

# Morphology and distribution of NADPH-d-positive neurons in the superficial layers of the rat superior colliculus and in the lateral geniculate nucleus relative to retinal projections

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## SUMMARY

Dihyronicotinamide adenine-dinucleotide phosphate diaphorase (NADPH-d) histochemistry is a good marker for nitric oxide synthase in the nervous system. NADPH-d activity is prominent in the primary visual centers of the adult rat. In the superficial layers of the superior colliculus NADPH-d positive neurons may be classified in different cell types; most cells are oriented along the dorsoventral axis of the SC. In the dLGN bipolar or tripolar NADPH-d-positive interneurons are rare and lightly labeled, whereas in the vLGN NADPH-d intensely positive neurons are frequent, located mainly in the lateral subdivision of the nucleus. By anterograde labeling of retinal axons following intraocular injection of the subunit B of cholera toxin (CT-B), combined with NADPH-d histochemistry, retinal terminals are found close to NADPH-d-positive somata and dendrites. Both reactions for CT-B and NADPH-d are intense in the superficial SC and in the lateral vLGN, whereas in the dLGN CT-B staining is uniformly dense contralaterally to the injection site, in contrast with the light NADPH-d staining and the paucity of NADPH-d positive neurons, mostly restricted in the lateral extent. This study will serve as a baseline for further studies on the effects of visual and chemical manipulations of retinal afferents and of nitric oxide synthase.

**Key words:** nitric oxide - visual system - retinal axons - dendrite - cholera toxin.

## INTRODUCTION

The rat primary visual centers, superior colliculus (SC) and lateral geniculate nucleus (LGN), strongly express dihyronicotinamide adenine-dinucleotide phosphate diaphorase (NADPH-d), an enzyme which has been demonstrated to be a nitric oxide synthase (NOS). It has been hypothesized that nitric oxide (NO), the product of NOS, is synthesized in neurons following NMDA receptor activation (Williams et al., 1994; Wu et al., 1994) by glutamatergic terminals, and that NO, in turn, may act retrogradely by stabilizing presynaptic terminals (Montague et al., 1991).

In our study, we describe the morphology and distribution of NADPH-d-positive neurons in the rat primary visual centers, in relation to the distribution of retinal axons and terminals as revealed by anterograde transport of the cholera toxin subunit B (CT-B) from the eye. This study is aimed to represent the basis for developmental, visual and chemical manipulations of NO synthesis in the visual system (Vercelli and Jhaveri, in preparation).

## MATERIALS AND METHODS

7 adult Wistar albino rats from our breeding colony in Torino were used for the study. All experimental procedures involving the use of live animals were done under the supervision of a licensed veterinarian, according to Italian guidelines for care and use of experimental animals (DDL 116/92), as approved by the Italian Ministry of Health. For surgical procedures, ani-

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mals were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg; Inoketam, Virbac, I) and xylazine hydrochloride (5 mg/kg; Rompum, Bayer, Leverkusen, D).

For NADPH-d histochemistry, 4 rats were killed with an overdose of ketamine hydrochloride and perfused transcardially with an initial rinse of 0.1 M phosphate buffer (PB), pH 7.4, followed by fixative (4% paraformaldehyde in PB). Brains were postfixed for 4 hours in the same fixative, cryoprotected overnight in buffered 30% sucrose, and cut on the cryostat in the coronal (N=3) or sagittal (N=1) planes, at a thickness of 50  $\mu$ m. Free-floating sections were reacted for localizing NADPH-d according to the protocol of Vincent and Kimura (1992). Briefly, sections were incubated at 37° C for 1 hour, in a solution of 1 mg/ml NADPH (Sigma, MO) and 0.2 mg/ml Nitroblue tetrazolium (Sigma) made up in PB containing 1% Triton X-100 (Fang et al., 1994). Sections were rinsed thoroughly in PB, mounted in two series on gelatin-chrome alum coated slides, air dried overnight, dehydrated, cleared in xylene and coverslipped in Eukitt mounting medium. For one series, the tissue was counterstained with 1% neutral red. Positive cells in the superficial layers of SC and in LGN were classified according previous reports (Langer and Lund, 1974; Tokunaga and Otani, 1976; Labriola and Laemle, 1977; Warton and Jones, 1985; Gonzalez-Hernandez et al., 1992; Vercelli and Cracco, 1994; and, respectively, Gabbott and Bacon 1994a and 1994b; Tenorio et al., 1996): narrow field vertical (NFV), wide field vertical (WFV), pyriform (PYR), stellate (STL), horizontal (HZ) and marginal (MA) neurons; all neurons which could not be ascribed to a particular cell type were considered as unclassified (UN). For each dendrite of the NFV, WFV and PYR neurons (which represent the great majority of neurons in the superficial layers of SC) we drew the orientation relative to the collicular surface, and used circular statistics to obtain the mean vector  $r$  of dendritic orientation (Zar, 1984).

For CT-B anterograde tracing (Angelucci et al., 1996; Ling et al., 1997), three adult rats had 3  $\mu$ l of 1% CT-B (List Biological Lab., CA; diluted in distilled water) injected in the vitreous chamber of the left eye, with the aid of a Hamilton microsyringe. Four days post-injection, rats were killed and perfused with 4% paraformaldehyde in PB, and their brains cut in two series of sections. In one series, NADPH-d histochemistry was performed as above before CT-B immunohistochemistry. For CT-B immunolocalization, free-floating sections were incubated for 30 minutes in 0.1M glycine in PBS, then in PBS containing 0.5% Triton X-100, 2% normal rabbit serum and 2.5% bovine serum albumin

(PBST) for 1 hour at room temperature (r.t.). This was followed by immersion overnight at r.t. in a solution containing goat anti CT-B antibody (List Biological Lab.) diluted 1:4000 in PBST containing 2% Triton X-100. Binding of primary antibody was visualized by incubating in biotinylated rabbit anti-goat IgG antibody (Vector, CA, diluted 1:200 in PBST) for 1 hour at r.t., followed by an avidin-biotin-peroxidase complex (Vector, Elite ABC Kit); the peroxidase was detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as chromogen, adding 0.5% cobalt chloride (Sigma) to intensify the reaction product. Slices were mounted as above.

The slides were viewed on a Leitz Dialux microscope and selected sections were examined and photographed using TMAX 100 black-white films (Kodak) or drawn using a camera lucida attached to the microscope.

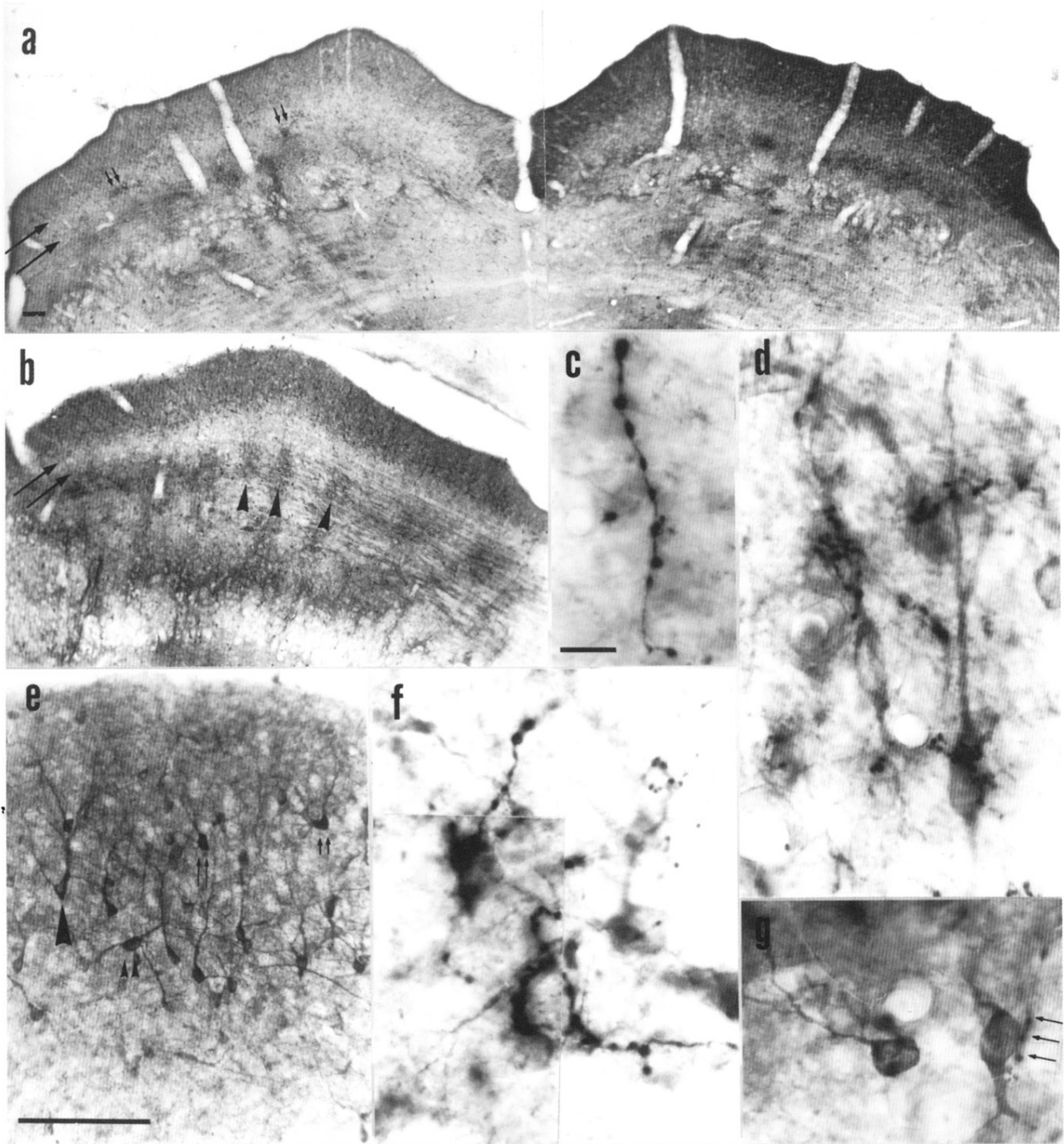
## RESULTS

### *NADPH-d in the superficial layers of the SC and in the LGN*

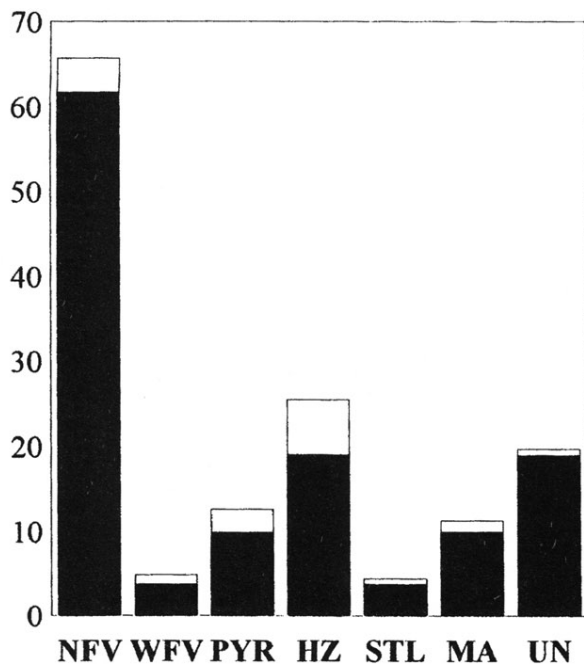
In the SC of the adult rat (Fig. 1b and 1e), NADPH-d histochemistry reveals either weakly labeled neurons (with only the cell soma being reactive and no visible dendrites, - unclassifiable), moderately labeled neurons [such as the horizontally-oriented ones that resided in the deeper part of the superficial gray layer (SGS) and whose dendrites can be followed for at least a couple of hundred microns], or intensely labeled cells (that appear to be fully stained, including spines on dendrites, which are also usually filled up to their terminal ends) which represent  $36.81 \pm 6.79\%$  of NADPH-d-positive neurons (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 classifications (Fig. 2). Axons of many of these cells can also be followed for several hundreds of microns, or to the edge of the section. A rich neuropil of densely labeled fibers is observed, primarily in the deeper (intermediate and deep gray) layers of the SC, whereas in the superficial laminae, a fine network of NADPH-d-positive fibers is seen both in the stratum zonale (SZ) and in the stratum griseum superficiale (SGS), but not in the stratum opticum (SO). The mean vector  $r$  of dendritic orientation for NFV, WFV and PYR neurons is oriented close to 0°, i.e. parallel to the dorsoventral axis and the value for  $r$  is  $0.77 \pm 4.79$ , i.e. most dendrites are aligned on this axis.

In the LGN, the expression of NADPH-d is markedly different in the different subnuclei. In the dLGN (Fig. 3a and 3b), sparsely stained fibers and scattered cells, both of moderate staining intensity, are localized in the superficialmost part of the nucleus. Most of the cells have a bipolar or tripolar morphology, with processes that emerge from opposite sides of

the cell soma, and that are often oriented along the dorsoventral axis of the brain. Some of the dendrites can be followed to their first bifurcations, after which the reactivity fades out. Rarely, cells are intensely labeled, including the full extent of their dendrites (Fig. 3b). In contrast, histochemical labeling in the vLGN is very intense in some parts of the nucleus and the



**Fig. 1.**— a) Superior colliculus, coronal plane: NADPH-d histochemistry combined to CT-B anterograde labeling of retinal projections. On the left the SC ipsilateral to CT-B injection. The long arrows indicate the limits of the stratum opticum, and the double small arrows retinal axon fascicles in the stratum opticum; b) SC, sagittal plane: NADPH-d histochemistry; arrowheads indicate patches of NADPH-d activity in the stratum griseum intermedium; left is caudal, right cranial; c, d, f and g) SC, NADPH-d histochemistry combined with CT-B: retinal terminals in the stratum griseum superficialis of the SC ipsilateral to CT-B injection, some of which are in close relationship with NADPH-d-positive neurons; e) SC, coronal plane, NADPH-d histochemistry; NADPH-d-positive neurons in the stratum griseum superficialis: double long arrows point to a NFV neuron, double short arrows PYR, double arrowheads HOR and big arrowhead WFV neuron. Reference bar = 100  $\mu$ m in a, b and e, and 10  $\mu$ m in c, d, f, and g.



**Fig. 2.**— Distribution of NADPH-d positive neurons of the superficial layers of SC in cell types. NFV = narrow field vertical, WFV = wide field vertical, PYR = pyriform, STL = stellate, HZ = horizontal, MA = marginal and UN = unclassified neurons.

overall pattern of NADPH-d expression reveals the sublaminae of this cell group (Figs. 3a). Thus, in the external lamina of the lateral vLGN, the labeling is dense, both in cells and in the neuropil. Labeled fiber fascicles traverse the cell group, forming dorso-ventrally-oriented stripes. Embedded within these stripes are intensely reactive neurons, most of which ( $72.75\% \pm 2.47$ ) appear to be of the class A variety (Gabbott and Bacon, 1994a) in that they have a multipolar or triangular soma and long, spine-bearing dendrites that usually course out of the  $50 \mu\text{m}$  sections. Many dendrites are oriented either dorsoventrally along the NADPH-d-positive stripes or are perpendicular to the optic tract, apparently spanning the distance between adjacent stripes. Smaller, bipolar class B neurons are more rare ( $5.25\% \pm 0.35$ ), have spine-free dendrites and are found scattered amongst the class A cells. In the optic tract that forms the lateral edge of the vLGN, large, intensely stained class C neurons ( $22\% \pm 2.12$ ) are identifiable - these cells give off one or more dendrites that are oriented medially, entering the external lamina of lateral vLGN. Medially to the densely labeled, outermost lamina of the lateral vLGN lies the internal lamina of lateral vLGN, in which there is virtually no labeling, except for some rare, NADPH-d-positive cells, and some light neuropil reactivity. Adjacent to this division, in the external portion of the medial vLGN, both cells and neuropil are densely labeled, although less

so than in the external division of the lateral vLGN. Finally, in the medialmost lamina of the vLGN, cells are only weakly labeled and a scattered neuropil is visible.

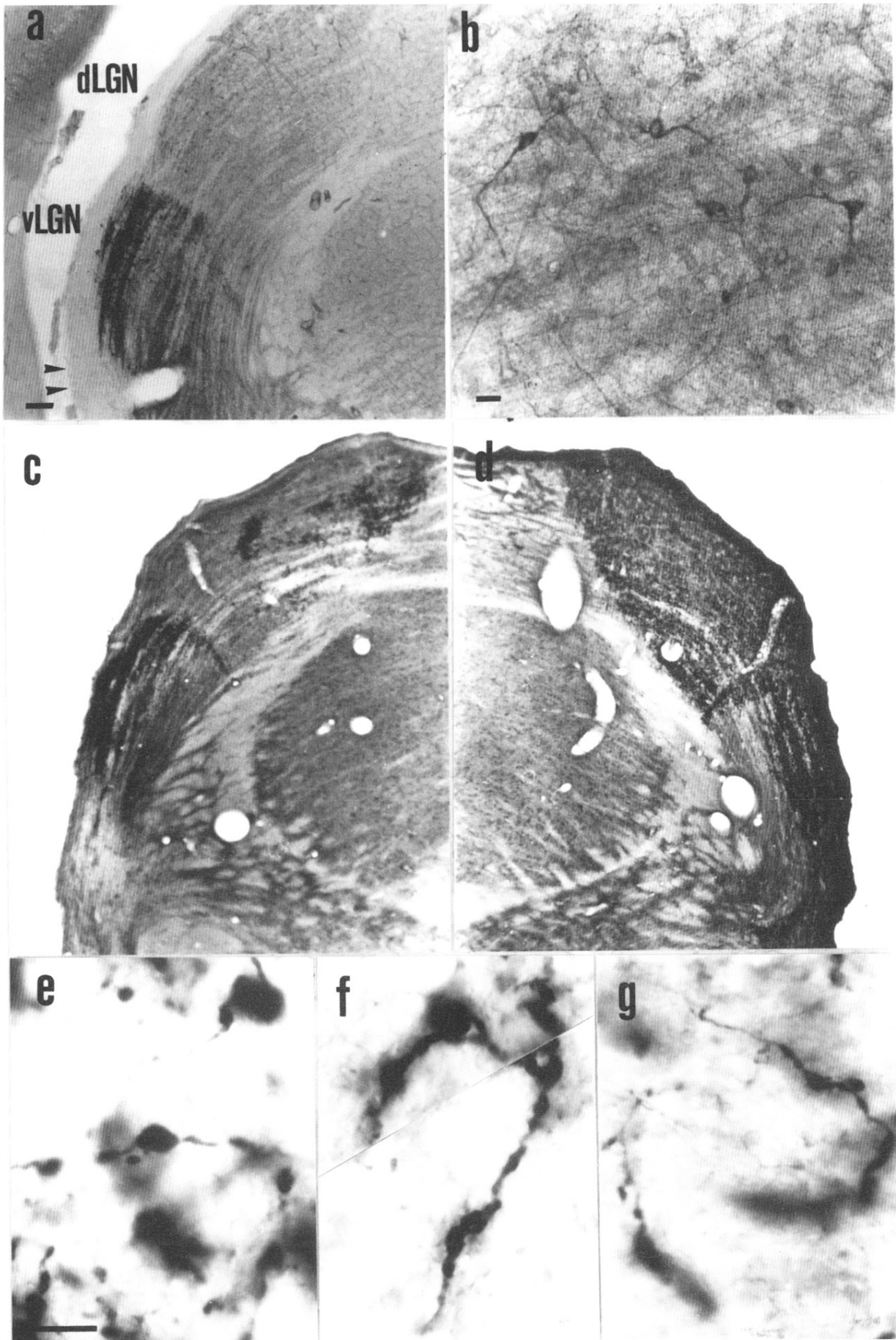
#### *Combined NADPH-d histochemistry and retrograde transport of CT-B.*

Intraocular injection of CT-B results in uptake and anterograde transport of the toxin by retinal ganglion cells. A dense net of labeled axons and terminals is observed in the contralateral LGN and superficial layers of the SC, whereas ipsilaterally the distribution of labeled fibers is more discrete and localized.

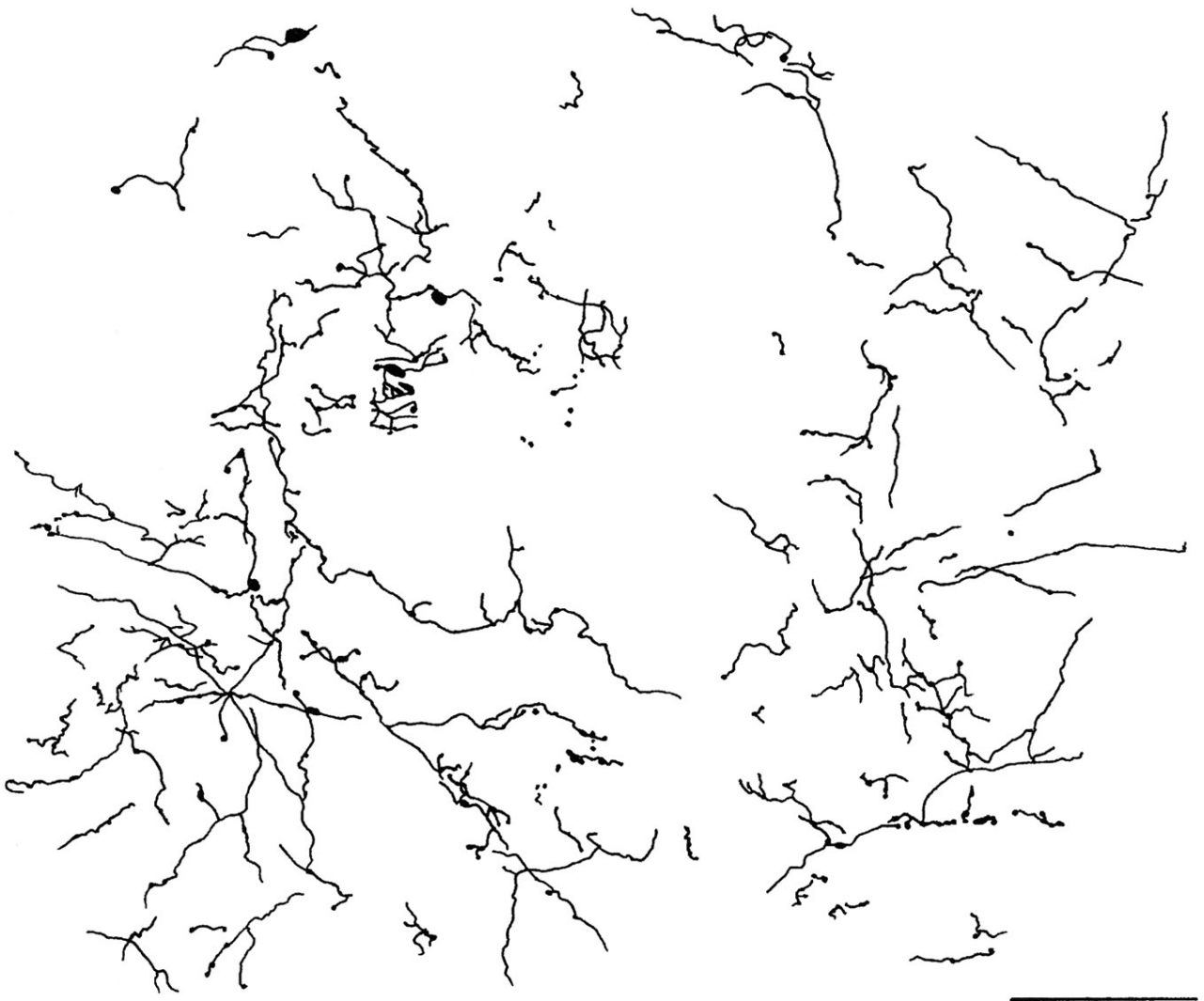
In the SC retinotectal axons constitute a dense net in the contralateral SO, and a dark band of termination in the superficial layers (SZ and SGS) in which single terminals are not easily resolved. On the ipsilateral side, retinal axons form fascicles in the SO, which are particularly evident in the cranial SC. Then, single fibers enter perpendicular to SC surface into the SGS. Ipsilateral optic axons are denser in the medial SGS; single axon branches or terminals ramify into the SGS, bearing and terminating in boutons more or less uniform in size ( $2-4 \mu\text{m}$  in diameter) and shape (ovoid) (Figs. 1c and 4). In some cases terminals and boutons form rosette-like structures and it is possible to resolve single terminals and boutons on the cell body or dendrites of a NADPH-d-positive neuron.

In the contralateral dLGN, labeled terminals fill the entire nucleus uniformly, whereas on the ipsilateral side they are concentrated in the rostro-dorsomedial portion of the nucleus. These terminals may be identified in three different types, as previously described in the hamster (Erzurumlu et al., 1988; Ling et al., 1997): type R1 are large, elliptical varicosities along the length of large caliber axons (Figs. 3e and 5); type R2 consist of medium and small varicosities forming rosette-like clusters of medium boutons (with more than two fine collaterals contributing to each cluster, Figs. 3f, 3g and 5); type R3 are made up of varicosities that have various shapes and sizes on medium or small caliber axons, and no specific clustering (Fig. 5). Optic terminals are rarely seen in direct relationship with NADPH-d positive neurons.

Both the contra and ipsilateral vLGN receive a dense retinal projection, especially in the lateral subdivision of the nucleus, which terminates with dense arbors around NADPH-d positive neurons (Figs. 3c and 3d). On the contrary, the medial vLGN is almost devoid of retinal fibers bilaterally. A particularly dense terminal projection is seen in the intergeniculate leaflet (IGL).



**Fig. 3.**— **a)** LGN, coronal plane, NADPH-d histochemistry; **b)** dLGN, sagittal plane; NADPH-d-positive neurons; **c)** and **d)** NADPH-d histochemistry combined with CT-B; LGN ipsilateral (**c**) and contralateral (**d**) to the CT-B injection; **e**, **f** and **g)** retinal terminals in the ipsilateral dLGN. Reference bar = 100  $\mu$ m in **a**, **c** and **d**, and 10  $\mu$ m in **b**, **e**, **f** and **g**.



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**Fig. 4.**— Camera lucida drawing of CT-B-labeled retinal terminals in the superficial layers of the SC ipsilateral to the injected eye, in a control adult rat. Reference bar = 50  $\mu$ m.

**Fig. 5.**— Camera lucida drawing of CT-B-labeled retinal terminals in the LGN ipsilateral to the injected eye, in a control adult rat. Reference bar = 50  $\mu$ m.

## DISCUSSION

Since neuronal NADPH-d colocalizes with the brain isoform of NOS in the peripheral and central nervous system (Bredt et al., 1991; Dawson et al., 1991; Hope et al., 1991; Schmidt et al., 1992; Vincent and Kimura, 1992), we used NADPH-d histochemistry to evidence NO-synthesizing neurons in the primary visual centers.

NOS- or NADPH-d-positive neurons have been detected in the primary visual centers of birds as well as of mammals. In the chick, numerous NADPH-d-positive neurons are found in the optic tectum (Williams et al., 1994). In adult rodents, prominent NADPH-d activity has been reported in the SC (Gonzalez-Hernandez et al., 1992) and in the cells and neuropil of the LGN (Bredt et al., 1991; Vincent and Kimura, 1992; Gonzalez-Hernandez et al., 1993; Gabbott and Bacon, 1994a and 1994b). In some species, such as ferrets, NADPH-d is transiently expressed in the lateral geniculate nucleus in development (Cramer et al., 1995). Therefore, neuronal cell bodies and fibers expressing NADPH-d are widely represented in the SC and LGN in the adult animal, suggesting one or more roles for NO in visual function.

The distribution of NADPH-d-positive elements in the superficial layers of the rat SC is characterized by neurons in the SZ and SGS, showing different levels of histochemical activity. All the cell types described in earlier Golgi studies (Langer and Lund, 1974; Labriola and Laemle, 1977; Warton and Jones, 1985) are identifiable within NADPH-d-positive neurons, even if only one subset is positive for NADPH-d (Vercelli and Cracco, 1994). NADPH-d activity is displayed by both intrinsic (STL, MA and HZ, Langer and Lund, 1974) as well as projection neurons in the upper layers of the SC (PYR, NFV and WFV, Langer and Lund, 1974; Mooney and Rhoades, 1993). A prominent feature of projection neurons (which constitute the large majority of NADPH-d-positive neurons) is their dendritic orientation, aligned to the dorsoventral axis, which changes after contralateral eye enucleation (Vercelli and Cracco, 1994; Vercelli et al., 1995), activity blockade (Vercelli et al., 1995, 1997) and in aging (Vercelli and Jhaveri, 1996).

CT-B labeling of retinal projections to the SC confirmed previous results on rodents (hamsters, Sachs and Schneider, 1984; Sachs et al., 1986; Erzurumlu et al., 1988; Ling et al., 1997). Retinal axon terminals intensely label the superficial layers of the SC contralaterally, in strict correspondence to the band of dense NADPH-d activity. Ipsilaterally, they are mostly distributed in the medial extent, whereas in the remaining SGS they are quite scattered and dispersed. Some of these retinal boutons and fibers surround and are close to NADPH-d positive neurons, both to the soma and the dendrites.

In dLGN, NADPH-d histochemistry reveals a diffuse neuropil and scattered neurons that are mostly bipolar, usually oriented on the dorsoventral axis, and located primarily in the superficial half of the nucleus, closer to the optic tract (Mitrofanis, 1992; Gabbott and Bacon, 1994a and b). Gabbott and Bacon (1994b) have documented with double labeling that NADPH-d-positive neurons in the dLGN are a subtype of class B, GABA-positive interneurons. CT-B labeled retinal fibers are uniformly distributed in the contralateral dLGN, in contrast with previous findings on hamsters and rats (Sachs and Schneider 1984; Erzurumlu et al., 1988; Ling et al., 1997) which reported a rostromedial area almost devoid of contralateral afferents. Actually, we attribute this discrepancy to species or rat strain differences. Ipsilaterally to the CT-B injection, labeling is restricted to the dorsorostromedial area, as reported by others (Ling et al., 1997).

In the vLGN, the lateral, magnocellular division receives a major input from both the contralateral and ipsilateral retinas as shown by CT-B labeling. Cells that are stained histochemically can be classified as belonging to class A, B or C, according to their morphology. Type B neurons represent a subpopulation of GABA-positive interneurons (Gabbott and Bacon, 1994a). Type A and C are relay cells (Mitrofanis, 1992; Gabbott and Bacon, 1994a; Gonzalez-Hernandez et al., 1994). By contrast, in the medial division of vLGN, the overall NADPH-d activity is lighter and the CT-B labeling scarce bilaterally.

Our results on the morphology of the terminations of retinal axons confirm previous studies on rodents (Lund and Cunningham, 1972; Sachs and Schneider, 1988; Erzurumlu et al., 1988; Jhaveri et al., 1991; Ling et al., 1997). Moreover, we provide evidence of a close relationship between retinal fibers and NADPH-d positive neurons. The distribution of retinal afferents parallels areas of intense NADPH-d activity in the superficial layers of the SC and in the lateral vLGN. On the other hand, the dLGN receives a dense retinal innervation, whereas NADPH-d activity is low.

In the adult animal, NO appears to play a role in the processing of visual information (Gabriel et al., 1985). In the dLGN in particular, NO is involved in regulating the oscillatory activity of thalamocortical projection neurons via an NO-mediated gating mechanism, and may alter the dynamics of local blood flow in response to local neural activity (Gabbott and Bacon, 1994b). Ionophoretic application of inhibitors of NOS in the dLGN decreases the visual responsiveness of geniculate neurons and results in a concomitant reduction in their responsiveness to NMDA, an effect which is reversed by coapplication of L-arginine, the natural substrate of NOS. In agreement with this result, NO donors lead to increased spontaneous activity and responsiveness of dLGN neu-

rons in cats (Cudeiro et al., 1996). NOS activity could be linked to light stimulation (Nucci et al., 1995). Therefore, retinal afferents would play a fundamental role in stimulating NOS activity.

Additionally, during development in both the SC and LGN, NO may be related to the reorganization of retinal projections after they reach their targets, acting as a retrograde signal (Montague et al., 1991; Williams et al., 1994; Cramer et al., 1995). It has also been speculated (Cramer et al., 1996) that NO may link neuronal activity to neurotrophin expression and alter the responsiveness of neurons to these neurotrophins.

This paper provides the baseline for further studies on the development of NOS-expressing neurons in the primary visual centers, and on the effects of visual manipulations on their maturation. It also provides a basis for studying the effects of NOS inhibition on the development of retinal axons in the primary visual centers of the rat.

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