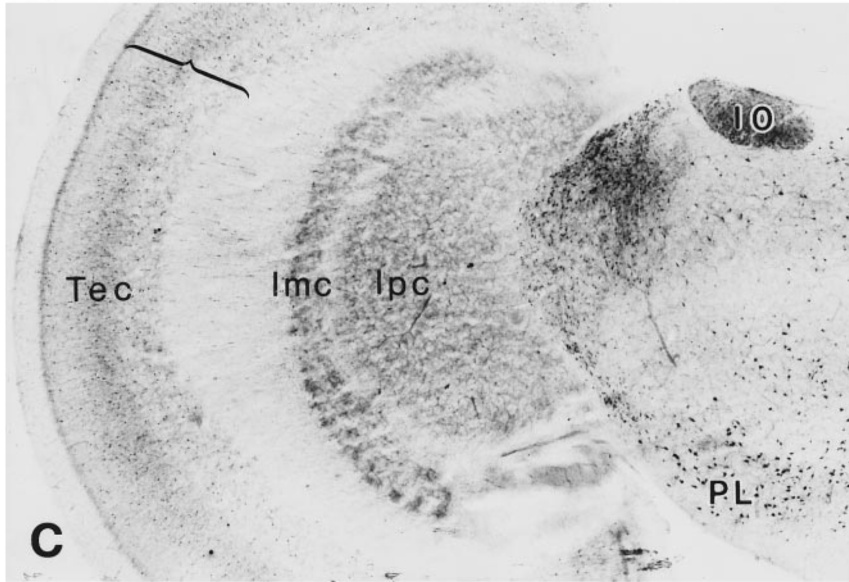
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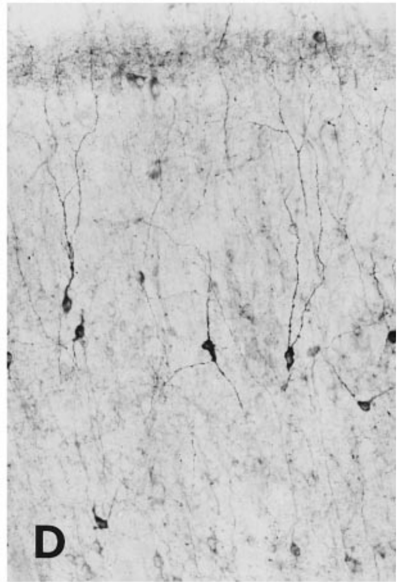
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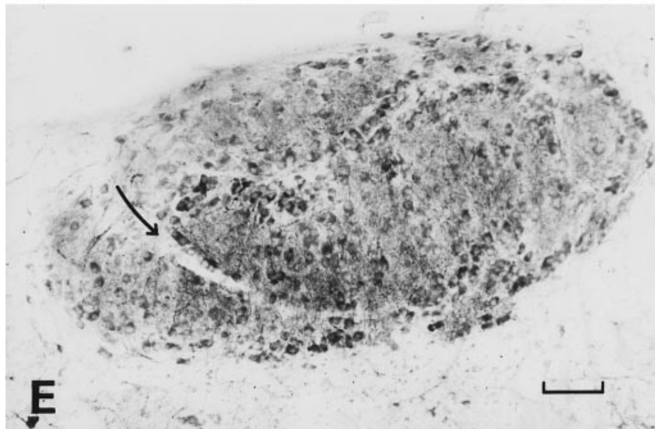
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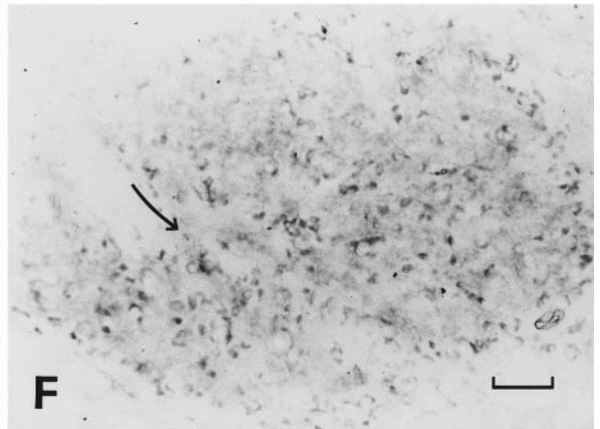
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intense staining in the Imc and Ipc was preserved. Since NADPH-d positivity further increased in the Slu, the most intensely stained structures were the Imc, the Ipc, and the Slu at the end of this sub-period (Fig. 3A). In the IO, the intensity of diffuse staining was maintained at a moderate level; cell staining, however, was more intense. This could be best seen in some sections of an E20 embryo. Some perikarya were intensely, others moderately stained (Fig. 2G), and the initial segments of cell processes were discerned under high power magnification.

Period III

This is the period after the chicks hatched. In the 7-day-old chick (H7) scattered and intensely stained cells were seen in the tectum (Fig. 3C), but fewer in number than in the freshly hatched chick (H0). The axon fascicles were very faintly stained. The Imc showed moderate, the Ipc and Slu somewhat weaker staining. The most prominent NADPH-d positive isthmic structure was the IO (Fig. 3C). The staining pattern, with somewhat higher intensity of staining, was similar to that shown in the E20 embryo (Fig. 3E). In the H14 and the H21 chickens the diffuse staining was faint in the tectum, but the scattered and intensely stained cells were present in the superficial layers (Fig. 3D). A very faint blue stripe indicated the site of the SAC; however, individual axons were not seen. The Imc, Ipc and Slu were only faintly and diffusely stained, with no enzyme-positive cells being identified. The IO was stained similarly as in the H7 chick, though diffuse staining was less intense. The NADPH-d-positive perikarya were clearly visible, and in the H21 chick short segments of tortuous cell processes, probably dendrites, also occurred. In the 4-month-old chicken (H120), the number of stained cells was further reduced in the superficial layers of the optic tectum; nevertheless the periventricular tectal cells were stained as intensely as in the E13 embryos. The NADPH-d positivity disappeared from the Imc, Ipc and Slu, but persisted in some cells of the IO (Fig. 3F).

Several NADPH-d-positive cells of the IO projected their axons into the tractus isthmo-opticus (TIO). These axons formed a tiny fascicle at the surface of the mesencephalon and ran towards the optic chiasm around the ventrolateral margin of the optic tectum. We identified the TIO in the E17 and E19 embryos and the H7 chick. Sagittal sections proved the best to show this tract (Fig. 4A).

We could not identify the tractus tectoisthmo-opticus (TTIO) with NADPH-d staining. In horizontal sections of the E19 embryo's brain, a well-defined unstained streak was seen in the very place where the TTIO locates, suggesting that TTIO axons were NOS negative (Fig. 4B).

We also failed to locate a transient ipsilateral pathway from the IO to the ipsilateral tectum (Wizenmann and Thanos 1990) and the projection from the IO to the ipsilateral retina (Clarke and Cowan 1976; O'Leary and Cowan 1982; Péquignot and Clarke 1992).

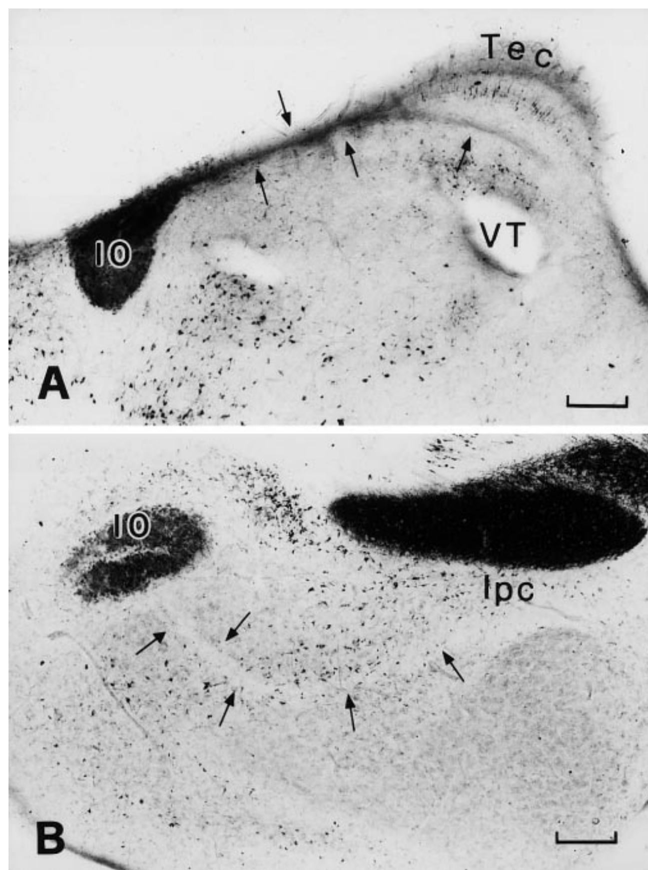

Fig. 4 **A** The course of the tractus isthmo-opticus (*arrows*) in a sagittal section cut from the brain of an E19 embryo. Dorsal is up (*VT* ventriculus tecti mesencephali). *Bar* 300 μm. **B** Horizontal section from the brain of an E19 embryo at the level of the tractus tectoisthmo-opticus (*arrows*), which is not stained. Rostral is to the right. To enhance staining the incubation time was 60 min in these cases. *Bar* 300 μm

Discussion

Technical considerations

We have followed developmental changes in the expression of NOS by showing NADPH-diaphorase in the isthmic nuclei. It was shown by several authors (see for references Wolf 1997) that NADPH-d and NOS colocalized in almost all brain areas investigated in various vertebrate species (exceptions were the olfactory system, the paraventricular organ and spinal parasympathetic neurons). Oermann et al. (1999) found 95% colocalization of NOS-I and NADPHd in the developing mouse cerebral cortex. According to Brüning et al. (1994) "...with certain reservations, NADPH-diaphorase histochemistry serves as a reliable marker for avian neuronal NOS". Studies on the visual system showed that NADPH-d and NOS colocalized in the turtle retina (Blute et al. 1997). Using biochemical techniques and NADPH-d histochemistry, Goureau et al. (1997) found NADPH-d-positive structures and measured Ca-dependent NOS activity in

the developing chick retina. Therefore, we also used the diaphorase enzyme histochemical technique, supposing that NOS and NADPH-d colocalize in the chick not only in the retina, but also in the central visual structures we investigated.

For processing the sections, the conditions of incubation were kept standard, and therefore we believe that this technique allowed us to make reliable comparisons of the preparations among different embryonic stages, and notice any changes in the characteristics of NADPH-d staining.

Because aldehyde fixation is a prerequisite to show NOS-dependent NADPH-d activity (Scherer-Singler et al. 1983; Gonzalez-Hernandez et al. 1996) we used perfusion fixation and discarded all brains that appeared subfixed during removal from the skull.

Since the isthmic nuclei are not formed before E9, we stained only one brain from each stage of the youngest embryos. Beginning with the pair of E9–E16, up to E12–E19 embryos, two well-stained brains were compared from each stage. The results of the second series of these investigations fully supported the observations we made in the first series. Staining properties of the investigated structures were similar in each brain of the embryos of the same age. Sex of the embryos older than E12 was determined. We did not find any sexual differences in NADPH-d staining of the tectum and the isthmic nuclei.

In the description of the results we did not mention NADPH-d positive structures other than the optic tectum and the isthmic nuclei. However, in several nuclei, including the locus ceruleus, nucleus subceruleus ventralis, nuclei of the lateral lemniscus and the central gray, multipolar neurons were intensely stained in embryos older than E10 and the hatched chicks. Their presence in the youngest embryos also proved that the very faint enzyme reaction in the isthmic nuclei was not the result of insufficient staining.

Correlation between the development of NOS expression in the tectum and the isthmic nuclei

We have confirmed the finding by Williams et al. (1994) that NOS is first expressed in the superficial cells of the rostral tectum in E8 embryos and further indicated that many of these cells will be related, during further development, with retinal axons and project to the isthmic nuclei (Woodson et al. 1991). No NADPH-d positivity is found in any of the isthmic nuclei in this embryonic stage. The first sign of NADPH-d expression appears in the Ipc of E10 embryo. By that time the migration of the cells forming the isthmic nuclei is finished, and the first axons of the IO reach the retina (Clarke 1992).

In E11 embryos, faint diffuse staining appears also in the Imc and IO. The presence of faintly stained tectal axons directed toward the isthmic nuclei suggests that NADPH-d positivity in these nuclei is related to tectal afferents.

The peak expression of NOS in the tectum is observed in E12–E15 embryos (Williams et al. 1994). It is the same period when the natural cell death occurs in the IO that lasts till E17 (Clarke 1992). Since the intensity of NADPH-d staining does not reduce in the IO during this time, natural cell death probably does not affect NADPH-d expression. It may imply that not all cells express NOS, and the NADPH-d negative cells die. Alternatively, reduction of the staining intensity is balanced by its increase due to axonal transportation of NOS from the tectum to the nucleus in this period of embryonic life.

Cells in the Imc, Ipc and Slu do not show NADPH-d staining either in the embryos or the hatched chickens. The bulk of brain NOS is cytosolic (Bredt and Snyder 1990; Smidt et al. 1991) and transported centrifugally in the axons (Lázár and Losonczy 1999). We have observed a 1-day delay between the peak expression of NOS activity in the tectum (beginning at E12) and the appearance of intensive NADPH-d staining in the isthmic nuclei (E13). Since NADPH-d-positive axons can be followed from the tectum to the Imc, Ipc and Slu, and cells are not stained in any of these nuclei, we may safely conclude in agreement with Dermon et al. (1998) that tectal axons deliver most NOS-related NADPH-d to these isthmic nuclei. This assumption is also supported by the observation that, parallel with the reduction in the intensity of NADPH-d staining in the tectum, the enzyme activity also reduces in the Imc and Ipc. However, it remains intense in the Slu, where NADPH-d staining appears last among the isthmic nuclei during development. The morphology of tectal neurons projecting to the Ipc is exactly the same (Woodson et al. 1991) as we have observed in our NADPH-d preparations.

In the IO, NADPH-d positivity should be mainly intrinsic. This conclusion is supported by the following facts; (1) NADPH-d positive cells can be identified in the IO; (2) The TTIO is unstained and only a few neurons show NADPH-d positivity in the tectal layer, where the cells of origin of the TTIO locate and their shapes are different from those of the enzyme positive cells here (Woodson et al. 1991; Uchiyama et al. 1996). Our preliminary results show that the tectoisthmo-optic pathway is glutamatergic (J. Hu, W.C. Li and S.R. Wang, unpublished observation); (3) In all coronal, sagittal and horizontal sections, no stained fibers are seen entering or bypassing the IO; (4) The stained TIO can be located; (5) NADPH-d staining in the IO is preserved during the obvious reduction in the intensity of tectal staining. In the 7-day-old chick, the IO is the most intensely stained isthmic nucleus as a result of NOS expression in its neurons.

In the adult pigeon, Miceli et al. (1999) showed in a double-labeling experiment that many NOS positive tectal cells projected to the IO. This observation may mean a species difference, or that in the chicks the concentration of NADPH-d in the TTIO axons is very low, rendering the enzyme undetectable in our preparations.

Possible role of NOS in the development of the isthmic nuclei

The transient expression of NOS in a large population of tectal cells suggests that NO may play a role in the differentiation of the optic tectum. Blocking NO synthesis during development, prevents the elimination of transient ipsilateral projection from the retina to the tectum (Wu et al. 1994), and this elimination is mediated by N-methyl-D-aspartate (NMDA) receptors (Ernst et al. 1999). The present study has failed to demonstrate a transient ipsilateral projection from the IO to tectum, probably due to the absence of NADPH-d activity in this pathway. We do not know whether the transient ipsilateral projection from the IO to the retina contains the enzyme, but we can surely show that the isthmo-optic pathway is NADPH-d positive. Since the peak expression of NOS coincides in time with the refinement and stabilization of retinotectal connections, it has been suggested that NO is necessary for this process, and this small freely diffusing molecule may act retrogradely (Williams et al. 1994; Wu et al. 1994; Posada and Clarke 1999). The involvement of NO in neuronal development and cell differentiation of other systems has been also discussed (Kalb and Agostini 1993; Bredt and Snyder 1994; Roskams et al. 1994; Oermann et al. 1999). The presence of a considerable population of NADPH-d-positive cells in those layers of the optic tectum that project to the isthmic nuclei, and the intense NADPH-d staining in three of these nuclei during the embryonic days E13–E20 suggests that NO is necessary for the differentiation of these nuclei and acts here as an anterograde signaling molecule.

NO is also probably a transmitter. Morgan et al. (1994) have suggested that in the retina isthmo-optic axons release NO. Since these axons reach the retina between E9.5 and E10.5 (Clarke 1992), and already in E13 embryos a few IO cells show NOS positivity, NO may be involved in the formation of the isthmo-retinal connections. Application of selective NOS inhibitors in morphological and electrophysiological experiments is needed to further elucidate the role of NOS during development of the visual system.

Conclusions

NADPH-diaphorase is temporally expressed in the majority of neurons in the optic tectum during the embryonic period of neuronal differentiation and synapse formation. With some delay, intense NADPH-d reaction appears in three isthmic nuclei, the Imc, Ipc and Slu that receive tectal afferents. The enzyme activity should be related to tectal axons and their terminals because cells are not stained in these nuclei. No other enzyme-positive fibers arrive here, and their staining disappears in the young chicks, parallel with a significant decrease in the number of NADPH-d-positive tectal cells. It is supposed that NO of tectal origin is necessary for neuronal differentiation in these isthmic nuclei and their formation.

The IO that projects to the contralateral retina also shows NOS positivity, and its efferent fibers my have some role in the differentiation of retinal cells.

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