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Invited Review

Substance P: A neurotransmitter of amacrine and ganglion cells in the vertebrate retina

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Summary. A short history and summary of the occurrence of substance P in the vertebrate body is presented. Substance P is now generally accepted to be a neurotransmitter and can be visualized by immunocytochemistry to occur in various nerve cells in the CNS. In the retina, substance P-immunoreactivity (SP-IR) occurs in amacrine cell populations in all the species so far studied. In some vertebrates retinas SP is also apparent in one or more ganglion cell types. Anatomical investigations have revealed the morphology and connectivity of SP-IR amacrine cells: they branch in several strata of the inner plexiform layer receiving input from bipolar and amacrine cells and making synapses upon bipolar and ganglion cells. Most commonly SP-IR amacrines emit axon-like process that pass to both the outer plexiform layer and the ganglion cell and nerve fiber layers. These processes often end upon the retinal vasculature.

SP-IR ganglion cells have been described in turtle, rabbit and human retinas. In turtle, intracellular dye injection has revealed the morphology of one type of SP-IR ganglion cell as being a large-field monostratified cell with a branches in the outer stratum of the inner plexiform layer. It may correspond to a «Dogiel cell» type. Intracellular investigation of SP-IR amacrine cells in turtle reveal their physiological responses to be ON-OFF in nature with some color-coding characteristics. In general SP acts as an excitatory neurotransmitter raising the spontaneous activity level of ganglion cell responses. The SP-IR ganglion cell is an OFF-center unit in the turtle retina and may be driven in the center of its receptive field by luminosity bipolar cells and in its surround by amacrine cells with color-opponent properties.

Key words: Tachykinins, Morphology, Amacrine cells, Synapses, Electron microscopy, Physiology

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General background

Until the 1960s, only a few neurotransmitters (acetylcholine, epinephrine, norepinephrine and γ-aminobutyric acid) were known, and the general belief was that the total number of neuroactive substances would be relatively small (less than 10). Today, the situation is entirely different, and it is now accepted that the number of transmitters is much larger, probably greater than 50 (Snyder, 1980; Potter et al., 1981). The main reason for this increased estimation is the recent discovery of a large number of neuroactive peptides: of the latter substance P (SP) is probably one of the best characterized. Thus our knowledge of SP could serve as a model for many other putative neuropeptides (Otsuka and Yoshioka, 1993).

SP is a neuropeptide which forms part of the group known as tachykinins. In the past decades, it has become firmly established that the tachykinins including SP, neurokinin A (NKA) and neurokinin B (NKB), all of which share the common C-terminal sequence Phy-X-Gly-Leu-Met-NH_2, are diversely distributed throughout the peripheral and central (CNS) nervous system.

Mammalian tachykinins are found in various organs of the body besides the nervous system. However they seem to be mostly contained in neurons, released therefrom probably upon excitation of the neurons, and act on the receptors of target cells to evoke various cellular responses (Otsuka and Yoshioka, 1993). Thus this mode of function of SP conforms with the definition of a neurotransmitter, although the term «neuromodulator» is frequently used in the literature, particularly for peptide functions. Since the term «neuromodulator» is used to mean various and different concepts depending on users (Dismukes, 1979; Krieger, 1983), we will follow in this review the terminology of Potter et al. (1981) who used «neurotransmitter» to refer to «any substance secreted by a neuron to control its target cells», independent of their excitatory or inhibitory effects, duration of action, or distance to the target cells. Thus we consider SP to be a neuro-

transmitter of the CNS and, as we shall see in this review, of particular importance in visual processing in the retina.

Early history of Substance P

In 1931 von Euler and Gaddum found a substance that occurred in equine brain and caused hypotension and smooth muscle concentration. Because they used a «standard preparation in the form of a dry powder ...» it was conveniently abbreviated to «P» (Chang and Gaddum, 1933). In 1936, its peptide nature became known (von Euler, 1936) and during the 50s and 60s there were several attempts to purify SP. It was actually not finally purified until 1971 when its structure was revealed by Leeman and her colleagues (Chang et al., 1971).

Soon after the discovery of SP, Dale suggested that the chemical transmitter secreted from the peripheral ending of a sensory neuron might be the same as the transmitter secreted from its central ending (Dale, 1935). Dale's statement also suggested that a sensory transmitter might be a vasodilator substance, and since SP was known to be an endogenous vasodilator it became a candidate for a sensory transmitter (Lembeck, 1953). However, this role was not generally accepted, mainly because the lack of evidence of any clear action of SP on central neurons. The first hint on this issue came from Konishi and Otsuka (1971), who in 1971 found that physalaemin, a substance similar to SP in structure and biological activity, exerted an excitatory activity on frog's spinal motoneurons. On the basis of this expectation, an extract of bovine dorsal root tissue was prepared and, a peptide fraction of it was found to exert the same excitatory action on frog's spinal motoneurons. This peptide was tentatively named «dorsal root peptide», and when it was compared with the newly discovered synthetic SP (Tregear et al., 1971) it proved to be identical (Takahashi et al., 1974). During the following years, data accumulated showing SP to fullfill the basic criteria for being considered a neurotransmitter.

SP and other tachykinins are active in both the CNS and the peripheral nervous system. In general tachykinins excite neurons, evoke behavioral responses, are potent vasodilators and secretagogues, contract (directly or indirectly via release of classical transmitters) many smooth muscle and could act as growth factors and as messengers between the nervous and the immune systems (Maggio, 1988; Felten et al., 1993).

Molecular studies have shown an important homology among different tachykinins. Therefore, the many conclusions about the functional role of SP in the mammalian nervous system, largely based on the demonstration of SP with immunohistochemistry or radioimmunoassay, must be interpreted with caution (Maggio, 1988). It is important to realize that some of the biological functions thought to be mediated by SP may in fact be mediated by another tachykinin. Hence a crucial goal is the development of antisera, antagonists, and radioligands that are specific enough to discriminate between the different mammalian tachykinins. In addition, the presence of SP need not imply the presence of a receptor for SP at any particular site. Of course both the ligand and the receptor are required for activity, however there is no apparent correlation across all CNS regions between the density of SP innervation and the density of SP binding sites (Beajouan et al., 1984; Dam and Quirion, 1986; Daniel et al., 1989).

Pharmacological and ligand-binding studies have led to the proposal that there are at least three distinct tachykinin receptors in mammals (Watling et al., 1994). These receptors named NK1, NK2 and NK3 receptors have been cloned and sequenced, and shown to be members of the G-protein linked superfamily of receptors (Guard and Watson, 1991; Nakanishi, 1991). thus SP displays highest affinity for NK1 tachykinin receptors, whereas Neurokinin A and Neurokinin B bind preferentially to NK2 and NK3, however the selectivity of these peptides for their preferred receptors is poor, and it is possible that their physiological actions would be mediated via their interaction with all three receptors (Watling, 1992). Although most of the effects of SP and other tachykinins on the NK1, NK2 and NK3 receptors appear to be dependent on their C-terminal, it is also possible that SP effects may be related to N-terminal directed receptors (Gergathy et al., 1990; Guard and Watson, 1991), the presence of yet undiscovered subtypes of tachykinin receptors, or to direct activation of G-proteins (Mousli et al., 1990).

Biosynthesis, storage, release, inactivation and actions of substance P

The complete primary structure of SP and its precursor peptides α-, ß- and γ-preprotachykinin) is now known (Kawaguchi et al., 1986; Nakanishi, 1987), as well as the mRNAs encoding these three SP precursors. Substance P is biosynthetized at the perikaryon where it is packaged into vesicles (Fort et al., 1989) and axonally transported down to the axon terminals. Immunohistochemical studies suggest that SP is stored primarily in large granular vesicles with diameters of approximately 100 nm. The terminals containing SP often lack synaptic structures though, suggesting nonsynaptic modes of release and sites of transmitter action in addition to synaptic modes (Barber et al., 1979; Zhu et al., 1986; Matthews et al., 1987; Ribeiro-Da-Silva et al., 1989).

Action of SP is terminated by inactivation mechanism. Several enzymes like enkephalinase (Turner et al., 1985), acetylcholinesterase (Chub et al., 1980) and some SP-degrading endopeptidases (Lee et al., 1981; Laufer et al., 1985; Endo et al., 1988) as well as uptake mechanisms (Nakata et al., 1981) seem to be involved in this inactivation. Usually SP produces membrane depolarization of slow time course in neurons and smooth muscle fibers. The main mechanism is probably

a decrease of potassium conductance (Minota et al., 1981; Fujisawa and Ito, 1982). Stanfield and coauthors (1985) and Masuko and coworkers (1986) have shown that SP raises neuronal membrane excitability by reducing inward rectification of potassium channels. Other effects of SP on postsynaptic membranes such as inhibition of muscarine-sensitive potassium current, inhibition and activation of calcium-activated potassium current, activation of calcium, and activation of sodium current have all been reported (Otsuka and Yoshioka, 1993). These effects of SP may also contribute to the generation and regulation of depolarization. Although in general the action of SP lasts between 10-20 sec (Dougherty et al., 1993), faster responses having a duration of 1-10 sec have also been observed upon short pulse iontophoretic or pressure application of SP (Hanani and Burnstock, 1984; Konishi et al., 1989). The membrane depolarization evoked by SP induces an elevation of intracellular calcium ion concentration. Thus SP appears to increase calcium through both the release of calcium from intracellular stores (Matthews et al., 1989) and the influx of external calcium (Matthijs et al., 1990), probably mediated through inositol phospholipid hydrolysis (Merrit and Rink, 1987). Molecular cloning studies have also shown the involvement of G proteins in SP actions.

Coexistence of SP with other neurotransmitters

Until the 1970s it was generally believed that a neuron contained and released only one transmitter. However, the possibility that a neuron contained more that one neurotransmitter substance was strongly advocated in the late 1970s by the observations of Chan-Palay et al. (1978) and Hökfelt et al. (1978) that SP and serotonin coexisted in neurons of the rat's CNS. Nowadays we know of a great many examples of coexistence of SP with other putative neurotransmitters and related substances such as Choline Acethyltransferase (Vincent et al., 1983), GABA (Kosaka et al., 1988; Pourcho and Goebel, 1988a,b; Vaney et al., 1989), Tyrosine Hydroxylase (Kosaka et al., 1988), 5-Hydroxytryptamine (Chan-Palay et al., 1978; Hökfelt et al., 1978; Chiba and Masuko, 1989; Halliday et al., 1988), glutamic acid and other excitatory amino acids (Battaglia and Rustioni, 1988; Dougherty et al., 1993), Neurokinin A and Calcitonin gene-related peptide (Lee et al., 1985; Cameron et al., 1988), Somatostatin (Murakami et al., 1989), Bombesin (Cameron et al., 1988), Enkephalin (Tashiro et al., 1989, Dynorphin (Anderson and Reiner, 1990), Corticotropin-releasing factor (Crawley et al., 1985; Hökfelt et al., 1994) and other newly discovered messengers such as Nitric Oxide (Hökfelt et al., 1994). However the functional significance of the coexistence of more than one neurotransmitter is still not fully understood (see Otsuka and Yoshioka [1993] for a recent review on these issues).

Function of Substance P

A wealth of evidence supports the involvement of SP in a variety of biological activities such as: pain transmission, vasodilatation and increase in capillary permeability, smooth muscle contraction, motor and secretory control of peripheral organs such as the intestines and the salivary glands, control of respiratory, cardiovascular, autonomic and endocrine functions, activation of the immune system, and neurogenic inflamation and memory processings (Pernow, 1983; Maggio, 1988; Otsuka and Yoshioka, 1993; Felten et al., 1993).

Substance P and neurodegenerative disease

SP is implicated in many psychoneurological diseases such as Huntington'disease, Parkinson's disease, schizopherenia, Alzheimer's disease, Riley-Day-syndrome, Hirschprung's disease, allergy, rhinitis and chronic urticaria, arthritis, headache, Herpes zoster, carcinoid syndrome and others (Leake and Ferrier, 1993; Kowall et al., 1993; Otsuka and Yoshioka, 1993). The causes of the neurodegenerative disorders of Parkinson's disease. Alzheimer's disease and amyotrophic lateral sclerosis are unknown. It is proposed that all these disorders result primarily from a loss of trophic peptidergic neurotransmitter, possibly SP (Barker, 1991). Constantidinis et al. (1983) have found that destruction of dopaminergic cells produces an increase of SP in substantia nigra, thus indicating the inhibitory action of dopamine on this peptide. In Huntington's disease, *decreased* SP levels are found in the pars reticulata and pars compacta in the substantia nigra (90%), in the glomus pallidus (80%) and in the putamen (70%) (Kanazawa et al., 1988). On the other hand, an increase of SP has been suggested to occur in these brain areas in patients with schizophrenia (Rimon et al., 1984).

Morphology of SP containing amacrine cells in the retina

In the vertebrate retina, SP-IR is primarily found in classes of amacrine cells (Karten and Brecha, 1980; Brecha et al., 1982; Brecha and Karten, 1983, 1985; Pourcho and Goebel, 1988a; Brecha and Sternini, 1989; Cuenca and Kolb, 1989; Marshak, 1989; Vaney et al., 1989). Figures 1 and 2 show camera lucida drawings of the different morphologies of SP-IR amacrine cells in vertebrate retinas. In most species SP-IR amacrine cells have their cell bodies located either in the inner nuclear layer (INL) or in the ganglion cell layer (GCL), and their dendrites are monostratified in the middle to lower strata of the inner plexiform layer (IPL) (the IPL is commonly divided into 5 equal thickness strata or sublayers as was first described by Cajal, 1892) (Fig. 1, toad, Type B cell in turtle). In some species such as the tiger salamander, bullfrog, and turtle, SP-IR is also seen in bistratified or multistratified cells (Fig. 1, turtle Type A cell; tiger

salamander) (Brecha and Karten, 1985; Yang and Yazulla, 1986; Cuenca and Kolb, 1989). Reptiles (i.e. lizards and turtles) seem to be rather unique in having two quite morphologically distinct types of amacrines cell containing SP. Type A amacrine cells have large somata and wide-field, tristratified dendritic trees in strata 1, 3 and 5 of the IPL while Type B cells have small somata and small-field, monostratified dendritic trees restricted to a plane of branching in S3 (Fig. 1) (Cuenca and Kolb, 1989; Hiscock and Straznicky, 1989).

In most mammalian retinas only one type of SP amacrine cell is consistently seen but other subtypes have been described, which may have been classified as separate from the single type due to confusions of

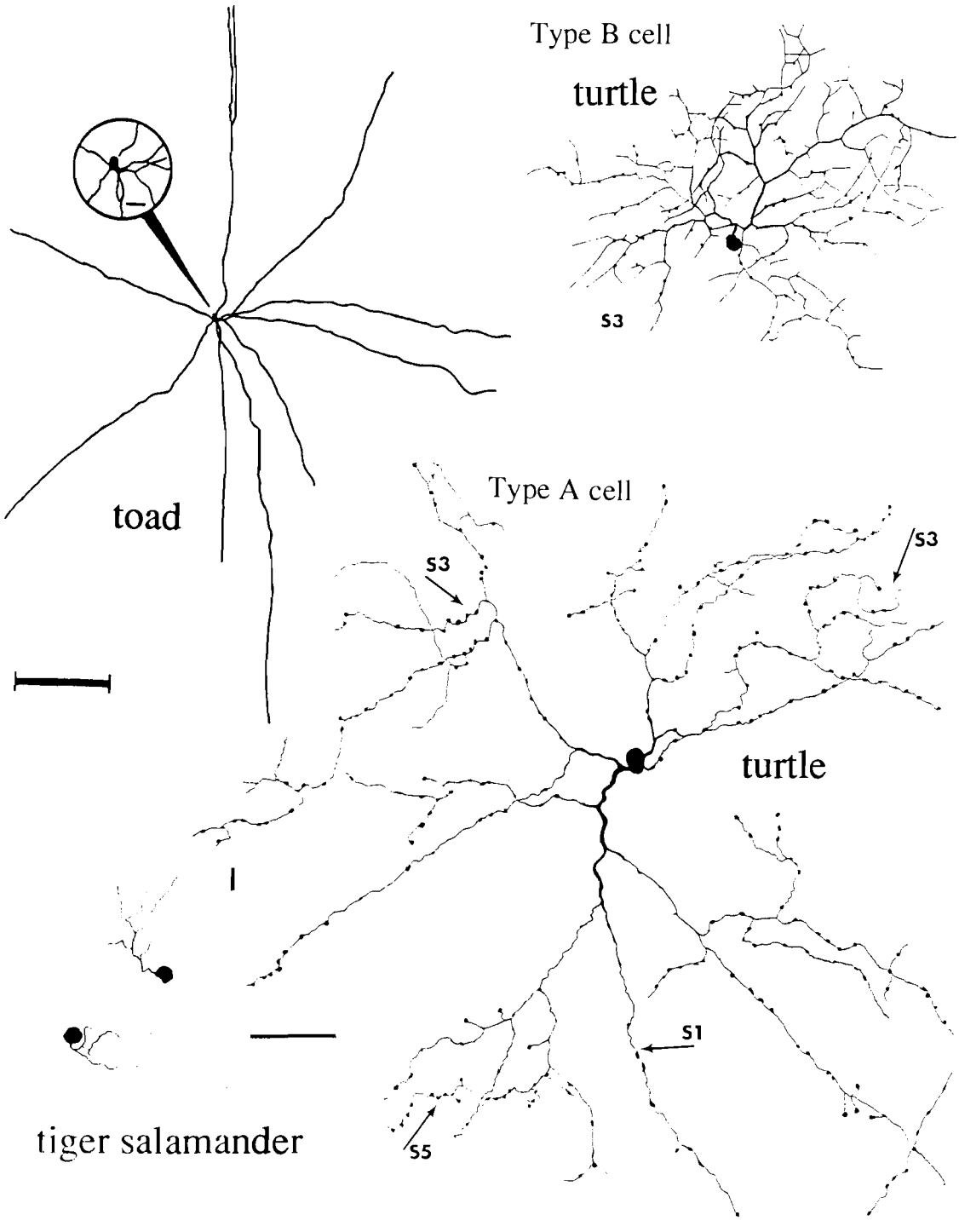

Fig. 1. Camera lucida drawings of SP-IR amacrine cells in the toad, turtle and tiger salamander, as seen in wholemount views. Turtle has two types of SP-IR amacrines: Type A and Type B. Toad cells from Hiscock and Strasnicky (1989). Scale bar= 200 µm. Turtle cells from Cuenca and Kolb (1989). Scale bar= 40 µm. Tiger salamander cells from Yang and Yazulla (1986). Scale bar= 20 µm.

dendritic stratification levels. For example, two subpopulations of amacrine cells containing SP have been described in the cat retina. One cell was reported as a monostratified amacrine with processes ramifying on the S3/4 border or in S4 of the IPL (dependent on author) (Fig. 2, cat) (Pourcho and Goebel, 1988a; Vaney et al., 1989). The other type was said to be bistratified with processes in S1 and S4 (Pourcho and Goebel,

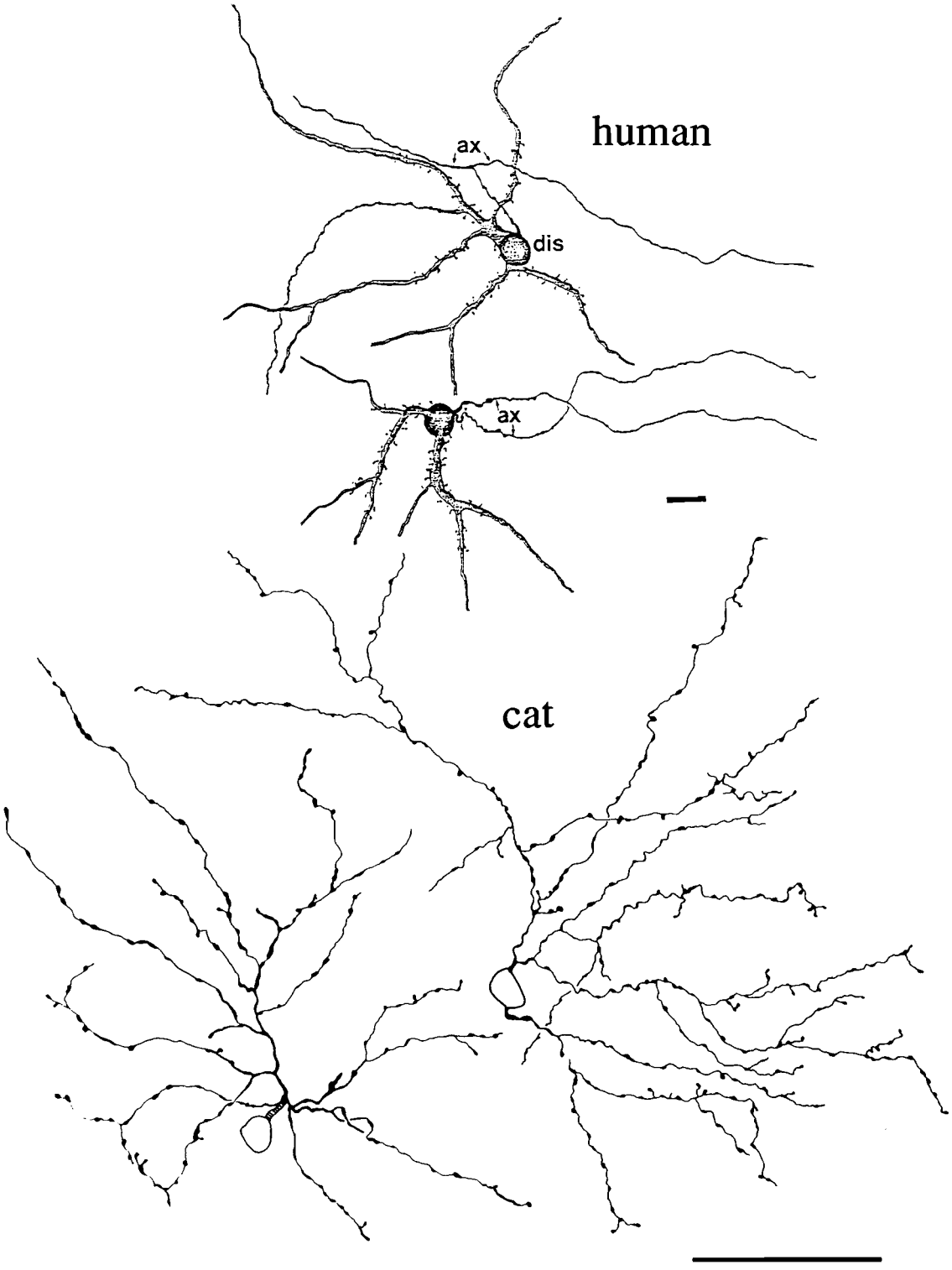

Fig. 2. Camera lucida drawings of SP-IR amacrine cells in the human and cat. Human cells from Cuenca et al. (1995). Scale bar= 10 μm. Cat cells from Pourcho and Goebel (1988a). Scale bar= 50 μm. In human retina, the top cell is displaced (dis) to the GCL, and both have axon-like processe (ax). In the cat, the right hand cell is displaced to the GCL.

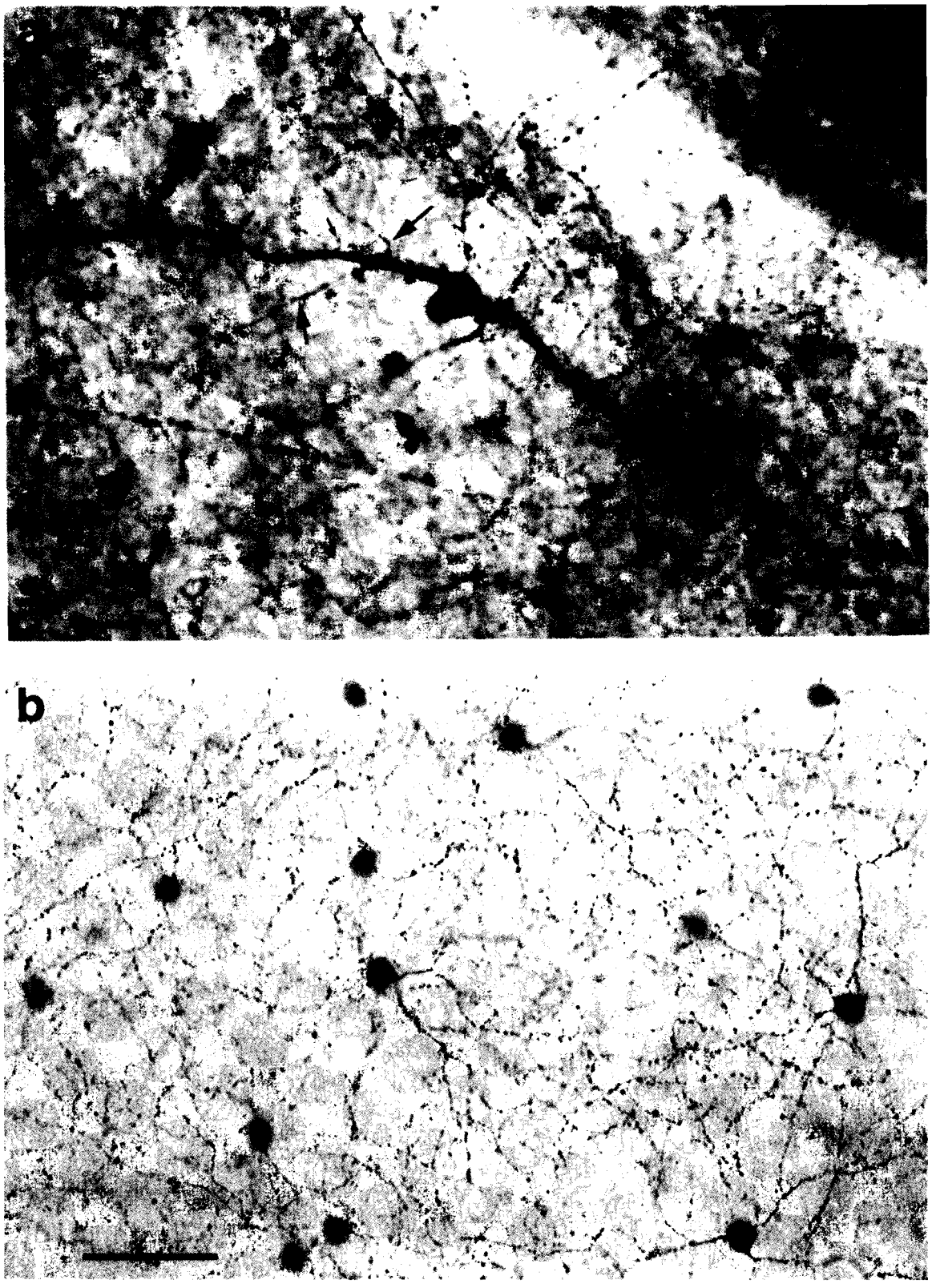

Fig. 3. a. Light micrograph of a SP-IR amacrine cell in human retina. Large arrow points to «axon-like» processes. Small arrows point to spines on the primary dendrites. **b.** Light micrograph of SP-IR amacrine cells in cat retina. From Vaney et al. (1989). Scale bar= 20 µm in both a and b.

1988a). As discussed in Vaney (1990) it is not clear whether these might not actually be the same cell type but differences in intensity of staining led to the appearance or non-appearance of S1 processes (Vaney et al., 1989). The morphology of the so called mono-or bistratified cells in cat are very similar. A drawing from Pourcho and Goebel's paper (1988a) shows them in Fig. 2. In Fig. 3b, a picture of a population of SP-IR amacrine cells, as seen in a wholemount micrograph from Vaney and coauthors work (1989), can be compared. Rather obviously the two publications are showing the same cell type yet the authors give them the slightly different

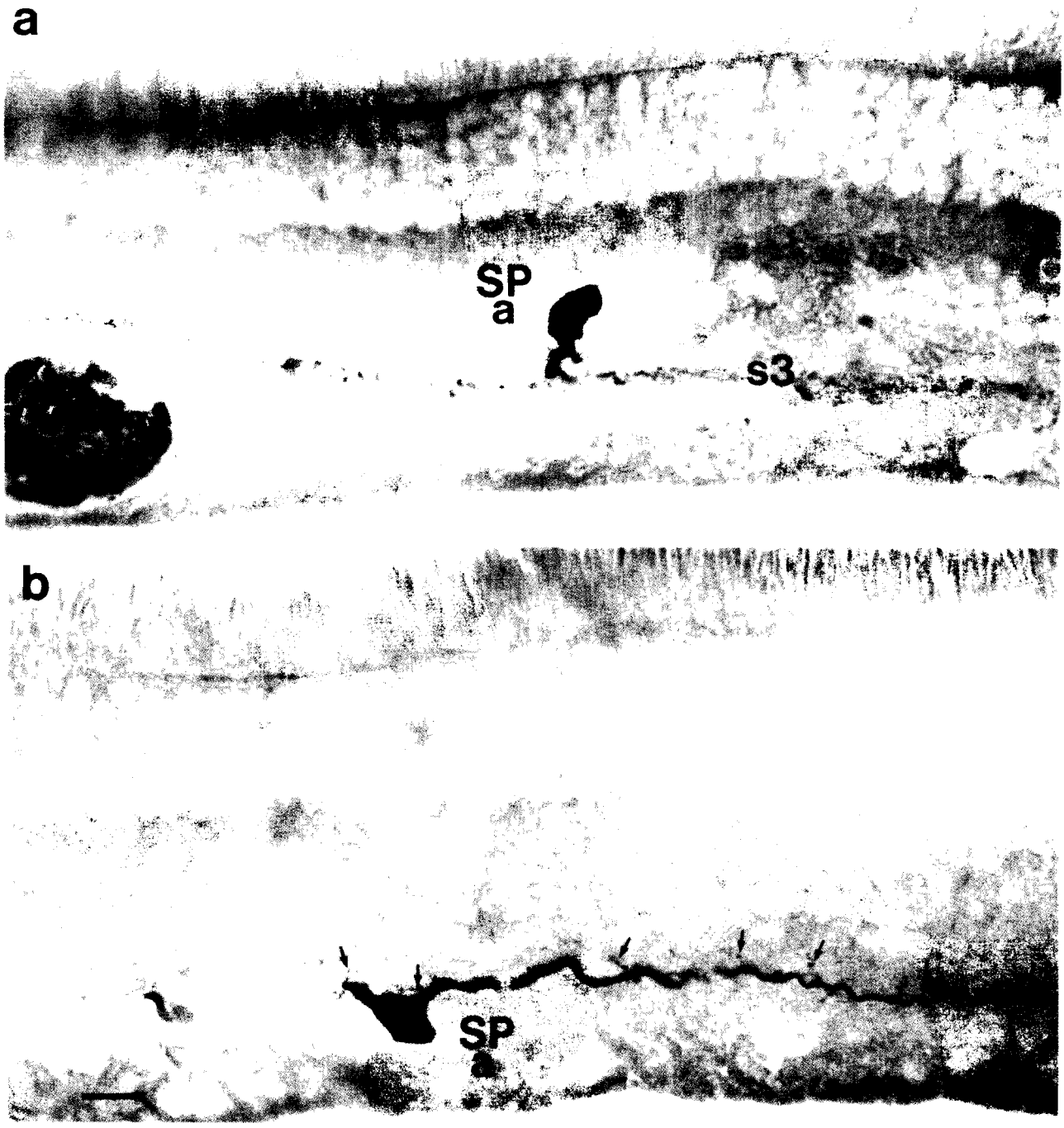

Fig. 4. a. Light micrograph of a SP-IR amacrine cell in a vertical section of human retina. The SP cell is normally placed in the INL and its major dendritic branching is in S3 of the IPL. **b.** Light micrograph of an SP-IR amacrine cell in a vertical section of human retina, with its cell body displaced to the GCL. The major dendritic branching is in S3 like the cell above. Small arrows point to spines on the major dendrites. Scale bar= 20 µm.

descriptions.

In the rabbit retina two types of SP-IR amacrine cells have been described: a monostratified amacrine ramifying in S5 of the IPL, and a multistratified amacrine with processes running in S1, S3 and S5 (Brecha and Sternini, 1989). Again it is possible that the description of a monostratified amacrine of S5 could have been interpreted from a partial section of a multistratified cell where the S5 ramifying dendrites are confused with an intensely stained SP-IR ganglion cell (see later section on SP-IR ganglion cells) Two subpopulations of SP-IR amacrine cells have been reported in the rat retina as well. These cells have somata in either the INL or the GCL. It is unclear whether these have different morphologies although they do appear to have topographical differences in distribution. The SP-IR cells of the GCL are limited to the superior temporal region while the SP-IR cells in the INL are found throughout the retina (Zhang and Yeh, 1992). However, the exact differences in branching patterns or overall morphologies of these two different cell types, if they are indeed different, is not yet well documented.

Even more confusion arrives when we consider SP-containing amacrine cells in the monkey and human retinas. In monkey retina the perikarya of the SP-IR amacrine cells have been described as being located in the proximal row of the INL, the middle of the IPL or in the GCL (Brecha et al., 1982). They are described as three different amacrine cell types with immunostained processes in three plexi in the IPL. In addition because SP-IR processes pass to the OPL and optic nerve fiber layer, SP-IR interplexiform cells and ganglion cells are claimed to be present (Brecha et al., 1982; Marshak, 1989). They may in fact all be variations of the same cell type and interpretations of differences may arise when viewing sectioned immunostained material as compared wholemounts.

A few authors have described SP-IR in the human retina (Tornqvist and Ehinger, 1988; Li and Lam, 1990) and their descriptions are essentially the same as those for monkey SP-IR amacrine cells. We have recently repeated immunocytochemical staining for SP in human retina and have obtained further information on morphology and neurocircuitry of these cells (Cuenca et al., 1994, 1995). In the human retina we have seen intensely stained, wide-field, large-bodied, amacrine cells to be SP-IR (Figs. 2, 3a, 4, 5). We interpret there to be only one morphological type. They can be displaced to the GCL or normally placed in the INL and stratify predominantly in S3 of the IPL (Fig. 4a,b). Their primary dendrites in S3 are studded with spines (Figs. 3a, 4a,b, small arrows). These SP-IR amacrines also have a few processes in S1 and some even pass into the OPL (Fig. 5a). In addition, they all appear to emit «axon-like» processes that run in S3 and S5 and into the nerve fiber layer to end on blood vessel walls (Figs. 2a, 5b). By light microscope observation, the SP-IR amacrine look as though they communicate directly with unstained amacrine cell bodies and with SP-IR ganglion cells (see later section) via a network of fine IR processes. These SP-IR amacrine cells in human retina correspond most closely in morphology to a wide-field thorny type 2 amacrine cell, described and classified in Golgi studies (Mariani, 1990; Kolb et al., 1992).

In terms of development, SP appears in amacrine cells in the human retina around 14 weeks of gestation (Jotwani et al., 1994) although, they may be present in the outer neuroblastic layer even as early as 10 weeks of gestations (Yew et al., 1991). SP thus appears early in development of the vertebrate retina and might help shape connections of the IPL in a growth factor role. Alternative or additionally, SP could be a secretagogue to the retinal vasculature as is suspected of SP fibers in other parts of the CNS (Wahl, 1985; Maggio, 1988). Indeed, Ye and coauthors (1990) studied specifically the peptidergic innervation of the vasculature of the optic nerve and retina in both rhesus monkey and rat. They found several peptidergic immunoreactive fibers including SP in the adventitia and perivascular space, along the course of the central retinal artery within the optic nerve and some in the lamina cribosa. However, they did not find immunoreactivity in the retinal arterioles nor stain well the SP-IR neurons (Ye et al., 1990). In the human retina, we find, the SP-IR amacrines themselves to emit long, fine processes that loop back and forth over blood vessels in the GCL and even terminate against blood vessel walls and in Müller cell end feet close to blood vessels (Cuenca et al., 1995).

Electron microscopy of SP-IR amacrine cells has been performed in goldfish (Yazulla et al., 1985), in monkey (Marshak, 1989) and in human (Cuenca et al., 1994). Some aspects of neurocircuitry of these cells has been demonstrated by these studies. Processes of SP-IR amacrine cells in goldfish and monkey retina make conventional synaptic contacts onto both amacrine and ganglion cell processes and receive synaptic input from amacrine and bipolar cells (Yazulla et al., 1985; Marshak, 1989). Our own studies confirm these findings as can be seen in Figure 6. In Figure 6a, two SP-IR amacrine cell processes are postsynaptic to a cone bipolar axon terminal in S3 of the IPL (CB with a synaptic ribbon). SP-IR processes are primarily postsynaptic to unspecified amacrine cell processe (Fig. 6b, A, arrows) but also to other SP-IR amacrine processes (not shown). The output of SP-IR amacrine cells is to ganglion cell dendrites (Fig. 6c, GC), other unspecified amacrine cells and reciprocal to their input bipolar axons.

Some of the input amacrine cells to SP-IR cells may be excitatory from ACh containing neurons in the retina. We say this because, in the rabbit retina, infusion of exogenous acetylcholine (ACh) into the vitreal chamber leads to an increase in the amount of SP immuno-reactivity in the retina (Goebel and Pourcho, 1992) suggestive of synaptic interactions between ACh and SP amacrine systems. We shall have to await further studies to confirm these putative connections.

Density and distribution of SP-IR amacrines in the vertebrate retina

SP-IR amacrine cells occur at quite high density in the vertebrate retina particularly in comparison to dopaminergic amacrine cells. But their density is typically less than cholinergic amacrine cells (Vaney, 1990). In turtle, we found that the SP-IR amacrine cell population, comprised entirely of the Type B SP-IR cells, peaked in density in the visual streak temporal to the optic nerve at 250 SP-IR cells per mm^2. The distribution appeared to fall off uniformly in elliptical isodensity lines parallel to the visual streak until a density of 50 cells per mm^2 was reached in far peripheral retina. We estimated that the total number of SP-IR amacrine cells in turtle retina to be 7000. Of these

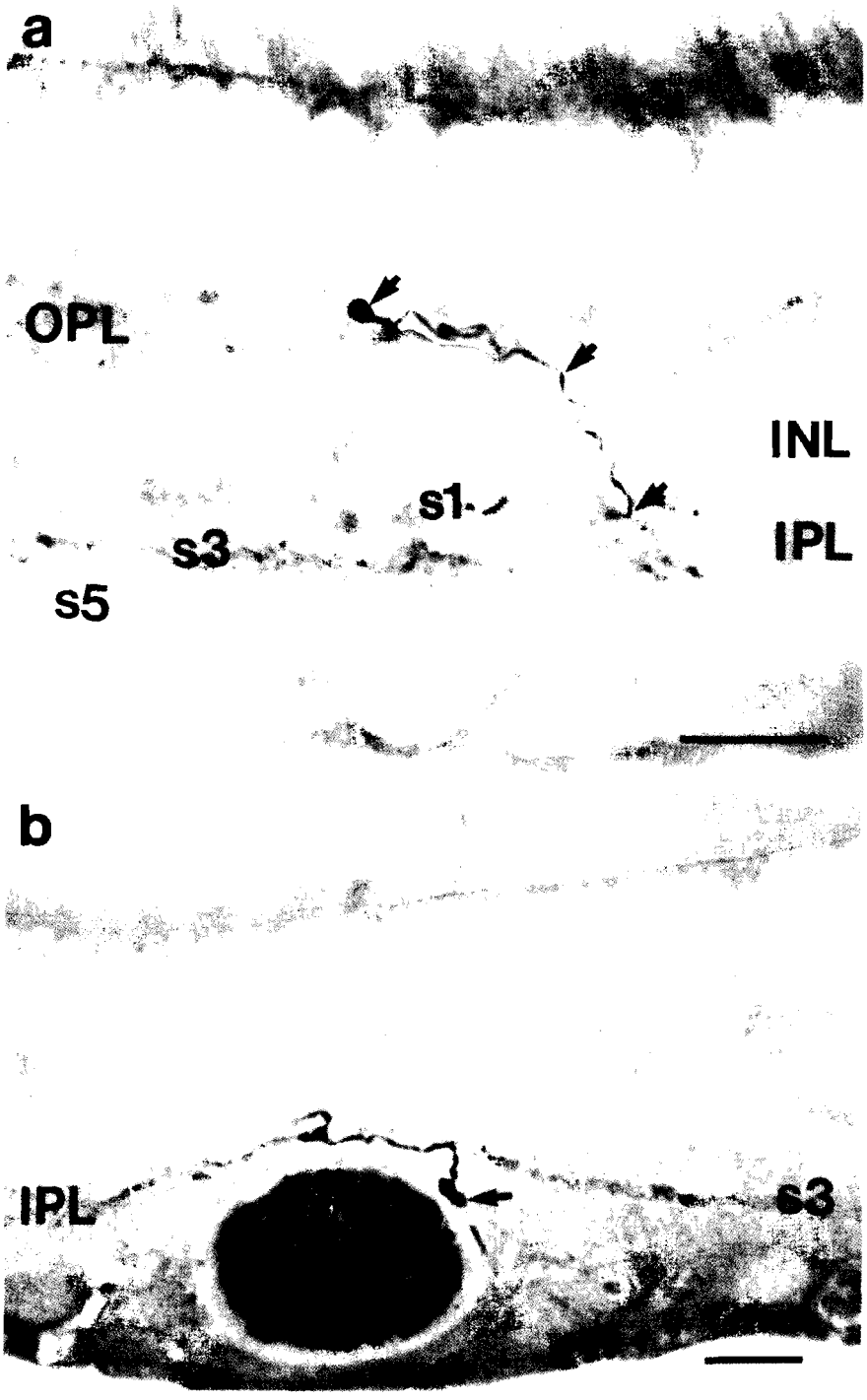

Fig. 5. Light micrographs of a vertical section of human retina immunostained with SP. **a.** SP-IR process passes from an S1 running process in the IPL to the OPL to end in a large varicosity (arrows). Most SP-IR dendrites run in S3 but some processe run down in S5 as well. INL: *inner nuclear layer.* **b.** SP-IR *process passes* down from the IPL to end as a varicosity on a blood vessel wall (bv) (arrow). Scale bars= 20 μm.

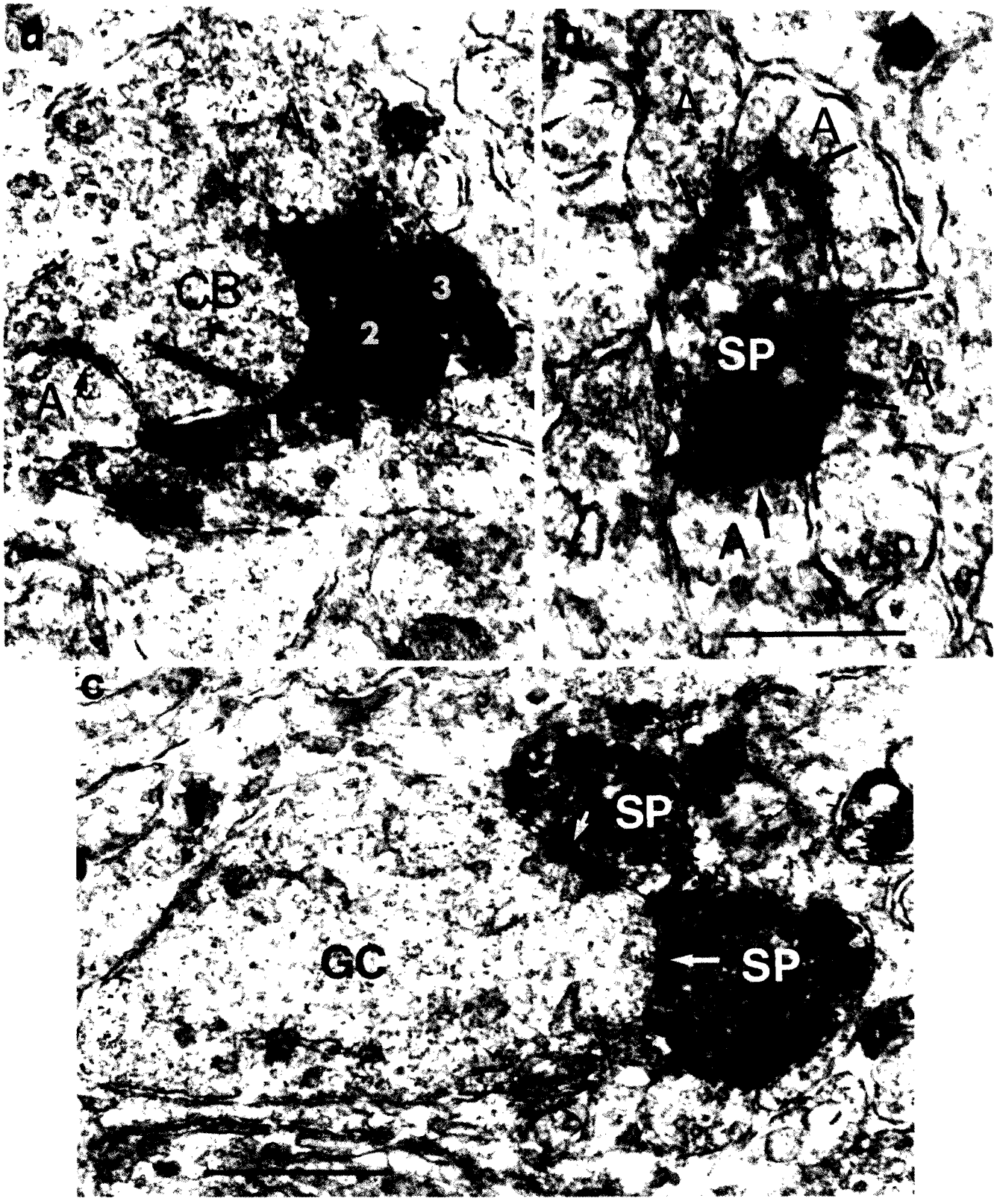

Fig. 6. Electron micrographs of SP-IR amacrine cell synapses in the human retina. **a.** Two SP-IR amacrine processes (1 and 2) are postsynaptic at a ribbon in a cone bipolar axon terminal (CB) in S3 of the IPL. Process 3 is probably postsynaptic to process 2. Process 2 is postsynaptic to a non-reactive amacrine profile (A, arrow). **b.** An SP-IR amacrine profile is postsynaptic to a cluster of non-reactive amacrine cell profiles (As, arrows). **c.** SP-IR processes are making synapse (white arrows) upon a large diameter ganglion cell dendrite (GC). Scale bar a, c is 1 μm but for b is 0.5 μm.

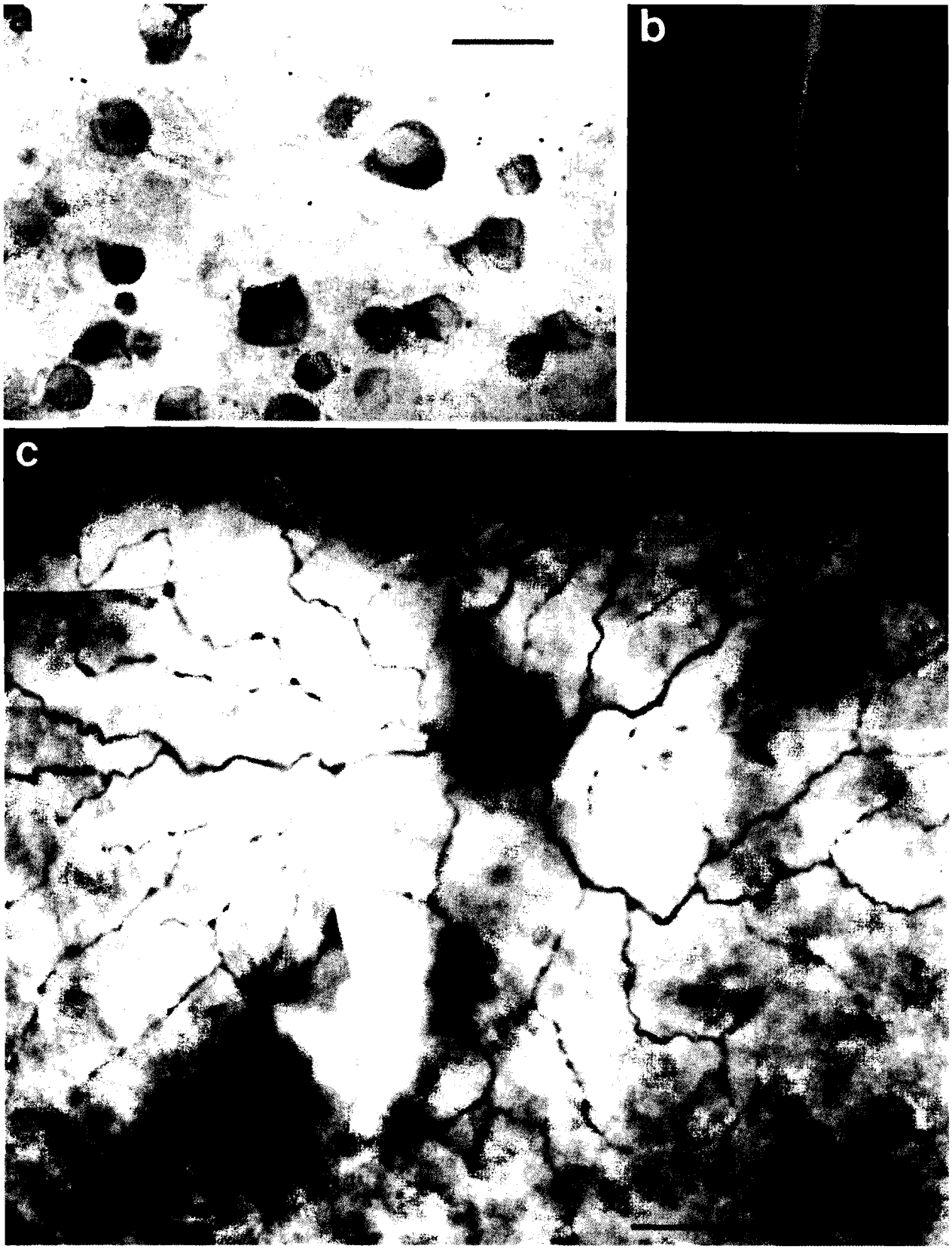

Fig. 7. Light micrographs of SP-IR ganglion cells in the turtle retina. **a.** SP-IR ganglion cell bodies, some of which are large diameter and have IR in their primary dendrites, can be seen. **b.** A microelectrode filled with lucifer yellow and neurobiotin approaches an FITC fluorescent SP-IR ganglion cell body and both ionophores are passed into the SP-IR cell body. **c.** The ganglion cell as seen in wholemount after staining with neurobiotin, and incubation with peroxidase-conjugated streptavidin and HRP histochemistry. It has a beautiful large-field dendritic tree that stratifies in S1 of the IPL. Scale bars 25 μm in a and b and 50 μm in c. From Fernandez and Kolb. (1993).

7000 cells, only 250 were thought to be Type A cells. The latter were found only in the peripheral retina and were not present in the linear visual streak at all (Cuenca and Kolb, 1989).

Vaney and colleagues (1989) found SP-IR amacrine cells in cat retina to have a density of 500 cells/mm^2 in the area centralis declining to a density of 25 cells/mm^2 in the peripheral retina. In cat like in human, the population of SP-IR amacrine cells is divided into normally placed cells in the INL and displaced cells in the GCL. The displaced cells predominate as 90% of the SP-IR amacrines in peripheral cat retina, but displaced and normal appear to occur in equal numbers at maximum density in the cat area centralis (Vaney et al., 1989). Cat retina apparently has as many as 39,000 SP-IR amacrine cells.

Similar total counts of SP-IR cells have not yet been possible in human retina (Cuenca et al., 1995). However, even with limited data, we have counted 35 cells/mm^2 SP-IR amacrine cells in an area 5-9 mm temporal superior from the fovea and 9 SP-IR amacrine cells/mm^2 in area 12-15 mm inferior temporal from the fovea. Thus the density distribution may be very similar in human and cat retinas, but taking into consideration the larger area of the human retina and the increased cell density at the fovea compared with the area centralis of the cat, it is likely that human has a larger total number of SP-IR amacrine cells. Again like in cat, in human, displaced SP-IR amacrines outnumber normally placed (59:41 for the central retinal piece and 71:29 for the more peripheral retinal piece) (Cuenca et al., 1995). Unlike cat though, our nearest neighbor analysis on human retina indicates the SP-IR amacrine cells lie in a close to random mosaic. It seems, however, that the irregularities in the amacrine mosaic are caused by the frequent occurrence of pairs of SP-IR amacrine cells with intervening longer distances to others.

Rabbit, apparently has a very large number of SP-IR neurons too, but it is not clear exactly which proportion of them are amacrine cells and which are ganglion cells. There are supposedly 1,000-1,400 SP-IR cells/mm^2 in the linear visual streak of this retina with a fall off to 125-200 cells/mm^2 in the peripheral retina (Brecha et al., 1987). The majority of the SP-IR cells lie in the GCL in rabbit. After optic nerve section the number of SP-IR cells are reduced by 90% in the visual streak and 45-60% in the peripheral retina. This equates to a density of SP-IR amacrine cells at the visual streak of approximately 120 cells/mm^2 and in the peripheral retina of approximately 90 cells/mm^2. Counts like these lead to the conclusion that a very high proportion of amacrine cells, and an even higher proportion, relative to other animals, of ganglion cells in rabbit retina use SP in a neurotransmitter role.

SP-IR ganglion cells

In turtle, frog, bird, rabbit and human retinas there is evidence for certain ganglion cells to immunostain for SP (Karten and Brecha, 1980, 1983; Kuljis and Karten, 1982; Brecha et al., 1982, 1984, 1987; Ehrlich et al., 1987; Cuenca and Kolb, 1989; Fernandez and Kolb, 1993; Cuenca et al., 1994). A subpopulation of ganglion cells that are displaced in the pigeon retina also seem to be specific for SP (Britto and Hamassaki, 1991). They may be the large cells of the amacrine cell layer described by Dogiel and referred to in Cajal (1892). The most positive evidence for a ganglion cell being SP-IR comes from studies of the rabbit retina. Brecha and co-workers (1987) showed that after optic nerve section, the SP-IR cells in the GCL, that branched in S5, disappeared. Furthermore retrograde tracing studies with Fast Blue from the superior colliculus revealed SP-IR ganglion cell bodies to be double stained. These SP-IR ganglion cells are estimated to form 25-35% of the total number of ganglion cells in the rabbit retina. They project to the superior colliculus, dorsal lateral geniculate body and the accessory optic nucleus (Brecha et al., 1987; Brecha and Sternini, 1989).

Two types of SP-IR cell bodies are stained in turtle retina in our hands (Cuenca and Kolb, 1989; Fernandez and Kolb, 1993). (Fig. 7a). The smaller-bodied one is less intensely SP-IR than the larger and little information is available on its morphology. However, the large SP-IR ganglion cell in turtle has often enough immuno-reactivity in the major dendrites that it can be seen to branch in S1-2 of the IPL and to bear an axon, so positively identifying it as a ganglion cell. Figure 8a shows the appearance of such SP-IR ganglion cells in wholemount preparations of turtle. With a technique of immunostaining for SP (Fig. 7a) and then intracellular injection of neurobiotin through a microelectrode into SP-IR cell bodies in wholemounts of turtle retina (Fig. 7b), we have been able to discover the exact morphology of one of the large-bodied SP ganglion cell types in turtle retina (Fernandez and Kolb, 1993). Thus in Figs. 7c, 8b,c such a SP-IR ganglion cell is fully revealed. Originally we considered this ganglion cell to be the G20 of the Golgi study (Kolb, 1982; Cuenca and Kolb, 1989; Fernandez and Kolb, 1993) however, with our latest evidence from intracellular dye injection and retrograde tracing studies, we are now convinced that this particular SP-IR ganglion cell is in fact a G18 cell type (Kolb, 1982; Zhang and Eldred, 1994; Ammermüller et al., 1995). Its dendrites branch on a plane in S1 to S1/2 border (Fig. 8c) and the tree consists of coarse, crenulated dendrites with curving side branches filling in between main dendrites. It has a large cell body (22 µm diameter) and a 300-500 µm dendritic tree diameter. Many of this kind of cell are displaced in turtle retina, and considered to be Dogiel cells (Kolb, 1982; Zhang and Eldred, 1994), although the one intracellularly stained with neurobiotin (Figs. 7b, 8b,c) was not (Fernandez and Kolb, 1993). SP-IR G18 cells project exclusively to the nucleus of the basal optic root in the turtle (Zhang and Eldred, 1994).

We have also immunostained a ganglion cell type with SP in the human retina (Cuenca et al., 1995). Figure

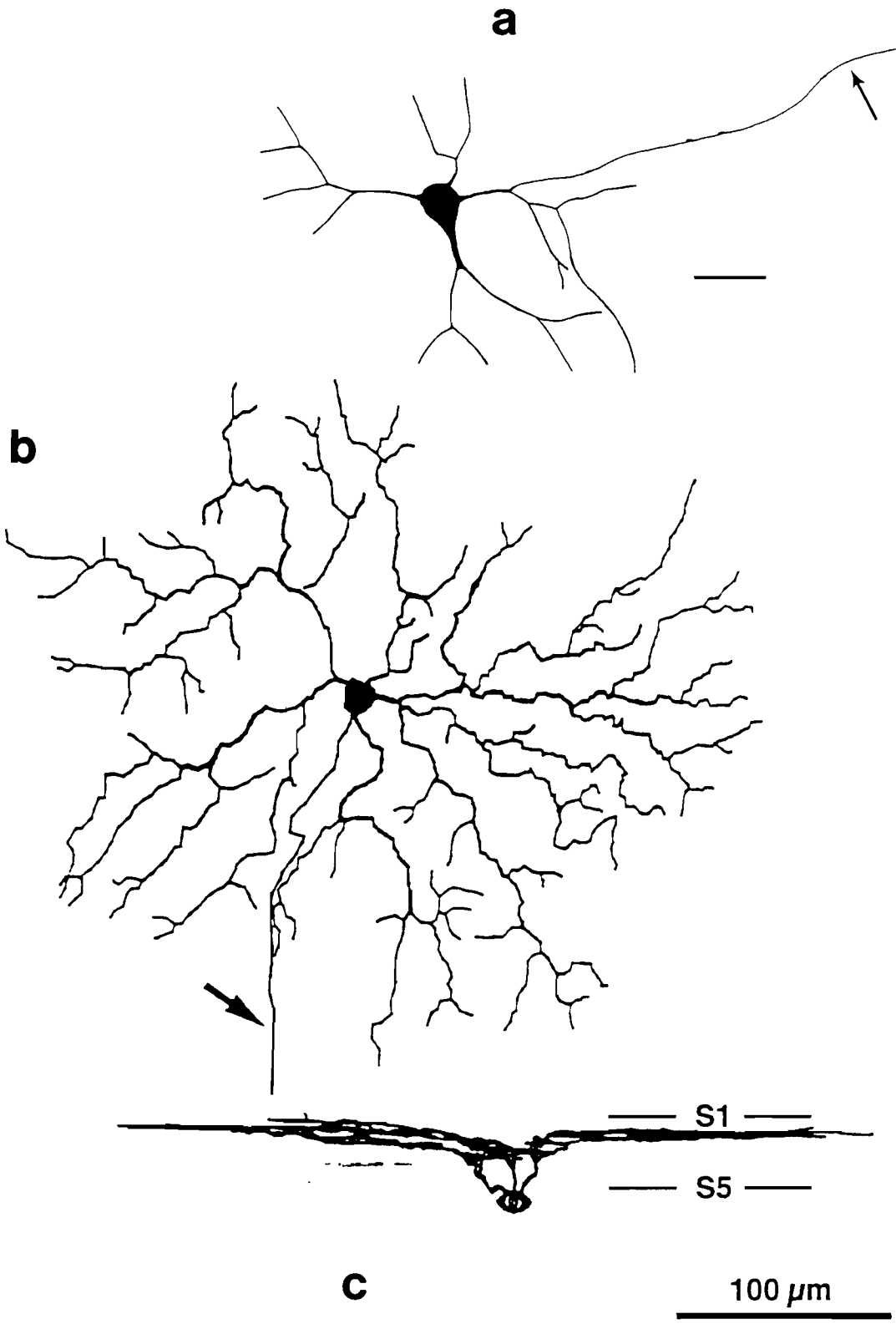

Fig. 8. Camera lucida drawings of an SP-IR ganglion cell and major dendrites as seen in wholemount. The axon is immunostained for some distance (arrow). From Cuenca and Kolb (1989). Scale bar= 40 μm. **b.** Camera lucida drawing made by the Eutectics neuron tracing system of the neurobiotin stained SP-IR ganglion of Fig. 7. **c.** Vertical rotation of the Eutectics drawings in b. Scale bar for b and c is 100 μm.

9 shows such a cell in a wholemount view of peripheral human retina. It appears as a lightly SP-IR cell body of large diameter (22 µm) and with immunoreactivity spreading only into the major dendrites. However, focusing up and down on the immunostained cell and its dendrites, we can determine that the main branches are in the same IPL stratum as the SP-IR amacrine cells primary dendrites. The more intensely stained fine processes are from the SP-IR amacrine cells as they wrap around the SP-IR ganglion cell body and its primary dendrites (Fig. 9, arrows). Electron microscopic observation will be needed to determine whether these fine processe and boutons are synaptic upon the SP-IR ganglion cell body or not. The size of the cell body and the branching level of the dendrites in S3, suggest that this human SP-IR ganglion cell is a G21 (Kolb et al., 1992). In turtle too, we noticed the same apparent interaction between SP-IR amacrine cells (in this case it

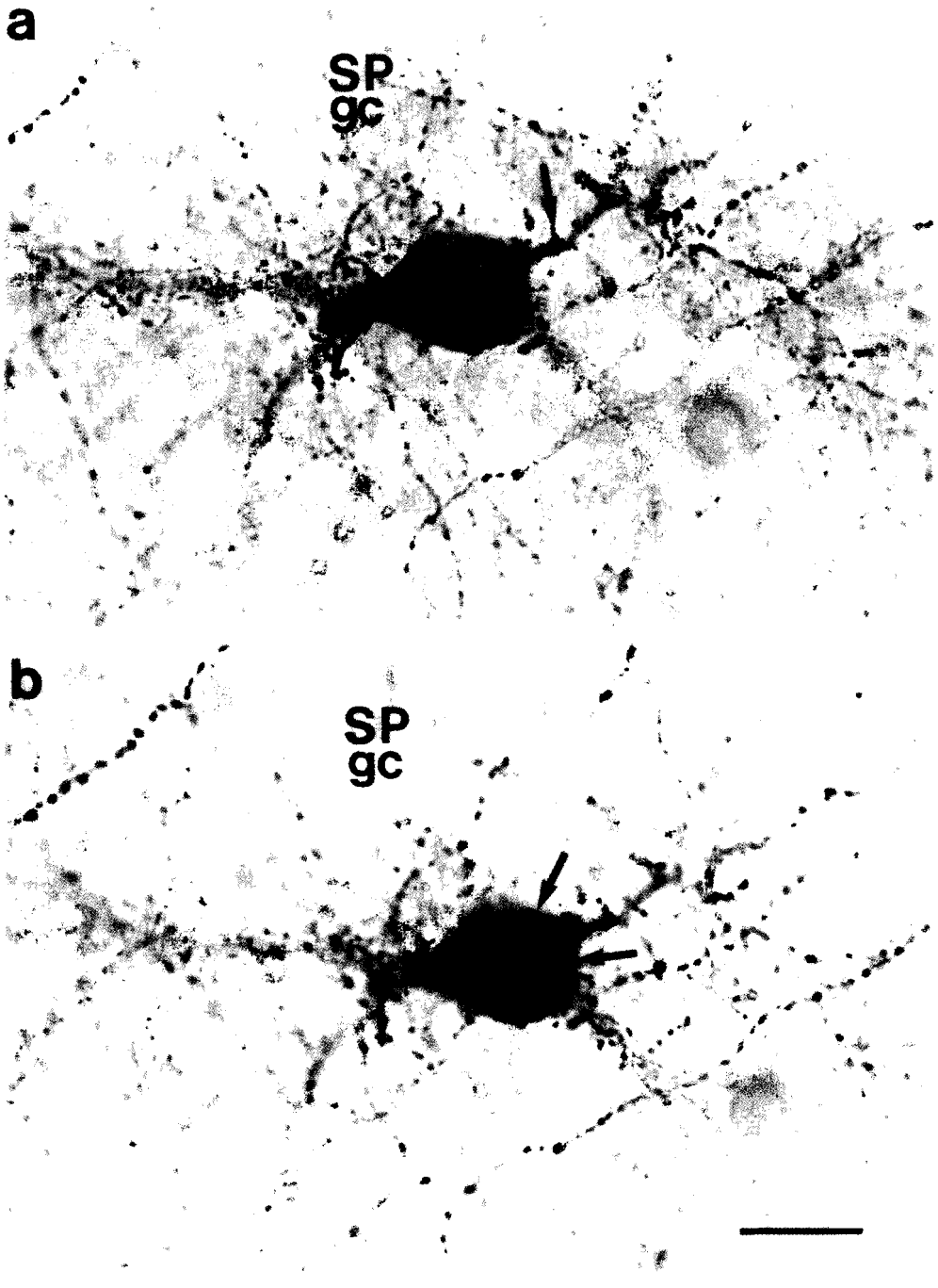

Fig. 9. Light micrographs at two different planes of focus on an SP-IR ganglion cell in human whole-mount retina. In **a.** The focus is on the cell body of the ganglion cell, and fine intensely SP-IR processes from amacrine cells run across the top of the cell body and over the primary dendrites. **b.** The cell at a plane of focus underneath the cell body where the SP-IR amacrine processes can still be seen running over the lower surface of the cell body. Arrows point to small intensely IR boutons of the amacrine cell fibers. Scale bar= 20 µm.

was the tristratified, Type A SP amacrine, Fig. 1) and ganglion cell bodies, both SP-IR and non SP-IR. Like in human, processes from the SP-IR amacrine cells in turtle wrap around ganglion cells, and end in clusters of boutons on ganglion cell somata, often far from the immunostained cells of origin (Cuenca and Kolb, 1989). This long-range interaction is particularly evident in turtle because the SP-IR Type A cell bodies are found in the peripheral retina, avoiding the linear visual streak, while their bouton endings are very noticeable on ganglion cells of the visual streak.

Substance P in the optic nerve

Some proportion of optic nerve fibers have been shown to be SP-IR in the different species where SP-IR ganglion cells have been suspected (Kuljis and Karten, 1982; Kuljis et al., 1984; Brecha et al., 1987; Ehrlich, 1987; Cuenca and Kolb, 1989). SP fibers from the retina are found to terminate in the optic tectum in anurans (Kuljis et al.,, 1984) but in the superior colliculus, dorsal lateral geniculate and the suprachiasmatic nucleus (SCN) of the accessory optic system in rabbit (Brecha et al., 1987; Takatsuji et al., 1991). This nucleus has been identified as a pacemaker for many circadian activities in mammals. SP apparently produces shifts in spontaneous activity in neurons of the SCN with a phase response curve that is similar to the effect of light pulses to the eye under constant darkness. In effect SP may be a transmitter for signals conveying environmental light-dark information from the retina to the SCN (Shibata et al., 1992; Shirakawa and Moore, 1994). In this respect, it is also interesting to note that SP-IR fibers have been seen to innervate the parietal eye visual system of the

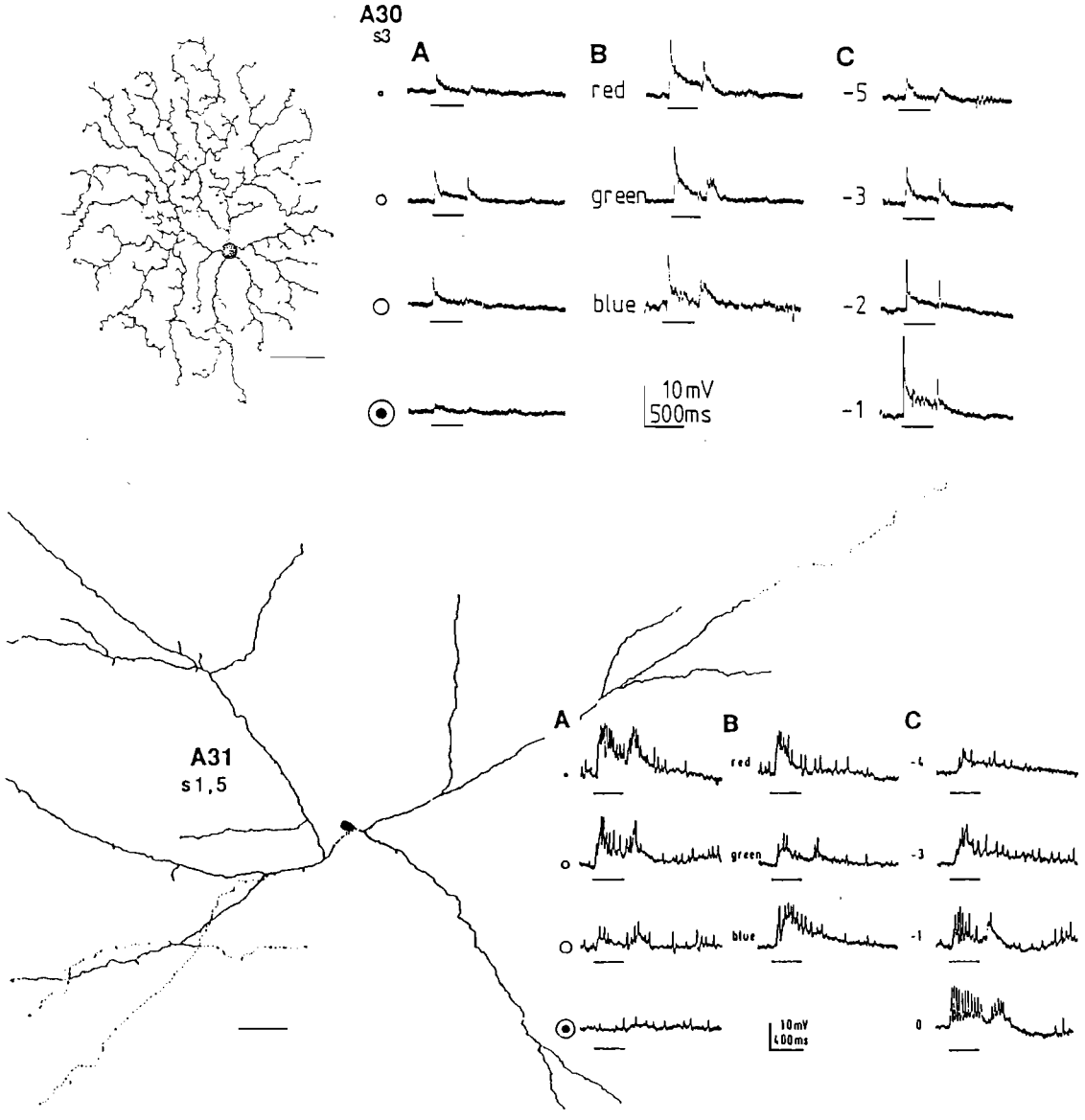

Fig. 10. Intracellularly stained and recorded amacrine cells in the turtle retina that have the morphology of SP-IR Type B (A30, top cell) and type A (A31, bottom cell) amacrines in the turtle retina. Both cells' physiological responses to **A**) spots and annuli of light and to **B**) colored and **C**) an intensity series of stimuli are shown. See text for full descriptions. From Ammermüller and Kolb (1995). Scale bar 25 µm for A30 and 50 µm for A31.

lizard (Engbretson et al., 1982).

Co-localization of substance P and other neuroactive substances in the retina

There is strong evidence for colocalization of SP and other neurotransmitters in the vertebrate retina. Thus, SP-IR amacrine cells also exhibit GABA-like immunoreactivity in the cat retina (Pourcho and Goebel, 1988b; Vaney et al., 1989). In larval tiger salamander retina one population of SP amacrine cells also express GABA-IR (Watt et al., 1993, 1994). In addition GABA-IR colocalizes in some of the SP-IR amacrine cells in another anuran retina, that of the toad, *Bufo marinus* (Main et al., 1993).

Also in larval tiger salamander retina one population of the SP-IR amacrine cells expresses glycine immunoreactivity and glycine high-affinity uptake (Watt and Florack, 1993). In contrast, there is no evidence for colocalization of glycine and SP in goldfish retina (Yazulla and Studholme, 1990). Mammalian SP-IR amacrines have not been tested for colocalization with glycine. It is almost certain that SP-IR cells in mammals do not colocalize ACh (Vaney, 1990). The morphology of the ACh «starburst» amacrine cell and the SP-IR amacrine cells is entirely different although the type b «starburst» cells do stratify in the same IPL stratum and thus they could very well talk to each other synaptically.

Physiological role of SP-IR neurons in the turtle retina

Since the SP-IR amacrine cells in the turtle are either monostratified in S3 of the IPL at the border of sublaminas a and b (sublamina a is thought to be the portion of the IPL neuropil where OFF-center ganglion cells are driven and sublamina b, where ON-center ganglion cells are driven (Nelson et al., 1978); we know that the ON/OFF border is in S3 in the turtle retina (Ammermüller and Kolb, 1995)) or tristratified with dendrites in both ON and OFF strata, one would predict that they would be «ON-OFF» responding neurons. Ammermüller and Kolb (1995) have recently intracellularly recorded and morphologically stained most of the known neurons of the turtle retina. Amongst the numerous physiological types, the three cells that are SP-IR in the turtle retina can be recognized. Thus, for the first time now, we have some information on the physiological response characteristics of neurons that may use SP as a neurotransmitter and test the prediction of response type.

The small-field, monostratified Type B SP-IR amacrine cell (see Fig. 1) corresponds in morphology and stratification level to an amacrine cell given the name A30 (Ammermüller and Kolb, 1995). Figure 10 illustrates the intracellular lucifer stain of an A30, whose physiological responses to light spots and annuli of various wavelengths and intensities are shown in the neighboring panel. A30's response consisted of a large depolarizing transient at light ON and another smaller transient at light OFF for all stimulus parameters used (Fig. 10, A30, A, B and C). This is a typical ON-OFF neuron of the retina. There was no antagonistic surround, as stimulation with an annulus (A, lowest trace) did not show any sign reversal of the response. Responses to all the three colored stimuli were equal and thus the A30 was not color-coded in any way (Fig. 10, A30, B). The dynamic range was quite flat, since the response amplitude increased steadily over the whole range of intensities used (5 log units) (Fig. 10, A30, C).

The A31 of figure 10, is considered to be the SP-IR Type A amacrine cell of turtle (compare Fig. 1, Type A and A31 in Fig. 10). The A31 is labeled as having wide-ranging dendrites in S1 and S5 but this was a far peripheral cell with a very large dendritic field size (1 mm diameter) and the dendrites started in S1 from the cell body position and descended to S5 to run for 600 µm. The dendrites branched sparsely and bore a few appendages. Some of the dendrites then migrated back up to S1 to run therein for several more 100 µm (Fig. 19a). The whole dendritic tree covered a 700 µm diameter field (Fig. 11) and many of the dendrites occupied S3 of the IPL, for much of the time. The beaded and appendage-bearing aspect of the terminal dendrites in S5 and the manner of passing up and down between S1 and S5 are particularly characteristic of the SP-IR Type A cells (Cuenca and Kolb, 1989) and convince us that this is the same cell type. Like SP-IR A30, A31 was an ON-OFF cell (Fig. 10, A31, A). However, unlike A30, an increasing spot size drastically reduced the amplitudes of both the ON and the OFF transient, indicating an inhibitory surround although no clear response sign reversal could be obtained with the annulus (Fig. 10, A31, A lowest trace). The responses to red and blue were sustained ON while green elicited a pronounced OFF transient (Fig. 10, A31, B). This may suggest some degree of color sensitivity. In the intensity series, low intensities produced sustained responses, while OFF transients appeared at higher intensities (Fig. 10, A31, C). Fast transients were superimposed on the slow potentials in all the responses. Thus A31, SP-IR Type A cell, seems to be a more complicated ON-OFF amacrine cell type than A30. Its physiology reflects the wide-field nature of the cell and its responses suggest dendritic spiking mechanisms are at play. A31 probably has a role in transferring activity across long distances in the retina and possibly controlling ganglion cell activity, including SP-IR ganglion cells, right at the latters cell body, by means of the encircling synaptic boutons.

The SP-IR ganglion that is the G18 type has also been intracellularly recorded (Ammermüller et al., 1995). An example of a G18 is shown in Fig. 11. As can be seen it has a remarkable similarity to the injected cell that had an SP-IR cell body in Figs. 7, 8. The intracellularly recorded G18 had a dendritic tree measuring 600 µm across that stratified narrowly in S1 (Fig. 11). The cell gave OFF-center type responses to light spots but use of an annulus revealed an

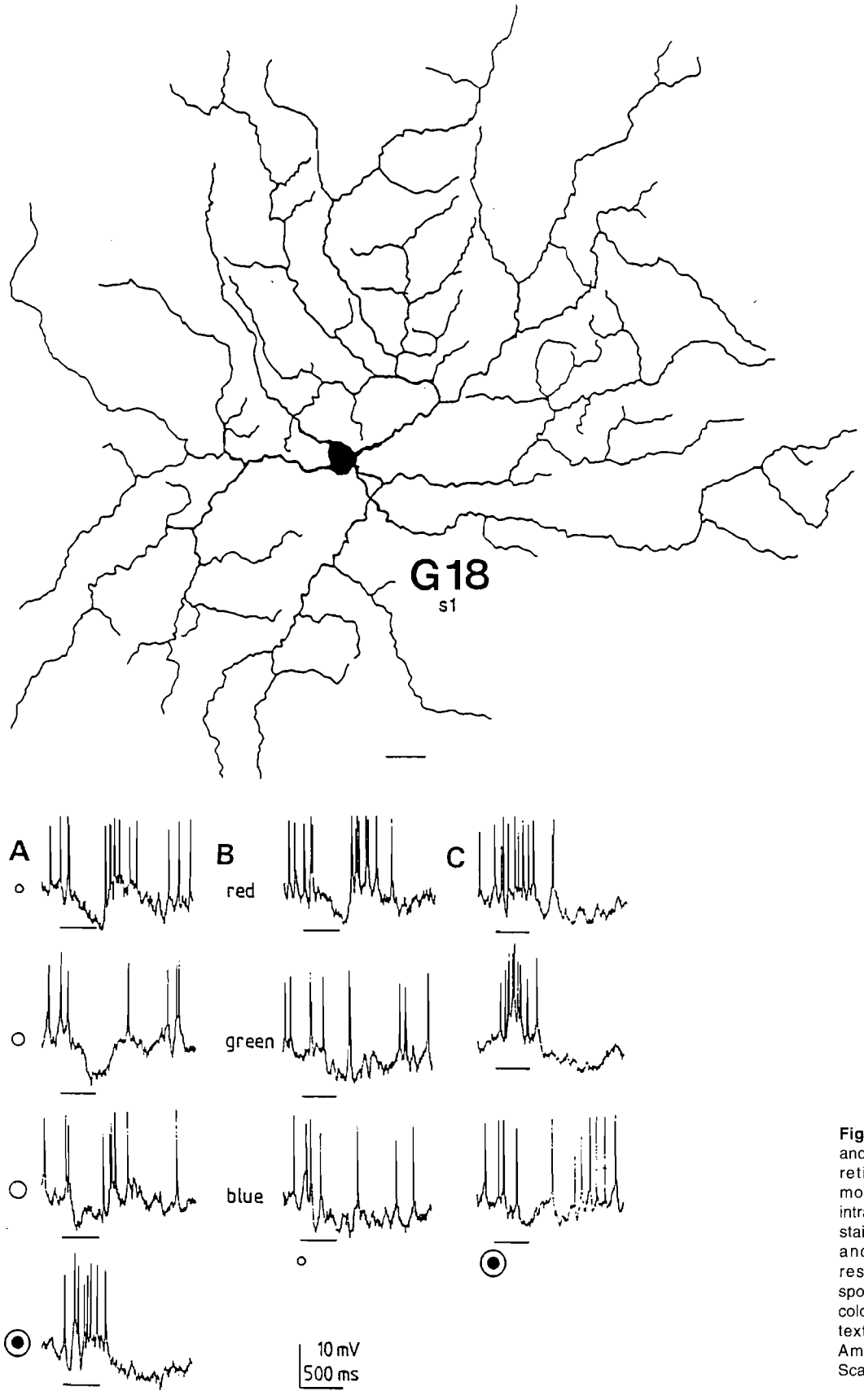

Fig. 11. Intracellularly stained and recorded G18 in the turtle retina. G18 has the same morphology as the SP-IR, intracellularly- and neurobiotin-stained, SP-IR cell of Figs. 7b and 8b. The physiological response to **A)** white light spots, **B)** colored spots and **C)** colored annuli are shown. See text from more details. From Ammermüller et al. (1995). Scale bar= 25 µm.

antagonistic, sustained depolarizing surround (Fig. 11, A, lowest trace). Red, green and blue small spots were all hyperpolarizing, non color-coded (Fig. 11, B). However, red and green annuli stimulating the surround (Fig. 11, C) produced sustained depolarizations, while blue annuli produced sustained hyperpolarizing (OFF) responses (Fig. 8C). This suggests that G18, one of the putative SP-IR ganglion cells, is driven in the center of its receptive field by luminosity (non color-opponent) bipolar cells and in its surround by amacrine cells with color-opponent properties.

In other species as well as in the turtle, physiological studies demonstrate that the application of SP to the retina has a direct excitatory action upon most physiological varieties of ganglion cells (Dick and Miller, 1981; Glickman et al., 1982; Downing and Djamgoz, 1983; Zalutsky and Miller, 1990). SP action is not restricted to only SP-containing ganglion cell varieties. For example, application of 1 μm substance P causes a slow and prolonged increase of background activity in various ganglion cells of the fish retina (where no SP-IR ganglion cell have yet been reported) and an enhancement of «ON» and «OFF» components of light-evoked responses (Glickman et al., 1982; Downing and Djamgoz, 1983). In the turtle retina, SP causes a general elevation of the excitable components of all ganglion cell types recorded and, in particular, increases spontaneous activity (Adolph, 1989).

Substance P has a strong excitatory effect on ganglion cells of mammalian retinas too. Such can be seen in Fig. 12b,c where SPs action on a brisk sustained and a transient ON-OFF directionally selective ganglion cell of the rabbit retina is documented (Zalutsky and Miller, 1990). In the ON-center brisk sustained ganglion cell, SP greatly increases the spontaneous activity but does not noticeably affect the light response. SPs effect on the ON-OFF directional cell is to dramatically excite the cell with a huge increase in spontaneous activity after the termination of the light response (Fig. 11c). According to Zalutsky and Miller's data, 15% of the rabbit ganglion cells are strongly excited and 60% show a moderate to large increase in spontaneous activity. These authors also found that SPs excitatory actions were limited to neurons of the inner retina, in the rabbit. Thus horizontal cells were not affected. They conclude that SP amacrine cells are playing a role in modulating the excitability of inner retinal neurons locally, and are probably not contributing to the actual receptive field organization of the ganglion cells (Zalutsky and Miller, 1990).

Whether the action of SP is a direct one via release of transmitter from SP-IR amacrine processes upon ganglion cell dendrites and cell bodies, or whether there is a feedback loop through bipolar cells thereby affecting ganglion cells secondarily, is not completely clear. Electron microscopy indicates that bipolar cells receive extensive synaptic feedback synapses from SP-containing amacrine cells in both goldfish and human retina (Yazulla et al., 1985; Cuenca et al., 1994). Furthermore, physiological evidence indicates that

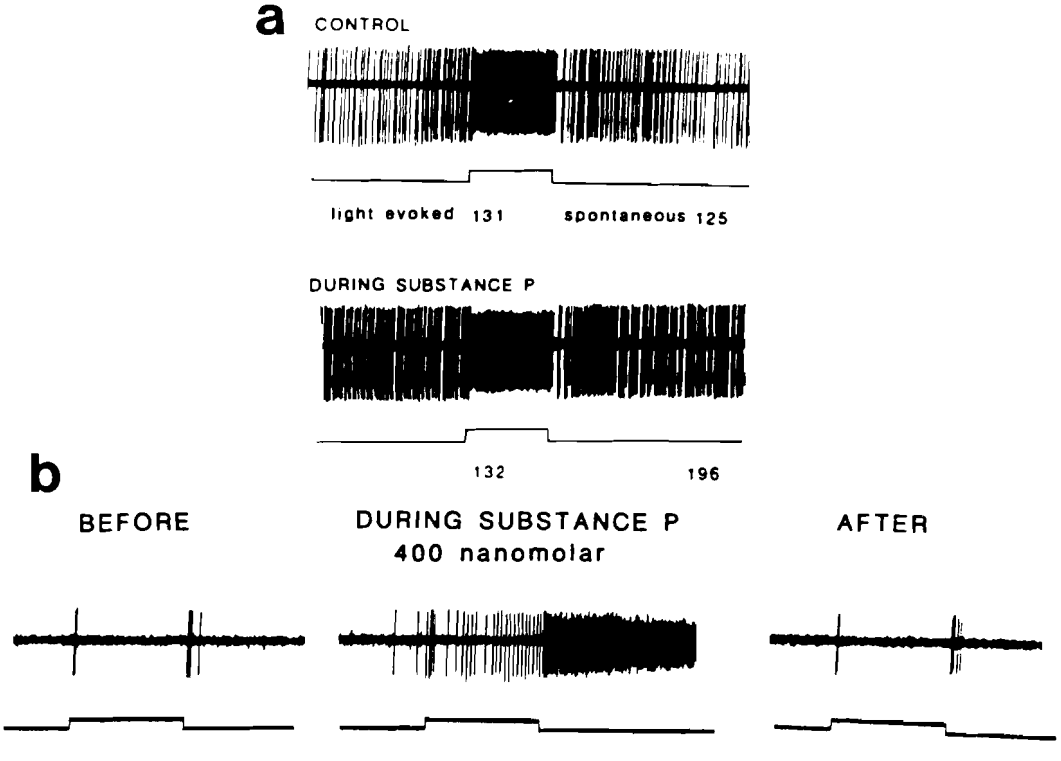

Fig. 12. Extracellular recordings of **a.** a brisk sustained ON-center ganglion cell and **b.** An ON-OFF directionally selective ganglion cell in the rabbit retina before and during applications of SP in a perfusate over the retina. The major effect of SP is to increase the spontaneous activity of both cells. Their receptive field characteristics are unaltered. See text for further details. From Zalutsky and Miller (1990).

bipolar cells in goldfish retina are affected by SP to produce voltage-dependent inhibition of calcium currents (Ayoub and Matthews, 1992) so a secondary bipolar route for SP effects on ganglion cells is feasible. However, selective blocking of SP effects on rabbit ganglion cells by using SP analogs and receptor blockers has also been demonstrated (Zalutsky and Miller, 1990), thus indicating a direct synaptic route as well, at least for some varieties of ganglion cells. Thus, taking all the evidence concerning SPs occurrence in specific neurons with specific neurocircuitry together with the recent knowledge on SPs physiological action, leads to a conclusion that SP is an important excitatory neurotransmitter in the vertebrate retina that deserves considerably more attention in future studies.

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