

Substance P-immunoreactive neurons in hamster retinas

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(RECEIVED September 2, 1997; ACCEPTED October 28, 1998)

Abstract

Light-microscopic immunocytochemistry was utilized to localize the different populations of substance P-immunoreactive (SP-IR) neurons in the hamster retina. Based on observation of 2505 SP-IR neurons in transverse sections, 34% were amacrine cells whose pear-shaped or round cell bodies (7–8 μm) were situated in the inner half of the inner nuclear layer (INL) or in the inner plexiform layer (IPL), while 66% of SP-IR somata (6–20 μm) were located in the ganglion cell layer (GCL) which were interpreted to be displaced amacrine cells and retinal ganglion cells (RGCs). At least three types of SP-IR amacrine cells were identified. The SP-IR processes were distributed in strata 1, 3, and 5 with the densest plexus in stratum 5 of the inner plexiform layer. In the wholemount retina, the SP-IR cells were found to be distributed throughout the entire retina and their mean number was estimated to be 4224 ± 76 . Two experiments were performed to clarify whether any of the SP-IR neurons in the GCL were RGCs. The first experiment demonstrated the presence of SP-IR RGCs by retrogradely labeling the RGCs and subsequently staining the SP-IR cells in the retina using immunocytochemistry. The second experiment identified SP-IR central projections of RGCs to the contralateral dorsal lateral geniculate nucleus. This projection disappeared following removal of the contralateral eye. The number of SP-IR RGCs was estimated following optic nerve section. At 2 months after sectioning the optic nerve, the total number of SP-IR neurons in the GCL reduced from 4224 ± 76 to a mean of 1192 ± 139 . Assuming that all SP-IR neurons in the GCL which disappeared after nerve section were RGCs, the number of SP-IR RGCs was estimated to be 3032, representing 3–4% of the total RGCs. In summary, findings of the present study provide evidence for the existence of SP-IR RGCs in the hamster retina.

Keywords: Substance P, Retinal ganglion cells, Amacrine cells, Dorsal lateral geniculate nucleus, Optic nerve section

Introduction

Retinal ganglion cells (RGCs) are the sole output of the retina to the brain. Characterization of their neurotransmitters and/or modulators is, therefore, of fundamental importance to the understanding of visual function. Substance P (SP) (for review, see Maggio, 1988) is one of the neurotransmitters used by subsets of amacrine cells (Karten & Brecha, 1980; Eskay et al., 1981; Brecha et al., 1982, 1987; Li et al., 1986; Pourcho & Goebel, 1988; Cuenca & Kolb, 1989; Cuenca et al., 1995) and in certain species, a small number of ganglion cells (Kuljis & Karten, 1986; Brecha et al., 1987; Ehrlich et al., 1987; Cuenca & Kolb, 1989; Vaney et al., 1989; Caruso et al., 1990). Turtle and bird retinas contain SP-IR RGCs with axons projecting centrally to the superficial layers of the optic tectum (Ehrlich et al., 1987; Cuenca & Kolb, 1989). The existence of SP-IR RGCs with their projections to the dorsal lateral geniculate nucleus (LGd), superior colliculus (SC), and accessory optic nuclei in rabbit has also been shown (Brecha et al., 1987). However, SP-IR RGCs have not been studied in most other mam-

mals or their existence remains controversial. It is still unclear if SP-IR RGCs exist in the rat retina (Caruso et al., 1990; Zhang & Yeh, 1992). The present study was undertaken to determine whether SP-IR RGCs are present in another mammalian species, the hamster, the RGCs of which have been extensively used for developmental and regeneration studies (So et al., 1986; Campbell & Frost, 1988; Carter et al., 1989; So et al., 1992).

In this study, we demonstrate SP-IR RGCs in the ganglion cell layer (GCL) and at least three types of SP-IR amacrine cells in the inner nuclear layer (INL) and GCL of the hamster retina. SP-IR projections from RGCs have been found in the LGd, and SP-IR RGCs were estimated to compose 3–4% of the total population of the RGCs.

Methods

Adult male hamsters (*Mesocricetus auratus*, Laboratory Animal Unit, University of Hong Kong) ranging from 1–1.5 months in age were used. Hamsters were maintained in a temperature-controlled environment on a 14-h light/10-h dark cycle. All of the operations were carried out following intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). The operated animals were kept warm until they recovered from the anesthesia before they were returned to their cage. An overdose of sodium pentobarbital was

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used when the animals were sacrificed. Bupreorphine (0.5 mg/kg, s/c) was administered if the animals were observed to be in pain during the survival period.

SP immunoreactivity in normal retinas

Nine hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital. Immunohistochemical detection of SP in cell bodies was enhanced by an intraocular injection of 3 μ l of colchicine (Sigma, St. Louis, MO; 0.2 % in saline) made posterior to the ora serrata (Rethely et al., 1991). Our pilot data showed that only a few SP-IR cells were observed in the non-colchicine treated retinas. The animals were deeply anesthetized 12–48 h subsequently with an overdose of anesthetic and then perfused transcardially with 50 ml 0.9% NaCl, followed with 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The retinas of both eyes were removed and postfixed in the same fixative overnight at 4°C. The right retinas of the animals were used as wholemounts and they were put through a freeze-thaw procedure (Eldred et al., 1983; Li et al., 1986). The left retinas were cut at 16 μ m using a cryostat.

Immunocytochemistry

A rat monoclonal antibody against SP developed by Cuello et al. (1979) was used for immunocytochemical staining. This SP antibody was obtained commercially (Pel-freeze Biologicals, Rogers, AK). The avidin-biotin procedure (Vector Laboratories, Burlingame, CA) was used for immunoperoxidase staining of brain sections, retinal wholemounts, and retinal sections using 3-3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Triton X-100 (0.3%) was included in the incubation steps and, unless stated otherwise, all steps were performed at room temperature. Cryosections of the brain and retina were incubated in 2% normal rabbit serum in phosphate-buffered saline (PBS) for 1 h. The sections were then washed in PBS for 30 min and incubated in the primary antibody (1:1000 in PBS) overnight at 4°C. Following three 10-min rinses in PBS, the sections were subsequently incubated in the biotinylated secondary antibody (rabbit anti-rat, 1:200 in PBS) for 1.5 h. After rinsing in three changes of PBS for 30 min, the sections were incubated in the avidin-biotin complex solution (1:100 in PBS) for 1.5 h. Following washing in the PBS, the sections were incubated with 0.15% DAB in PBS for 10 min. H₂O₂ was added to the incubation medium to make a final concentration of 0.5% H₂O₂, and after 5–10 min, as determined by the degree of tissue stain, the reaction was halted with several washes of PBS. The protocol for retinal wholemounts was as described above for cryosections except that incubation in the primary antibody was 5 days at 4°C and 5 h at room temperature for the secondary antibody.

Existence of SP-IR RGCs revealed by HRP retrograde labeling

Five hamsters were used in this group. Under anesthesia, small openings in the skull were made bilaterally over the SC; cortical tissues covering the SC were removed with an aspirator. Small pieces of gelfoam (Upjohn, Rochester, NY) soaked with 25% horseradish peroxidase (HRP, Sigma Type VI) in saline were applied to the surfaces of both SC, a primary visual center in hamster receiving most of the projecting fibers from the RGCs (Linden & Perry, 1983). This procedure did not damage the retinocollicular projections or the SC. After 2–3 days, colchicine was injected intravitreally and the animals were sacrificed with an overdose of

anesthetic 12 h later. Retinas of both eyes were obtained and fixed as described above.

The retinas were processed for HRP histochemistry as described by Mesulam (1978) using tetramethyl benzidine (TMB) as the chromogen. The TMB reaction product formed was stabilized by DAB-cobalt reaction: the retinas were rinsed in PBS for 2–5 min and then incubated in a solution containing 0.05% DAB, 0.3% cobalt chloride, and 0.005% hydrogen peroxide (H₂O₂) for 15 min. Afterward the retinas were processed for SP immunocytochemistry.

Estimation of the number of SP-IR RGCs

Four hamsters underwent unilateral section of the optic nerve. Under anesthesia, the right optic nerves of the hamsters were cut 1–2 mm behind the eyeball. Colchicine was injected intravitreally at the end of the survival period of 2 months. The retinal wholemounts of the right eyes were fixed and processed for SP immunocytochemistry.

Central projections of SP-IR RGCs

In this group, six hamsters underwent monocular enucleation and two intact hamsters were used as controls. Under anesthesia, eyes of the animals were removed and the socket was filled with gelfoam, then the eyelids were sewn up. The brains of the hamsters were examined at 2, 4, and 8 weeks after enucleation, two animals for each time point. Transverse frozen sections of the brain were cut at 50 μ m and were processed for SP immunocytochemistry.

Controls

To examine the specificity of primary antibody, SP antibody was incubated with 10⁻⁶ M synthetic SP before application to the tissue and specific staining was abolished completely. Absence of staining was also noted when replacing the primary or the secondary antibodies with normal rat serum and/or PBS, respectively.

Data analysis

A systematic, randomized sampling method was employed to obtain an estimate of the number and size of SP-IR cells in whole-mount preparations of normal retinas and retinas 2 months following axotomy. A systematic non-overlapping series of fields (25–40) were examined across the whole retina at a magnification of 680 \times . An unbiased counting frame with a grid was superimposed on the retina through a camera lucida. The number of cells lying within the counting frame were recorded. This resulted in an estimate of density, which was in turn combined with the total area of the retina to obtain the total number of SP-IR cells in the retinas. Concurrently, the size of each SP-IR cell was recorded by a point-counting technique (Mayhew, 1991). Using the same counting frame, the area of the soma was estimated by counting the number of squares that fell on the profile of the cells. The number of squares was then converted into area by multiplying the number of squares with the area of a single square.

One of the normal retinas and one of the retinas 2 months following optic nerve section were used to illustrate the distribution pattern of SP-IP cells in wholemounted retinas (Fig. 2). The outline of the retina was drawn using a projector and the SP-IR cells were plotted onto the appropriate locations of the outline of the retina with the aid of a camera lucida.

Results

The study of the SP-IR cells was carried out using transverse sections of the retinas and wholemount retinas.

Localization of SP immunoreactivity

SP-IR staining was present within neural processes that ramified in distal, middle, and proximal portions of IPL. The distribution of these processes corresponds to strata 1, 3, and 5 of the IPL as described by Cajal (1892). The most prominent immunoreactive plexus was observed in stratum 5 where SP-containing processes formed a broad, continuous, and intensely stained band; immunoreactive bands in strata 1 and 3 were less dense than in 5 and they were often discontinuous in sections (Fig. 1A).

SP-IR somata were located within the INL, IPL, and GCL. Of the 2505 SP-IR cells examined in transverse sections, 34% of the somata were located in the INL and IPL and 66% in the GCL. Those in the INL and IPL were identified as amacrine cells, whereas those in the GCL were RGCs and displaced amacrine cells (see Discussion).

The SP-IR somata located in the GCL were 6–20 μm in diameter and were oval or round in shape. These somata gave rise to

either one or more processes that ascended to ramify in the IPL. However, it was difficult to determine in which strata they ramified because it was not possible to trace their processes for extended distances, although the process could sometimes be traced to the outer portion of the IPL (Figs. 1B and 1C).

Three types of amacrine cells were identified. The somata of the first type were found in the GCL, and they were displaced amacrine cells. The somata of the second type were located in the INL. They were round or pear-shaped and were 7–8 μm in diameter. Most of these amacrine cells gave rise to a single stout process that descended to stratum 3 of the IPL where it possibly arborized to other strata (Fig. 1D). Occasionally, amacrine cells with multiple primary dendrites could also be observed (Fig. 1E). The third type were tentatively designated as interstitial amacrine cells whose cell bodies and processes were located within the IPL (arrowhead in Fig. 1A).

From the wholemount preparation, the SP-IR cells were found to be distributed throughout the entire retina (Fig. 2A). The somata of the SP-IR cells in the GCL were clearly stained but the processes could only be observed in some of the cells (Fig. 3A). The mean of the total number of SP-IR cells in retinal wholemounts was estimated to be 4224 ($n = 4$, S.E. = 76). The diameter of these cells varied from 6–20 μm (Fig. 4A).

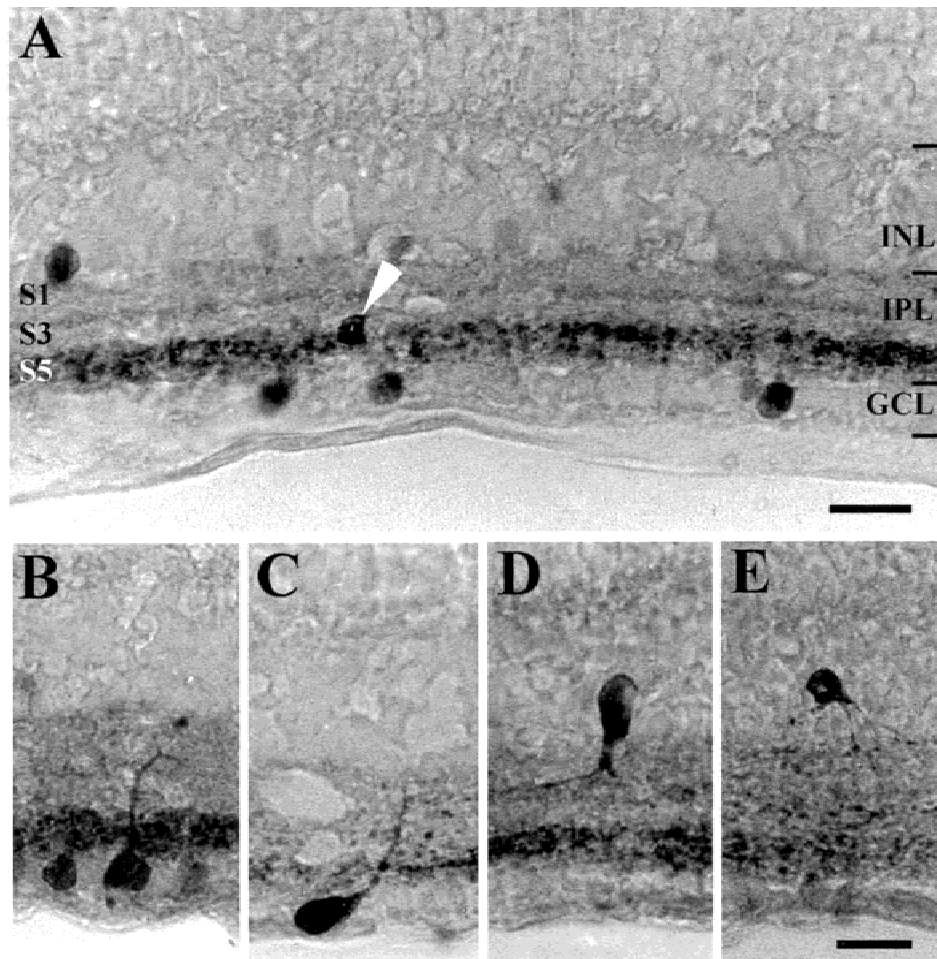


Fig. 1. Transverse sections of retinas showing SP-IR neurons and processes. SP-IR processes originating from cell bodies in the inner nuclear layer (INL) and the ganglion cell layer (GCL) form three distinct bands within strata 1, 3, and 5 (s1, s3, and s5) in the inner plexiform layer (IPL). An arrowhead in (A) is pointing to a presumed amacrine cell located in the IPL. (B) and (C) show SP-IR cells in the GCL. (D) and (E) show amacrine cells in the inner nuclear layer. Scale bars = 20 μm .

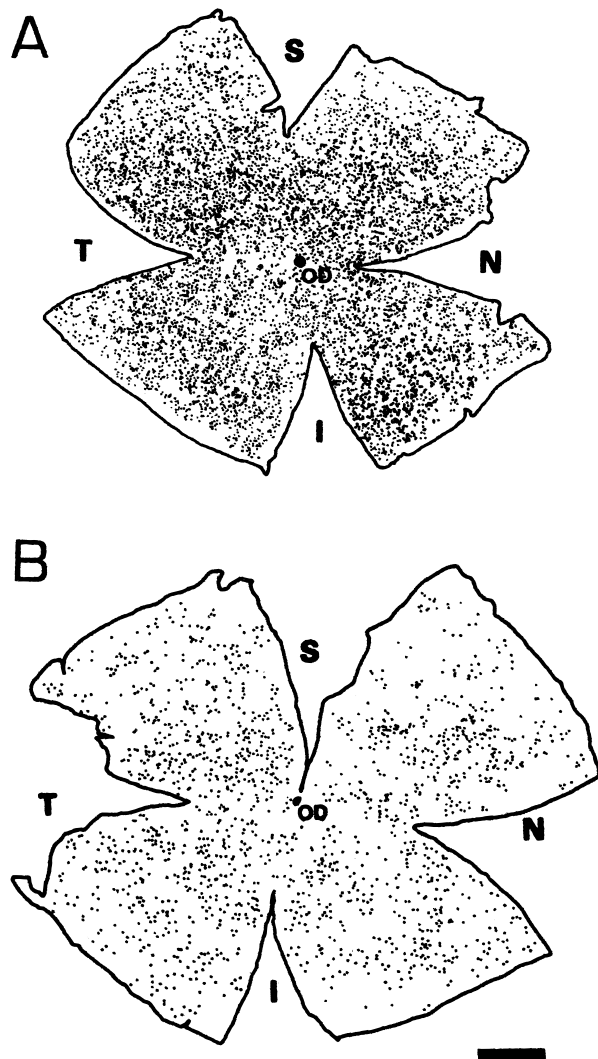


Fig. 2. Distribution maps showing the localization of SP-IR neurons in the wholemount preparation of retinas. The cells were drawn with the aid of a camera lucida. (A) normal retina; (B) retina from an animal with optic nerve section two months before sacrifice. Note the distribution of SP-IR cells throughout the entire retinas in both maps and also the dramatic reduction of SP-IR neurons following optic nerve section shown in map (B). S: superior quadrant; I: inferior quadrant; T: temporal quadrant; N: nasal quadrant; and OD: optic disc. Scale bar = 1 mm.

SP-IR RGCs identified by retrograde labeling and SP immunocytochemistry

Some of the SP-IR neurons in the GCL were found to be RGCs by double labeling. RGCs were first labeled by HRP applied at the SC. The HRP-labeled cells containing dark-blue reaction products were distributed throughout the retina and exhibited a wide range of sizes in the GCL suggesting that all three major morphological classes of RGCs (Boycott & Wässle, 1974; Fukuda, 1977) had taken up HRP from the SC. Following SP immunocytochemical staining, three disparate populations of cells were found: one contained only dark-blue HRP granules (Fig. 3B, filled star), one contained only brown homogenous staining of SP (Fig. 3B, empty stars); a considerable number of cells, however, possessed both dark-blue HRP granules and brown homogenous SP staining

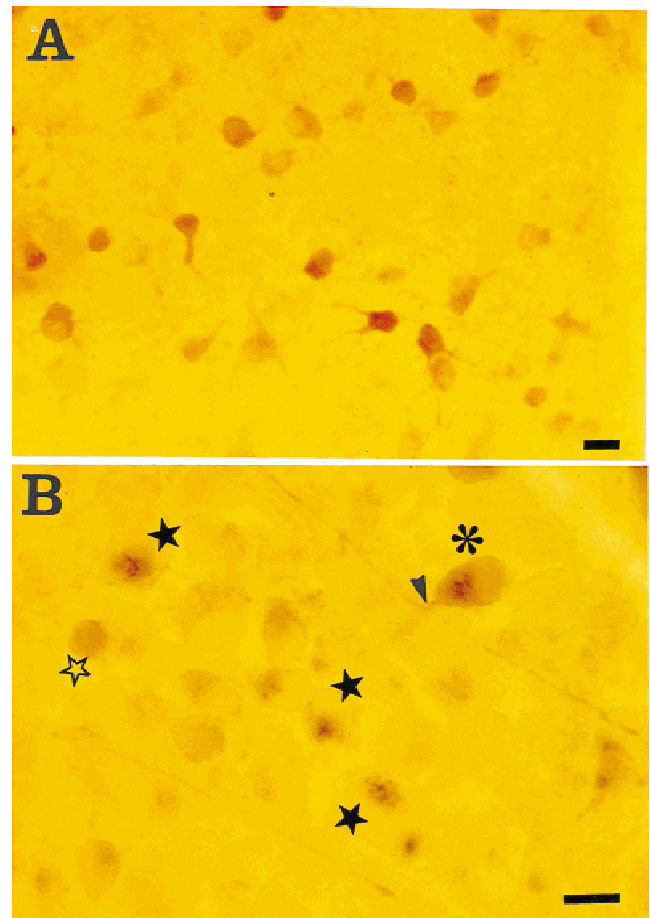


Fig. 3. (A) SP-IR neurons observed in the wholemount preparation of normal retina. The proximal dendritic arborization can be observed in some of the neurons. Scale bar = 20 μ m. (B) HRP-labeled and SP-IR neurons obtained by combining HRP retrograde tracing and SP immunocytochemistry. Empty star denotes a SP-IR neuron; filled stars denote HRP-labeled retinal ganglion cells. Asterisk denotes a SP-IR retinal ganglion cell that was labeled with HRP. The arrowhead points to the axon of the double-labeled cell. Scale bar = 20 μ m.

(Fig. 3B, asterisk). These double-labeled neurons represent the population of RGCs which exhibited SP immunoreactivity. Most of the double-labeled cells were among the largest and most of the single-labeled SP-IR cells were among the smallest in the retina.

Number of SP-IR RGCs and their central projections

A major loss of SP-IR neurons in the GCL and processes in the IPL was found 2 months following optic nerve section (Fig. 2B). SP-IR cells, which remained in the retina, were found to reside primarily in the INL. The mean number of SP-IR cells decreased from 4224 ($n = 4$, S.E. = 76) in normal retinas to 1192 ($n = 4$, S.E. = 139) in retinas 2 months after optic nerve section (Fig. 4). Cells of all sizes had reduced in number (Fig. 4B) compared to the normal (Fig. 4A) but cells with diameters ranging from 16–20 μ m disappeared completely after axotomy. Previous reports have suggested that SP-IR amacrine cells are not affected by axotomy in terms of number and size (Brecha et al., 1987; Ehrlich et al., 1987). Reduction of SP-IR cells following axotomy in the present study,

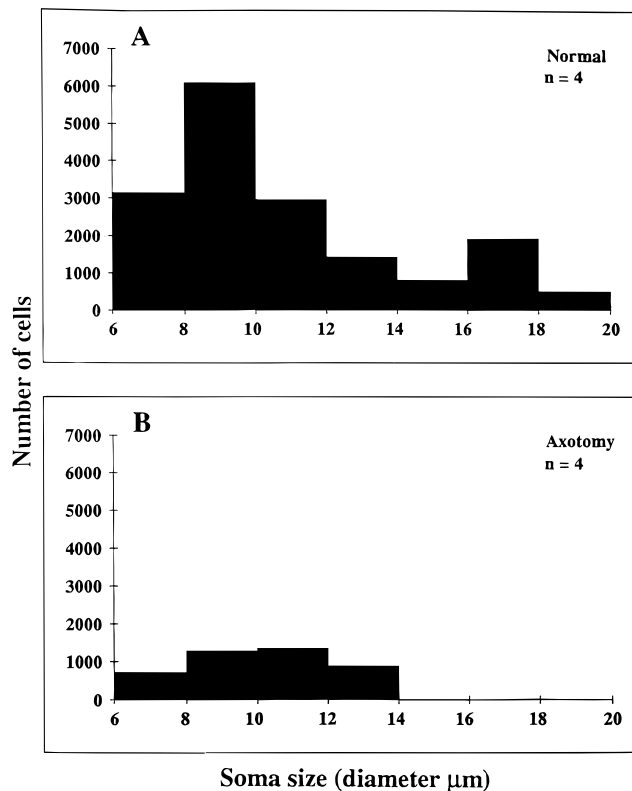


Fig. 4. Two histograms showing profile of sizes of SP-IR neurons in normal retinas (A) and retinas from animals with optic nerve section 2 months before sacrifice (B). The height of rectangles represents the number of cells estimated by the stereological method.

therefore, appeared to be due to the disappearance of SP-IR RGCs. Therefore, the number of SP-IR RGCs would be equal to the number of SP-IR cells that had disappeared at 2 months following axotomy, which was 3032.

SP-stained sections through the LGd, SC, and accessory optic nuclei were examined in the present study. We were able to demonstrate the presence of a prominent plexus of SP-stained axons and varicosities which formed a narrow band just below the optic tract in the LGd (Fig. 5A). Some SP-IR axons could be traced into the LGd from axons in the optic tract. A similar but not as obvious SP-IR plexus was present in the upper part of the stratum griseum superficiale of the SC and in the accessory optic nuclei in the pretectum. In all hamsters with eye enucleation, disappearance of SP immunoreactivity was noted in fibers of the LGd contralateral to the enucleated eye. Fig. 5B shows a complete elimination of SP-stained fibers in the LGd 4 weeks after eye removal.

Discussion

We have observed three types of SP-IR amacrine cells and demonstrated the existence of SP-IR RGCs in the hamster retina in the present study. Our findings are in general agreement with the results reported in earlier studies which have examined the localization of SP-IR neurons in other mammalian retina (Brecha et al., 1982, 1987; Pourcho & Goebel, 1988; Li & Lam, 1990; Caruso et al., 1990; Cuenca et al., 1995).

Comparison with previous studies

SP-IR RGCs have been found in other mammalian retinas (rabbit: Brecha et al., 1987; cat: Vaney et al., 1989). The present study, based on the localization of SP-IR cells, depletion of SP neurons in the GCL after optic nerve section, detection of SP-IR in retrogradely labeled RGCs, and the presence of SP-IR central retinal projections, provide evidence for the existence of SP-IR RGCs in the hamster retina. A similar observation was made in the rat retina, where 3% of ganglion cells were found to be SP-IR neurons (Caruso et al., 1990).

The distribution of SP-IR processes in the hamster retina is similar to that observed in the rabbit (Brecha et al., 1987), rat (Caruso et al., 1990), cat (Pourcho et al., 1988), monkey (Brecha et al., 1982), and human (Li & Lam, 1990; Cuenca et al., 1995).

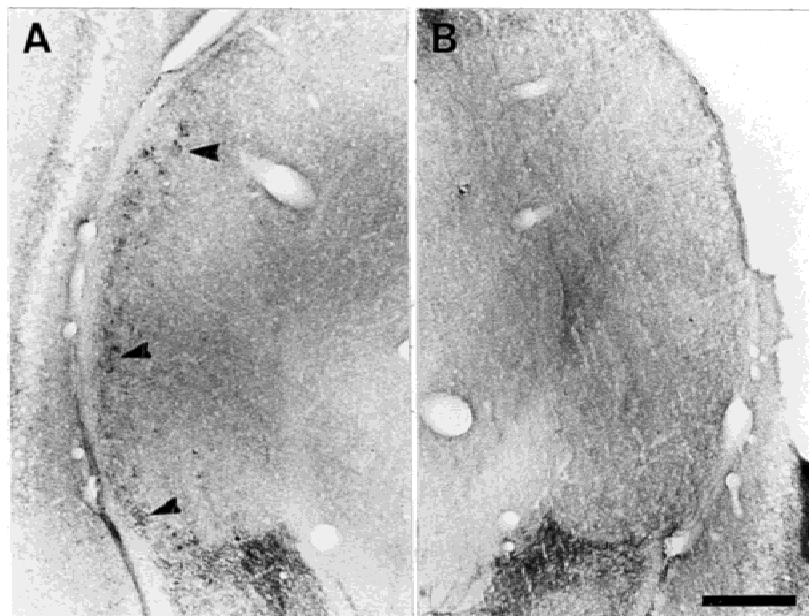


Fig. 5. Photographs showing two sections of the dorsal lateral geniculate nucleus (LGd) from an animal with monocular enucleation 4 weeks before sacrifice. (A) shows the LGd ipsilateral to the enucleated eye. Note the intensely stained SP-IR fibers in the outer laminae of the nucleus (arrowheads). Similar fibers are absent in the corresponding laminae of the LGd contralateral to the enucleated eye (B). Scale bar = 20 μm .

retinas in that the SP-IR processes were localized mainly in strata 1, 3, and 5. Moreover, the existence of multiple populations of SP-IR amacrine cells in the hamster is similar to that observed in the rabbit and monkey. In the rabbit retina at least two types of SP-IR amacrine cells have been found: one unistratified type with processes ramifying in stratum 5 of the IPL and one multistratified type with processes ramifying in strata 1, 3, 5, and possibly with some processes going to the outer plexiform layer (Brecha et al., 1987). In the monkey, both interstitial and displaced amacrine cells with processes ramifying in strata 1, 3, and 5 have been described (Brecha et al., 1982). However, the multiple populations of SP-IR amacrine cells observed in the present study is not seen in species like goldfish, lizard, pigeon (Brecha et al., 1984), and anuran (Hiscock & Straznicki, 1989) for they have unistratified SP-IR amacrine cells only. Bistratified SP-IR amacrine cells have been described earlier in some species (Brecha et al., 1984; Pourcho et al., 1988).

The significance of these differences in stratification for different types of SP-IR amacrine cells still remains obscure until the functional role of SP is better understood. However, some hints on the laminar distribution of SP have been derived from the electrophysiological studies which showed that SP has influences on both ON and OFF central pathways in the retina (Dick & Miller, 1981; Glickman et al., 1982).

Evidence for SP-IR RGCs

In this study, depletion of SP neurons in the GCL following axotomy, double labeling of RGCs, and the demonstration of a SP-IR projection suggest the existence of SP-IR RGCs in the hamster retina.

A substantial loss of SP-IR cells was observed following axotomy. At 2 months after axotomy, the total number of SP-IR cells decreased by 72%. The profile of diameter of the remaining cells also changed dramatically from that of the normal (Fig. 4). It is apparent that cells of all sizes had reduced in number. However, cells with soma diameter from 16–20 μm , which are mostly RGCs, disappeared completely. Previous studies have suggested that more than 90% of RGCs would degenerate by 1 month following axotomy (Bray et al., 1992) and expression of SP in RGCs virtually disappears completely beyond 2 weeks after axotomy (Brecha et al., 1987; Ehrlich et al., 1987). Taken all these into consideration, the results from the axotomy study tend to suggest that SP-IR RGCs were present among the SP-IR neurons in the GCL and they disappeared following axotomy.

A direct and definitive evidence for the existence of SP-IR RGCs was obtained from the results of the RGC double labeling. It was found that SP-IR cells in the GCL could be labeled retrogradely by HRP applied to the SC, a major central projecting area of the RGC. Therefore, RGCs must be present among the SP-IR cells. This is supported by our other findings that SP fibers in the LGd disappeared completely after transection of the optic nerve. Since RGC is the sole output of the retina to the brain and most of the retinocollicular projections send collaterals to the LGd (Sefton, 1968), this result provides further evidence for the existence of SP-IR RGCs.

Number of SP-IR RGCs

The reduction of SP-IR cells following transection of the optic nerve has been used to estimate the number of SP-IR RGCs in the rabbit retina (Brecha et al., 1987). In this study, the mean number of SP-IR cells decreased from 4224 ± 76 in normal animals to 1192 ± 139 in animals 2 months following axotomy. Those

cells that remained were most likely to be amacrine cells in the INL and displaced amacrine cells in the GCL (Linden et al., 1987). However, we cannot rule out the possibility that some specific subpopulations of amacrine cells might die following axotomy but were not detected by our method. Thus, there were about 3000 SP-IR RGCs in the normal retina representing 3–4% of the 72,000–81,000 RGCs in hamsters (Linden et al., 1987; Lau et al., 1991). This percentage is appreciably lower than that reported in the rabbit retina in which SP-IR RGCs constituted 25–35% of the total RGCs (Brecha et al., 1987). However, the present result is quite consistent with the percentage of SP-IR RGCs reported in the chicken retina (3%) (Ehrlich et al., 1987), turtle retina (0.4%) (Cuenca & Kolb, 1989), and rat retina (3%) (Caruso et al., 1990).

A small number of SP-IR RGCs might persist for 2 months after optic nerve section. It has been reported that a small number of RGCs in the cat may survive up to 15 months after optic nerve section (Hollander et al., 1984). These RGCs contribute only a minute number to the total number of RGCs, and whether this population of RGCs is SP positive remains a matter of speculation. Hence, even if this population exists in hamsters, the estimation of the proportion of SP-IR RGC would not be affected to a significant extent.

SP-IR central projection

The SP-IR central projection observed in this study is similar to that observed in the rabbit retina (Brecha et al., 1987). In both animals, SP-IR RGCs possess SP-IR projecting fibers to the contralateral LGd. Previous studies have shown that SP-IR RGCs in the retinas of the turtle and chicken with projections terminating in the tectum (Cuenca & Kolb, 1989; Ehrlich et al., 1987). In frogs which possess SP-IR RGCs, SP-positive fibers were observed in the optic tectum which were reduced or eliminated after contralateral eye enucleation (Kuljis & Karten, 1983).

In conclusion, the present study, along with other recent reports (Brecha et al., 1987; Ehrlich et al., 1987; Cuenca & Kolb, 1989; Caruso et al., 1990), provides evidence for the existence of SP-IR RGCs in many species of vertebrate retinas. Thus, SP might be exploited in the future as a cellular marker for a specific population of RGCs, and its expression can reflect the activity of peptide synthesis in different biological statuses, such as in the course of degeneration following axotomy (Aguayo et al., 1991), regeneration into a peripheral graft (So & Aguayo, 1985; Vidal-Sanz et al., 1987; Watanabe et al., 1993; So & Yip, 1998), synapse reestablishment, and functional reconnection (Carter et al., 1989; Thanos, 1992; Sawai et al., 1996; Sasaki et al., 1996).

Acknowledgments

We would like to thank Dr. H.K. Yip and the reviewers for their comments on the manuscript. This study was supported by research grants from the University of Hong Kong (K.-F.S.) and the National Natural Science Foundation of China (H.-B.L.). H.-B. Li was awarded a Sino-British Trust Visitorship from the University of Hong Kong.

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