
SHORT COMMUNICATION

NADPH-diaphorase reactivity in ciliary ganglion neurons: A comparison of distributions in the pigeon, cat, and monkey

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Abstract

Ciliary ganglia from the pigeon, cat, and monkey were investigated for the presence of NADPH-diaphorase reactivity by use of a standard histochemical method. In the pigeon, where the ganglion is known to control lens and pupil function, and the choroidal vasculature, about one-third of the ganglion cells were densely stained and most other somata were lightly stained. In some cases, preganglionic terminals with a cap-like morphology were also darkly stained. The pattern of NADPH-diaphorase staining in mammals was very different from that seen in pigeons. In both mammalian species, where the ganglion is known to control lens and pupil function, a small number (less than 2%) of the ganglion cells were shown to be densely NADPH-diaphorase positive, revealing their neuronal processes. The presence of NADPH-diaphorase positive cells in pigeon, cat, and monkey ciliary ganglia suggests that nitric oxide may be used for intercellular communication in this ganglion, or in light of the known importance of nitric oxide in vascular control, some of these positive neurons may participate in the control of choroidal vasodilation.

Keywords: Oculomotor system, Nitric oxide, Vasomotor, Pupil, Ciliary muscle

Introduction

Small discrete populations of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase positive cells have been found in many areas of the vertebrate brain, as well as in the peripheral ganglia (Bredt & Snyder, 1992; Grozdanovic et al., 1992; Yamamoto et al., 1993). The functional significance of these neurons has recently become clearer with the demonstration that one NADPH-diaphorase is a nitric oxide synthetase, which catalyzes the synthesis of nitric oxide (NO) in response to calcium level changes in postsynaptic neurons (Dawson et al., 1991; Hope et al., 1991). Besides its proposed role as an endothelium-derived relaxing factor mediating vasodilation (Ignarro et al., 1990), NO is now suggested to be a neuronal messenger molecule, or a special co-transmitter, in the nervous system (Bredt & Snyder, 1992, for review).

The presence of NADPH-diaphorase has been reported in the choroid and retina of the rat and bird (Bredt et al., 1990;

Fitzgerald & Reiner, 1993; Stanforth et al., 1993; Yamamoto et al., 1993), where NO helps maintain choroidal and retinal vessel dilation (Mann et al., 1993; Gray et al., 1993). Furthermore, NO has recently been demonstrated to modulate calcium currents in avian ciliary ganglion neurons and thus change the physiological status of these neurons (Khurana & Bennett, 1993). In light of these studies, and the fact that the avian ciliary ganglion contains choroid neurons that control the choroidal vasculature (Hess, 1965; Reiner et al., 1983; Fitzgerald et al., 1990), it was of interest to determine whether endogenous NADPH-diaphorase activity was present in pigeon ciliary ganglion neurons. Moreover, in light of the rat findings (Yamamoto et al., 1993), the present study sought to determine whether this enzyme might be found in other mammalian ganglia. The results indicate that NADPH-diaphorase positive neurons are present in pigeon, cat, and monkey ciliary ganglia (Sun et al., 1993).

Materials and methods

Ciliary ganglia from six White Carneaux pigeons (*Columba livia*), five cats, and three monkeys (*Macaca fascicularis*) were used in this study. The ganglia were harvested from animals sac-

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rificed in nonconflicting studies. Animals were first deeply anesthetized (sodium pentobarbital, 50 mg/kg, iv), and then perfused with 0.1 M phosphate-buffered saline (pH 7.2), followed by a fixative containing paraformaldehyde (1.0–4.0%) and glutaraldehyde (0–1.25%) in 0.1 M phosphate buffer (pH 7.2). The ganglia were removed immediately and postfixed in the same fixative for at least 1 h. Ganglia were sectioned on a vibratome at a thickness of 35–50 μm . In some cases, pigeon ganglia were cut on a cryostat at a thickness of 15 μm . Sections were processed for NADPH-diaphorase histochemistry following a modified standard protocol (Hope & Vincent, 1989). Briefly, sections were incubated in 0.1 M phosphate buffer (pH 7.2) containing 0.3% Triton X-100, 1.5 mM NADPH, and 0.25 mM nitro blue tetrazolium. The incubation was conducted at 37°C for 45–120 min, as determined by inspection for an optimal reaction that stained positive cells dark blue.

Cell soma size analysis was undertaken on all cells labeled for NADPH-diaphorase, and a similar number of randomly chosen unlabeled cells. Outlines of somata were drawn, and the long and short axes of each outline were calculated using an image analysis system consisting of a solid-state camera and 386 PC equipped with an Imaging Research Inc. (St. Catherines, Ontario, Canada) imaging board and software. These data were expressed as means of the average of the long and short axes.

Results

In the pigeon, the majority of ganglion cells were stained with the blue reaction product. As shown in Figs. 1A and 1B, the degree of staining varied from cell to cell. Since adult avian postganglionic cells lack conspicuous dendrites, and because these cells did not display an obvious dichotomy in labeling intensity, it was difficult to categorize them. An arbitrary criterion for NADPH-diaphorase positive staining (as demonstrated in Fig. 1B) indicated that about one-third of the cells in the pigeon ciliary ganglion were darkly stained. Examples of both putative choroid cells (i.e. those with relatively smaller somata) and non-choroid cells (i.e. ciliary neurons with relatively larger somata and those receiving a single cap-like ending) were densely labeled. In fact, the somatic dimensions of these darkly staining diaphorase positive cells were not statistically different from those of lightly staining cells (Table 1). In addition to the cell staining, preganglionic terminals with a cap-like morphology were, in some cases, darkly stained with the NADPH-diaphorase reaction product (Fig. 2). Labeled cap-like endings were not only found contacting labeled postganglionic neurons (Fig. 2B), but were also found on lightly labeled cells (Fig. 2A).

The mammalian ganglia displayed a pattern of labeling that was distinctly different from that of the pigeon. Small populations of intensely NADPH-diaphorase positive cells (dark blue) were found in all cat and monkey ciliary ganglia examined in this study. The remaining ganglion cells displayed only a very light background level of staining. The number of strongly NADPH-diaphorase positive cells was estimated to be less than 2% of the total population of ganglion cells in either the cat or monkey, and they were sporadically distributed in the ganglion (Figs. 1C and 1E). However, tightly packed clusters of positive cells were occasionally present in cat ganglia (Fig. 1G). Most of the NADPH-diaphorase positive somata were ovoid in shape, and their average soma size was significantly smaller than that of other unlabeled ganglion cells, as indicated in Table 1.

All NADPH-diaphorase positive cells found in both the cat

and monkey had at least one visible neuronal process, and many of them had two or more processes (Figs. 1D and 1F). In both the cat and monkey, the processes of these cells follow a tortuous course within the ciliary ganglion. In some cases, they extend nearly across the ganglion (Fig. 3). In two monkey ciliary ganglion sections, labeled processes from NADPH-diaphorase positive cells were found extending within the short ciliary nerve (Fig. 1H). Labeled terminals were not observed in mammalian ganglia.

Discussion

The present results demonstrate that NADPH-diaphorase positive neurons are present in the ciliary ganglion of these three species. These cells may represent postganglionic motoneurons that control choroidal vasculature, postganglionic motoneurons that modulate their presynaptic input, or interneurons that modify transmission through the ganglion. Each of these possibilities is discussed below.

Nitric oxide has a well-known role in the relaxation of blood vessels (Ignarro et al., 1990; Toda & Okamura, 1990), including choroidal and retinal capillaries (Mann et al., 1993). It has been established that the bird ciliary ganglion contains a major population of cells controlling choroidal vasodilation (Reiner et al., 1983, 1991), and NADPH-diaphorase positive fibers are present in the avian choroid (Fitzgerald & Reiner, 1993). The presence of NADPH-diaphorase positive neurons in the pigeon ganglion appears to provide further evidence of NO control over vessels *via* this ganglion. However, the fact that numerous non-choroid cells in pigeon ciliary ganglion contain NADPH-diaphorase (present results; Fitzgerald & Reiner, 1993) indicates that NO must have functions other than the control of choroidal vasodilation. It is possible that NO may also influence the activity of the muscles in the ciliary body and iris of birds. However, these muscles are striated in the avian system, and positive endings have only been observed in relation to the choroid and the ciliary processes (Fitzgerald & Reiner, 1993). In the rat, while NO synthetase activity is also present in choroidal tissue (Bredt et al., 1990; Stanforth et al., 1993), NO synthetase-positive nerve fibers that distribute within the rat choroid derive instead from the pterygopalatine ganglion (Yamamoto et al., 1993). Our results suggest the possibility that a small portion of the NO synthetase activity present in the mammalian choroid may derive from the ciliary ganglion. The observation of NADPH-diaphorase positive neurons whose nerve processes extend within the short ciliary nerve (see Fig. 1H) supports this view. However, it is also possible that they target cells in the accessory ganglion (Grimes & von Sallmann, 1960; Kuchiiwa et al., 1989).

NADPH-diaphorase positive cells that produce NO have also been implicated in presynaptic modulation within the CNS. NO is proposed as a messenger molecule for systems displaying synaptic plasticity, such as long-term potentiation (LTP) in the hippocampus (Bohme et al., 1991; O'Dell et al., 1991) and long-term depression (LTD) in the cerebellum (Shibuki & Okada, 1991). In these systems, glutaminergic NMDA receptors activate NADPH-diaphorase catalyzed NO synthesis, and NO mediates the enhancement of presynaptic glutamate release. There is, to the best of our knowledge, no evidence of glutamate being used by preganglionic motoneurons. Thus, if NO has a local effect in the ciliary ganglion, it is likely to be *via* non-glutaminergic modulation.

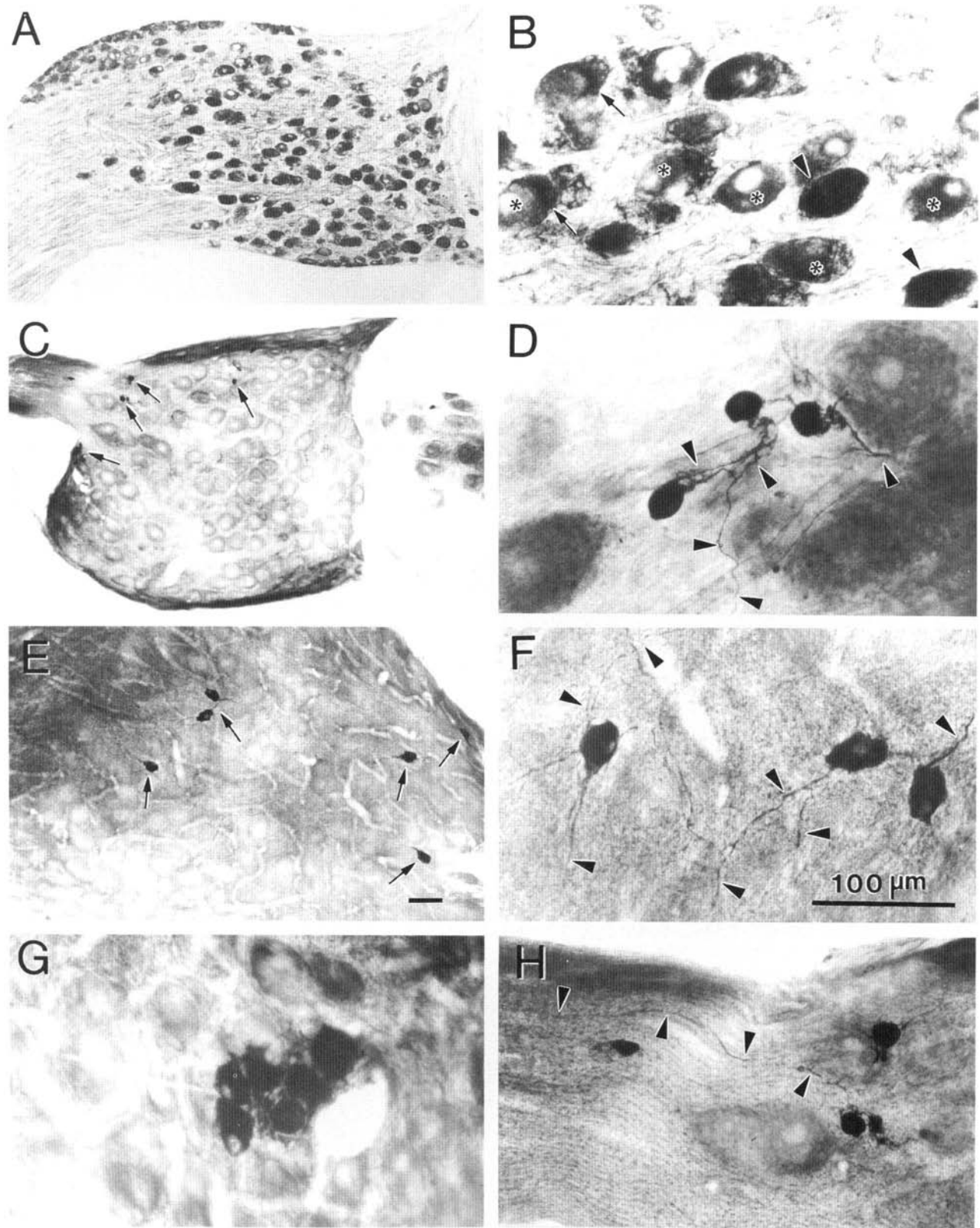


Fig. 1. NADPH-diaphorase reactivity in pigeon (A and B), monkey (C,D, and H), and cat (E-G) ciliary ganglia. A: Most cells are labeled in the pigeon ganglion. B: Higher magnification showing darkly stained diaphorase-positive cells (arrowheads), lightly stained diaphorase-negative cells (asterisks), and labeled terminals (arrows). Scattered labeled cells (arrows) are present in the monkey (C) and cat (E) ganglia, and their processes (arrowheads) are shown at higher magnification (D and F). G: Example of tightly clustered labeled cells. H: A process (arrowheads) extends from a labeled cell into the short ciliary nerve. Scale bars = 100 μ m. Magnification in E = A and C; F = B, D, G, and H.

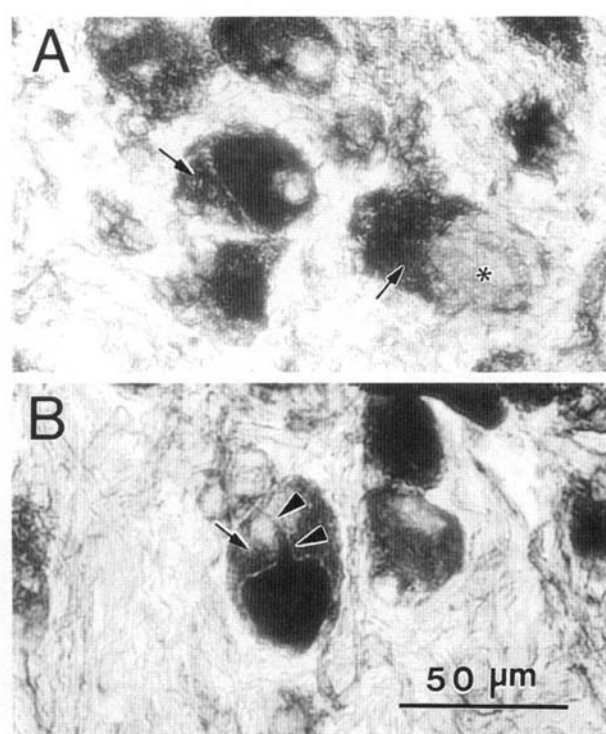


Fig. 2. High magnification of pigeon ciliary ganglion cells showing NADPH-diaphorase activity in cap-like preganglionic terminal (arrows). Terminals are shown in contact with a lightly labeled cell (asterisk) (A) and a densely labeled cell and axon (arrowheads) (B).

In birds, recent studies of avian ciliary ganglion have suggested that NO is synthesized in the ciliary ganglion, have described its role in modulating calcium channels, and have shown that it mediates a form of LTP within the ganglion (Khurana & Bennett, 1993; Scott & Bennett, 1993*a,b*). Since the present results demonstrate NADPH-diaphorase activity both pre- and postsynaptically in the bird, it remains to be determined whether this LTP is due to NADPH-diaphorase in the pre- or postsynaptic elements, or both. Previous studies have shown that cholinergic preganglionic terminals on the choroid neurons in the pigeon ciliary ganglion contain co-transmitters, including substance P and enkephalin (Erichsen et al., 1982). The present study indicates the NO may also be among the co-transmitters influencing cholinergic transmission in this ganglion.

In mammals, it is possible that NO modulates ganglionic activity as a neurotransmitter in an interneuronal population. This possibility receives support from the small size and scattered distribution of the labeled cells. The distinct differences in cell size between NADPH-diaphorase positive cells and unlabeled ganglion cells in mammals is also in agreement with prior studies, which suggested that a minor subpopulation (less than 5%) of small neurons may exist in the monkey ciliary ganglion (Warwick, 1954; May & Warren, 1993). Presumably, at least some of these small cells contain NO synthetase. Interestingly, the present finding that less than 2% of cells in the mammalian ciliary ganglion are NADPH-diaphorase positive parallels results observed in the cerebral cortex, striatum, and hippocampus. Only 1–2% neurons in these areas contain NO synthetase (Bredt & Snyder, 1992). NO may have some common function in different areas of the brain and ciliary ganglion that is accom-

Table 1. Means of the averages of the cell long and short axes \pm s.d. (μ m)

	<i>n</i>	Negative cells	<i>n</i>	Positive cells
Pigeon	96	34.1 \pm 3.7	92	34.5 \pm 4.3
Cat	86	45.4 \pm 6.2	85	30.3 \pm 4.7 ^a
Monkey	104	53.5 \pm 8.2	110	29.2 \pm 5.1 ^a

^aSignificant difference between positive and negative cells ($P < 0.01$, unpaired *t*-test).

plished by a sparsely distributed subpopulation of cells. Specifically, it may be a small molecular messenger used for regional modulation of neuronal activity or vascular tone.

In summary, the small size, the morphology, and the limited number of neurons with NADPH-diaphorase reactivity in both the cat and monkey ciliary ganglion suggest they might be interneurons. On the other hand, they may be a minor choroidal cell population. The large size and number of positive cells in the pigeon ganglion strongly indicate they are motoneurons, not interneurons. Furthermore, the avian distribution indicates that NO may have more diverse roles than the modulation of choroid vascular tone, perhaps including mediation of ganglionic LTP. The reasons for these striking differences between the patterns of labeling in mammals and birds are yet to be determined.

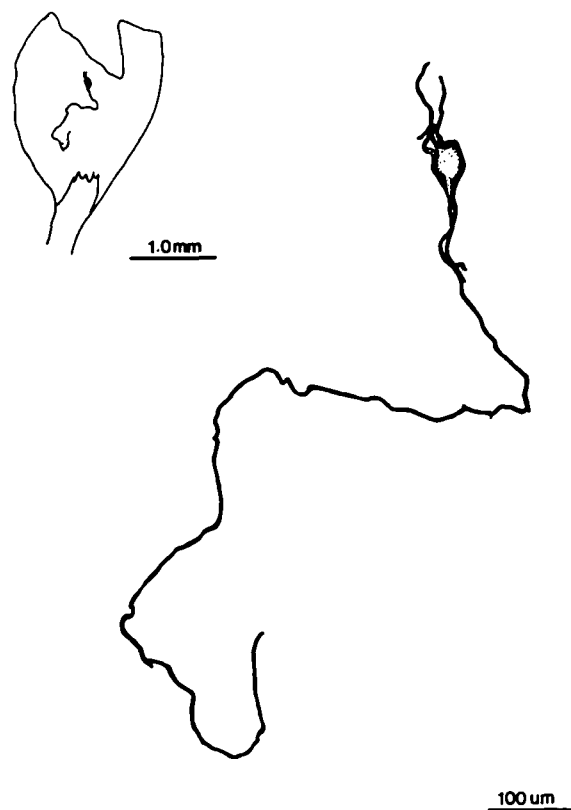


Fig. 3. A serial reconstruction of a labeled cell in the monkey ciliary ganglion showing one process that extends over 1.30 mm within the ganglion.

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