

Research Article: Histology And Cell Biology

NADPH diaphorase expression in superior colliculus of developing, aging and visually deafferented rats

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Submitted January 18, 2012; accepted February 9, 2012

Summary

We have studied the development of NADPH-diaphorase activity in the retinorecipient layers of the superior colliculus (SC) in rats from embryonic day 17 to adulthood, during aging, and following neonatal tetrodotoxin injection or unilateral eye removal in the neonatal or in the adult animal. In the superficial SC, NADPH-d activity is first seen in neurons on postnatal day (P) 4; over the next two weeks, enzyme expression increases gradually, in cells as well as in the neuropil. By P12-14, around the time of eye opening, NADPH-d reactivity increases dramatically. In parallel, the dendrites of many NADPH-d-positive neurons in the superficial gray layer, more or less randomly distributed at first, gradually align their orientation relative to the dorsoventral axis. The pattern of NADPH-d activity in the superficial layers of the SC (i.e. stratum griseum superficiale and stratum opticum) is adult-like by the fourth week of age. Deafferentation of the superficial SC, both in the neonatal and adult rat, and block of retinal activity lead to reduction in the size of the SC and changes in NADPH-d-positive neurons, including dendrite misorientation, decreased cell size and reduced number. Some of these changes are seen also in the aging animal.

These results document a protracted and progressive increase in the development of NADPH-d expression in the SC. Our results suggest a strong influence of retinal afferents and activity on the development and maintenance of NADPH-d-positive neurons in the retinorecipient layers of the SC, where NO can act as a retrograde signal to carve the terminal arbors of retinal axons.

Key words

Nitric oxide; eye enucleation; retinotectal projection; terminal axon refinement; activity.

List of abbreviations:

dLGN dorsal lateral geniculate nucleus

HZ horizontal neuron

MAR marginal neuron

NADPH-d dihydronicotinamide adenine-dinucleotide phosphate diaphorase

NBT nitroblue tetrazolium

NFV narrow field vertical neuron

NOS nitric oxide synthase

PYR pyriform neuron

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SC superior colliculus
SGS stratum griseum superficiale
SO stratum opticum
STL stellate neuron
UN unclassified neuron
vLGN ventral lateral geniculate nucleus
WFV wide field vertical neuron

Introduction

Nitric oxide (NO) is produced as a result of the conversion of arginine to citrulline, a reaction that is catalyzed by the enzyme nitric oxide synthase (NOS). NO in the CNS has received attention because of its potential contribution to events such as long term potentiation in the hippocampus (Schuman and Madison, 1991), long term depression in the cerebellum (Shibuki and Okada, 1991), and also in regeneration (Wu and Scott, 1993). Increased synthesis of NO is detected in neurons subsequent to activation of the N-methyl-D-aspartate (NMDA) receptor (Montague *et al.*, 1991; Moncada *et al.*, 1991; Vincent, 1994; Garthwaite and Boulton, 1995). This observation, along with the short half-life of NO within the brain, has led to the hypothesis that NO acts as a local retrograde signal between postsynaptic neurons and afferent fibers, in response to afferent activity; this hypothesis is particularly intriguing in relation to connection formation in the immature CNS: in fact, NO is believed to be the retrograde messenger that signals activity-dependent stabilization of synapses during development (Montague *et al.*, 1991).

In the visual system, transiently exuberant, ipsilaterally projecting retinotectal fibers are eliminated during development, an event that is known to be dependent upon activity-mediated competition between axons from the two eyes. In chicks, NOS is present in tectal neurons during the time of elimination of the transient ipsilateral projection (Williams *et al.*, 1994); administration of N-nitro L-arginine or N-nitro L-arginine methyl ester (both of which inhibit NO production) leads to an abnormal retention of the ipsilateral retinotectal projection (Wu *et al.*, 1994), leading to the conclusion that, following activation of retinotectal synapses, NO serves as the retrograde signal that passes from tectal neurons to retinal fibers; the absence of NO production thus blocks the normal activity-dependent elimination of ectopically projecting retinal fibers. We have shown that NOS block in developing rats also disturbs the refinement of retinocollicular axons (Vercelli *et al.*, 2000; Vercelli *et al.*, 2001), which results in the maintenance of aberrantly projecting ipsilateral retinocollicular axons. In chicks, the topographic mapping of the developing retinotectal projection is initially crude, and undergoes a process of refining, a process that involves the pruning of mistargeted, exuberant connections (Nakamura and O'Leary, 1989; Wu *et al.*, 2001).

Here we employ a simple histochemical reaction to visualize the expression of NADPH-d in the superior colliculus of developing aging and visually deafferented rodents. Our results document a protracted up-regulation of enzyme activity within the neurons of the SC, followed by a marked increase in enzyme expression around the time of eye opening. Our results confirm earlier reports on the co-localization of expression of NADPH-d (Gonzalez-Hernandez *et al.*, 1993; Tenório *et al.*, 1995) and

NOS (de Bittencourt-Navarrete *et al.*, 2004; Giraldo-Guimarães *et al.*, 2004) in many cells of the rodent SC, and extend these prior findings by providing a quantitative analysis of various NADPH-d-positive collicular cell types.

These observations in the developing animal provide the basis for examining how the NADPH-d-positive SC neurons are altered in the absence of visual input: we have studied the effects of removing one eye (at P0, as well as in the adult animal) of intraocular injections of tetrodotoxin (TTX), or of eyelid suture, on the morphology of NADPH-d-positive SC neurons. We show that NADPH-d expression in the SC is highly sensitive to the presence of retinal fibers, and to retinal activity; however, eyelid suture, or lack of patterned activity, during adulthood do not influence enzyme expression. We also show that changes in NADPH-d-positive SC neurons are similar to those seen after blocking retinal activity or in the absence of retinal afferentation.

Finally, we studied the expression of NADPH-d in the SC of aged rats, which may be considered both as an end point of development and a condition of impaired retinal activity.

Methods

Brains from 75 Wistar albino rats (from the animal colony bred at the University of Torino) were processed for visualizing NADPH-d. The animals ranged in age from embryonic day (E) 17 to adulthood (3-6 months) and senescence (24-36 months; see Table 1). Timed-pregnant dams were obtained from our breeding colony; the day of impregnation is referred to as E0, and the day of birth as P0 (=E22). Postnatal rats were maintained on a 12 h light/12 h dark cycle, and were allowed to drink and eat *ad libitum*. They were sacrificed by an overdose of ketamine hydrochloride and were perfused transcardially with 0.1M phosphate buffer, pH 7.4 (PB), followed by fixative solution (4% paraformaldehyde in PB). The brains were removed and immersed in the same fixative solution for 4 h. The tissue was cryoprotected by immersion in 30% buffered sucrose overnight and cut in the transverse plane on a cryostat, at a thickness of 50 μm .

Experimental procedures on rats

Eye enucleation. i) The left eye was enucleated at P0 in 15 rat pups: 7 of these were killed as adults, the other eight were killed at various stages of development (at 3 and 4 weeks of age); ii) five adult rats were enucleated on the left side, and killed after different survival times. For eye enucleation, an incision was made at the edges of the eyelid, the optic nerve with the ophthalmic artery was exposed and ligated with a 6-0 silk suture, the nerve was cut, and the eye removed. The orbit was filled with Gelfoam embedded in xylocaine, and the eyelids were sealed with a 4-0 silk thread.

Eyelid suture. For three additional rats, the left eyelid was sutured at P12 (a couple of days prior to the time of eye opening); the animals were killed at four weeks of age.

Intraocular injections of tetrodotoxin. To block activity in retinal axons, 100 nl of 1.6×10^{-3} M tetrodotoxin (TTX; Sigma, MO, USA) were injected into the left eye of P1 rats via a glass micropipette inserted behind the temporal ridge of the *ora serrata*, accord-

Table 1 – Number of rats used for this study.

| Age | Rats | Age | Rats | Age/treatment | Rats |
|-----|------|---------|------|-------------------|------|
| E17 | 3 | P12 | 2 | 4-36 mos | 7 |
| E19 | 2 | P13 | 1 | | |
| P0 | 2 | P14 | 4 | TTX | 3 |
| P3 | 1 | P15 | 2 | Suture | 3 |
| P4 | 2 | P16 | 1 | P0 enucleation | 15 |
| P5 | 2 | P19 | 1 | Adult enucleation | 5 |
| P6 | 1 | P20 | 1 | | |
| P7 | 2 | P22 | 3 | | |
| P8 | 2 | P30 | 3 | | |
| P10 | 2 | 3-6 mos | 5 | | |

ing to the protocol of Galli-Resta *et al.* (1993). This procedure was repeated every 7 days (at P1, P8 and P15) until P22, at which point the rats were killed.

All surgical procedures were performed under hypothermia (for newborn rats) or under general anesthesia achieved by intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg; Inoketam, Virbac, Milan, Italy) and xylazine (5 mg/kg; Rompum, Bayer, Leverkusen, Germany). Rats were killed as above and the tissue was fixed by perfusion and subsequent immersion as described above.

Histochemistry and immunohistochemistry

Sections through the SC of young pups (up to P12) were mounted on gelatin-chrome alum coated slides; sections from older rats were processed free-floating in 24-well dishes. All tissues were immersed for 1 h in a solution of 1 mg/ml NADPH (Sigma, St. Louis, MO) and 0.2 mg/ml Nitroblue tetrazolium (Sigma) in PB with 1% Triton X-100 at 37 °C (Vincent and Kimura, 1992; Vercelli and Cracco, 1994). A high concentration of Triton X-100 is critical for achieving a consistently good histochemical reaction (Fang *et al.*, 1994, and also our experience), as is a short time of postfixation in paraformaldehyde (the intensity of histochemical reaction decreases with overnight postfixation). Sections were rinsed in PB; free-floating sections were mounted on gelatin-chrome alum-coated slides; all the sections were air dried overnight, dehydrated, cleared in xylene and mounted with Eukitt (Bioptica, Milan, Italy). In some cases, the tissue was counterstained with 1% neutral red.

To assess the reliability of NADPH-d histochemistry for visualizing neuronal NOS (nNOS), alternate series of sections from a few adult rats were incubated overnight at room temperature with an antibody against nNOS (rabbit anti-nNOS, 1:500, Affiniti, Mamhead, UK), or sections were first reacted for 10 min in NADPH-d reaction medium, rinsed thoroughly, and then further labeled for the immunolocalization of nNOS. Binding of primary antibody was visualized by incubating sections for 2 h with a biotinylated goat anti rabbit secondary antibody, followed by reaction

for 2 h in avidin-biotin-horseradish peroxidase complex (ABC Kit Elite, Vector Labs, Burlingame, CA); peroxidase was revealed with use of 0.05% 3-3'-diaminobenzidine (Sigma) as chromogen.

Microscopic analysis and quantification

For each age, sections from mid-SC levels were photographed; also, at selected ages, NADPH-d-positive cells in the superficial layers of the SC were drawn at 100 X with a drawing tube attached to the microscope (Leitz Dialux or Nikon; Figs 1 and 3). Neurons in the superficial layers of the SC were classified as marginal (MAR), horizontal (HZ), stellate (STL), wide field vertical (WV) or narrow field vertical (NFV), and pyriform (PYR) according to the criteria described by Vercelli and Cracco (1994); neurons that did not fall into these categories were designated "unclassified". Multipolar neurons present in the SO (*stratum opticum*) are not included in this classification.

To study the dendrite orientation of SC neurons, the angle of each dendrite relative to the dorsoventral axis of the brain was measured on drawings made at 20x magnification. Thus, a dorsally directed dendritic arbor was considered to be at 0°, a medially directed one at 90°, etc. The frequency distribution of the angles of dendrite orientation was calculated at 30° intervals for enzyme-positive neurons in each colliculus, and circular diagrams were drawn to represent the mean frequencies (Fig. 4). Statistical analysis of the data was done as appropriate for circular distributions, according to Zar (1984; see also Vercelli and Cracco, 1994 for more details). Briefly, the mean angle of dendrite orientation and its circular standard deviation were calculated, together with the vector r along the mean angle: this number (from 0 to 1) is directly proportional to the number of dendrites oriented along the mean angle.

A quantitative analysis of age-related increase in number of NADPH-d-positive neurons in the different visual centers was completed with use of the disector method on rats at P12, P14, P19, P33 and adult (P90) (one at each age-point). The total volume of the superficial layers, *i.e.* optic fiber layer (SO) and superficial gray layer (SGS: *stratum griseum superficiale*) of the SC was calculated with the aid of NeuroLucida and Neuroexplorer software (Microbrightfield Inc., Vermont) by measuring the area of the layers in each section and multiplying the result by the thickness of the section. Cell density was measured with StereoInvestigator software (Microbrightfield Inc.). Neurons were counted at the final magnification of 400x at approximately 10 to 20 locations (counting fields chosen at random by the software) within three different sections through each colliculus. Within each counting field, the number of neurons in an optical box of 100 $\mu\text{m} \times 100 \mu\text{m} \times 50 \mu\text{m}$ (the last figure corresponding to section thickness) were quantified according to the optical disector method (Coggeshall and Lekan, 1996). The total numbers of NADPH-d positive cells, and of all neurons, were estimated by multiplying the density of the cells (cells/mm³) by the volume (mm³).

The results are given as mean \pm standard deviation. Statistical analysis consisted in the unpaired t test, with two tails. Values of $p < 0.05$ were considered significant.

Results

NADPH-d expression in adult SC

In the mature animal, NADPH-d histochemistry revealed cells labeled with different intensity. Intensely labeled cells, that appear to be fully stained including spines on dendrites that are usually filled up with reaction product to their terminal ends; moderately labeled neurons, such as the horizontally-oriented ones that reside in the deeper part of the SGS and whose dendrites can be followed for at least two hundred micron; weakly labeled neurons, with only the cell soma being reactive and no visible dendrites - these cells cannot be classified on morphological grounds (see details in Vercelli and Cracco, 1994). Taken together, the intensely and moderately stained cells can be characterized as belonging to the MAR, HZ, STL, PYR, NFV or WFV classes

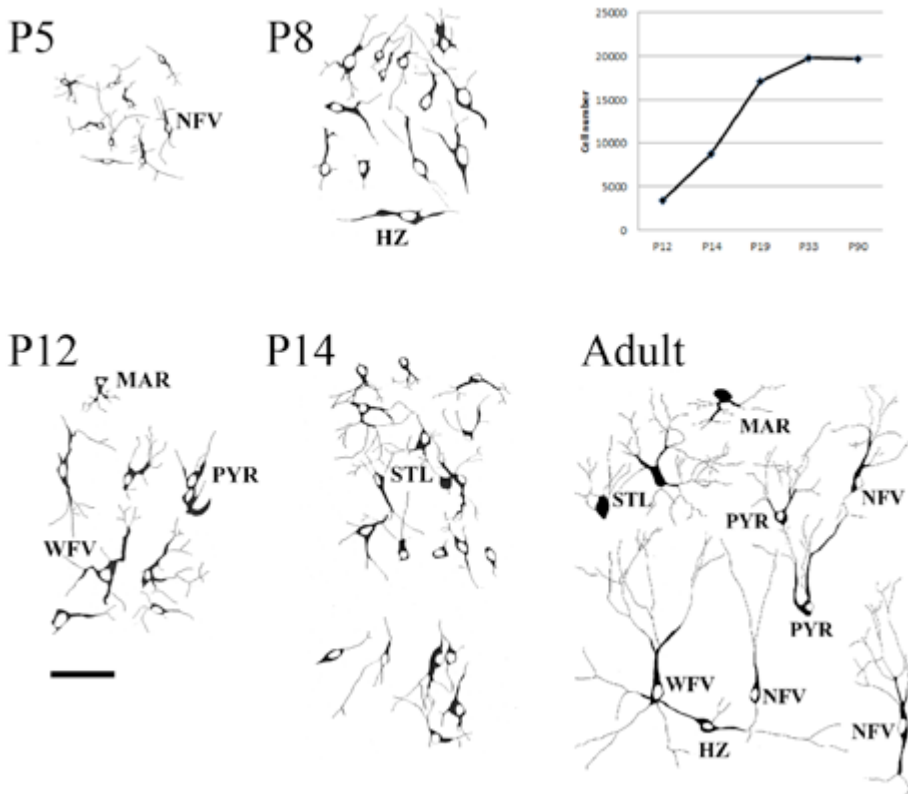


Fig. 1 – Morphology and number of NADPH-d-positive neurons in the developing and adult retinorecipient layers of rat SC. Camera lucida drawings of NADPH-d-positive neurons in the superficial layers of the SC in developing and adult rats. Marginal (MAR), stellate (STL), horizontal (HZ), pyriform (PYR), narrow field vertical (NFV), and wide field vertical (WFV) cells are indicated (putative at developmental stages). Scale bar = 50 μ m. Upper right corner: total number of NADPH-d-positive neurons in the retinorecipient layers of the developing SC at different postnatal ages.

(Fig. 1). The axons of many of these cells can be followed for several hundreds of microns, or to the edge of the section. Please note that multipolar neurons present in the SO are not included in our classification scheme. A rich neuropil of densely labeled fibers is observed primarily in the deeper (intermediate and deep gray) layers of the SC, whereas a fine network of NADPH-d-positive fibers is seen in the *stratum zonale* and in the SGS, but not in the SO.

Anti nNOS immunohistochemistry revealed that the overall distribution of immunoreactive cells is similar to that seen with NADPH-d histochemistry; however, at a quantitative level, there were fewer nNOS-positive cells than those stained for NADPH-d (data not shown). Because the dark blue reaction product for NADPH-d tended to obscure the oxidized diaminobenzidine immunostaining, it was not possible to identify all NADPH-d-positive cells as being immunoreactive, but we can state with certainty that in double-labeled sections none of the nNOS-positive SC neurons were void of NADPH-d activity. A marked difference between the two histochemical methods, however, was that we observed almost no neuropil staining with the NOS antibody – this was true not only in the SGS, but in deeper layers as well, where histochemistry normally revealed an intense, patterned distribution of fibers. Moreover, dendrites were not as fully filled (to the tips) with the immunoreactive product as they were with NADPH-d reaction product. Finally, NADPH-d histochemistry revealed blood vessels, which were also immunoreactive for nNOS. Therefore enzyme histochemistry apparently revealed more than one form of NOS, whereas the antibody is stated by the producer to be specific for nNOS (Bredt *et al.*, 1991b; Vincent, 1994).

NADPH-d expression in the developing SC

In the SC of E17 fetuses, virtually no diaphorase-labeled neurons or fibers were detected - the only sign of NADPH-d-reactivity was at the midline, where cells near the ependymal lining of the developing ventricle were stained; at E19, a few neurons in the periaqueductal gray matter and rare cells in the deep gray layer of the SC were labeled; by P0 (=E22), numerous cells showing intense reactivity for NADPH-d were present in each section through the deep tectal layers, and a few positive cells were also visible in the intermediate gray layer (data for these early times not shown).

It was not until P4 that NADPH-d histochemistry revealed a few, lightly-labeled neuronal somata in the SGS; light staining of the neuropil was also visible in the SGS (Fig. 2). However, either the incomplete labeling of processes, or their immaturity, of the diaphorase-expressing cells at this age did not permit categorization of the cells on a morphological ground. In P6 and P8 pups (Fig. 2), however, dendrites of scattered, diaphorase-reactive neurons expressed enough enzymatic activity to allow these newly differentiated cells to be classified as putative HZ, NFV, WFV or PYR cells, on the basis of the size and overall orientation of their processes (Fig. 1). The SO was completely devoid of axonal as well as cellular labeling at this early age (Fig. 2). From P7 onward, progressively more SGS neurons were NADPH-d-positive.

In pups aged P10-12, the neuropil and many cells in SGS were darkly enzyme-positive (Figs 1 and 2), and most could be classified on the basis of the orientation of their dendrites. MAR neurons in P12 SC had vertically-directed dendrites, although these were relatively short and wide compared to the ones seen in the adult ani-

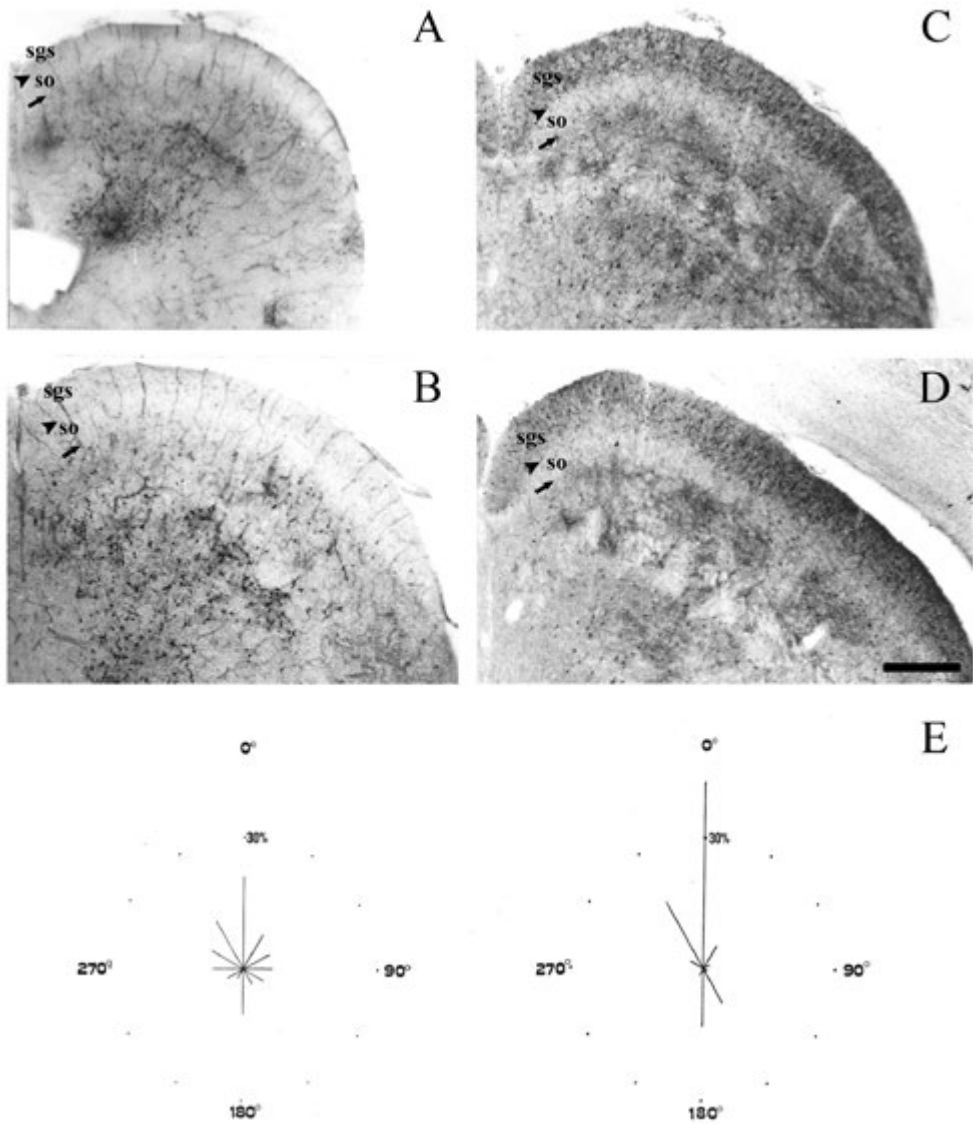


Fig. 2 - NADPH-d reacted SC at different ages and dorsoventral orientation of main positive cell types. Low magnification photomicrographs of coronal sections through the SC of rats aged P4 (A), P6 (B), P14 (C) and P22 (D). All sections are stained histochemically for NADPH-d. Arrowheads and arrows lines indicate respectively the upper and lower borders of the stratum opticum (so); sgs: stratum griseum superficiale. Scale bar = 200 μ m. E): Representation of the mean percent circular distribution of overall dendritic orientation for NFV, WFV and PYR neurons at P14 (left) and in adult rats (right). 0° is dorsal, 90° is medial. The length of each line is proportional to the percentage of dendrites aligned in a 30° interval.

mal; the perikarya of these cells were located close to the pial surface of the SC. HZ neurons had large, lightly labeled cell bodies, were located primarily in the deeper part of the SGS, and displayed two primary dendrites that run mediolaterally; STL neurons, visible as of P12, had cell bodies of different sizes, radially directed dendrites, and were found located mostly in the superficial SGS; NFV neurons were small, located in the superficial part of SGS, and displayed one or two dendrites that emerged from opposite sides of the soma; at P10-12 these dendrites were not as precisely oriented in the dorsoventral direction as in adult animals (Fig. 2). The cell bodies of WFV cells were larger than those of NFV neurons, were located in the deep SGS and had long dendrites. Finally, PYR neurons could be classified on the basis of their medium size cell somata from which two dendrites emerged along the dorsal aspect. By the time of eye opening on P14, the cells and also the neuropil in the SGS were intensely labeled (Figs 1 and 2). All cell types found in adult rats were clearly identifiable in the P14 tectum, although the orientation of dendrites of NGF, WFV and PYR neurons (strictly dorsoventral in adult rats) was still immature (Fig. 1). The circular standard deviation from the mean angle was much higher in P14 rats (Fig. 2), and the mean vector r of dendrite orientation was significantly lower, than in adults (Table 2). It should be recalled that r would tend to 1 if dendrites were all oriented along the same direction (i.e. along the mean angle of dendrite orientation) whereas it would tend to 0 if the distribution of dendrite orientation were to be uniform in all directions. Only after the third week of postnatal life (Fig. 1) did NADPH-d staining in the SC acquire an adult-like pattern. Quantitation shows that the number of NADPH-d-positive cells increased gradually over the first two weeks of life, with an abrupt increase in number of enzyme-positive neurons around the time of eye opening (four-fold in the week between P12 and P19, Fig. 1); the adult pattern of enzyme staining in NADPH-d-positive cells was observed by the end of the first month of postnatal life.

Effects of P0 eye enucleation

Unilateral eye enucleation done at P0 affects NADPH-d activity in the developing superficial layers of the contralateral SC, at 3-4 weeks of age. The ipsilateral side is not affected relative to normal SC, and thus has been used as control: the neuro-

Table 2 – Dendrite orientation of NADPH-d positive neurons in the SC superficial layers of each of four young (Y; P14) and four adult (A) rats. For each animal at least one hundred neurons were analyzed. Circ. st.dev. = circular standard deviation, r = mean vector of dendrite orientation ($r = 1$ when all dendrites are dorsoventrally oriented). The mean value for r is significantly lower in P14 than in adult rats (0.180 ± 0.034 vs 0.772 ± 0.048 , $p < 0.0001$).

| Rat | Mean angle | Circ. st. dev. | r | Rat | Mean angle | Circ. st. dev. | r |
|-----|------------|----------------|------|-----|------------|----------------|------|
| Y1 | 320° | 103° | 0.19 | A1 | 344° | 43° | 0.75 |
| Y2 | 353° | 113° | 0.14 | A2 | 352° | 34° | 0.83 |
| Y3 | 42° | 106° | 0.17 | A3 | 351° | 39° | 0.79 |
| Y4 | 348° | 99° | 0.22 | A4 | 348° | 46° | 0.72 |

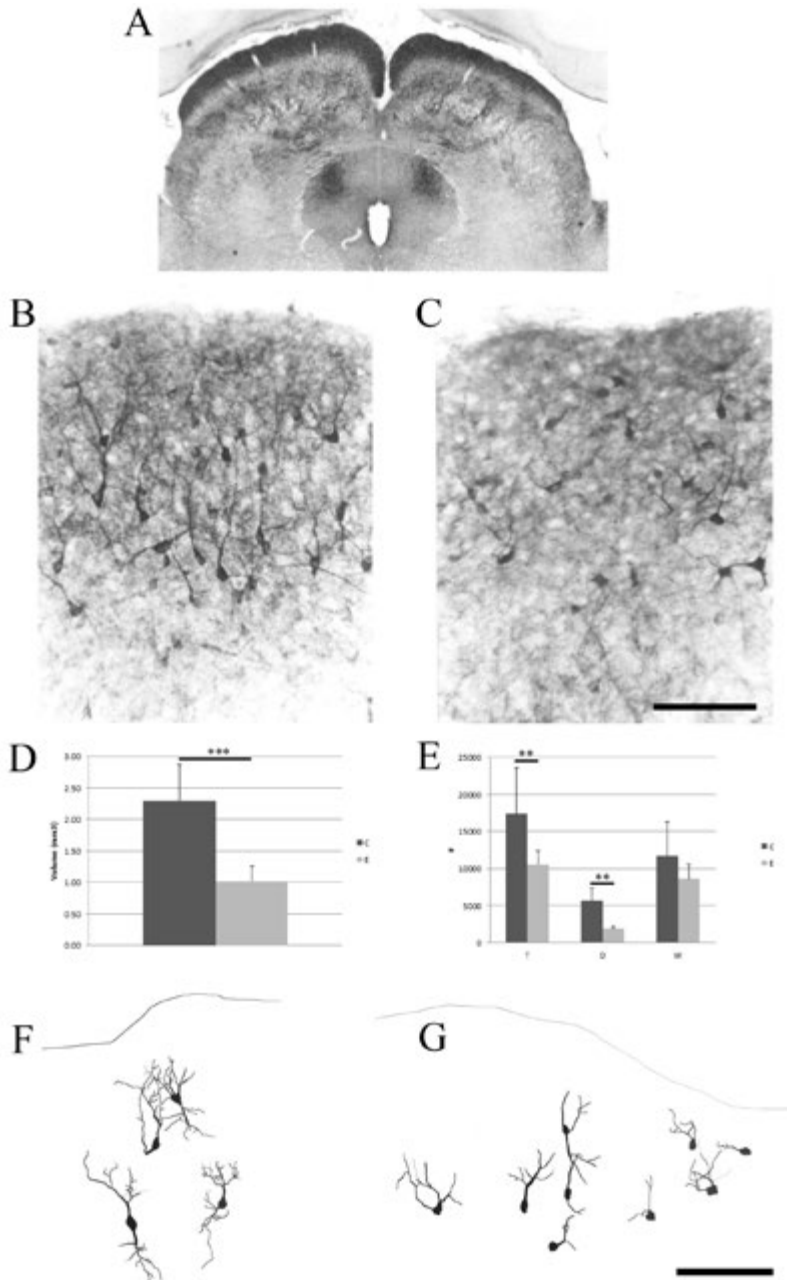


Fig. 3 – Effects of neonatal eye enucleation on the superficial layers of the SC. Low magnification of a coronal section through the SC of a rat which had undergone eye enucleation at P0 : deafferented side on the right. B) and C): Higher magnification of the stratum griseum superficiale of the control and deafferented side, respectively. D) and E): Histograms related to the total volume of the superficial layers of the SC (D) and the number of all, darkly and lightly stained neurons (E) on the control (black bars) and deafferented (grey bars) side; ** $p < 0.01$; *** $p < 0.001$. In F) and G) Neurolucida drawings of NADPH-d-positive neurons in the stratum griseum superficiale on the control (F) and deafferented (G) side, in which dendrites are less intensely labeled, atrophic and misoriented. Scale bar = 1 mm in A, 100 μ m in B-C and F-G.

pil shows a lower intensity of labeling, and fewer neurons are NADPH-d-positive on the contralateral (deafferented) side, where the neuropil is less intensely labeled than on the ipsilateral side (control) side, and labeled collicular neurons usually show the only cell body or the stem dendrites (Fig. 3A-C).

When the SC is denervated by removal of the opposite retina on P0, the volume of the superficial layers of the deprived SC is decreased by 61% (Fig. 3D). The number of NADPH-d-positive neurons also decreases significantly in the superficial layers of SC (by 70% for intensely labeled cells – $p < 0.01$ – and by 26% for weakly labeled ones; Fig. 3E). The size of NADPH-d positive neurons is decreased in the deprived side: from 81.22 ± 13.77 to 61.55 ± 10.98 ($p < 0.0001$; Fig. 3F-G). In order to exclude the possibility that the differences in cells size between the two sides were due to a different cell shape in the 3 axis, we also made some measurements in the brain of one adult rat in which the eye was removed at P0 and the brain was cut in the sagittal plane at day P90. The results confirmed a decrease in cell size in the SC by 30% (SC $88.93 \pm 11.09 \mu\text{m}$ vs $62.07 \pm 8.95 \mu\text{m}$; $p < 0.01$).

In summary, the dendrite orientation of NADPH-d-positive SC cells was highly disrupted (Table 3), and there was a strong increase in circular standard deviations of mean angles of dendrite orientation; the mean r vector was also significantly decreased (control: $r = 0.51 \pm 0.15$; enucleated: $r = 0.22 \pm 0.15$; $p = 0.0015$).

Effects of postnatal TTX treatment

TTX eye administration causes a reduction in NADPH-d activity in the superficial layers of the contralateral SC. As with denervation, it is difficult to classify labeled neurons on the deprived side, opposite to the control side (left). NADPH-d positive neurons were decreased in size (TTX= 76.93 ± 14.67 ; control = 90.31 ± 11.85).

Table 3 – Dendrite orientation of NADPH-d positive neurons in the SC superficial layers of adult rats (enucleated at P0), comparing the control (C) and the enucleated (E) side. For each animal at least one hundred neurons were analyzed.

| Rat | Mean angle | | Circular st. dev. | | r | |
|-----|------------|------|-------------------|------|------|------|
| | C | E | C | E | C | E |
| 1 | 356° | 13° | 46° | 114° | 0.72 | 0.13 |
| 2 | 354° | 16° | 50° | 110° | 0.68 | 0.16 |
| 3 | 1° | 345° | 61° | 74° | 0.57 | 0.42 |
| 4 | 357° | 4° | 67° | 76° | 0.51 | 0.41 |
| 5 | 355° | 312° | 71° | 95° | 0.46 | 0.25 |
| 6 | 340° | 340° | 69° | 93° | 0.48 | 0.27 |
| 7 | 4° | 27° | 97° | 131° | 0.24 | 0.07 |
| 8 | 7° | 315° | 78° | 166° | 0.40 | 0.02 |

Effects of eyelid closure

The superficial layers of the SC contralateral to eyelid suture (right) did not show gross alterations in the expression of NADPH-d activity, in the distribution of cell types and or in the orientation of dendrites, as compared with the opposite side.

Effects of adult eye enucleation

Removal of one eye in the adult animal leads to a decrease in the volume of the superficial layers of the SC on the deprived side: the decrease could be observed already after one week post-enucleation (Fig. 4). The size of NADPH-d-positive SC neurons decreased two weeks after enucleation (Fig. 4A). The overall dendrite orientation of NADPH-d-positive cells in the superficial layers of SC was more dispersed on the deprived side, starting from 7 days post enucleation (Fig. 4B).

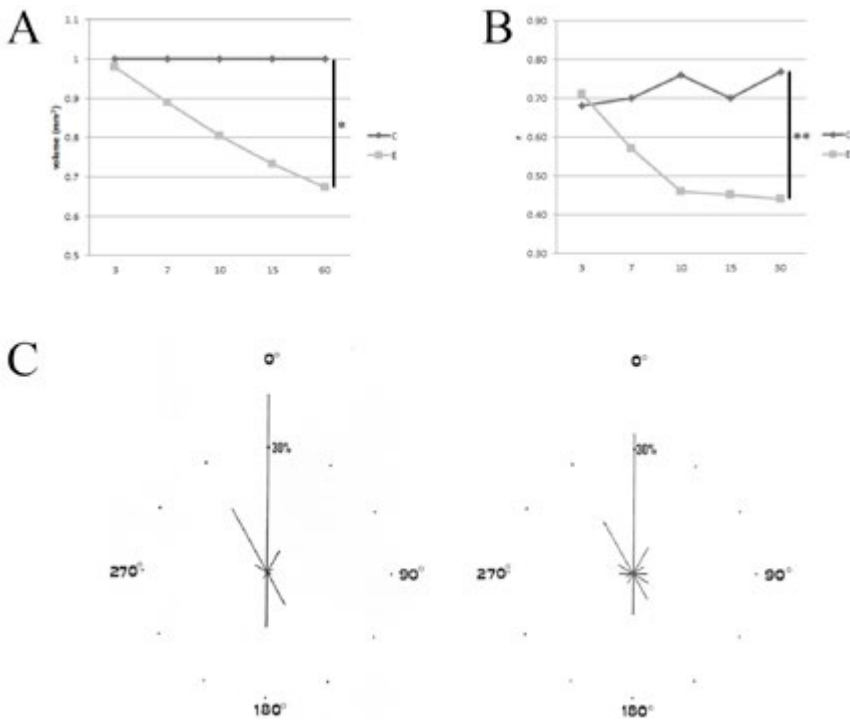


Fig. 4 – Effects of adult eye enucleation on the superficial layers of the SC. Histograms of the changes induced through time (expressed as days) by eye enucleation in adult life. Volume of the superficial layers (A) and dendrite orientation (r) (B) in control (C) and deafferented side (E); already 7 days after enucleation the two values diverge significantly: * $p < 0.05$; ** $p < 0.01$. On the abscissa time interval from eye enucleation (days). In C, orientation of dendrites in control (left) and enucleated (right) side one month after adult eye enucleation.

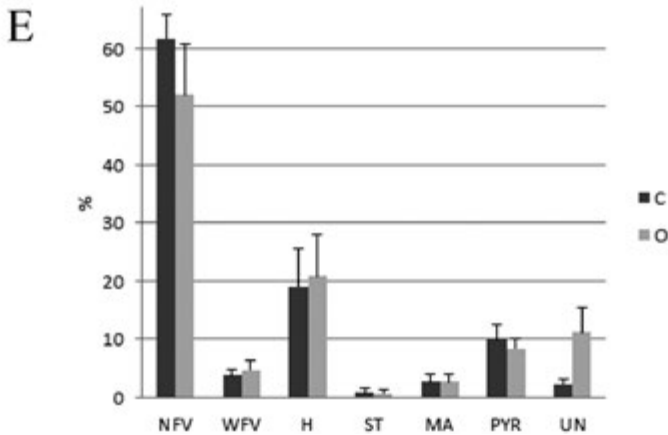
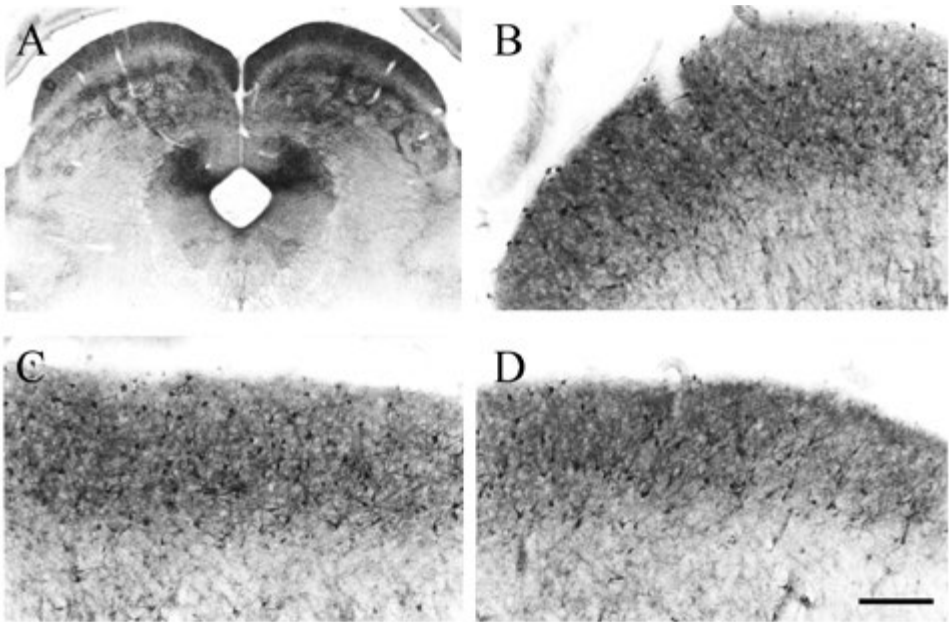


Fig. 5 – NADPH-d activity in the aging SC. Coronal section through the SC at low magnification (A), and higher magnification of the stratum griseum superficiale on the medial (B), intermediate (C) and lateral (D) parts. E): distribution of neurons in the different classes in control and old rats. Scale bar = 500 μ m in A, 100 μ m in B-D

Effect of aging on the superficial layers of the superior colliculus

Aged rats showed considerable inter-animal variability in the superficial layers of the SC; in general, we made the following observations: i) no changes in volume of

Table 4 – Dendrite orientation of NADPH-d positive neurons in the SC superficial layers of adult controls (A) and aged rats (O). For each animal at least one hundred neurons were analyzed.

| Rat | Mean angle | Circ. st. dev. | Rat | Mean angle | Circ. st. dev. |
|-----|------------|----------------|-----|------------|----------------|
| A1 | 344° | ± 43° | O1 | 353° | ± 48° |
| A2 | 351° | ± 39° | O2 | 345° | ± 55° |
| A3 | 348° | ± 46° | O3 | 350° | ± 87° |
| A4 | 352° | ± 34° | O4 | 343° | ± 69° |
| | | | O5 | 359° | ± 57° |
| | | | O6 | 334° | ± 97° |
| | | | O7 | 333° | ± 82° |

the superficial layers (control $2.08 \text{ mm}^3 \pm 0.188$ vs old $2.17 \text{ mm}^3 \pm 0.363$); ii) no changes in the overall density of NADPH-d-positive neurons, although with a remarkable decrease in density at the medial and lateral edges of the SC; consequently the total number of neurons was unchanged (control 13459 ± 3641 vs old 16400 ± 2732); iii) a tendency to a decrease in the percentage of the intensely stained NADPH-d positive cells (control $36.84\% \pm 6.80$ vs old $30.029\% \pm 7.86$); iv) a significant decrease in the soma size of intensely NADPH-d-positive neurons (control $135.178 \mu\text{m}^2 \pm 13.123$ vs old $103.279 \mu\text{m}^2 \pm 16.904$; $p < 0.05$); v) a shift in the distribution of cell types, with a decrease in the percentage of narrow field vertical neurons (Fig. 5); vi) a rearrangement of the overall dendrite orientation with a significant decrease in the mean vector of dendrite orientation: control 0.778 ± 0.051 vs old 0.475 ± 0.176 ($p < 0.01$; Table 4); vii) shorter overall length of dendrites of NADPH-d-positive neurons.

Discussion

By analyzing the patterns of NADPH-d expression in the SC of developing and aged rats we could document a gradual differentiation of neurons and neuropil that extends well into postnatal life and a temporal relationship between NADPH-d expression in the SC and what is known about the development of retinocollicular axons. Moreover, by experimental manipulation of visual activity, we have highlighted the role of retinal axons on shaping the morphology of neurons in the superficial layers of the SC and on their NADPH-d activity.

Technical considerations

NADPH-d colocalizes with the brain isoform of NOS in neurons of the peripheral and central nervous systems (Bredt *et al.*, 1991a; Dawson *et al.*, 1991; Hope *et al.*, 1991; Schmidt *et al.*, 1992) and, in particular, of the visual system (Hope *et al.*, 1991; Vincent and Kimura, 1992). Our double labeling experiments confirm that NADPH-d and nNOS are co-localized in many collicular neurons and along their major dendrites. Thus NADPH-d histochemistry can be used as a simple and reliable method

to identify nNOS. However, NADPH-d histochemistry is more efficient than immunohistochemistry in that it labels more of the finer distal dendrites than the Affiniti antibody. Also, in our hands, axons and elements of the neuropil were revealed by enzyme histochemical staining but not by immunostaining. Our results partially disagree with those of Cork *et al.* (2000) in the mouse: these authors report that nNOS-positive neurons (detected by immunohistochemistry) are visible earlier in development than NADPH-d positive neurons (detected by enzyme histochemistry). We find NADPH-d-positive cell bodies in the superficial layers of the SC of P4 rats, which is a comparable, if not earlier, stage than that reported for NOS-positive neurons in the P5 mouse (Cork *et al.*, 2000).

In preliminary experiments, we tried various dilutions (from 0.1 to 1%) of Triton X-100 in the reaction mixture and found that a relatively high concentration (1%) of Triton X-100 gave optimal staining, while still preserving morphological details. Lower concentrations of Triton X-100 led to decreased histochemical reactivity and increased precipitation of the nitroblue tetrazolium (NBT), in keeping with the report by Fang *et al.* (1994), who show that NBT solubility is proportional to concentration of Triton X-100. In our hands, tissue processed with short exposure (4 h) to paraformaldehyde and higher concentration of Triton X-100 (1%) gave reliably good staining. This was verified in several cases, when sections from brains of different ages were processed together, to minimize potential differences in staining. The possibility that lower intensity of staining in the younger brains arose from the on-slide staining of the immature tissue (versus staining of free-floating sections of more mature tissue) had been previously excluded by observations on other regions (e.g., cortex, striatum), which showed comparably stained cells between tissue processed free-floating and on slide (Vercelli *et al.*, 1999).

nNOS in the visual system

Neuronal NOS- or NADPH-d-positive neurons have been detected in the visual system of birds as well as mammals. In the chick, numerous NADPH-d positive neurons are found in the optic tectum (Williams *et al.*, 1994), appearing as early as E5 in the neuroepithelial layer I; they are detected in more superficial tectal layers as of E8, before the refinement of the final location of axonal terminal zones along the rostro-caudal axis takes place (Nakamura and O'Leary, 1989; Wu *et al.*, 2001). In the superficial layers of the rat SC, enzyme activity is detected a few days after the arrival of retinal afferents (Bunt *et al.*, 1983), and when cortical afferents are growing into the SC (Ramirez *et al.*, 1990; Rhoades *et al.*, 1991; see also, Tenório *et al.*, 1995, 1996). In adult rodents, prominent NADPH-d activity has been reported in the SC (Gonzalez-Hernandez *et al.*, 1992), in the cells and neuropil of both the dLGN (dorsal lateral geniculate nucleus) and vLGN (ventral lateral geniculate nucleus), as well as in the visual cortex (Bredt *et al.*, 1991a; Vincent and Kimura, 1992; Gonzalez-Hernandez *et al.*, 1993; Gabbott and Bacon, 1994a, 1994b). In ferrets, NADPH-d expression in cells of the lateral geniculate nucleus is transient, detected between 1-5 weeks of postnatal life (Cramer *et al.*, 1995), after which enzyme activity diminishes. NADPH-d is also expressed by amacrine cells in the rodent retina (Mitrofanis, 1989; Palanza *et al.*, 2002; Palanza *et al.*, 2005) and by interneurons of the visual cortex (Vincent and Kimura, 1992). Therefore, neuronal cell bodies and fibers expressing NADPH-d are widely

represented in the visual system, both in the adult animal and during development, suggesting one or more roles for NO in visual function.

Adult pattern and development of NADPH-d activity in the SC

Cells in the *stratum zonale* and SGS display various levels of intensity of NADPH-d staining. All cell types described in earlier Golgi studies (Langer and Lund, 1974; Tokunaga and Otani, 1976; Labriola and Laemle, 1977; Warton and Jones, 1985) can also be identified in histochemically stained tissue (Gonzalez-Hernandez *et al.*, 1992; Vercelli and Cracco, 1994). However, we have shown in a previous paper that only a subset of the collicular neurons are positive for NADPH-d (Vercelli and Cracco, 1994), implying that only a subset of each of the tectal cell types expresses the enzyme: the reason for this selectivity is not understood. We do know, however, that NADPH-d activity is displayed by both intrinsic neurons, as well as by projection neurons in the upper layers of the SC. Specifically, PYR, NFV and WFV neurons project to the lateralis posterior nucleus or to vLGN (Langer and Lund, 1974; Mooney and Rhoades, 1993) while STL, MAR and HZ cells have axons which remain in the SGS (Langer and Lund, 1974) - all of these cell types express NADPH-d.

The source of the NADPH-d positive fibers in the neuropil of the different visual targets in rodents is also not known. In the SC, the neuropil in the superficial layers may derive primarily from NADPH-d-positive neurons *in loco*, rather than from outside the midbrain (the superficial SC receives projections from the retina, visual cortex, lateral geniculate nucleus and parabigeminal nucleus - Albers *et al.*, 1988). Our preliminary studies show no reduction in neuropil staining intensity 5 days following contralateral eye removal in the adult animal, nor after a lesion of the ipsilateral cortex. And while the large class A cells of the vLGN may be a possible source of NADPH-d positive input to the SC (these cells are intensely positive for NADPH-d activity - unpublished data), we saw no alterations in patterns of NADPH-d histochemical staining in the SC in one animal in which we made a deep wound just anterior to the SC to cut incoming axons from anterior cell groups to the superficial SC (unpublished observations - however, it may be that not all fibers between the diencephalon and the tectum were severed in this animal). This finding would also negate the possibility that the projection from the SC to the dLGN is a source of NADPH-d positive input to this dorsal thalamic nucleus (see Harting *et al.*, 1991).

In E17 rats, NADPH-d positive cells are found along the midline where the cell bodies of radial glia, implicated in barricading immature retinal axons from crossing the tectal midline (Wu *et al.*, 1995; see also, Mize *et al.*, 1996 for staining in embryonic kitten SC), are located; similar findings are reported in the neuroepithelial cell layer of the chick tectum at E5 (Williams *et al.*, 1994). As of E19, NADPH-d reactivity in the SC shows an inside-out gradient of development, following the same gradient as the neurogenesis of collicular cells (Altman and Bayer, 1981). Thus, deep tectal neurons are the first to express the enzyme; NADPH-d positive cell bodies are not seen in the superficial layers until P4, a few days after the arrival of retinal afferents (Bunt *et al.*, 1983; Edwards *et al.*, 1986a and 1986b for mouse, Jhaveri *et al.*, 1991, for hamster). Thereafter, enzyme levels in tectal neurons increase gradually over the first two weeks of postnatal life, and it is not until P12-P14, just prior to the time of eye opening, that a marked up-regulation in staining intensity and in the number of positive

cell profiles is noted (see also, Tenório *et al.*, 1995, 1996). The adult pattern of staining is obtained after the third week of age, as shown also by biochemical analysis (Giral-di-Guimarães *et al.*, 2004).

Effects of visual deafferentation

We show that retinal axons influence the differentiation of NADPH-d-positive neurons in the superficial SC: following removal of one eye during the first few days after birth, histochemical staining of tectal neurons in the contralateral SC of the adult animal is less intense, and we have evidence of distinct changes in the dendrite orientation of the NADPH-d positive SC cells resulting from deafferentation; we also note that dendrite atrophy is detectable in NADPH-d-positive cells within one week following contralateral eye enucleation in the adult animal. Moreover, blocking activity by intravitreal TTX administration produces results similar to eye enucleation. These findings support the report by de Bittencourt-Navarrete *et al.* (2009), who blocked activity with MK801 (a NMDA receptor blocker) and saw that the extent of dendritic labeling for NADPH-d histochemistry was reduced by 45% when compared to the opposite (unaffected) SC of the same animals, and by 64% when compared to the SC of control animals. In contrast, eyelid suture, which prevents vision-related but not spontaneous retinal activity (Galli-Resta *et al.*, 1993) does not significantly affect NADPH-d activity in neurons. However in the adult cat NADPH-d activity is upregulated in neurons of the LGN in response to contralateral eyelid suture, whereas it is normally expressed only in axons of mesencephalic cholinergic cells that project to this nucleus (Günlük *et al.*, 1994).

Thus retinal input has a profound effect on the maintenance of dendrite orientation on SC neurons; this influence is not related merely to afferentation, since injection of TTX into one eye of postnatal rats also leads to dendrite atrophy, or to a reduction in the amount of enzyme in distal dendrites, on NADPH-d-containing tectal neurons in the adult animal. Whether these effects of retinal deafferentation are direct or indirect still needs to be determined. Our findings disagree in part with the biochemical data of de Bittencourt-Navarrete *et al.* (2004), who found no biochemical changes in NOS activity or in NOS protein levels after neonatal binocular enucleation, and no significant changes in nNOS isoforms in western blots. On the other hand, Tenório *et al.* (1998) reported that visual deafferentation of the SC at birth does not alter the developmental sequence of nNOS expression in the SC superficial layers, but changes the intracellular distribution of this enzyme: in the normal SC, nNOS is distributed along the entire dendritic tree of neurons, but after removal of one eye at birth, nNOS staining disappears from the distal portions of the dendrite tree in the deafferented neurons (*ibidem*). The intracellular distribution of nNOS, its subcellular localization and the association of nNOS with elements of cytoskeleton have also been investigated in normal and enucleated animals, showing that post-synaptic labeled regions were often associated with presumptive retinal unlabeled terminals (Batista *et al.*, 2001).

Based on analysis of Golgi-impregnated collicular neurons, an early study by Warton and Jones (1985) documented that distinct neuron types can be identified in the superficial SC of the rat already at P3 (especially for NFV and WFV neurons), and that by P9 some of these cells bear long, branched dendrites that sport mature-

looking spines. In our study the appearance of NADPH-d-positive neuron types in the superficial layers of SC followed the same age gradient: at the very early ages the histochemical reaction was weak and we saw few enzyme-reactive neurons, while NADPH-d positive primary dendrites were visible during early postnatal life. Thus it is likely that dendritic differentiation at the morphological level slightly precedes the histochemical visualization of their processes. At older ages, the histochemical results allowed quantitative analyses on large numbers of neurons and documented that the dendrite orientation of some cell types (namely NFV, WFV and PYR) matures only after P14, once the eyes have opened.

The effect of aging on the expression of NADPH-d in the superficial layers of the superior colliculus was similar to that seen following eye enucleation, though much less pronounced: the staining intensity of NADPH-d-positive cells decreases, especially in NFV cells, and dendrites appear to be stunted, either as a result of dendritic atrophy or because of decreased enzyme expression in the distal dendrites. These data are in agreement with those of Díaz *et al.* (2006) who described, in the aging SC somatic atrophy in NFV and WFV neurons, an increase in dendritic processes with dorsoventral orientation and a reduction of mediolaterally oriented processes on WFV; they also reported that marginal neurons undergo somatic hypertrophy at 26 months relative to "control" 3-month-old adult rats. These authors concluded that the increase in the total dendritic length of NFV neurons compensates for the age-related atrophy of neighboring neurons. The changes we observed are also in agreement with recent reports on the aging mouse retina, where the dendritic arbor of retinal ganglion cells shrinks, as well as their terminal arbor in central target zones (Samuel *et al.*, 2011): the latter finding supports the hypothesis of a partial deafferentation of the superficial layers of the SC, associated with aging.

Collectively, these data suggest that retinal axons impact the superficial layers of the SC in three major ways: i) they alter the dorsoventral orientation of NFV and WFV neurons; ii) they seem to provide trophic support for tectal neurons, with regard to cell size and dendrite extent; and iii) they appear to regulate the distribution of NOS activity within the dendrites of tectal neurons. Whereas the first of these influences might be due mostly to mechanical forces, due to the preferential dorsoventral orientation of retinocollicular axons, the other two are cellular events probably due to excitatory activity at the synaptic level.

Possible Role(s) of NADPH-d in the SC

We have documented that NADPH-d activity appears in the retinorecipient layers of the SC as early as P4, in agreement with Gonzalez-Hernandez *et al.* (1993) and three days earlier than reported by Tenório *et al.* (1996; see Mize *et al.*, 1996 for related developmental events in cats). The earlier time point is important in view of the possible role of NADPH-d activity during the development of retinocollicular connections. Retinal axons invade the immature SGS around the time of birth (Lund and Bunt, 1976; Bunt *et al.*, 1983) and the process of synaptogenesis continues for many days thereafter (Lund, 1969; Lund and Lund, 1972). Cortical projections enter the tectum soon after birth (Thong and Dreher, 1986; Ramirez *et al.*, 1990; Rhoades *et al.*, 1991). Experiments in which nitro-arginine was systemically administered in developing hamsters show that segregation of the ipsilateral and con-

tralateral retinofugal projections occurs normally in the absence of NO production (Frost *et al.*, 1994); these projections also segregate along a normal sequence in mice that have a targeted deletion of the gene for nNOS (Frost *et al.*, 1994) indicating that NO may not have the same function in rodents as it does in chicks (Williams *et al.*, 1994; Wu *et al.*, 1994). However we know little about the detailed topography of retinogeniculate or retinotectal projections in rodents with these perturbations (Yhip and Kirby, 1990) and it cannot be excluded that NADPH-d activity is associated with the fine tuning of the retinotectal map, or with the pruning of exuberant retinal terminal arbors (Sachs *et al.*, 1986; Vercelli *et al.*, 2000). With regard to the first option, Simon *et al.* (1992) reported that NMDA receptor antagonists disrupt the fine tuning of the retinotectal map; and since NO release by the postsynaptic cell can be stimulated by activation of NMDA receptors (Schuman and Madison, 1994; Vincent, 1994), NO could very well be involved in retinal arbor refinement (cf. Montague *et al.*, 1991; Cramer and Sur, 1995; Cramer *et al.*, 1996, Vercelli *et al.*, 2000; Vercelli *et al.*, 2001). We had previously studied the effect of NOS blockade on primary visual projections of in 4-6 week-old postnatal rats, and showed significant alterations in ipsilateral retinotectal projections, in the mediolateral and anteroposterior axes: the density of fibres entering the SC, branch length, and the numbers of boutons on retinotectal arbors were increased in the treated group; however, when animals were allowed to survive for several months after stopping treatment, these changes were much less striking. Thus, our findings here support the hypothesis that NO released from target neurons in the mammalian SC serves as a retrograde signal which feeds back on retinal afferents, influencing their growth. The effects of NOS inhibition were partially reversed after treatment was stopped, indicating that lack of NO synthesis delays the maturation of retinofugal connections (Vercelli *et al.*, 2001). Our results on rats are in keeping with those obtained on NOS knockout mice (Wu *et al.*, 2000a and b).

NADPH-d activity in the superficial layers of the SC increased significantly at the end of the second week, around the time of eye opening. Although it would be intriguing to associate this finding with a role of visually-evoked activation in the maturation of NADPH-d activity, it should be noted that the sharp increase in levels of NADPH-d staining occurred immediately prior to, or on the day of, eye opening - it seems more likely that the expression of mature levels of NADPH-d is a precondition to visual stimulation. This idea is confirmed by our results in which eye closure failed to induce significant changes in NADPH-d activity, and is strongly supported by the report of Williams *et al.* (1994) on the chick optic tectum, where NO influenced the elimination of ipsilateral retinotectal projection.

The strong increase in NADPH-d activity between P12 and P14 allowed us to visualize most cell types in the superficial layers of the SC by the time of eye opening, since the histochemical reaction delineated the dendrites morphology of tectal neurons as well as a Golgi stain. In particular, we have used this technique to examine the development of dendritic arbors of vertically oriented neurons (NFV, WFV, PYR). Mooney and Rhoades (1993) reported that a subpopulation of vertically oriented collicular neurons changes its dendrite orientation after contralateral eye enucleation, the dendrites becoming aligned along the course of non-retinal afferents (e.g., trigeminal axons). The findings presented here on the time course of maturation of the dendrites of SGS neurons, along with results on enucleated rats (Vercelli and Cracco,

1994, and present paper), suggest that a normal retinal innervation is necessary to align dendrites of tectal neurons relative to the dorsoventral axis.

The development of NADPH-d activity in the superficial layers of the SC is reminiscent of the development of AChE staining in the SC; AChE labeling gradually increases over the first 4 postnatal weeks (Harvey and MacDonald, 1985). In the superficial layers an increase in AChE activity is first seen at P6 in the rostromedial SC, then throughout the upper tectal layers by P10. However, in contrast to NADPH-d reactivity, AChE expression is unaffected by eye removal in the adult and only slightly affected by P0 eye enucleation. It also develops in embryonic tectum transplants that do not receive retinal inputs (Harvey and MacDonald, 1985).

Hess *et al.* (1993) have suggested that NO, via its action on growth associated protein 43, may be involved in the inhibition of growth cone motility. In the rat visual system, high levels of NADPH-d expression occur well after the elongation phase of retinal axon growth is over (Bunt *et al.*, 1983; see also, Jhaveri *et al.*, 1991), but our observations do not rule out a contribution of NO in signaling the termination of arbor formation (Vercelli *et al.*, 2000). Whether NO production along the primary visual pathway also participates in the process of synapse formation, via a putative influence on ADP-ribosylation of the growth associated protein GAP-43 (Coggins *et al.*, 1993; Luo and Vallano, 1995), remains to be determined. The suggested link provided by NO between neuronal activity and neurotrophin expression and the altered responsiveness of neurons to these neurotrophins (Cramer *et al.*, 1996) are issues that remain to be tested in the rat visual system.

In conclusion, while NADPH-d is widely expressed in the CNS (Vincent and Kimura, 1992), we show that its distribution in specific visual centers is related to distinct subpopulations of cells. Our results document that in the rodent NADPH-d expression along the primary visual pathway increases in a progressive fashion, and does not exhibit developmentally ephemeral expression. The spatio-temporal distribution described here suggests different roles for NO at various times in the life of the animal. Our data are consistent with the possibility that NOS expression in both the SC and LGN is involved in the final stages of refinement of retinal axon arbors (occurring toward the end of the second week of postnatal life). However, the enzyme is expressed in only a few cells, and not at very high levels, during the early stages of retinal arbor formation. Also, the progressive increase in NADPH-d expression along the maturing visual pathway suggests that either the enzyme does not participate in temporally isolated developmental events, or that its role undergoes a gradual transition from involvement in developmental processes to influencing responses of mature cells.

Acknowledgments

Supported by MURST grants (AV), by NIH grant EY 05504 (SJ) and EY 02621 (M.I.T. Vision Core Grant) and by a grant from NATO. We are grateful to Dr. S.Biasoli for excellent technical assistance.

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