Quantitative Analysis of the Optic Nerve of the Horse (Thoroughbred)

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ABSTRACT. Three optic nerves (L1, R2, R3) 12–18 mm behind the eyeball of the horse (Thoroughbred) were investigated quantitatively under light and electron microscopes. Thin sections at the thickness of 0.35 μm were cut, stained by toluidine blue and observed under the light microscope. The areas of the optic nerve and the axon bundles were 20.03 ± 1.04 and 16.59 ± 0.79 mm² (mean ± SD, n=3), respectively. The axon numbers for optic nerve L1, R2 and R3, estimated from light micrographs, were about 481 × 10³, 543 × 10³, and 494 × 10³, respectively. Axons of optic nerve L1 were also counted from electron micrographs and the total number of 488 × 10³ was received. Furthermore, axon diameters of optic nerve L1 were also measured from electron micrographs. The diameter of a circle with the same peripheral length as an axon, was regarded as its diameter. The medullary sheath of the axon was not included during measuring. Altogether 5,744 axons were measured and axon diameters were in a range of 0.23–12.69 μm, with a mean of 2.56 ± 1.45 μm (mean ± SD). A regional difference of axonal diameters was found across the optic nerve; the mean diameter of axons in the centro-dorsal region (2.28 μm) was the smallest, and had significant difference with those in several peripheral regions (P<0.05).

KEY WORDS: equine, optic nerve axon, quantitative analysis.

As the only pathway from the eye to the brain, optic nerves have been well studied anatomically and physiologically in varied animals [4, 7, 13, 14, 19, 21]. Some quantitative characteristics of axons of optic nerves, such as their sizes and total numbers were received [4, 14, 19, 21] and axon arrangement across the optic nerve were investigated extensively. Axons in the mammalian optic nerve are generally thought to be organized retinotopically, while there is also evidence that topography is degraded along the length of the mammalian optic nerve [7, 13]. However, on the contrary to the huge studies on optic nerves of other animals, there were nothing investigated on the optic nerve of the horse and the majority of visual system in the horse are still unclear. In the present study, we investigated three optic nerves by a light microscope and one of them also by a transmission electron microscope. The regional difference of axonal diameters across the optic nerve is also discussed. The present study, along with the previous one [5], is expected to provide anatomical and physiological backgrounds for further study of the visual system in the horse.

MATERIALS AND METHODS

Three optic nerves (L1, R2, R3) from different adult horses (Thoroughbred), were used in the present study. After the horse was killed in a local slaughter house, the eyeballs were enucleated quickly with the optic nerves. The attachments including optic nerve sheath, were removed as soon as possible. And orientations of optic nerves were marked with a marking pen. The optic nerve 12–18 mm distal to the eyeball was used. One block at the thickness of 1 mm was separated from the optic nerve, scanned to a computer quickly and its area was measured by an area-measuring software (Area properties 3.2, a shareware). Since the optic nerve of the horse was very large and a cross section of the optic nerve could not be loaded on a grid completely, the block, after being scanned, was further divided into ten regions (as in Fig. 4). The ten regions were immersed into 2.5% phosphate buffered glutaraldehyde for 48 hr at 4°C, postfixed in 2% osmium tetroxide for 2 hr, dehydrated and embedded in Epon 812. Semi-thin sections at the thickness of 0.35 μm were cut with an ultramicrotome (ULTRACUT N, Reichert-Jung Optische Werke AG, Wien, Austria) from every region, mounted onto glass slides and stained with 0.3% toluidine blue. Ultra-thin sections (70 nm) were also cut from optic nerve L1, mounted onto 200-mesh grids and stained by acetate uranium and citrate lead. For estimating the shrinkage during processing, the length and width of a region were measured before immersed into the fixation and after embedded in resin. And the shrinkage from a fresh region to an embedded region was by a linear factor of about 0.8 and was corrected during calculations. Furthermore, we also estimated the shrinkage during making ultra-thin sections by comparing the length and width of an ultra-thin section from an embedded region with the corresponding length and width of the embedded region. And in the ultra-thin sections an additional linear compression of about 0.85 was found along axes perpendicular to the knife’s edge and was also corrected during calculations.

Analyses: Semi-thin sections from optic nerve L1, R2 and R3 were observed under a light microscope and micrographs were taken at low (Fig. 1a) and high magnification (Fig. 1b). With the area-measuring software, areas of regions containing axon bundles were measured from low magnification micrographs. While in high magnification micrographs, the areas of the optic nerve and the axon bundles were estimated from low magnification micrographs. The areas of the optic nerve and the axon bundles were 20.03 ± 1.04 and 16.59 ± 0.79 mm² (mean ± SD, n=3), respectively. The axon numbers for optic nerve L1, R2 and R3, estimated from light micrographs, were about 481 × 10³, 543 × 10³, and 494 × 10³, respectively. Axons of optic nerve L1 were also counted from electron micrographs and the total number of 488 × 10³ was received. Furthermore, axon diameters of optic nerve L1 were also measured from electron micrographs. The diameter of a circle with the same peripheral length as an axon, was regarded as its diameter. The medullary sheath of the axon was not included during measuring. Altogether 5,744 axons were measured and axon diameters were in a range of 0.23–12.69 μm, with a mean of 2.56 ± 1.45 μm (mean ± SD). A regional difference of axonal diameters was found across the optic nerve; the mean diameter of axons in the centro-dorsal region (2.28 μm) was the smallest, and had significant difference with those in several peripheral regions (P<0.05).
micrographs, the number of axons in 0.0039 mm² sampling areas was counted. And total sample areas in L1, R2 and R3 were summarized in Table 1. The total number of axons across the optic nerve was estimated by proportionality.

Ultra-thin sections from optic nerve L1 were observed under a transmission electron microscope (JEM-100 SX, Japan Electron Optics Laboratory Co., Ltd, Tokyo, Japan). Electron micrographs were taken in upper left and bottom right corners of each window of the mesh and printed. The number of axons was counted from electron micrographs and areas of those electron micrographs were measured. And the total number of axons across the optic nerve L1 was estimated by proportionality. Furthermore, sizes of axons in the electron micrographs were measured with the area-measuring software. The diameter of a circle with the same peripheral length as an axon was regarded as its diameter. The medullary sheath of the axon was not included during measuring. The distribution histogram of axon sizes in every region and the total distribution histogram across the optic nerve were made finally. Statistical analysis for axon sizes among regions was done using one way analysis of variance followed by Tukey’s studentized range test, and P values less than 0.05 were considered as significant.

The criterion for the identification of a cross-sectioned axon under the electron microscope is that its axoplasm contains both microtubules and neurofilaments, while the presence of medullary sheath provided an adequate single criterion for identification.

RESULTS

Total axon count from light micrographs: The cross section of the optic nerve was approximately circular, and its area was 20.03 ± 1.04 mm² (mean ± SD, n=3). Axons of the optic nerve were grouped together into bundles by glial processes and many connective tissues (Fig. 1a), and the area of the axon bundles was 16.59 ± 0.79 mm² (mean ± SD, n=3). The vascularization was entirely by small capillaries, and there was no central artery. Axons with varied sizes were found in high magnification microphotographs (Fig. 1b).

For estimating the total number of axons accurately, only the area of the axon bundles was used while the area of non-axon regions was excluded during calculation. Table 1 was the summary of axon counts for optic nerve L1, R2 and R3 from light micrographs. The average total axon count was about 506 ± 32,679 (n=3).

Total axon count from electron micrographs: The great majority of axons in the optic nerve were myelinated (Fig. 2a) and only a few unmyelinated axons which appeared singly were found (Fig. 2b). The total count for optic nerve L1 was listed in Table 2. Since the number of axons in 0.301197 mm² was 8,994, the number of total axons was about 488 ± 10³.

Nerve axon diameter spectrum: Altogether 5,744 axons from 10 regions of optic nerve L1 were measured for their diameters. Axon diameters were in a range of 0.23–12.69 µm, with a mean diameter of 2.56 ± 1.45 µm (mean ± SD). The spectrum of total axon diameters across the optic nerve was found to be unimodal and positively skewed (Fig. 3). There was only an axon with its diameter less than 0.25 µm among the axons measured, so it was not showed in the spectrum. The diameter was recorded in steps of 0.25 up to 5 µm and 0.5 up to 8 µm (Fig. 3). The rest of axons (>8 µm) were analyzed as a group. The percentage of axons with diameters larger than 5 µm was 7.5%, with diameters larger

| Table 1 | Total axon counts for nerve L1, R2 and R3, estimated from light micrographs |
|-----------------|-----------------|-----------------|-----------------|
| Area of the axon bundles (mm²) | Total sample area (mm²) | Total no. of axons sampled | Total count for nerve |
| L1 | 16.36 | 0.6474 | 19,053 | 481,475 |
| R2 | 17.47 | 0.8775 | 27,283 | 543,173 |
| R3 | 15.95 | 0.8151 | 25,227 | 493,646 |
| Average no. of axons (mean ± sd) | 506,098 ± 32,679 (n=3) |
than 8 \( \mu m \) was 0.57%. A few axons were found with diameters larger than 10 \( \mu m \). The axon diameter distribution in every region was showed in Fig. 4. Since diameters of the great majority of axons were less than 5 \( \mu m \) (92.5%), only frequencies of axons with diameters less than 5 \( \mu m \) were showed in the spectra for convenience. Axons in the centro-dorsal region were the smallest with a mean diameter of 2.28 \( \mu m \) (Fig. 4), and had significant difference with several peripheral regions with mean diameters larger than 2.55 \( \mu m \) (P<0.05). It was noted that axons in dorsal regions were relatively large (Fig. 4).

**DISCUSSION**

Characteristics of the optic nerve of the horse

The large optic nerve: The nerve area of the horse with a mean of 20.03 mm\(^2\) was the largest among animals studied. It was about 148 times of 0.135 mm\(^2\) of the honey possum [4], 18 times of 1.11 mm\(^2\) of the rabbit [19] and 8 times of 2.6 mm\(^2\) of the largest section taken some 7 mm from the globe of the cat [8]. In the electron microscope studies of other animals' optic nerves, the whole nerve cross sections were loaded on grids completely. However, the optic nerve of the horse was too large to be loaded completely on a grid. We had to divided the block of the optic nerve into 10 regions, which made the study more difficult and time-consuming than others.

Axons of the optic nerve: In birds [3, 12], the existence of centrifugal axons was well established: the perikaryon is situated in the isthmo-optic nucleus, and the axon passes to the contralateral eye to terminate presynaptically on amacrine cell perikarya. In the optic nerve of the pigeon [3], the number of centrifugal axons was approximately 10,000, forming 1% of the total number of axons. The reports on mammals showed that at least some mammalian retinas receive cen-
trifugal innervation [2, 10, 18]. However, in the present study, it can not be known whether there were centrifugal axons or not in the optic nerve of the horse.

In quantitative assessments of mammalian optic nerves [9, 15], the regions of the optic nerve studied were reported to be almost completely myelinated. The exceptions were the marsupial South American opossum [6], in which 20% of axons are unmyelinated at a level 2 mm behind the eye, and the honey possum [4], in which 26–43% of optic axons were unmyelinated at the retrobulbar level. In the optic nerve of the horse, only a few unmyelinated axons were found. Furthermore, unmyelinated axons of the horse appeared singly, not like the pigeon [1] and the anurans [11] in which unmyelinated axons occurred singly or in groups.

Axon diameters: There were two particularities on axon diameters of the optic nerve of the horse. One is the much larger size range (0.23–12.69 µm) compared with those of other animals. For example, the range of axon diameters extended from 0.19 to 4.13 µm in the monkey [16], from less than 0.25 to 7 µm in the rabbit whose retinal ganglion cell sizes were in a range of 5–32 µm [19], from 0.1 to 2.4 µm in the honey possum whose retinal ganglion cell sizes were in a range of 8–20 µm [4], from 0.2 to 3.9 µm in the golden hamster [17] and 0.2 to 3.6 µm in the chipmunk [20]. One exception was 0.5 to 13.6 µm in the cat [8]. Axon size has been suggested as one of the triggers for myelination, with a minimum diameter of 0.2 µm above which axons become myelinated. So the axon size range of the horse also indicated that the majority of axons in the optic nerve were myelinated. The other particularity was the large mean diameter (2.56 µm). Mean diameters of the monkey [16], rabbit [19], cat [8] and honey possum [4] were less than 1.5 µm, respectively. In previous study [5], we also found the large size range (5–53.8 µm) and mean diameter of retinal ganglion cells. The above two particularities in the optic nerve of the horse were supposed to be correspondent with results on retinal ganglion cell sizes.

The total axon count

There are two classic approaches to estimate the number of retinal ganglion cells. The direct one is through investigating the topography of ganglion cells in a retina, and the other one is to estimate the number of axons in the optic nerve. In a previous study [5], we had investigated the topography of ganglion cells in the retina of the horse, estimated the total number of retinal ganglion cells and measured sizes of ganglion cells. Our present result of the number of axons with a mean of 506 × 10³ was similar to our previous result of the number of retinal ganglion cells with a mean of 441 × 10³ [5], which reassured us about our identification of ganglion cells.

The total axon number for optic nerve L1 from light micrographs (481 × 10³) was similar to that from electron micrographs (488 × 10³). Two factors were supposed to induce the similarity. One was the minority of unmyelinated axons, and the other was the relatively large sizes of axons. So the great majority of axons of the horse were within the limit of resolution of the light microscope, which indicated that the failure to resolve small axons which happened in the cat, pigeon and anurans almost did not exist in the horse.

Axon distributions across the optic nerve

Although axon diameters in the centro-dorsal region had significant difference with several peripheral regions with mean diameters larger than 2.55 µm, no significant differences were found between the centro-dorsal region with centro-nasal, centro-temporal and ventro-nasal regions (Fig. 4). That is, only crude centro-peripheral difference was found across the optic nerve of the horse. The centro-dorsal region with smaller axons might be the axonal outflow of the visual streak which showed small ganglion cells [5]. It may be concluded that crude retinotopy presents across the optic nerve of the horse. Furthermore, since there are evidence that topography is degraded along the length of the mammalian optic nerve [7, 13], it was supposed that axon distributions might change in the other part of the optic nerve which were not investigated in our study.

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