A horseradish peroxidase study of the innervation of the internal structures of the eye

Evidence for a direct pathway

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The sources of innervation of the internal structures of the eye in monkey and rabbit were investigated by injecting horseradish peroxidase (HRP) intraocularly and monitoring its retrograde transport. HRP-labeled cells were found in the ciliary ganglia and in the midbrain throughout the dorsoventral extent of the midline of the oculomotor complex. The results suggest that a direct pathway exists from the midbrain, and it is argued that this pathway is for the control of accommodation. Either this pathway is direct, with no synapse interposed, or the postsynaptic neurons are near or in the intrinsic musculature. A third but not so likely possibility is that retrograde transsynaptic transport of HRP occurred across electrotonic synapses in the ciliary ganglion. These findings may challenge the commonly held view that the entire pathway for the control of the intrinsic musculature synapses in the ciliary ganglion. On the other hand, it may be that this direct path is a sensory system much like the mesencephalic nucleus of the trigeminal nerve.

Key words: accommodation, ciliary ganglion, Edinger-Westphal, horseradish peroxidase, oculomotor nuclei, parasympathetic, pupil reflexes, intrinsic musculature, lens, third nerve

The afferent and efferent pathways related to control of the intrinsic musculature of the eye for pupillary and accommodative responses are not fully agreed upon or understood. The most generally accepted view of the final efferent path is based on observations that surgical removal of the short ciliary nerves in macaque monkeys produced chromatolysis in 97% of the cells in the ciliary ganglion whereas iridectomy alone caused only 3% of these cells to become chromatolytic.
ic. These results have been taken to mean that practically all the cells in the ciliary ganglion innervate the intrinsic musculature of the eye, with the vast majority innervating the ciliary muscle. It is not clear why relatively few cells seem to be involved in the innervation of the pupil. However, the existence of other pathways such as via the episcleral ganglion for pupillary accommodative innervation of the pupil. However, the existence of other pathways such as via the episcleral ganglion for pupillary accommodative reflexes (the near response) rather than light reflexes has been postulated. This pathway, although anatomically distinct, would be functionally the same parasympathetic pathway in which the synapse occurs in the ciliary ganglion.

In contrast, recent electrophysiological and pharmacological studies in monkeys challenge the view that a postganglionic pathway is involved in accommodation. These investigators found that by raising the frequency of electrical stimulation of the preganglionic fibers to over 150 Hz or by local application of nicotine to the ciliary ganglion, the pupillary response could be abolished. On the other hand, accommodative responses were still obtained from preganglionic stimulation with frequencies from 10 to 1000 Hz.

In the present study, we wished to investigate the hypothesis that fibers arise in midbrain and project directly to the intrinsic musculature with no synapse interposed, by allowing the nerve terminals which innervate the intrinsic musculature to take up horseradish peroxidase (HRP) and by monitoring the retrograde transport to the ciliary ganglion and brainstem. This report is one of a series of studies which use modern neuroanatomical tracing techniques in order to elucidate the pathways concerned with the intrinsic musculature of the eye.

Materials and methods

Four white rabbits and three macaque monkeys (Macaca fascicularis) were used in the present study. For all animals, sodium pentobarbital anesthesia was given, in appropriate dose, either intraperitoneally or intravenously. The rabbits were further maintained on halothane. The head was held in a stereotaxic instrument, and local anesthesia (Xylocaine) was applied at all pressure points. Topical anesthetics and antibiotics were applied to the eye. The needle of a 50 μl Hamilton microsyringe was inserted through the sclera 2 to 3 mm posterior to the limbus and angled anteriorly such that 20 μl of a 30% HRP solution (Worthington) could be deposited on or in the ciliary body and iris. A series of 1 to 3 μl deposits of HRP were made following the circumference of the lens and pupil. Deposits were made in the posterior and anterior chambers by forcing the needle past the edge of the lens. The surface of the bulb was flushed with ophthalmic solutions immediately after puncture and withdrawal to minimize extraocular leakage of HRP. In addition, Neosporin ointment was applied liberally to minimize possible backflow of HRP from the puncture site.

In one monkey and one rabbit, both eyes were injected with HRP. In two monkeys and rabbits, injections were made in one eye only. In a fourth control rabbit, topical applications and subfascial injections of HRP were made in the same area of the sclera or conjunctiva that the needle had to pierce for the intraocular injections in both eyes. Although we believe that our results are probably not due to extraocular spread of HRP, we have no way of knowing how extensive was the extraocular spread of the HRP. Consequently any nerve terminal in the extraocular structures was a candidate for take-up of HRP.

After a 24 hr survival time, the animals were deeply anesthetized and perfused transcardially with 0.1M phosphate buffer, pH 7.4, followed by 1% paraformaldehyde and 1.25% glutaraldehyde fixative. The brains and ciliary ganglia were then removed and washed for 24 to 48 hr in phosphate buffer containing 15% to 30% sucrose. Frozen sections were cut at 40 μm and were reacted according to the benzidine method. Some of the ganglia were cut at 60 μm. All sections from the ganglia were analyzed, whereas for the brainstem every second or fifth section, from the upper medulla to the middle of the thalamus (i.e., anterior to the third nucleus), was analyzed. Sections were mounted on gel subbed slides and lightly counterstained. Sections were traced at 8X magnification and systematically searched for labeled cells at 100X using a microscope with an x-y plotter attachment. Regions of the brainstem in which cells were observed were traced at 20X magnification for the purpose of mapping the exact location of the labeled cells.

Results

Our results are summarized as follows. In the monkey, labeled cells were found in the midbrain and the ciliary ganglia (Figs. 1 and
Innervation of eye internal structures

Fig. 1. HRP-filled cells in ciliary ganglion of macaque monkey. A, Low-power photomicrograph of longitudinal section of monkey ganglion. (Neutral red stain; ×14.) C to F, High-power (×320) photomicrographs of HRP-filled cells indicated by letters C through F in A. The cells C to F were selected to show the various degree of HRP labeling seen in our material, i.e., from very dark (C) to light (F). In this and following figures the thickness of the HRP sections precludes more crisp photomicrographs. B, Unlabeled cell from a cresyl violet-stained section not processed for HRP is shown for comparison. Same animal as in Figs. 2 and 3.

2). In the rabbit, labeled cells were also found in the midbrain (Fig. 4). We were unable to successfully process the rabbit ciliary ganglia for HRP because of attrition of the tissue during dissection or processing. However, one of the main purposes of the rabbit studies was to determine whether or not labeled cells would indeed be found in the brainstem of a mammal, prior to doing such experiments in macaque monkeys, and this purpose was realized.

Figs. 1 and 2 illustrate the labeled cells observed in the ciliary ganglion and brainstem of one of the monkeys. Although it was not likely that we had the entire ganglion, due to attrition of material during dissection
Fig. 2. HRP-filled cells in the midbrain of macaque monkey. A, Low-power photomicrograph of oculomotor complex. (Neutral red; ×14.) III, Somatic nuclei. B to D, HRP-filled cells in the midline and next to the somatic nuclei which are also shown at high power (×320) in B to D. Note sparse, elongated dendrites.
and HRP processing, we have estimated that 15% to 70% of the ganglion cells were labeled. This percentage may represent the effectiveness of our technique in applying HRP to the nerve terminals in the intrinsic musculature. In the brainstem, labeled cells were found scattered throughout the dorsoventral extent of the middle of the oculomotor complex, perhaps including the Edinger-Westphal nucleus (EW). They were located primarily between the somatic nuclei but also between the borders of the somatic and accessory oculomotor nuclei (Fig. 3). In the case illustrated, binocular injections were made (Fig. 2), and a total of 96 clearly labeled cells were observed in the sections throughout the oculomotor region. The cells were distributed in such a way that it was not possible to collectively assign them to a particular nucleus (Fig. 3). The cells did not have the usual appearance of the somatic cell types of the oculomotor complex but had smaller

Fig. 3. Line drawings at four levels of monkey brainstem through the oculomotor region. HRP-filled cells are indicated by heavy black dots. Each panel shows labeled cells from the section that was traced plus the two immediately adjacent sections, one rostral and one caudal. A is most caudal, and D is most rostral. Same animal as in Fig. 2. CA, cerebral aqueduct; CG, central gray; DSP, decussation of superior cerebellar peduncle; III, third nerve nucleus; INC, interstitial nucleus (Cajal); MLF, medial longitudinal fasciculus; ND, nucleus of Darkschewitsch; RN, red nucleus.
Fig. 4. HRP-filled cells in the midbrain of a rabbit. A, Low-power photomicrograph of a section of the brainstem in the region of the oculomotor complex. (Neutral red stain; ×14.) Two HRP-labeled cells are indicated at B, and one labeled cell is indicated at C. B and C, High-power photomicrographs (×320) of the labeled cells in A. Note the spindle appearance of the cells, especially in C. Monocular injection. III, Somatic nuclei.

somata with sparse and often elongated dendritic fields (Fig. 2, B and C).

Fig. 4 illustrates the brainstem results from a case in which a unilateral injection was made in the rabbit. The results were similar to those in the monkey. The spindle-shaped labeled cells appeared to be of the visceral type and were again found in the midline adjacent to the somatic nuclei. As explained previously, we were unable to obtain any HRP data from the rabbit ciliary ganglia.

In both the rabbit and monkey the cells appeared to be confined to one side of the midline with monocular injections (Fig. 4) and appeared on both sides with binocular injections (Fig. 3).

Control experiments (see Methods) were undertaken in order to determine whether the labeled cells in the brainstem were due to extraocular spillage. No labeled cells were observed in the brainstem of the control.

Discussion

Our results, taken with those from earlier physiological work, indicate that the classic parasympathetic concept applied to intraocular innervation is incomplete and support the hypothesis of a direct projection from the midbrain to the intrinsic musculature of the eye. Frankly, we were surprised by our results because the fact that cell bodies of a final parasympathetic efferent path could be found in the brainstem rather than in a peripheral ganglion is an exception to the organization of parasympathetic outflow. However, a similar type of exception also exists in the trigeminal sensory system where the first-order unipolar ganglion cells are found in the central mesen-
cephalic nucleus of the trigeminal nerve rather than peripherally. In the ciliary ganglion of some animals such as the chick, both electrotonic and chemical synapses have been shown to exist. If electrotonic synapses exist in mammals, it would explain the fast accommodative pathway seen in the physiological experiments on monkeys and cats. However, such synapses do not easily explain our results. Although remote, the possibility of selective retrograde transsynaptic transport of HRP across gap junctions in the monkey ciliary ganglion would produce labeled cells in the brainstem. If this were true, then our results would stay in harmony with the classic view that this pathway synapses entirely in the ciliary ganglion. On the other hand, it might also be hypothesized that a ganglionic plexus of some sort exists near or actually in the intrinsic musculature of the eye that is similar to, for example, the plexi of Miessner and Auerbach in the gut. In this case, the autonomic ganglia are very close to or in the innervated structure, and presumably the HRP would easily be taken up by preganglionic terminals. The existence of such a plexus would be in keeping with the concept of the organization of the parasympathetic nervous system and could exist in parallel with the ciliary ganglion. However, we do not know of any reports of such structures in the intrinsic musculature of the eye, and such a plexus would presumably not be consistent with the physiological demonstrated fast pathway.

Undesired labeling of cells in the oculomotor nuclei due to leakage of peroxidase from the scleral puncture, with subsequent diffusion of the uptake by the extraocular muscles or other structures in the orbit, must be considered. This possibility was minimized by the application of sterile saline and topical ophthalmic solutions which would have flushed any spilled peroxidase out of the area. In addition, the application of Neosporin ointment to cover the scleral puncture minimized possible backflow of peroxidase. Other investigators dealing with a similar problem of peroxidase leakage in the orbit while studying the innervation of extraocular muscles in the carp specifically noted that obvious artifactual labeling of the oculomotor nuclei in the brain was never observed if leaking HRP was washed away. Results from the control animals in the present study support the contention that no undesired or artifactual labeling was taking place in these experiments. As mentioned earlier, in this paper we believe that our results are probably not due to extraocular spread of HRP, but we have no way of knowing the extent of intraocular distribution of the label. Thus our results can be due to HRP being picked up by nerve terminals present in the ciliary muscle and iris and any other intraocular structure. But it is difficult to suggest the presence of nerve terminals in intraocular structures other than the ones in the ciliary muscle and iris that would have picked up HRP and transported it to the middle of the oculomotor complex. However, it may be that we are not dealing with a motor system, but with a sensory pathway which is organized much like the trigeminal proprioceptive system with a centrally located mesencephalic nucleus, whose afferent processes could have taken up the HRP. These sensory receptors need not necessarily be located in the ciliary muscle or iris.

Relatively few labeled midbrain cells were seen in rabbits compared to monkeys. This could suggest that the accommodative pathway is better developed in primates. Although more effective HRP injections in the rabbits might have produced better results, it has been noted that the rabbit has a poorly developed ciliary body and has only weak accommodative power. Also it has been shown that neurons in this parasympathetic pathway vary in their uptake and retrograde transport of various substances from species to species.

The problem of limited uptake and transport of HRP in our experiments must also be considered in light of recent experiments in the cat where direct application of HRP to the preganglionic branch of the III nerve or to the ciliary ganglion produced labeled neurons in the EW, periaqueductal gray, and ventral tegmental area.
rabbit we studied all levels throughout the brainstem and located labeled cells only in the midline of the oculomotor complex. Whether we have failed to label all sources of input or have isolated only those sources concerned with the intrinsic musculature by intracocular rather than extracocular applications may remain to be seen. The use of a retrograde tracer was thought by us to be the best and perhaps only way to approach the problem of the anatomical demonstration of an oculomotor parasympathetic pathway whose cell bodies are in the brain and whose axons do not terminate in the ciliary ganglion. Anterograde autoradiographic tracing methods following injection of radioactive amino acid into the oculomotor region could also be used to define this pathway. However, these experiments would be much more difficult from a technical standpoint, and their interpretation could also be complicated by the possibility of transsynaptic transport within the ciliary ganglion.

It is interesting to point out that ours is not the first anatomical experiment to report such results. An earlier retrograde degeneration study reported that chromatolytic cells were found in the midline of the oculomotor complex following removal of the intrinsic but not extrinsic musculature of the eye in the subhuman primates. This study had been neglected in favor of more recent studies.7

Experiments in which accommodative responses were evoked by electrical stimulation of the midbrain in macaque monkeys also seem to fit well with our data.22 These experiments have shown that accommodative responses in a given eye can only be evoked by ipsilateral stimulation. Furthermore, it was suggested that the cells involved with the intrinsic musculature of the eye were located close to the midline over an elongated portion of the dorsorostral portion of the oculomotor nucleus.

In conclusion, then, it appears that there may be two pathways for the innervation of the intrinsic musculature: one which synapses in the ciliary ganglion and one which does not. It could be speculated that the direct and presumably faster pathway is for voluntary control of accommodation whereas the other is for reflex control of the intrinsic musculature. Accommodation is readily controlled voluntarily.23 However, accommodation does have a reflex component in the near response. On the other hand, the pupil is believed not to be under voluntary control and responds reflexively to both light and the near response. Simultaneous recording of pupil area and accommodation of the lens in man have shown that the lens appears to have a better dynamic response than the pupil.24 This might be due to the existence of a direct pathway. It would not be surprising if a faster pathway is needed in accommodation so that the speed of adjustment of the lens and perhaps pupil can match that of the relatively fast response of the somatic extraocular musculature which converge the eyes. On the other hand, a slower system which synapses in the ciliary ganglion may be more appropriate for pupillary responses to light in which changes in pupil size should parallel changes in the slowly adapting photochemical processes of the retinal receptors to shifts in levels of illumination.

As pointed out earlier, there is more known about the sensory side of accommodative and pupillary reflexes than there is about the motor pathways to the oculomotor complex and the intrinsic musculature of the eye.1 In the present study, the location of the labeled cells along the lateral edge of the EW in the monkey is a potential terminal zone for both the crossed and uncrossed fibers from the pretectum and other nearby regions of the midbrain.1,4,6 Thus it is possible that the results of the present study describe the final efferent path for a number of accommodative and possibly pupillary reflexes. These regions of the oculomotor nuclei also can receive direct input from the cerebellum,16 an input which seems appropriate for the proposed role of these pathways in the near response.

While this paper was in press a study appeared25 which reported no axonal degeneration in the ciliary muscle following intracranial section of the third nerve. However, degenerating fibers were found in the
short ciliary nerves, and the iris was not checked for degeneration. In this regard, it is necessary to note that pupillary responses to convergence, but not light, have been obtained in apes following removal of the ciliary ganglia.26 Thus the nerve terminals of the direct path may be located in the iris. Alternatively, lack of degeneration in the ciliary muscle25 might support the notion that we may be dealing with a sensory system whose receptors are not located in the intrinsic musculature, as mentioned above.

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REFERENCES


