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Immunoreactivity of Protein Gene Product 9.5 and Synaptosomal-associated Protein 25 in the Retina of the Formosan Rock Monkey Following Optic Nerve Transection

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Shur-Tzu Chen, Jiang-Ping Wang, Chi-Hsien Chien and Ching-Liang Shen (1997) Immunoreactivity of protein gene product 9.5 and synaptosomal-associated protein 25 in the retina of the Formosan rock monkey following optic nerve transection. *Zoological Studies* **36**(4): 333-339. To investigate the retrograde degeneration of primate retinal neurons, the left optic nerves of 3 Formosan rock monkeys were transected intraorbitally. The pattern of organization of protein gene product 9.5 (PGP 9.5), a specific neuronal marker, and synaptosomal-associated protein 25 kDa (SNAP-25), were examined 1 mo after axotomy. A severe loss of retinal ganglion cells and their axons was observed, while photoreceptors became reactive in the lesioned retina as detected by PGP 9.5 immunoreactivity. A significant decrease of PGP 9.5 and SNAP-25 immunoreactivity in the plexiform and nerve fiber layers was also detected in the retina ipsilateral to the axotomized retina. The sublayers were mixed and indistinguishable in some areas of the lesioned retina, indicating that degeneration occurred not only in the retinal ganglion cells but also in other populations; further, the alteration was more severe in the monkey retina as compared with results from our previous studies performed in the rodent optic system. Our findings also show that PGP 9.5 and SNAP-25 are good markers for investigating the effects of neuronal injury and the mechanisms that underlie the processes of neuronal degeneration.

Key words: PGP 9.5, SNAP-25, Retina, Degeneration, Monkey.

Axotomy has been commonly used as a procedure for studying the effect of neuronal injury and changes of nerve cells in response to trauma. The ganglion cells in the retina, because they are part of the CNS, are particularly suitable for characterizing responses of central neurons. In the rat, cutting the optic nerve at birth results in rapid degeneration of the vast majority of retinal ganglion cells within a few days after the lesion (Miller and Oberdorfer 1981, Beazley et al. 1987). In adult rats, about 50% of ganglion cells degenerate by 1 wk after intraorbital transection of the optic nerve, and approximately 90% of ganglion cells die within 2 wk after the nerve is cut (Berkelaar et al. 1994). However, although optic nerve transection in adult rat results in severe degeneration of ganglion cells over a period of time, there are no detectable changes or loss of cells in the inner and outer nuclear layers (Beazley et al. 1987). In the primate, transneuronal retrograde cell degeneration in the monkey retina following striate cortex removal has also been observed (Weller et al. 1979). Further, some studies have shown that the amount of retinal degeneration is correlated to differences in age (Dineen and Hendrickson 1981). While these observations indicate that ganglion cell death may affect other neurons or synaptic zones in the retina, the results obtained so far appear confusing and the information available concerning transneuronal effects of ganglion cell degeneration on chemical changes, and on non-ganglionic cells, is fragmentary, especially in non-rodent experimental animals. Further investigation is needed to produce a clearer picture of the response of non-ganglionic neurons and their characteristic chemical changes.

Our studies have interestingly shown that a protein gene product 9.5 (PGP 9.5) is a useful marker

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for studying, not only the normal developing retina, but also the effect of lesion or transplantation on specific neuronal populations (Chen et al. 1994 1997). As several investigators have indicated, PGP 9.5 corresponds to a carboxyl-terminal hydrolase isozyme for ubiquitin (Wilkinson et al. 1989), and it may therefore play a role in disassembling ubiquitin protein conjugates formed during protein metabolism. Further, a defect or alteration in the expression of ubiquitin-catabolizing enzymes, such as PGP 9.5, has been suggested as a characteristic feature of certain neurodegenerative diseases (Wilson et al. 1988, Lowe et al. 1990). Synaptosomal-associated protein 25 kDa (SNAP-25), one of the synaptic vesicle-related and neuron-specific proteins, is localized primarily presynaptically in particular nerve terminals of neurons (Oyler et al. 1989, Geddes et al. 1990). Osen-Sand et al. (1993) have also shown that SNAP-25 plays a key role in axonal growth and it may contribute to nerve terminal plasticity by constitutive exocytosis. The association of SNAP-25 with membranes of synaptic terminals makes this protein an interesting potential marker of synaptointegrity and synaptogenesis (Geddes et al. 1990). In the visual system, SNAP-25 can be detected in the retina of rodent species and monkeys (Catsicas et al. 1992), as well as in optic axons, and it has been shown to be transported to their terminals (Hess et al. 1992). Therefore, in the present study SNAP-25 was used as a specific marker to investigate whether axotomy affects the activity of retinal synapse-rich zones.

Our previous study showed the topographic distribution of the retinal projection in the visual system of the Formosan rock monkey *Macaca cyclopis*, the only species of macaque in Taiwan (Chen et al. 1987). These observations indicate that the Formosan monkey has a pattern of optic fiber distribution similar to that of other macaques. In this paper, we investigate the effect of lesioning on the primate retina by using an available animal model. The aims of this study are: (1) to determine the spatiotemporal distribution pattern of PGP 9.5 and SNAP-25 in Formosan monkey retinae, (2) to study the effects of lesion-induced retrograde degeneration of retinal ganglion cells on the whole retinal cell population by the expression of these proteins, and (3) to assess the role of endogenous neuronal substances on the retinal cells and their synaptic organization.

MATERIALS AND METHODS

Three Formosan monkeys (*Macaca cyclopis*) weighing 10-15 kg were used in the study of the effects of axotomy on retinal cells. With the monkeys under deep anesthesia by intravenous injection of Nembutal, a hole was made through the eyelid of the left eye which was then retracted with sutures to expose the entire eyeball. An incision was made through the fascia, the lateral rectus muscle was isolated, and soft tissue around the left optic nerve was removed. The dural sheath was cut open and the left optic nerve was transected 2-3 mm behind the eyeball. No sign of hemorrhage was detected and the blood supply to the retina was checked under an operation microscope. The surgical procedures were similar to those in our previous studies on the rat (Shen and Baisden 1986, Chen et al. 1994). The experimental animals with lesions were then allowed to survive for 1 mo before sacrifice.

All animals were sacrificed with vascular rinse followed by 4% paraformaldehyde in 0.1 M phosphate buffer via cardiac perfusion. The eyes were dissected out and post-fixed for 1 h before they were transferred to 0.1 M phosphate buffer saline (PBS, pH 7.4) containing 30% sucrose at 4 °C for 3 d; at this time they were sectioned at 15 μm on a cryostat. The sections were then collected on poly-L-lysine coated slides, and processed for immunocytochemistry using polyclonal antibodies PGP 9.5 (dilution 1:4000; UltraClone, UK) or SNAP-25 (1:2000; a kind gift of Professor LJ Garey). These antibodies have been characterized previously (Wilkinson et al. 1989, Catsicas et al. 1992). Control sections were incubated with primary antibodies replaced by normal rabbit or goat serum, or PBS. All sections were then reacted by avidin-biotin-complex and developed by the glucose oxidase nickel-diaminobenzidine enhancement method (Chen et al. 1994). No positive immunoreactivity was observed in control sections. In some cases, adjacent sections were stained with cresyl violet to reveal the cytoarchitecture of the retinal tissue.

RESULTS

The results obtained from use of the Nissl stain show there are 10 sublayers in the normal retina or retina contralateral to the transected optic nerve, including the retinal ganglion cell layer (GCL) with single-layer cells and the inner and outer nuclear layers (INL, ONL) with multiple-layer cells (Fig. 1A) as well as 2 synaptic zones, i.e., inner and outer plexiform layers (IPL, OPL), located between the GCL, INL, and photoreceptors, respectively. In the lesion-induced degenerative retina, a severe loss of neu-

rons in the GCL and their axons in the optic nerve fiber layer (NFL) was found, while the inner limiting membrane (ILM) could be still detected in the innermost part of the retina (Fig. 1B). Compared to the dense population in the INL of controls, cellular numbers in the lesioned INL were reduced dramatically and the distribution became sparse. In contrast, cell bodies of photoreceptors located in the ONL seemed to increase significantly. It is evident by comparing the lamination of the 2 retinae (Fig. 1A, B), that the major cellular populations had changed. The main difference with regard to structural organization was that the GCL and INL had mixed, and they were not divided by the IPL which had also degenerated.

In sections reacted for PGP 9.5, distinctly labeled cells in monkey retina similar to those observed in normal rat retina were clearly identifiable

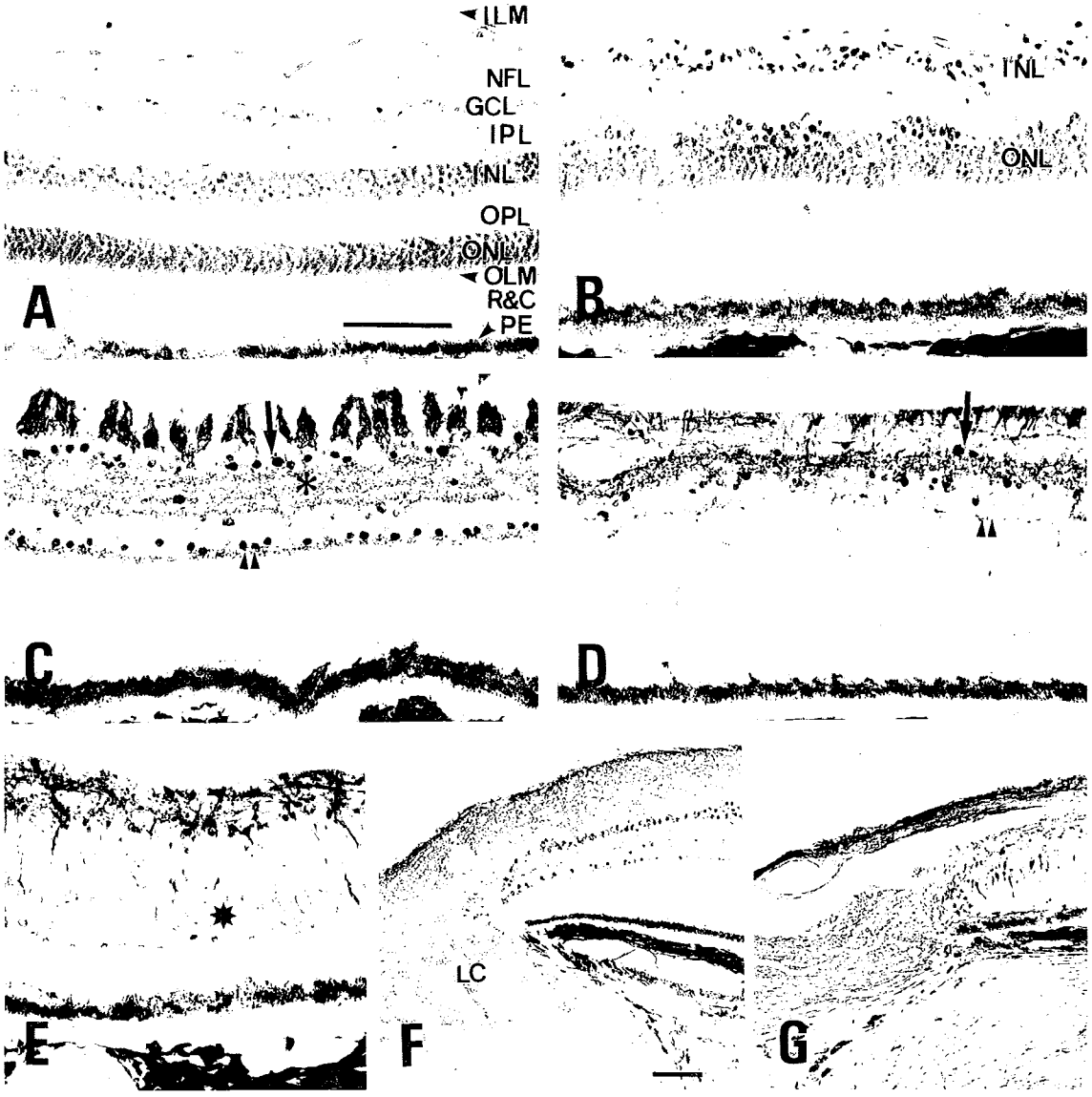

Fig. 1. Photomicrographs showing Nissl stain (A, B) and PGP 9.5 immunoreactivity (C-G) in normal control retinae (A, C, F) and retinae with the optic nerve cut (B, D, E, G). Cresyl violet-stained lesioned retinae (B) show 2 nuclear layers (INL, ONL), while normal retinae (A) show well-laminated structures including optic nerve and plexiform layers (NFL, IPL, OPL), inner and outer limiting membranes (ILM, OLM), 3 nuclear layers (GCL, INL, ONL), and a layer of rods and cones (R&C). In PGP 9.5 immunoreactivity (C and D), the arrows indicate neurons in the GCL while the asterisk indicates the IPL. Many darkly immunostained cells can be observed on the outer parts of the INL close to the PGP 9.5-positive OPL (arrowheads). In (E), a few PGP 9.5-immunoreactive cells appear in the ONL (star). The bar in A refers to all figures except F and G. F and G are at the same magnification. Scale bars: 200 μm.

(Chen et al. 1994). In the normal retina, a distinct population of darkly stained cells and numerous immunoreactive fascicles of fibers were observed in the GCL and NFL (Fig. 1C). Two or 3 immunoreactive sublaminae were visible in the IPL in which a few darkly stained neurons were found. These immunoreactive cells appeared to have small, round cell bodies characteristic of displaced amacrine cells, or to be more elongated in shape; thus we cannot rule out the possibility that they are displaced retinal ganglion cells. A large number of moderately to darkly immunoreactive cells was observed in the INL. Most of these cells, from their location, are likely to be amacrine cells in the inner border of the INL and horizontal cells in the outer part close to the OPL. No PGP 9.5 immunoreactivity was detected in the cell bodies of photoreceptors and their segments.

One month after axotomy, there was a great reduction in the number of PGP 9.5-immunoreactive cells in the GCL in the retina ipsilateral to the transected optic nerve (Fig. 1D). The fiber bundles disappeared, while sparse disordered densities of stained fibers appeared in the NFL. The thickness of the IPL was reduced significantly and the sublaminae were indistinct although the staining intensity had become stronger. In the INL of the lesioned retina, moderately staining cells in the inner border could be observed, while a large number of horizontal-like cells had disappeared from the outer INL. The reduction of immunoreactive horizontal cells was associated with a severe loss of nerve fibers in the OPL. In one case, the degeneration was more severe and the retinal sublayers including the NFL, GCL, IPL, and INL were mixed (Fig. 1E), although a few PGP 9.5-positive fibers and cells were still detected in the inner retina. Very surprisingly, many immunostaining cells were present in the middle part of the ONL and are likely cell bodies of rods or cones. Compared to the normal retina (Fig. 1F), the whole thickness of the optic disc area was also reduced in the lesioned side (Fig. 1G). In the optic disc of both retinae, the PGP 9.5-immunoreactive nerve fibers were expressed around an annular ridge and lamina cribrosa, but not at the beginning of the optic nerve where the axons are myelinated.

In general, most of the SNAP-25 immunoreactivity was present in the nerve fibers and synaptic zones, but no labeled cells were identified in the retinal cellular layers (Fig. 2A). In other words, darkly immunoreactive IPL and OPL as well as numerous stained fiber bundles in the NFL were visible, and the immunoreactivity was distributed throughout the whole retina including the optic disc area (Fig. 2C). In monkeys with optic nerve transection 1 mo before sacrifice, a severe loss of immunoreactivity was observed in the NFL and IPL as compared to the staining pattern in the normal retina (Fig. 2B). In high magnification, distinct SNAP-25-positive fibers were present in the OPL but only a few immunostained fibers appeared in the degenerated NFL and IPL (Fig. 2D). In addition, faintly to moderately staining nerve fibers could be traced from the NFL to the optic nerve head region, as shown in Fig. 2E. In addition, a distinct labeled layer in the inner OPL was detected between the INL and ONL; the latter became wider as described above. No SNAP-25 immunoreactivity was observed in the photoreceptors or their segments in either normal or lesioned retinae.

DISCUSSION

The results obtained from mature Formosan rock monkeys of the present study under experimental conditions are similar in part to those reported in rats in previous studies as detected by specific markers (Bonfanti et al. 1992, Catsicas et al. 1992, Chen et al. 1994). However, a number of new findings arise in this study and deserve comment. First, the results from this study indicate that PGP 9.5 can be expressed in the transection-induced degenerative retina and can be used as a neuronal marker to investigate the chemical activity of specific retinal populations under injury. For example, severe loss of PGP 9.5 ganglion cells and their axons and PGP 9.5 and SNAP-25 immunoreactivity in the NFL and plexiform layers was observed, indicating that the severe retrograde degeneration in retinal cells had occurred by 1 mo after optic nerve lesion. As previous studies in rodents (Osborne and Perry 1992) have shown, most ganglion cells die and the cells that survive in the GCL are mainly displaced amacrine cells. This is supported by our observation that most of the remaining PGP 9.5-positive cells in the GCL had small somata. In addition, the majority of immunoreactive cells were distributed in the inner border of the INL, indicating that they are conventional amacrine cells and that PGP 9.5 is a good marker for labeling certain specific populations.

However, the lesion effect in the monkey retina is present not only in retinal ganglion cells but also in cells of the INL and ONL. Transneuronal degeneration can be detected by the great reduction of PGP 9.5-immunoreactive cells in the outer border of the INL, which are likely the entire number of horizontal cells. The loss of horizontal cells may further reflect a severe degeneration of the synaptic zone in the OPL and may induce PGP 9.5 expression in the

photoreceptors. The transneuronal effect on the photoreceptors is not common and the up-regulation of PGP 9.5 immunoreactivity may indicate that the protein represents a physiological state or a characteristic feature of the maintenance of neuronal survival (Wilson et al. 1988, Chen et al. 1994 1997). On the other hand, the severe degeneration of horizontal cells suggests that certain subpopulations are damaged selectively, which is another issue that deserves further investigation.

Second, as our findings that an alteration of PGP 9.5 and SNAP-25 immunoreactivity in the NFL, IPL, and OPL have shown, these proteins are good markers for studying synaptic activity and neuronal transport following optic nerve injury. In general, the distinct IPL immunostained for PGP 9.5 at the inner part of the INL is observable while the OPL disappears. In contrast, the pattern for SNAP-25 immunostaining in the IPL is lost but moderate staining in the OPL remains. The severe loss of PGP 9.5 expression in the NFL and OPL may be the cause of the degeneration of retinal ganglion and horizontal cells. Moreover, the sublaminae of the IPL becoming indistinct suggests that ganglion cells may contribute to the immunoreactivity in these laminae zones. On the other hand, in light of our observations that transneuronal degeneration in the INL and SNAP-25 expression down-regulates in the IPL but not the OPL, SNAP-25 may be a presynaptic and vesicle-associated protein (Osen-Sand et al. 1993). The differential changes are interesting, further indicating that PGP 9.5 and SNAP-25 play various roles in the retinal system although both are present in the nerve fibers and plexiform layers.

Finally, many studies have shown that retinal ganglion cells of fish and amphibians have the capacity to regenerate functional optic axons after axotomy (Barron et al. 1985). Previous studies in rats have found that half of PGP 9.5-immunoreactive cells are lost in the GCL (Chen et al. 1994) and approximately 90% of ganglion cells die 1 mo after optic nerve cutting (Berkelaar et al. 1994). The degeneration of retinal ganglion cells induced by axotomy can be prevented by intraocular injection of

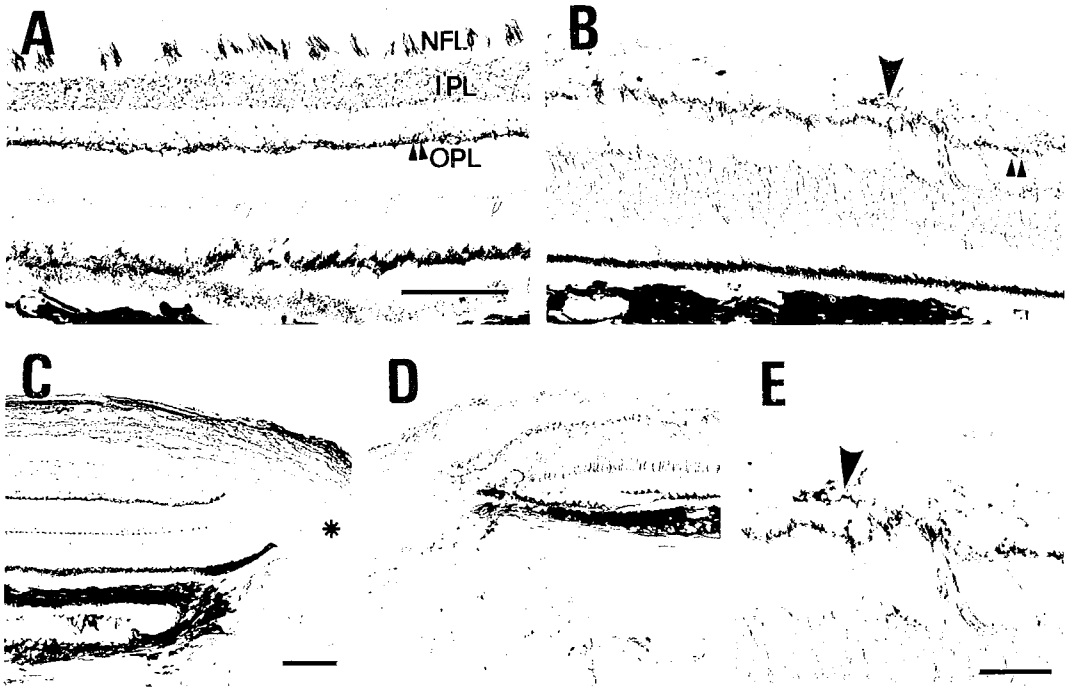

Fig. 2. Photomicrographs showing SNAP-25 immunoreactivity in normal retinae (A, C) and retinae with the optic nerve cut (B, D, E). In (A) are moderately to intensely immunostaining fibers in the NFL, IPL, and OPL (small arrowheads). Sublamination in the IPL is not distinctive in certain parts of the retina. Positive reactivity can also be detected around the optic disc and traced to at least the optic nerve head (asterisk) (C). In the lesioned retina (B), only a few immunoreactive fibers are distributed in the IPL (large arrowhead) which has degenerated severely while SNAP-25 immunoreactivity is clearly expressed in the OPL (small arrowheads). In (D) positive nerve fibers in the lesioned retina are present around the optic disc but not the lamina cribrosa. (E) is a higher magnification of (B). Note that the immunoreactivity in the NFL is absent; only some positive fibers are present in the degenerated IPL (arrowhead). Magnification in A and B is the same while those in C and D are equal. Scale bars in A and C = 200 μm. Scale bar in E = 70 μm.

trophic factors or through a peripheral nerve graft in the rat retina (So and Aguayo 1985, Berkelaar et al. 1994, Hull and Bähr 1994); other factors causing cellular death and regeneration await further investigation. In the present study, axotomy in the Formosan rock monkey resulted in almost complete degeneration in the retinal ganglion cells and the interneurons in the INL as well as severe loss of synaptic activity. The findings seem to indicate that the central nervous system in the primate is vulnerable to damage and may be not repaired, in contrast to the rodent nervous system.

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神經性物質 PGP 9.5 及 SNAP-25 在臺灣獼猴視神經切斷後之視網膜之表現研究

陳淑姿[1] 王建平[2] 簡基憲[1] 沈清良[1]

本實驗是利用兩種神經物質，PGP 9.5 及 SNAP-25作為神經細胞及突觸的標示物，來探討臺灣獼猴視網膜細胞在視神經切斷後的退化情形。結果發現，在視神經傷害一個月後，表現 PGP 9.5 免疫反應性的網膜節細胞大量減少，相反地，視覺受器細胞出現 PGP 9.5 的反應，而表現在叢狀層和視神經束的 PGP 9.5 及 SNAP-25 的免疫反應降低，說明了神經細胞間突觸及物質的傳導作用明顯下降。我們也發現，視神經切斷造成的視網膜細胞及纖維化的退化，在猿猴類比在嚙齒動物更為嚴重，而網膜內 PGP 9.5 及 SNAP-25 在正常表現及傷害後的遽減，可以說明這兩種蛋白不僅是神經病變的標示物，在維持網膜細胞正常功能上，也可能扮演重要角色。

關鍵詞： PGP 9.5，SNAP-25，視網膜，退化，獼猴類。

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