A Retinohypothalamic Pathway in Man: Light Mediation of Circadian Rhythms

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It has been proposed that, in animals, a retinohypothalamic pathway exists which mediates the synchronization of the diurnal light-dark cycle with the central neural components regulating endogenous rhythms. There have been numerous anatomic, physiologic and behavioral investigations to substantiate this proposed connection in experimental animals.

Morphologic investigation of a retinohypothalamic tract in man has awaited the development of a technique capable of axonal tracing in the human brain. The paraphenylenediamine method was applied to 7 post-mortem human brains. Degenerated axons were found in the suprachiasmatic nuclei of the hypothalamus in each of the 4 patients who had incurred prior optic nerve damage. The retinoseuprachiasmatic pathway may be the anatomical substrate for the integration of retinal light information with endogenous rhythms in man.

INTRODUCTION

In 1862 Wagner proposed that nerve fibers deriving from the optic chiasm entered the hypothalamus in man31. Since then, the existence of a retinohypothalamic connection has been a controversial issue. Support for this pathway was gained when investigators found changes in circadian rhythms in experimental animals following certain lesions of the visual pathways. Bilateral transection of the optic nerve in the rat resulted in desynchronization between endogenous circadian rhythms and the diurnal light-dark cycle21, 34. Yet, more distal destruction of the retinal ganglion projections, by transection of the primary or accessory optic tracts, did not alter circadian rhythms23, 24. It was, therefore, hypothesized that there exists a retinofugal pathway terminating in the region of the optic chiasm which is involved in the regulation of endogenous rhythms in animals.

Evidence of a retinohypothalamic pathway has also been provided by earlier morphological studies in a large number of animal species, including those of Herrick16 in amphibia and by Jacobs and Morgan19 in cetacea. However, Armstrong3 failed to confirm the pathway in reptiles.

Subsequent studies employing silver impregnation methods for identifying degenerated axons have done little to settle the controversy. Blümcke5 and Rieke27 used the ammoniacal silver methods of Bielschowsky and Glees to demonstrate optic fiber terminations in the periventricular system of the third ventricle, supraoptic nucleus and tuberal nuclei in the cat and rat, respectively. Polyak26 utilized the Marchi method, following a lesion of the retinal ganglion cells, and noted the existence of a direct retinohypothalamic connection in rat and guinea-pig, but not in cat and pigeon. Some investigators, using the Nauta method, failed to demonstrate the retinohypothalamic pathway in the cat, pigeon and monkey6,11, 32. However, Sousa-Pinto and Castro-Correia33 used the Fink-Heimer modification of the Nauta method to demonstrate a retinohypothalamic pathway in the rat. In a comprehensive study, Kiernan20 employed a variety of degeneration tracing methods in 5 mammals, including rat, rabbit and an amphibian, yet he failed to demonstrate optic fibers terminating in the hypothalamus of these animals.

The development of the more contemporary trac-
ing method of autoradiography allowed for a direct retinohypothalamic pathway to be firmly established in the rat. Further autoradiographic studies have identified this retinal pathway to the suprachiasmatic nucleus of the medial hypothalamus in several mammals, including cat, tree shrew and monkey. Among all the mammals studied to date, the retinohypothalamic projection has maintained some consistent features. Each suprachiasmatic nucleus has been shown to receive a bilateral retinal projection, with predominantly contralateral innervation. Additionally, the ventral and lateral portions of the nuclei are most densely innervated. Evidence of the retinohypothalamic pathway in the many mammals studied to date suggests the possibility of its existence in man as well.

Documentation of the retino-suprachiasmatic pathway in man was not feasible until a technique capable of tracing neural pathways in the human brain was found. Fortunately, the recently developed para-phenylenediamine (PPD) method reliably stains degenerating axons and axon preterminals in the visual system in human autopsy brain tissue. Thus, a direct investigation of human visual neuroanatomy is now possible.

MATERIALS AND METHODS

Autopsy brain specimens were obtained from 7 patients, 4 of whom had documented optic nerve damage prior to death. A 72-year-old woman had an aneurysm of the left carotid artery which had compressed the left optic nerve. She was followed for 4 years during which progressive visual field defects were documented, with subsequent loss of visual acuity and optic atrophy. Further tissue specimens were obtained from a 62-year-old woman who developed a melanoma in the right eye, which was enucleated 7 years prior to her death. Brain tissue was also obtained from a 45-year-old male with marked loss of vision and documented bilateral optic atrophy from retinitis pigmentosa diagnosed 10 years prior to death. Similarly, tissue was received from a 63-year-old male blinded and with bilateral atrophy due to a central artery occlusion which occurred 7 years prior to death. Three control autopsy brains without known visual or neurological damage were also examined.

The brain tissue was processed by the PPD method. This is a modification of a technique which employs PPD as a stain for degenerating axons and axon preterminals. The PPD method is adapted for use in human brain tissue obtained at autopsy. It reliably stains degenerated axons even after long survival periods in man.

The brain specimens, obtained at autopsy 4–24 h after death, are routinely fixed in 10% formalin for 1–20 days. The brains are cut into blocks with the smallest width less than 0.5 cm thick. These brain specimens are then placed into a fixative solution of 2% glutaraldehyde, 2% paraformaldehyde, 2.5% dimethylsulfoxide (DMSO) in 0.1 M sodium cacodylate buffer (pH 7.4). The brain blocks are stored in fixative at 4 °C for periods ranging from a few days to several weeks. These blocks are then further sectioned to slices of tissue approximately 0.5 mm by 4 mm by 6 mm. These tissue slices are stored in fixative in the cold for another 2–4 days.

These small tissue blocks are repeatedly rinsed in 0.1 M sodium cacodylate and then placed overnight in 0.5% osmium tetroxide in a 0.1 M sodium cacodylate solution. After several rinses in distilled water, the tissue is dehydrated through a graded series of alcohols and propylene oxides before infiltration with Embed 812 (previously Epon 812). After polymerization at 60 °C for 2 days, the blocks are trimmed of excess plastic and semi-thin sections 1–2 μm thick are cut on the ultramicrotome (Sorvall MT2B). The sections are placed on distilled water drops, dried at approximately 90 °C, kept warm overnight at 50 °C and cooled before staining. The slides are stained by immersion in a solution of 1% PPD in methanol for 5–10 min and then rinsed and washed in 95% ethanol for 2–5 min. The slides are dried on a warm plate and allowed to cool before mounting. The staining intensity does not vary with PPD concentration or with the duration of immersion in the PPD solution. Rather, the darkness of the stain can be varied only by the thickness of the section. Ethanol does not decolorize PPD, so the rinse time is not critical.

The sections are examined with a Zeiss 16-standard light microscope. The degenerated axons are visible with a ‘high dry’ objective (40 ×) or an oil immersion objective (63 ×).
Fig. 1A: cross-section of normal, right optic nerve shows fascicles of retinal ganglion cell axons separated by septae (s). The PPD method stains the myelin sheaths of intact axons, producing dark rings. (PPD; 1400 x). B: cross-section of lesioned, left optic nerve from a patient (same as in A) who incurred complete unilateral transection of this optic nerve one year prior to death. The degenerated axons (arrows) are seen as dark profiles. Normal axons are not seen. (PPD; 1400 x).
RESULTS

Sections through the optic nerves, optic chiasms, optic tracts and the suprachiasmatic nuclei (SCN) of the hypothalamus were stained with PPD. Our criterion for degenerated axons is the appearance of dark brown circular profiles. Preterminal degeneration is thought to be represented by smaller, homogeneously pale brown circular profiles adjacent to neuron somata or large dendrites.

In examination of sections through intact optic nerves, we noted many dark rings. These PPD-stained myelin sheaths were seen encircling the unstained cytoplasm of normal axons (Fig. 1A). Few, if any, degenerated profiles were seen in these undamaged optic nerves. In contradistinction, many degenerated axons were observed in the optic nerves from patients who had a lesion involving the retinal ganglion cells or the retinal ganglion cell axons (Fig. 1B).

Application of the PPD method to the decussating fibers of the optic chiasms from the patients with unilateral optic nerve damage show degenerated axons interspersed among normal axons. Similarly, both degenerated and normal axons were observed intermingled within the optic tracts from patients with unilateral optic nerve damage.

The SCN of the hypothalamus were cut in multiple coronal planes, and processed by the PPD method. Degeneration was observed bilaterally throughout the SCN from those 4 patients who sustained either unilateral or bilateral optic nerve damage prior to death. The degenerated axon profiles found in the SCN were somewhat smaller than those noted in the optic nerves (Fig. 3). Degenerated axon preterminals were noted, at high magnification, adjacent to the neuron somata and dendrites of the SCN (Fig. 4). Very few, if any, degenerated axons and no degenerated axon preterminals were found in the SCN from the normal (control) patients (Fig. 2).

DISCUSSION

The present morphological investigation provides evidence for the existence of a retino-suprachiasmatic pathway in man. This study employed a recently developed method utilizing paraphenylenediamine (PPD), an agent which stains by a different principle than does silver in standard impregnation methods.

Reduced-silver methods stain the neurofibrillar components of nerve cells, allowing for the evaluation of fibrillar changes that occur during degeneration. Optimal silver impregnation of neurofibrillar degeneration of axons is transient, and is typically applied 4–7 days after the lesion. Therefore, silver impregnation techniques, such as the Nauta and Fink-Heimer methods, are difficult to apply to human tissue.

PPD, on the other hand, chelates osmium which precipitates in lipids. Thus, PPD marks lipid elements which accumulate in degenerating axons. It probably also stains the lipid debris from pre-existing axons, found much later in the course of degeneration, and which we presently term degenerated axons.

While the exact biochemical and morphological course of neuronal degeneration remains unknown, the conventional notion has been that products of degeneration are not seen in brain tissue after long survival periods. However, there is an increasing body of evidence that remnants of degeneration can be found in brain tissue of certain species, including man, years after injury. Evans described in bird and rabbit CNS the time course of the degeneration process, and noted a persistence of lipid compounds as part of the stained material.

We postulate that in certain species lipid remnants become ensheathed by oligodendrocytes and, in this state, may be preserved for years. It is possible that each PPD-stained dark profile represents the compilation of several degenerated axons within one sheath. This may explain the apparent large size of some PPD-stained degenerated profiles in comparison to normal myelinated axons.

The PPD technique reliably stains both degenerating and degenerated axons in animals and man. Comparison of the PPD technique with the Fink-Heimer method and electron microscopy has been made in cat. This showed the PPD method to be simpler and less capricious than the Fink-Heimer technique. Moreover, the PPD method was powerful enough to resolve degenerated axon terminals, further demonstrating the sensitivity and reliability of this relatively unconventional staining technique.

Utilizing the PPD method, degeneration was noted bilaterally in the human suprachiasmatic nu-
Fig. 2. Section through the suprachiasmatic nucleus from a normal (control) patient. Note neuronal somata and absence of degenerated profiles. Rings of myelin are seen ensheathing normal axons. (PPD; 600 ×).

Fig. 3. Section through the suprachiasmatic nucleus from a patient with a damaged ipsilateral optic nerve. Note degenerated axons (arrows) throughout field. (PPD; 600 ×).

Fig. 4. Higher magnification of a section through the suprachiasmatic nucleus from a patient (same as in Fig. 3) with a damaged ipsilateral optic nerve. Note degenerated axons (large arrows) and degenerated preterminals (P) adjacent to neuronal somata and dendrites (PPD; 1000 ×).
clesus (SCN) of the hypothalamus. This suggests that some retinal ganglion cell axons branch off at the level of the optic chiasm to project directly to both SCN, which lie just dorsal to the chiasm. Other retinofugal fibers continue past to form the primary optic tracts.

A direct retinohypothalamic connection has been proposed in animals to mediate the synchronization of the diurnal light–dark cycle with the central neural components regulating circadian rhythms. This pathway in animals is demonstrated by the neuroanatomical studies previously discussed. It is also supported by numerous behavioral and physiological studies in various experimental animals.

Environmental illumination has been demonstrated to be a major regulator of circadian rhythms in animals. Non-physiological light conditions, such as constant lightness or darkness, have been shown to significantly alter the normally expressed circadian rhythms. The period of hamster estrous, melatonin secretion in rats and locomotor activity in sparrows proved to be sensitive to cycles of environmental lighting. Similarly, in the human, abnormalities of the neuroendocrine regulatory system have been attributed to the exclusion of diurnal light–dark cycles due to blindness. For example, it has been noted that blind women experience menarche at an earlier age than normal sighted women. It has also been shown that blind persons have differences in their water, carbohydrate and insulin balances as compared to sighted persons. Thus, it is possible that the absence of a retinal input to the hypothalamus in a blind person is the primary defect resulting in the disruption of endocrine rhythms.

It is likely that in man, as in other mammals, the diurnal light–dark cycle acts to entrain the endogenous circadian rhythms. The retina-suprachiasmatic pathway, identified by the PPD method, provides a neuroanatomical substrate for the integration of retinal light information with those hypothalamic neuroendocrine mechanisms which may regulate circadian rhythms in man.

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