Carol L. Shurlow. MORPHOLOGICAL EVIDENCE FOR A SUBSTANCE P PROJECTION FROM MEDIAL SEPTUM TO HIPPOCAMPUS. (Under the direction of Gary M. Peterson, Ph.D.) Department of Biology, May, 1991.

The medial septal nucleus (MS) provides one of the major afferents to the hippocampal formation and the hippocampus projects back to the lateral septum. The two major types of neurons present in the MS are cholinergic and GABAergic, but other types of neurons are also present. A small population of substance P (SP) containing neurons are present along the border between the medial and lateral septum but it is unclear whether these project to the hippocampus. The present study, by employing both anterograde and retrograde tracing techniques, combined with immunocytochemistry for SP, provides direct morphological evidence for a SP projection from MS to the hippocampus.

The anterogradely transported lectin PHA-L was iontophoretically injected into the vicinity of the SP-containing cells in the MS. Using the indirect fluorescent immunocytochemical method and two different fluorochromes, PHA-L and SP were co-localized within fibers in the CA2 region of the hippocampus but only in the mid-septo-temporal portion. To provide confirmation of this projection, the retrogradely transported marker WGA/HRP was injected into the suspected region of termination of MS SP fibers in the hippocampus. Using two different colored chromagens, SP and WGA-HRP were co-localized in cell bodies in the lateral portion of the MS. Together, the evidence from anterograde and retrograde tracings demonstrate that the SP containing cells in the lateral region of MS project to a region of CA2 which is restricted to the mid septo-temporal portion of the hippocampus.

MORPHOLOGICAL EVIDENCE
FOR A SUBSTANCE P PROJECTION
FROM MEDIAL SEPTUM TO HIPPOCAMPUS

A Thesis

Presented to

the Faculty of the Department of Biology

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In Partial Fulfillment

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Master in Biology

by

Carol L. Shurlow

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MORPHOLOGICAL EVIDENCE

FOR A SUBSTANCE P PROJECTION

FROM MEDIAL SEPTUM TO HIPPOCAMPUS

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Carol L. Shurlow
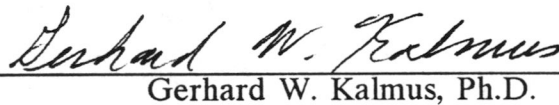
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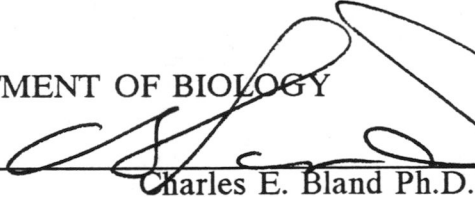Charles A. Singhas Ph.D.

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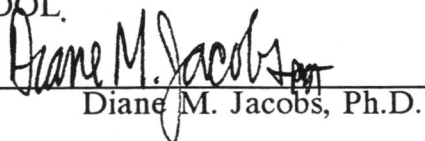Gerhard W. Kalmus, Ph.D.

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ACKNOWLEDGMENT

LIST OF FIGURES

LIST OF ABBREVIATIONS

apoHRP-gold   apohorseradish peroxidase conjugated to colloidal gold particles

DAB   diaminobenzidine

FITC   fluorescein isothiocyanate

GABA   gamma amino butyric acid

KPBS   potassium phosphate buffered saline

MS   medial septal nucleus

MSDB   medial septum and diagonal band of Broca

PBS   phosphate buffered saline

PHA-L   *Phaseolus vulgaris* leucoagglutinin

SP   substance P

SP-ir   substance P immunoreactivity

TBS   tris buffered saline

TMB   tetramethyl benzidine

TR   Texas Red

WGA/HRP   wheat germ agglutinin conjugated to horse radish peroxidase

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INTRODUCTION

The connections between the septal nuclei and the hippocampus provide a major two-way pathway between these two forebrain structures. The medial septum (MS) and the associated vertical limb of the diagonal band of Broca (together referred to as MSDB) provide afferents to the hippocampus. The lateral septum is efferent to the hippocampus and projects, in turn, to the medial septum (Swanson and Cowan, 1979). These pathways have been traced by a number of methods beginning in the 1950s and 1960s with degeneration techniques (Daitz and Powell, 1954; McLardy, 1955; Powell, 1963; Raisman, 1966). In the 1970s the connections of the septum were studied in greater detail using the more sensitive autoradiographic tracing methods (Lynch et al., 1978; Meibach and Siegel, 1977; Rose et al., 1976; Swanson and Cowan, 1979). The septohippocampal projection has been studied in the 1980s with even more sensitive methods employing anterograde transport of horseradish peroxidase (HRP; Crutcher et al., 1981) and more recently with anterograde transport of the lectin *Phaseolus vulgaris* leucoagglutinin (PHA-L; Nyakas et al., 1987).

In the rat brain the MSDB projects to the hippocampus via three routes: (1) a dorsal pathway over the corpus callosum, through the cingulum bundle, (2) through the fimbria-fornix, and (3) a ventral route through the amygdalar region (Gage et al., 1983; Milner and Amaral, 1984). Nyakas and co-workers (1987) demonstrated a topographical projection of the MSDB in which the medial regions project to the septal (or dorsal) regions of the hippocampus and the lateral extent of the MSDB projects to more temporal regions. A recent study by Peterson (1987), which utilized the retrograde transport of fluorescent dyes, demonstrated a small contralateral projection of MSDB neurons to the hippocampus in addition to the

well-studied ipsilateral projection. Within the hippocampus the MSDB projects primarily to strata oriens and lacunosum-moleculare of regio superior (CA1; Nyakas et al., 1987) and to strata oriens and radiatum of regio inferior (CA2/3; Crutcher et al., 1981). All regions within the dentate gyrus receive projections from the MSDB (Crutcher et al., 1981; Nyakas et al., 1987; Swanson and Cowan, 1979).

Several neurotransmitters and peptides have been demonstrated in neurons of the septohippocampal system. Choline acetyltransferase (ChAT), the enzyme which synthesizes acetylcholine (ACh), has been localized in approximately half of the septohippocampal projection neurons (Amaral and Kurz, 1985; Baisden et al., 1984; Wainer et al., 1985; Woolf et al., 1986). The other major population of septohippocampal neurons contains glutamic acid decarboxylase (GAD; Köhler et al., 1984), the enzyme which synthesizes the inhibitory neurotransmitter gamma amino butyric acid (GABA). Several peptides and hormones have also been biochemically localized in the neurons or fibers of the medial septum and/or hippocampus. These include SP, luteinizing hormone-releasing hormone, prolactin, cholecystokinin, enkephalin, neurotensin, bradykinin, delta-sleep inducing peptide, somatostatin, vasoactive intestinal polypeptide, thyrotropin releasing hormone, pancreatic polypeptide, endorphin, vasopressin, oxytocin, neuropeptide Y, and corticotropin releasing hormone (see reviews by Palkovits, 1984; Roberts et al., 1984; Lamour and Epelbaum, 1988). The role of these peptides in the neurons of the MSDB is not entirely known. Some of them, such as SP appear to act as neurotransmitters (see review by Nicoll et al., 1980) and/or neuromodulators (Payan and Goetzl, 1987; Malthe-Sørenssen et al., 1978).

Substance P was the first neuroactive peptide discovered in the brain. Von Euler and Gaddum (1931) isolated this substance from equine intestine and found

that it stimulated smooth muscle cells. Later, Chang and Leeman (1970) isolated this same substance from bovine hypothalamus and showed that it stimulated the salivary gland. Then in 1971, Chang and co-workers established the sequence for this peptide.

Substance P is comprised of 11 amino acids (Fig. 1) and belongs to a family of peptides called tachykinins. Mammalian as well as nonmammalian tachykinins have been observed. The tachykinins that have been extracted from nonmammalian tissue are: kassinin, physalaemin, and eledoisin. The four mammalian tachykinins that have been described thus far are SP, neuropeptide K, neurokinin A, and neuropeptide gamma (see review by Krause et al., 1989).

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$

<u>Fig 1</u>. Amino acid composition of SP.

The SP peptide can be synthesized from three different preprotachykinin (PPT) mRNAs: α-PPT, β-PPT, and gamma-PPT. These mRNAs have been found to be coded on the same gene (see reviews by Nakanishi, 1987; Krause et al., 1989). There are also several SP receptor subtypes: NK-1, NK-2, and NK-3 which bind variably to the different mammalian tachykinins (see reviews by Nakanishi, 1987; Krause et al., 1989).

Substance P neurons and fibers can be observed in the mucosa and submucosa along the length of the digestive tract (Barthó and Holzer, 1985). The function of SP in the GI tract is to excite the smooth muscle contraction of peristalsis (Barthó and Holzer, 1985). SP can also cause smooth muscle contraction of blood vessels in the circulatory system as well as bronchoconstriction in the respiratory tract (see review by Payan and Goetzl, 1987).

Substance P has several effects on the immune system. It can act as a chemotactic agent for mononuclear and polymorphonuclear leukocytes, it can cause a release of histamine from connective tissue mast cells in the rat or regulate tissue repair by increasing proliferation of connective tissue cells and it can stimulate the proliferation of T lymphocytes (see review by Payan and Goetzl, 1987).

In the central nervous system SP has been found to have several different functions, and increased or decreased levels have been observed in certain diseases. Jessel (1981) showed that SP is released from the spinal cord into the spinal fluid after electrical stimulation of peripheral nerves. SP administered to the medulla can enhance respiration (Yamamoto et al., 1981) and may also have a role in respiratory control in Sudden Infant Death Syndrome because of increased levels of SP in the hypothalamus and medulla (see review by Lembeck, 1988). In Huntington's disease, a decrease in staining in SP fibers has been observed in the substantia nigra and globus pallidus (Grafe et al., 1895). Furthermore, a 48% to 92% decrease of SP levels in several areas of the basal ganglia was found (Buck et al. 1981). SP has been found to be depleted in axons of the spinal cord in postmortem tissue from patients with familial dysautonomia (Pearson et al., 1982). Levels in the hippocampus have been found to be increased in some schizophrenics (Roberts et al., 1983; Takeuchi et al., 1988). Furthermore, a reduction in the concentration of

SP in the hippocampus and cortex in the brains of Alzheimer's patients has been observed (Coyle, 1987). SP fibers have also been observed in some senile plaques in the hippocampus and amygdala of patients with Alzheimer's disease (Armstrong et al., 1989).

Substance P has been found to have several behavioral effects when injected into the amygdala, substantia nigra, lateral hypothalamus, and medial septum (Huston and Stäubli, 1981). Post-trial injections of SP into the amygdala or the substantia nigra in rats resulted in amnesia, whereas, injections into the hypothalamus facilitated memory (Huston and Stäubli, 1981). Post-trial injections into the medial or lateral septum also increased passive avoidance learning in rats (Stäubli and Huston, 1980).

Substance P has been identified in many areas of the central nervous system of the rat and several SP pathways have been described (see reviews by Shepherd, 1988; Huston and Stäubli, 1981). Substance P-immunoreactive (SP-ir) cell bodies have been described in the medial aspect of the lateral septum (Gall and Moore, 1984). However, the position of these cells is actually on the border between medial and lateral septum and other authors have described them as being in the medial septum (Ljungdahl et al., 1978; Senut et al., 1989). This is an important distinction because if the cells are in the medial septum they would be expected to send fibers to the hippocampus, whereas if they are in the lateral septum they would receive from the hippocampus and possibly project to the medial septum.

Substance P immunoreactive fibers have been described in the hippocampus (Ljungdahl et al., 1978; Iritani et al., 1989), especially in the CA2/3 regions (Roberts et al., 1984), within the strata oriens, pyramidale, radiatum and lacunosum-moleculare (Davies and Köhler, 1985; Shults et al., 1987). The origin of the SP

projection to the hippocampus is poorly understood (Davies and Köhler, 1985; Lamour and Epelbaum, 1988; Roberts et al., 1984). A substance P projection from the supramammillary nuclei to the hippocampus has been described in the guinea pig (Gall and Selawski, 1984), cat (Ino et al., 1988), and monkey (Veazey et al., 1982), but has not yet been described in the rat (Haglund et al., 1984). Yanagihara and Niimi (1989) have demonstrated a substance P projection to the hippocampus from the posterior hypothalamus in the cat. Regarding the possibility that the SP neurons in the septum project to hippocampus, Vincent and McGeer (1981) demonstrated a 40% decrease in hippocampal SP levels one week after electrolytic lesions of the septal area. They concluded that a SP innervation to the hippocampus originates in the septum. In support of this, a recent study of retrograde degeneration by Peterson et al. (1987) reported a loss of SP cell bodies in the MS following fimbria-fornix transection. While these observations suggest that the SP cells in the MS project to the hippocampus, the observed reduction in SP levels could be the result of damage to non-septal fibers passing through the lesion site and the degeneration could result from damage to axons which do not project to the hippocampus.

The present study was designed to provide direct morphological evidence for a SP projection from MS to hippocampus. Both anterograde and retrograde tracing techniques were used in conjunction with immunocytochemical methods for the detection of SP. As a first approach, the anterograde tracer PHA-L was used to label cells in the medial septum, since the position of the SP-ir perikarya was known. This was followed by an immunocytochemical procedure designed to detect SP as well as the PHA-L labeled fibers. Once the terminal field of these fibers in the hippocampus was determined, the projection was confirmed by retrograde tracing.

Wheat germ agglutinin conjugated to horse radish peroxidase (WGA/HRP) was injected into the putative terminal region. Several days later, brains were sectioned and processed for WGA/HRP and stained immunocytochemically for SP. Co-localization of the anterograde or retrograde tracer with immunoreactivity for SP in cell bodies or fibers was taken as evidence for a projection of SP-containing medial septal cells to the hippocampus.

PHA-L was chosen as the anterograde tracer because of its many advantages over other tracing methods: it is not transported by fibers in or around the area of the injection site that do not originate there (fibers of passage), it labels neuronal perikarya at the site of injection, the axons are labeled throughout their extent, terminal fields are labeled, and the technique can be combined with immuno-cytochemical methods (Gerfen and Sawchenko, 1984; see also Luiten et al., 1988 for review). PHA-L is a plant lectin obtained from the red kidney bean. It binds to the carbohydrate components galactose and mannose (Luiten et al., 1988). It is thought that these carbohydrate components may only be present on membranes of cell bodies. When PHA-L binds to the carbohydrates on the cell membrane it is thought that the PHA-L is then internalized by endocytosis and transported in the anterograde direction (Luiten et al., 1988). The lectin is transported throughout the neuron and can then be stained by immunocytochemical techniques which will label the soma, dendrites, axons and terminals.

For retrograde tracing, WGA/HRP was chosen as the labeling substance. WGA is a lectin that has been isolated from *Triticum Vulgaris* which binds to the carbohydrates N-acetyl-D-glucosamine and N-acetylneuraminic acid present on neuronal membranes (Mesulam, 1982). HRP can be conjugated to WGA. The conjugated HRP (WGA/HRP) is more effective as a tracer than HRP alone for

several reasons. First, WGA/HRP is 20 to 30 times more effective in tracing than an equal amount of HRP and second, more than one molecule of HRP can be bound to one WGA, thus increasing its ability for detection (Mesulam, 1982). Once the WGA/HRP is bound to the neuronal cell membrane it is endocytosed and can travel in the retrograde, as well as anterograde direction (Mesulam, 1982; Lechan et al., 1981; Schwab et al., 1978; Crutcher et al., 1981; Condé and Condé, 1979). WGA/HRP was injected into the hippocampus where the PHA-L / SP studies had indicated the septohippocampal SP fibers to be located. Following transport of the WGA/HRP to the cell bodies, the SP and/or WGA/HRP cells in the septum could be visualized using double labeling techniques similar to those of Lechan and co-workers, (1981), and Amaral and Kurz (1985).

MATERIALS AND METHODS

SURGERY

Twenty Sprague-Dawley rats (Charles River), nine female and eleven male (200 to 400 gm), were used for combined substance P immunohistochemistry and tract tracing studies. Five additional female rats were used for the WGA/HRP tracing studies. Prior to surgery, rats were anesthetized by a cocktail of Ketamine, xylazine and acepromazine (25, 1.3, 0.25 mg/ml saline, respectively) injected i.m. at a dosage of 0.25 ml/kg. Additional anesthesia was injected during the surgical procedure if needed. The animals were then placed in a David Kopf stereotaxic frame in preparation for injections of the tracer or colchicine, as described below. Stereotaxic surgery was performed under aseptic conditions. Following surgery, animals were monitored and cared for by the animal care technicians in the Department of Comparative Medicine at the East Carolina University School of Medicine.

1. PHA-L Injections

The method used was similar to that described by Gerfen and Sawchenko (1984). Glass pipets with a tip diameter of 10 to 16 μm were pulled and then vacuum filled with 2.5% PHA-L (Vector Labs) in 0.05 M sodium phosphate buffer (pH 7.4) just prior to injection. The pipet was lowered into the medial septum through a hole in the skull. The location of the site was determined from the stereotaxic atlas of Paxinos and Watson (1986) to lie at a point 0.2 mm anterior to bregma, 0.4 mm lateral to midline, and 6.5 mm below the dura. The PHA-L was iontophoretically ejected using a Midgard CS 3 High Voltage Precision current source. A cathode wire of silver, silver plated with chloride, or platinum was

inserted into the PHA-L in the pipet. The current was set for either 3 or 5 μA. The current pulse ranged from 3 sec to 8 sec in a 50% duty cycle and was applied for 15 to 30 min. After completion of iontophoresis the pipet was left in place an additional 10 min before removal.

In an attempt to compare the efficacy of pressure injections with iontophoretic application and to test the various antisera necessary to detect PHA-L, two animals received pressure injections of PHA-L via a 1 μl Hamilton syringe. In one animal, 30 nl of PHA-L was injected over a period of three min. For the other pressure injection, a glass pipet (40 μm tip diameter) was glued to the end of the syringe needle. In this case 100 nl of PHA-L was injected over 35 min. The needle was again left in place for 10 min following the injection.

2. WGA/HRP Injections

WGA/HRP was injected bilaterally into the mid-septo-temporal portion of CA2, the region in which PHA-L / SP fibers had been observed. Coordinates for this site were 4.3 mm posterior to bregma, 5.4 mm lateral to midline, and 3.8 mm below the dura (Paxinos and Watson, 1986). Following the procedure of Amaral and Kurz (1985) and Mesulam (1982), 50 nl of a 2% aqueous solution of WGA/HRP (Sigma) was slowly injected over several minutes via a 1 μl Hamilton syringe. The syringe was left in place for an additional 5 min before removal to reduce leakage of the solution up the needle track.

3. Colchicine Injections

The immunocytochemical localization of SP cell bodies in the septum requires the intraventricular (ICV) injection of colchicine (Gall and Moore, 1984). Thus, bilateral ICV injections of colchicine were placed in animals previously injected with WGA/HRP. Twenty-four hours after WGA/HRP injections animals were

anesthetized and 5 μl of colchicine (10 mg/ml saline; Sigma) was injected into each lateral ventricle. The stereotaxic coordinates for these injections were 0.7 mm posterior to bregma, 1.3 mm lateral to midline, and 3.2 mm below the dura (Paxinos and Watson, 1986). The syringe was left in place several minutes before and after the injections.

TISSUE COLLECTION AND PROCESSING

Animals which received injections of PHA-L were allowed to survive for one week. They were then deeply anesthetized with Nembutal (60 mg/kg, i.p.) in preparation for transcardial perfusion. Upon exposure of the heart, 1 ml of sodium nitrite (1% in PBS) was injected into the left ventricle to induce vascular dilation. Next, approximately 50 ml of normal saline was perfused through the ascending aorta, followed by 300 ml of ice cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were then removed and post-fixed 2 to 24 hours before being transferred to 25% sucrose in 0.1 M sodium phosphate buffer (pH 7.4). To reduce freezing artifact, brains were stored in 25% sucrose in PBS at 4° C until equilibration occurred before they were sectioned on a sliding microtome. Brains were then frozen in dry ice and sectioned on an American Optical sliding microtome. Sections were cut in the coronal plane at 40 μm and every section saved in a 1-in-4 or 1-in-5 series. Sections through the MS and hippocampus were collected in 24-well tissue culture plates, in 0.1 M sodium phosphate buffer (pH 7.4). Sections that were not stained that day were saved in a cryoprotectant solution of sucrose, ethylene glycol and phosphate buffer (Watson et al., 1986) and stored at -20° C.

Animals given injections of WGA/HRP followed by colchicine were only allowed to survive 1 day longer before sacrifice. The transcardial perfusion was similar to that used in the animals injected with PHA-L. After the WGA/HRP animals had been injected with sodium nitrite, 200 ml of normal saline was perfused for 2 min through the ascending aorta and followed by 500 ml of ice cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for approximately 25 min, as described by Rye and co-workers (1984). The heads of the animals were packed in ice after the saline was perfused. An ice cold 5% aqueous solution of sucrose was then perfused for approximately 25 min to rinse out the paraformaldehyde and prevent over-fixation which is detrimental for the detection of HRP (Mesulam, 1982). Brains were then removed and placed in 25% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 2 to 3 days before being sectioned. Brains were frozen in dry ice and sectioned as described above. The sections were kept in the 24-well plates in 0.1 M sodium phosphate buffer (pH 7.4) at 4° C up to a week before being stained.

IMMUNOCYTOCHEMISTRY FOR PHA-L AND SUBSTANCE P

Antibodies to PHA-L and/or SP were used for the detection of immunoreactive cell bodies and fibers. Three sets of adjacent sections were stained immunocytochemically for PHA-L, SP, or both. The other sections were used for controls. Three labeling methods were used: one using a fluorescent label together with peroxidase, (see Lechango et al., 1979) and the other two using two different fluorescent labels as recommended by Wessendorf and Elde (1985). The fluorescent markers were fluorescein isothiocyanate (FITC) and Texas Red (TR). In most cases FITC was used with PHA-L and TR was used with SP, although occasionally these

were reversed. Several sets of sections from various brains were processed for only PHA-L to locate the injection site. Some were processed for only SP to locate immunoreactive fibers. Several controls were performed to demonstrate the absence of cross-reactivity of the antisera and the absence of fluorescent "bleed-through": (1) To detect any non-specific binding of the fluorescent dyes and/or the biotinylated antibody to the tissue itself, the antibody diluent was used in place of the primary antibody in the procedure for immunofluorescent labeling for PHA-L or SP; (2) For the combined technique, PHA-L and/or SP was omitted from the diluent to detect any cross-reactivity between antibodies in subsequent steps. For example, when SP is eliminated from the diluent, any binding between the FITC rabbit anti-goat and the biotinylated goat anti-rabbit IgG can be detected. The binding between these two antibodies is known to be 1% or less (personal communication, technical services, Vector Labs). TR was used instead of rhodamine because of the absence of overlap of excitation and emission spectra with fluorescein, because it fades more slowly than fluorescein and tetramethyl rhodamine, and because it can be conjugated with proteins (Titus et al., 1982). The procedure for indirect immunofluorescence was similar to that described by Sternberger (1979). The avidin-biotin protocol was modified from that of Hsu et al. (1981).

Immunohistochemical staining was done on free floating sections from the hippocampus and MS. The staining and rinsing procedures were done in 24-well tissue culture plates at room temperature with constant agitation with the exception of the primary antibody incubations which were done at 4° C with agitation in microcentrifuge tubes. The staining protocols are described below:

Method 1 (Fig. 2)

Sections were rinsed in 0.02 M potassium phosphate buffered saline (KPBS, pH 7.4) 3x10 min. Sections were solubilized for 30 min in 0.3% Triton X-100 diluted in KPBS and then rinsed again in KPBS 3x10 min. Three to four sections were incubated in microcentrifuge tubes containing an antibody cocktail consisting of: goat anti-PHA-L (1:1000, Vector) and rabbit anti-SP (1:4000, gift of J. Hong, NIEHS), together with 0.01% bovine serum albumen (BSA), diluted in KPBS. Sections were incubated for 2-3 days in primary antibody at 4° C and then rinsed in KPBS 3x10 min. Sections were next incubated for 45 min in FITC-labeled rabbit anti-goat IgG (1:50; Sigma) and rinsed before being incubated in biotinylated goat anti-rabbit IgG (1:200, Vector) diluted in KPBS for another 45 min. Sections were rinsed in KPBS 3x10 min and then incubated in avidin-conjugated TR (1:50, Vector) in 1.0 M Hepes buffer (pH 8.2) for 45 min. Sections were rinsed again in KPBS 2x10 min and then mounted onto gelatin coated slides from 0.1 M phosphate buffer and dried briefly to allow adhesion to the gelatin. Slides were then dipped back into the phosphate buffer and coverslipped with Aqua-Mount (Lerner Laboratories).
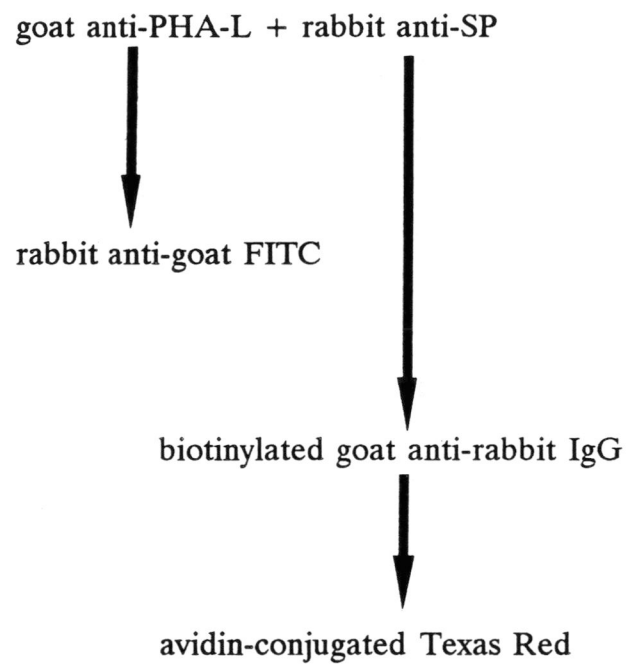
goat anti-PHA-L + rabbit anti-SP

rabbit anti-goat FITC

biotinylated goat anti-rabbit IgG

avidin-conjugated Texas Red

<u>Fig. 2</u>   Schematic illustration of the procedure for combined immuno-cytochemistry for PHA-L and SP (Method 1).

Method 2 (Fig. 3)

The procedure was similar to that of the combined immunofluorescence technique in Method 1 except that it combined immunofluorescence with immunoperoxidase (Lechango et al., 1979). Sections were rinsed, incubated in the primary antibody cocktail (anti-PHA-L + SP) for 2-3 days, and rinsed again. Next, they were incubated in biotinylated anti-rabbit IgG for 45 min followed by 3 more 10 min rinses. Then, an avidin-biotin-peroxidase complex (Vector Labs) was applied for 45 min, followed by three rinses, 10 min each, in 0.05 M Tris buffered saline (TBS, pH 7.6). Sections were then placed in a 0.05% solution of diaminobenzidine (DAB) containing 0.03% hydrogen peroxide until sections darkened. Sections were rinsed again in TBS 2x10 min and then in KPBS 3x10 min. Sections were then incubated in rabbit anti-goat FITC for 45 min. Finally, sections were rinsed, mounted onto gelatin coated slides, dried, and coverslipped with Aqua-Mount.

goat anti-PHA-L + rabbit anti-SP

biotinylated goat anti-rabbit IgG

avidin-biotin-peroxidase complex
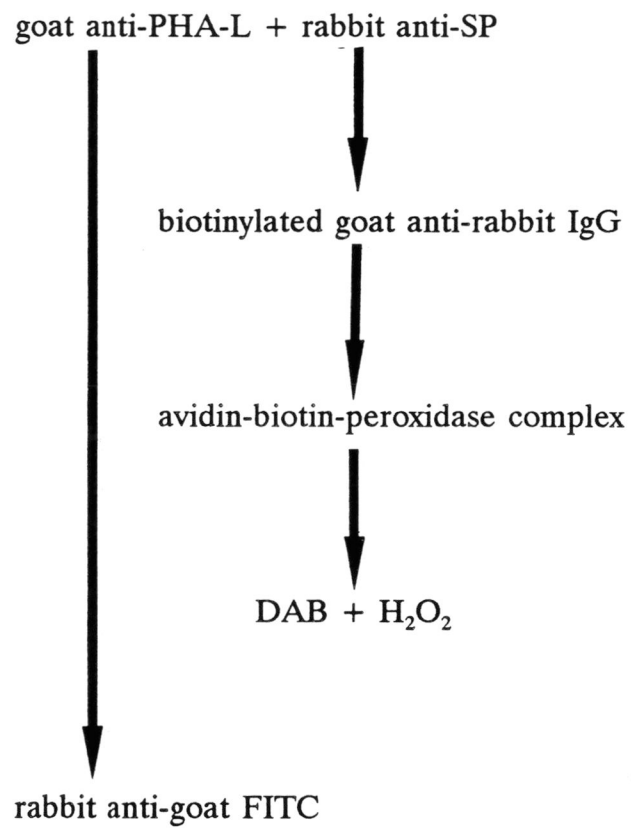
DAB + H_2O_2

rabbit anti-goat FITC

Fig. 3   Schematic illustration of the procedure for combined immuno-cytochemistry for PHA-L and SP (Method 2).

Method 3 (Fig. 4)

To reduce the chance of cross-reactivity between the two secondary antisera used in Method 1, antisera from two different species (goat and swine as opposed to goat and rabbit) were used. Furthermore, the time for solubilization in Triton X-100 was increased from 30 min to 24 h. Sections for this procedure were rinsed in 0.02 M KPBS (pH 7.4) 3x10 min, solubilized in 0.3% Triton X-100 in KPBS for 24 h (20° C) and then rinsed again in KPBS 3x10 min. Sections were incubated for 2-3 days at 4° C in an antibody cocktail consisting of biotinylated goat anti-PHA-L (1:100, Vector) plus rabbit anti-SP (1:4000, gift of J. Hong) together with 0.01% BSA diluted in KPBS. Sections were then rinsed 3x10 min in KPBS and incubated for 90 min in swine anti-rabbit FITC (1:40, DAKO) in 0.02 M KPBS. After another series of KPBS rinses, sections were incubated in avidin conjugated TR (1:50, Vector) in 1.0 M Hepes buffer (pH 8.2) for 90 min. Sections were rinsed again in KPBS 2x10 min, then mounted onto gelatin coated slides from 0.1 M phosphate buffer and dried briefly to allow adhesion to the gelatin. Slides were then dipped back into the phosphate buffer and coverslipped using Aqua-Mount.
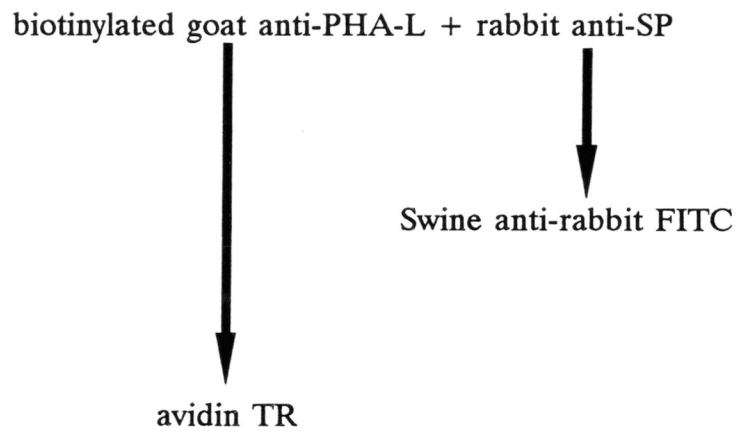
biotinylated goat anti-PHA-L + rabbit anti-SP

Swine anti-rabbit FITC

avidin TR

<u>Fig. 4</u>   Schematic illustration of the procedure for combined immuno-cytochemistry for PHA-L and SP (Method 3).

WGA/HRP PROCESSING

Sections from brains which had been injected with WGA/HRP and colchicine were stained by the tetramethyl benzidine (TMB) procedure of Mesulam (1982). TMB has been shown to be the most sensitive chromogen for the detection of HRP (Mesulam and Rosene, 1979) however, the TMB reaction itself is not very permanent and needs to be stabilized by further incubation in DAB plus cobalt chloride (Rye et al., 1984). This technique converts the blue TMB staining to a black granular pattern where WGA/HRP is present. These sections were prepared next for SP immunocytochemistry. To visualize SP cell bodies in the septum, sections were rinsed in PBS and incubated in 0.3% Triton X-100 in PBS for 5 min. This step was followed by incubation in 3% normal goat serum in PBS for 30 min. Sections were then rinsed in PBS 3x5 min followed by 2 to 3 days in rabbit anti-SP (1:4000, gift of J. Hong) in 0.3% Triton X-100 in PBS at 4° C. Sections were rinsed again 3x5 min and incubated in biotinylated anti-rabbit IgG in 0.3% Triton X-100 in PBS for 45 min. Three more 5 min rinses followed before sections were incubated in an avidin-biotin-peroxidase complex (Vector) in PBS for 45 min. Sections were rinsed again and placed in 0.05% DAB containing 0.03% hydrogen peroxide until the sections darkened. Sections were next rinsed in TBS, then in 0.1 M phosphate buffer. Sections were mounted onto gelatin-coated slides and air dried overnight before being dehydrated through a series of graded ethyl alcohols beginning with 70% and ending in 100%. Sections were cleared in xylene for several min and then coverslipped using DPX (Gallard-Schlessinger).

Controls consisted of omission of the TMB and stabilization steps or the omission of rabbit anti-SP from the diluent. With the exception of the above omissions the immunoperoxidase WGA/HRP and SP double labeling procedure (Fig. 5) was followed.
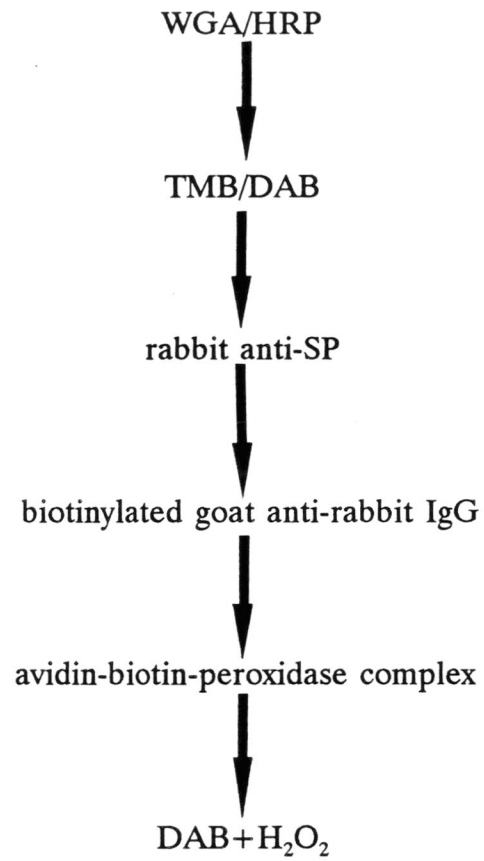
WGA/HRP

↓

TMB/DAB

↓

rabbit anti-SP

↓

biotinylated goat anti-rabbit IgG

↓

avidin-biotin-peroxidase complex

↓

$DAB + H_2O_2$

Fig. 5   Schematic illustration of the procedure for combined immuno-cytochemistry for WGA/HRP and SP.

PHOTOGRAPHY

Fluorescent labeled sections were viewed and photographed with a Nikon Optiphot photomicroscope. Sections were observed using a blue filter for FITC (excitation 450-490 nm, emission to 520 nm) and a custom-made yellow filter for TR fluorescence (excitation 560 nm, emission 595-635 nm). Sections stained using fluorescent labels were observed through both types of filter cubes whether sections were single or double labeled to insure the absence of cross reactivity and "bleed-through".

WGA/HRP stained sections were viewed and photographed with an Olympus Vanox photomicroscope.

RESULTS

INJECTIONS

Iontophoretic injections proved to be the better method for delivering the PHA-L. Injections using the Hamilton syringe caused tissue damage at the insertion site in the medial septum and along the needle track. Pressure injections of PHA-L also resulted in a less well defined area of labeled cell bodies. Iontophoretically applied PHA-L resulted in specific labeling of cell bodies and their processes in a very circumscribed area (Fig. 6). The labeled area was confined to a small circular area (200 to 320 μm in diameter) in the region on the border between medial and lateral septum.

PHA-L LABELED FIBERS

Method 1

PHA-L labeled fibers, stained by Method 1, could be observed in the fimbria and in regions of the hippocampus. The fibers observed in the fimbria were thick with few branches or swellings and they could also be followed for several hundred microns. The fibers in the hippocampus were found in CA2 and CA3 in the middle to temporal portions of the septo-temporal axis. There were more labeled fibers at the temporal pole than in the mid portion. The fibers in the hippocampus were thinner, had more branches, swellings, and shorter fiber projections than those in the fimbria. In the mid portion of the hippocampus the fibers were observed in strata oriens and radiatum. A few fibers were observed in stratum lucidum. Fibers observed in the more temporal pole were located primarily in strata oriens and radiatum although a few could be traced from stratum oriens to stratum radiatum through stratum pyramidale.
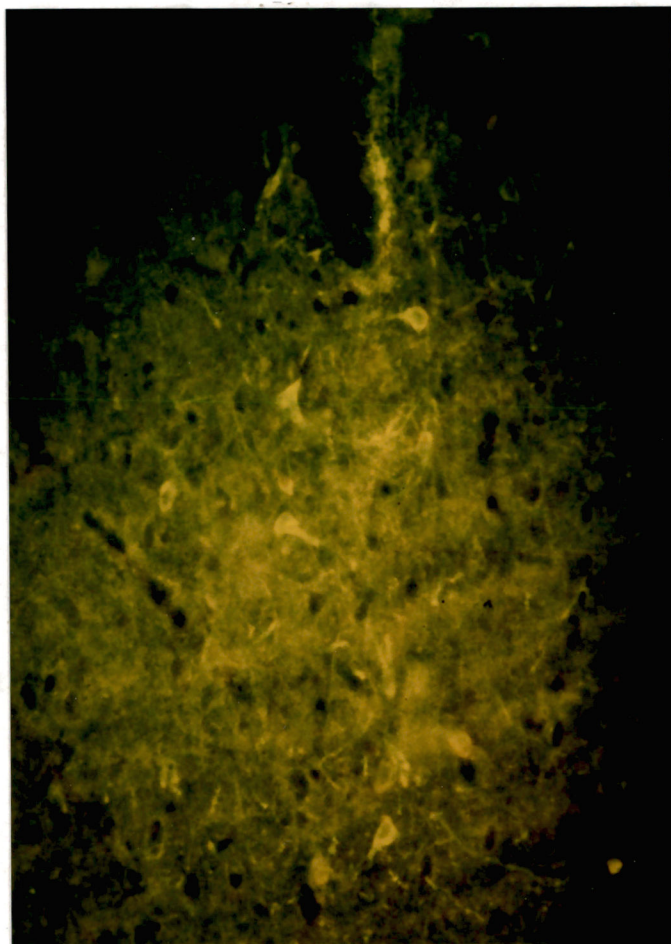
Fig. 6   PHA-L labeled cell bodies at the site of injection on the lateral border of the medial septum. FITC. 195 x.

Method 2

The PHA-L fibers observed with Method 2 were similar to those described for Method 1, however, when SP fibers were stained with the immunoperoxidase method this tended to obscure or block the immunofluorescence of PHA-L.

Method 3

Incubation of sections in Triton X-100 for 24 h prior to the immuno-cytochemical incubations resulted in an increased number of PHA-L labeled fibers in the hippocampus as compared to the 30 min incubations of Method 1. The appearance of labeled PHA-L fibers was similar to those described for Method 1 but they were present in a much larger area.

SP LABELED FIBERS

Method 1

Substance P-ir fibers labeled with TR were found in several areas of the hippocampus. Most of the fibers were short, thin, and branched. The fibers at the more septal pole were found in CA1, CA2, and CA3 as well as a few in the dentate gyrus. The few fibers in the dentate gyrus were either in the infra- or supra-pyramidal blade of the molecular layer. The labeled fibers in CA1, CA2, or CA3 were primarily found in strata oriens and radiatum. Most of the fibers coursed parallel to stratum pyramidale. A few fibers were observed in stratum pyramidale and traversed between stratum oriens and strata radiatum. In the middle to temporal regions of the hippocampus, fibers were observed in CA1, CA2, and CA3. Fibers in these three regions were restricted to strata oriens and radiatum. The majority of fibers in the middle to temporal regions coursed more perpendicular than parallel to strata pyramidale.

Method 2

SP fibers labeled with DAB that were observed with Method 2 were similar to those labeled with the TR as described in Method 1.

Method 3

The SP fibers in the hippocampus which had been labeled with FITC were similar in appearance and location to those labeled with TR as described for Method 1. However, there appeared to be considerable autofluorescence (fibers which appeared yellow-green instead of the apple-green which would be expected). This autofluorescence made it difficult at times to determine FITC-labeled SP fibers from autofluorescent fibers.

PHA-L / SP LABELED FIBERS

Fibers labeled for SP and PHA-L were only observed using Method 1. Those fibers which were immunoreactive for both SP and PHA-L were limited to the middle to temporal region of CA2 in the hippocampus. The double labeled fibers were also located only in strata oriens and radiatum. The few double labeled fibers that were observed were heavily branched. An example of the short branching double labeled fibers in stratum radiatum can be observed in Figure 7. Figure 8 summarizes the location of SP fibers in CA1, 2, and 3 at several levels throughout the hippocampal formation (blue fibers). In this same figure are those SP fibers (pink) which were also labeled with PHA-L in the CA2 region of strata oriens and radiatum.

The combined fluorescence and peroxidase technique (Method 2) resulted in FITC labeled cell bodies in the septum and SP fibers labeled with DAB in the

hippocampus. However, this method did not reveal PHA-L / FITC labeled fibers in the hippocampus.

Results from the third fluorescence method revealed PHA-L-immunoreactive cell bodies in the vicinity of the medial septum. The fibers in the hippocampus were labeled with PHA-L or SP but not for both. The PHA-L and SP fiber patterns were similar to those observed when only one of the antibodies was being detected, as described under results for PHA-L or SP labeled fibers.
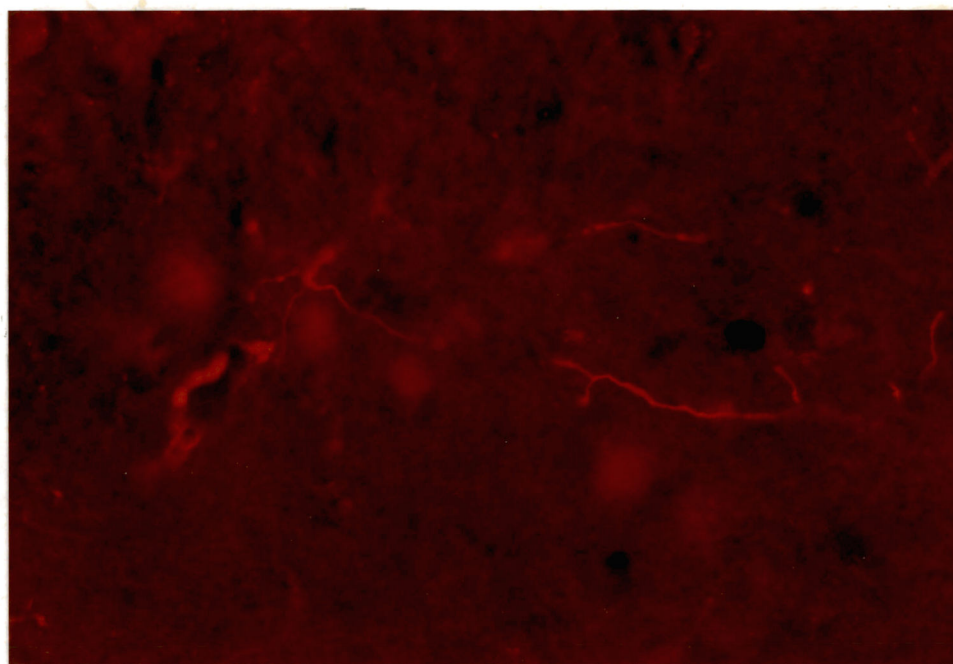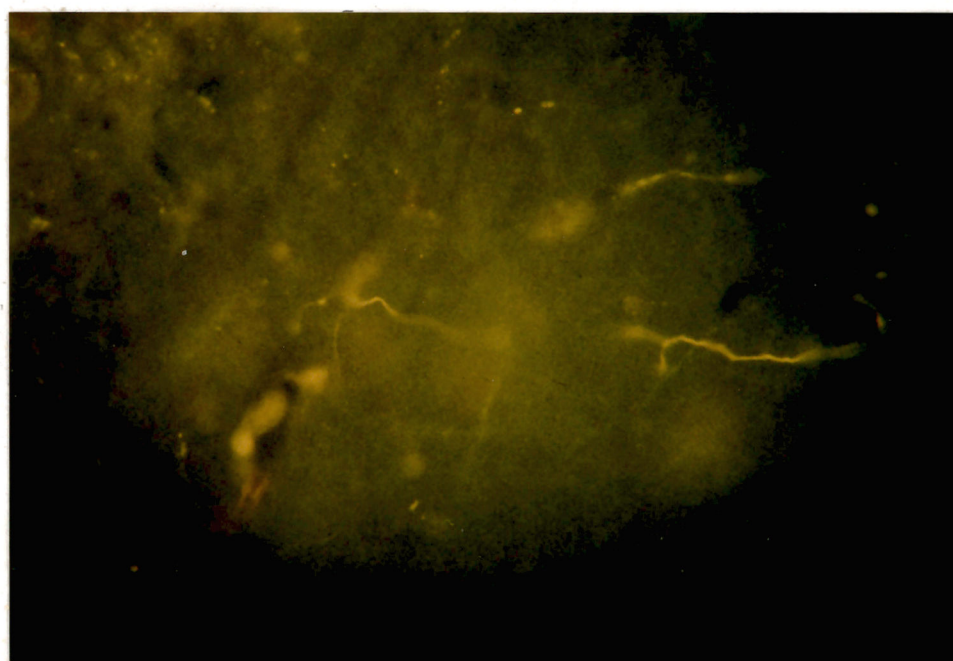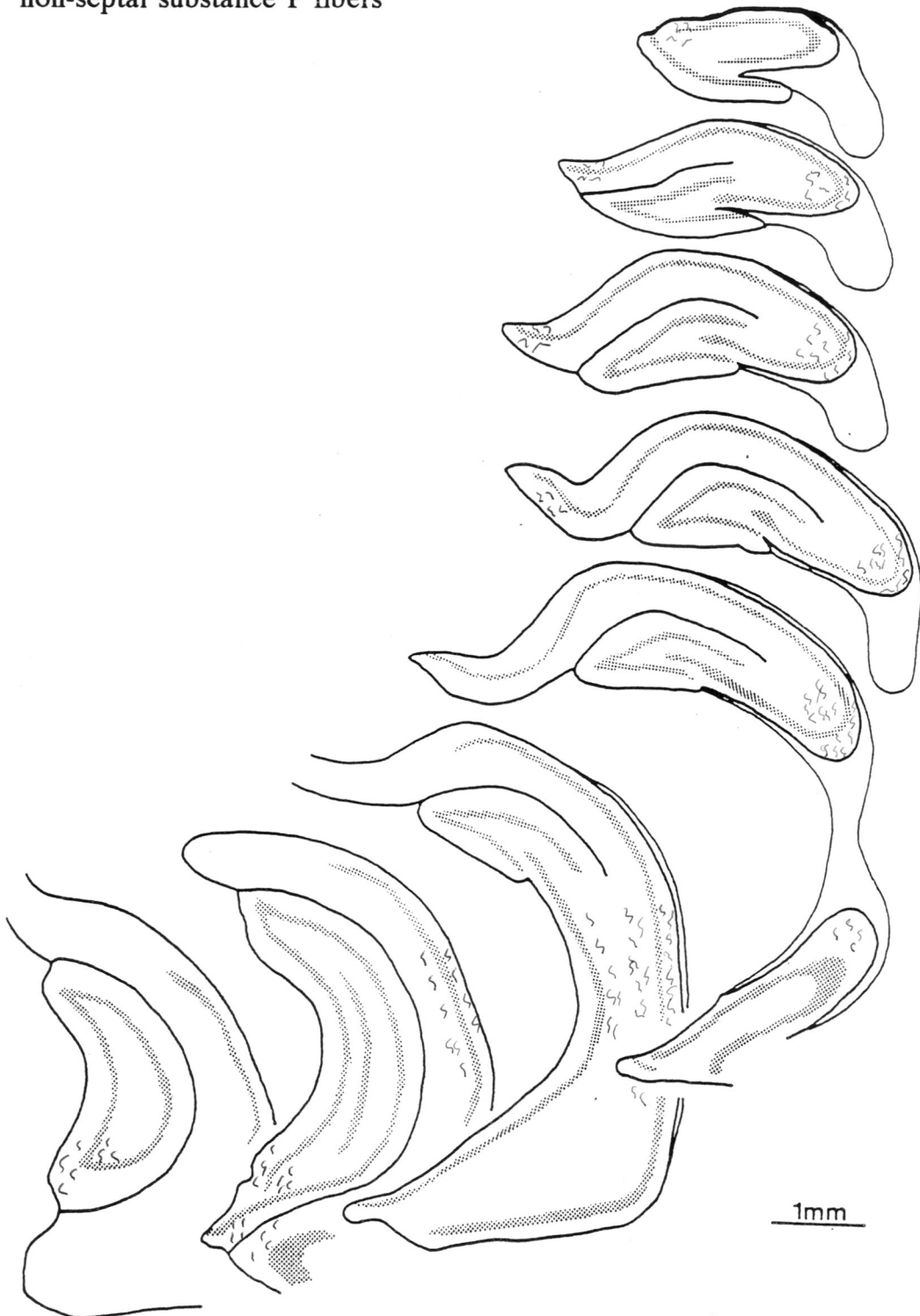
Fig. 7   Double labeled fibers in stratum radiatum of CA2. A Substance P-ir fibers as indicated by TR. B PHA-L labeled fibers as indicated by FITC. 390 x.

Fig. 8 **Substance P Innervation of the Rat Hippocampus.**

Substance P fibers from MS
non-septal substance P fibers

1mm

CONTROLS

FITC labeled fibers or cell bodies stained by Method 1 and observed under the TR filter showed no "bleed through" fluorescence (Fig. 9) nor did those fibers labeled with TR and observed under the FITC filter (Fig. 10). Sections in which the single labeled technique was used and anti-SP or anti-PHA-L was omitted showed no fluorescent fibers. Sections in which anti-PHA-L was omitted from the combined fluorescence technique showed only TR labeled SP fibers. Using the combined fluorescence technique, but omitting anti-SP, red and green fibers were observed within the thicker fibers as well as in some of the cell bodies, however, the thinner fibers did not show red fluorescence with the combined technique in which anti-SP was omitted.

Double labeled fibers and fluorescence "bleed-through" were not observed in Method 3 controls. Controls omitted either the biotinylated goat anti-PHA-L or the rabbit anti-SP antisera from the combined technique. The only problem with Method 3 was the observance of autofluorescent fibers. Autofluorescence is due to substances in fibers that fluoresce naturally without being labeled. The autofluorescent fibers appeared yellow-green when using the FITC filter and these fibers were hard to differentiate from the green SP FITC labeled fibers. This autofluorescence was not observed in fibers while observing sections through the TR filter.

WGA/HRP LABELED CELLS

Injections of WGA/HRP into the hippocampus resulted in a well defined ovoid area of labeling approximately 0.5 mm in diameter (Fig. 11). This region was limited to CA2/3 of the mid-septo-temporal portion of the hippocampus.
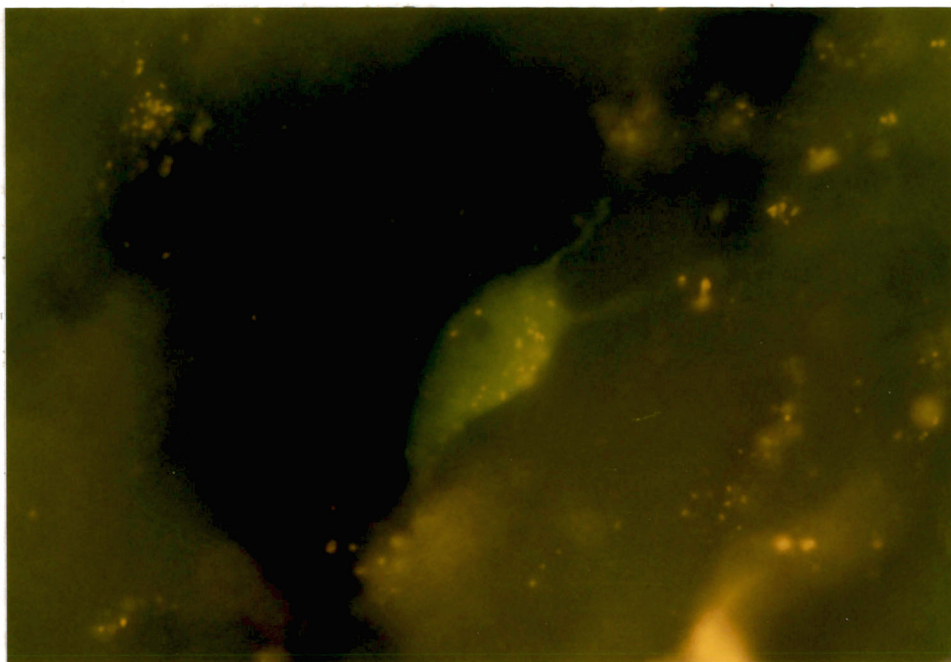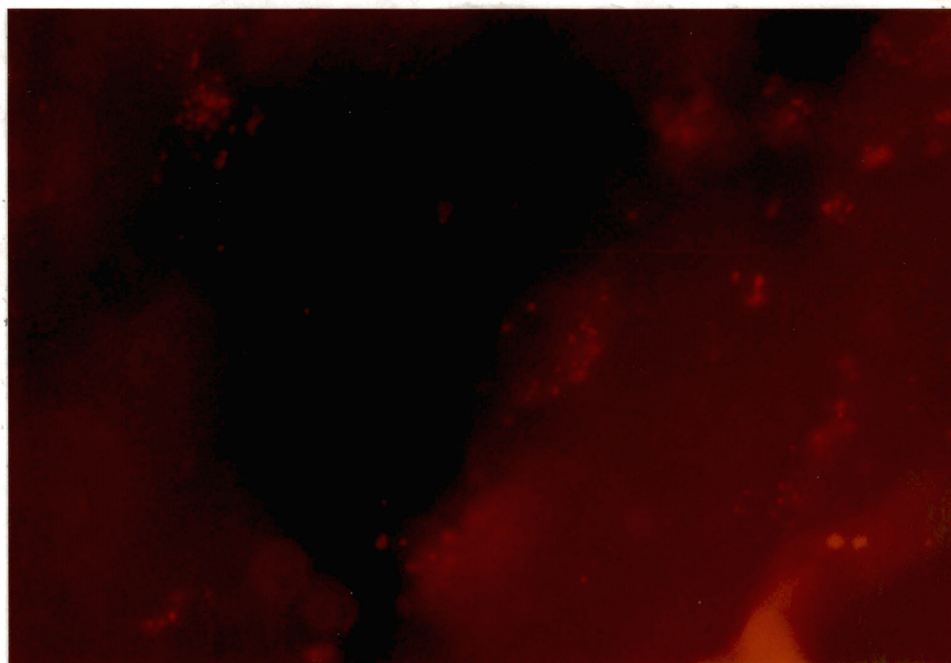
Fig. 9   Control section labeled for PHA-L only, showing labeled soma in septum
(**A**).   Note the absence of red fluorescence in **B** but the presence of
autofluorescent granules.   This demonstrates the specificity of the filters
and the lack of "bleed-through" of the FITC fluorescence into the TR
emission spectrum.   985 x.

Fig. 10 Control section labeled for substance P only, showing immunoreactive fibers in stratum radiatum of CA2 (**A**). The fibers are not present in **B**, indicating the absence of TR "bleed-through" into the FITC emission spectrum. 390 x.

Fig. 11   WGA/HRP injection site in CA2/3 region of the hippocampus.   20 x.

These injections resulted in the observations of WGA/HRP labeled cell bodies on the border between the medial and lateral septum. In some brains WGA/HRP cells w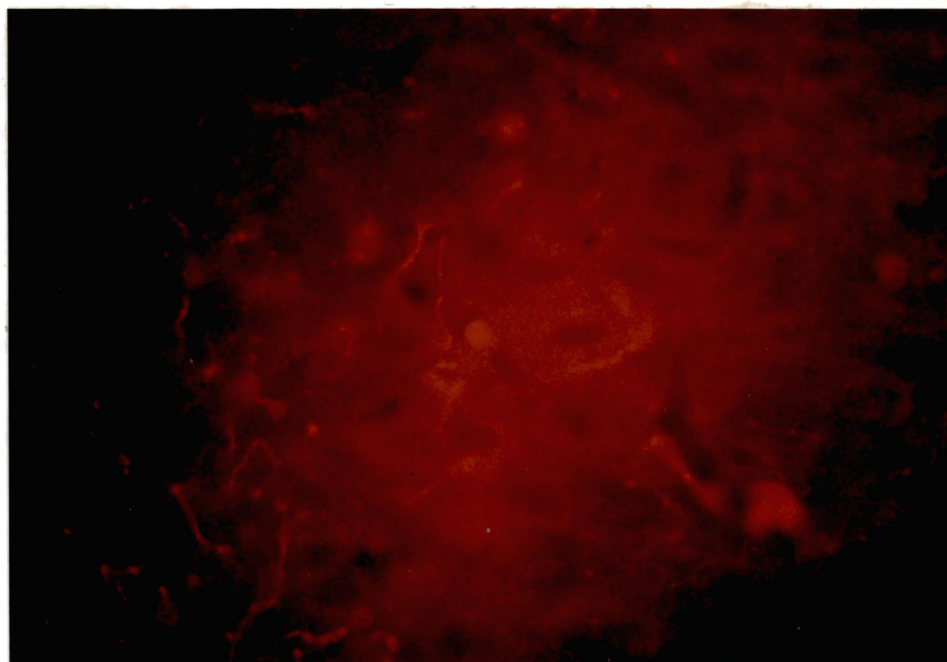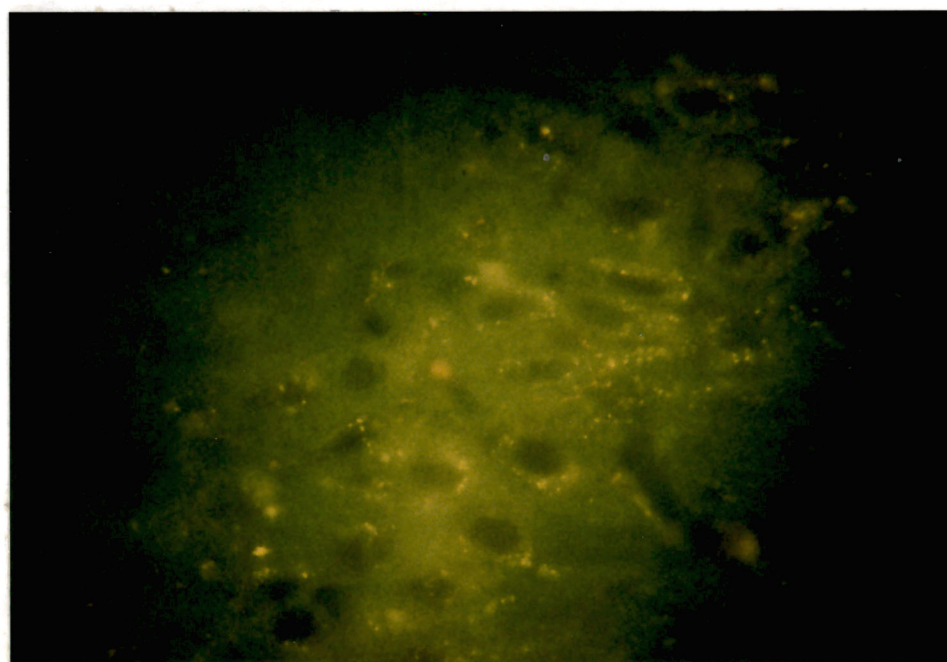ere also observed in the vertical limb of the diagonal band of Broca. Cell bodies labeled with SP were located along the lateral borders of the medial septum in a position similar to that described by Gall and Moore (1984; Fig. 12). Using the double labeling technique, scattered WGA/HRP (non-SP) and SP (non WGA/HRP) cell bodies could be observed in the middle and lateral portions of the medial septum (Fig. 13). A few cells were labeled for both SP (brown reaction) and WGA/HRP (black granular precipitate). These were located along the lateral border of the medial septum. A double labeled cell is shown in Figure 14B and the location of this cell in the medial septum can also be seen (Fig. 14A).

Controls in which anti-SP was omitted resulted in labeling of only WGA/HRP cell bodies and fibers. Those controls in which the TMB cobalt reaction was omitted resulted in SP and WGA/HRP cell bodies and fibers stained brown with the DAB reaction.

<u>Fig. 12</u> Septal section stained with SP only, showing SP-ir cells laying along the lateral borders in the medial septum. 98 x.

A

B

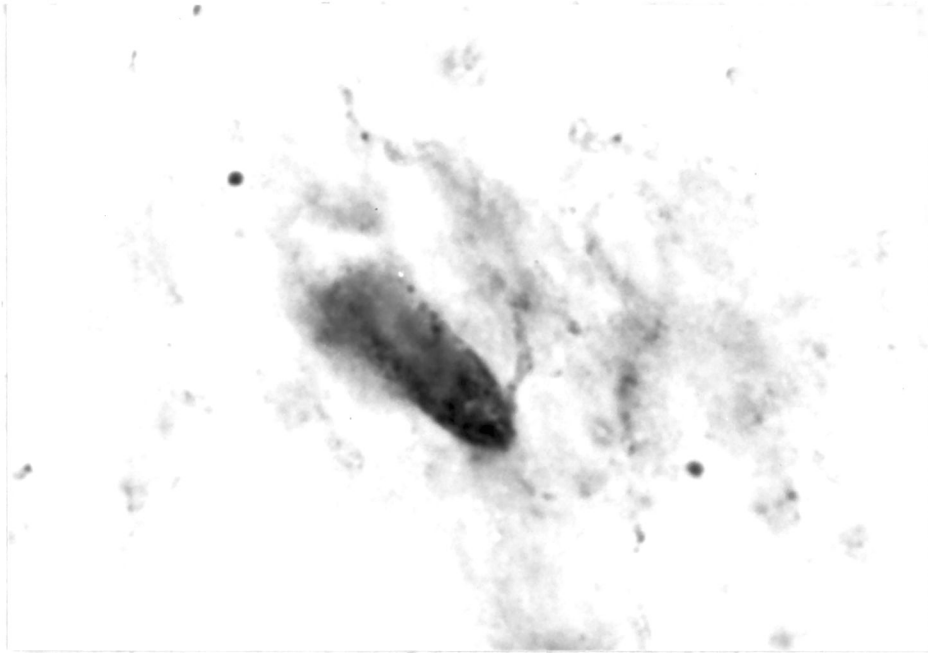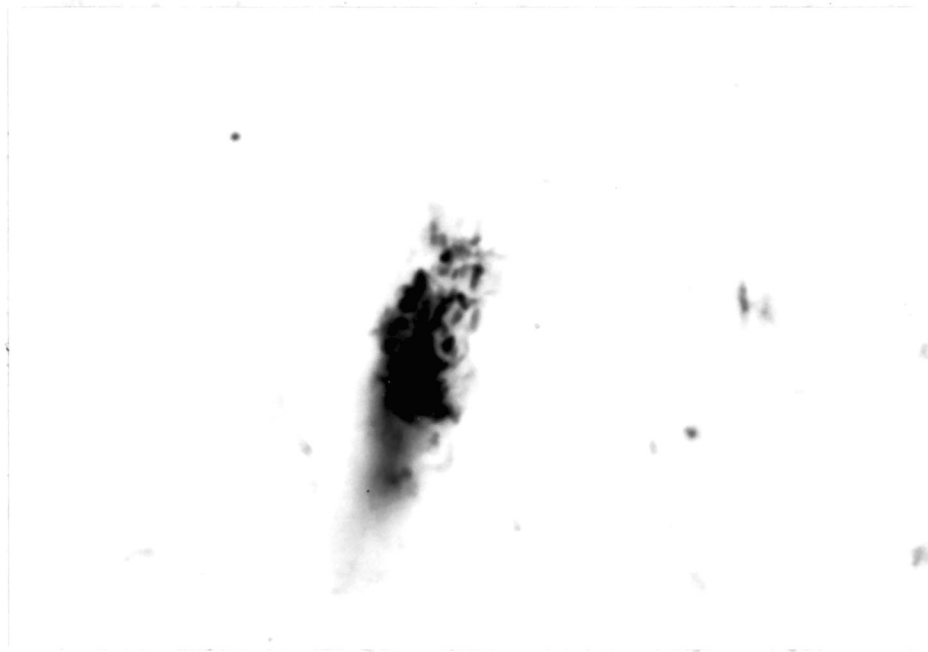<u>Fig. 13</u>   SP labeled cell (**A**) and WGA/HRP cell (**B**) from the same section having the double labeled cell body which is shown in Figure 14. 2160 x.
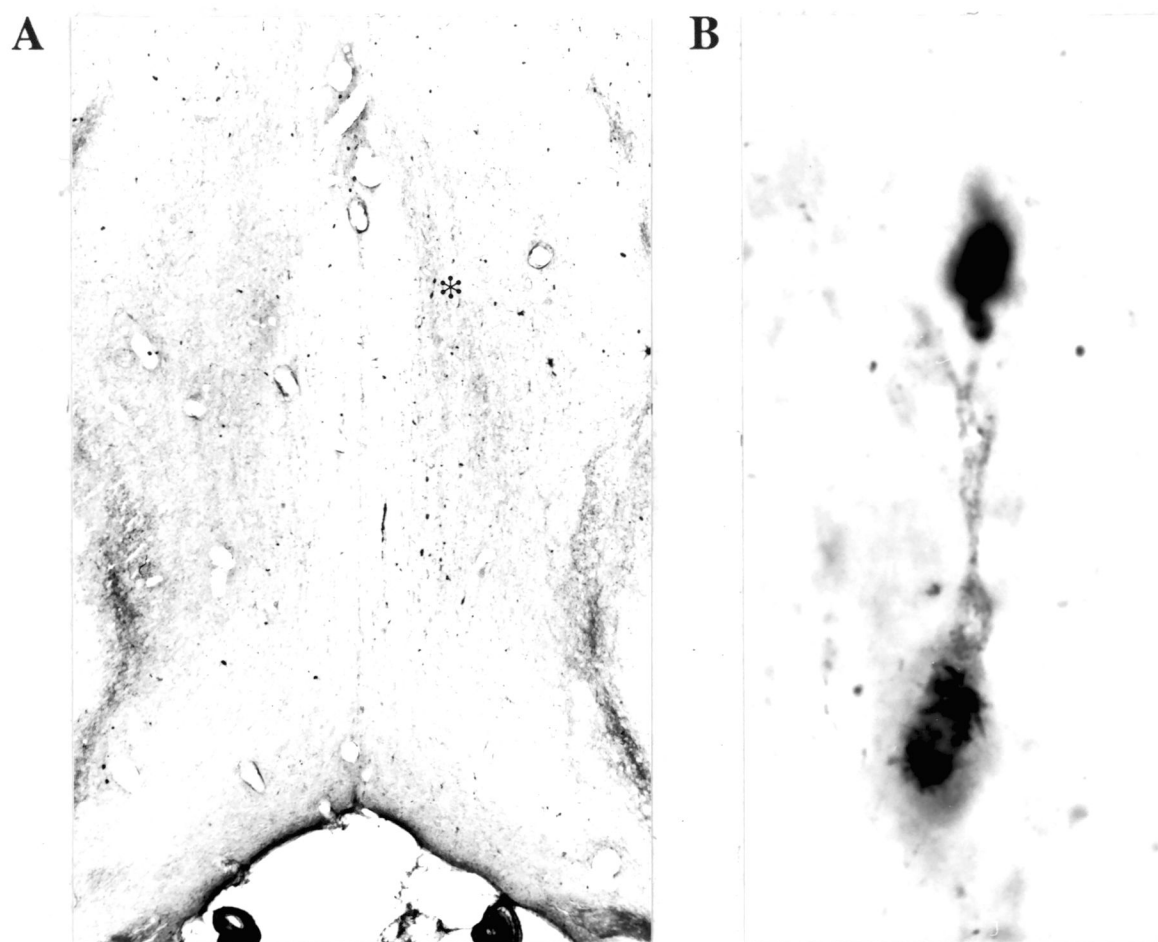
Fig. 14 **A** Section from medial septum from which double labeled cell in **B** was taken. Asterisk marks location of cell. 52 x.

B Double labeled cell (lower one of two); dark granules : WGA/HRP, dark cytoplasm : SP. 1600 x.

DISCUSSION

The major finding of this study is that SP-immunoreactive neurons, whose cell bodies lie along the border between medial and lateral septum, project to a discrete region of the hippocampus. Using injections of the anterograde tracer PHA-L into the lateral portion of the medial septum, fibers which contained both PHA-L and SP-ir were found in a limited region of CA2. This region was restricted to the mid septo-temporal portion of the hippocampal formation. Using the reverse approach, injections of the retrograde tracer WGA/HRP into this region of CA2 resulted in the labeling of numerous cells in the lateral portion of the medial septum, some of which also showed SP-ir.

The demonstration of a SP pathway from the septal area to the hippocampus began with the injection of the anterograde tracer PHA-L into the area of known SP cell bodies located between the border of medial and lateral septum. This was followed by combined immunocytochemical techniques using two fluorescent labels (Method 1) or the combination of fluorescence and peroxidase methods (Method 2) for the detection of both the PHA-L and SP. Since the combined fluorescence and peroxidase method did not reveal any double labeled fibers and the various controls indicated little chance of "bleed-through" but a slight chance of cross reactivity between antibodies using Method 1 it was determined that another fluorescence method should be attempted. In this method (Method 3) no cross reactivity was found between antibodies in control sections but, due to slight inaccuracies in the placement of PHA-L injections, double labeled fibers were not observed in the CA2 region of the hippocampus. Based on the initial findings of double labeled fibers containing PHA-L and SP in the CA2 region in the hippocampus (Method 1) another study was undertaken to see if the pathway could

be traced in reverse by the retrograde tracer, WGA/HRP. This was done by injecting WGA/HRP in the region of CA2/3 in the hippocampus where the double labeled fibers were located in the anterograde studies. Staining methods were used to stain for both SP and WGA/HRP in cell bodies in the septal area. The results from this study showed a few WGA/HRP and SP co-labeled cell bodies along the lateral border of the medial septum, as well as, cells labeled only with SP and WGA/HRP.

The position of SP fibers within the hippocampal formation was similar to that previously reported by Davis and Köhler (1985) and Shults and co-workers (1987). SP-ir fibers were found predominantly in the CA2 region throughout the septo-temporal extent of the hippocampal formation and those labeled by PHA-L were restricted to areas CA2 and CA3 in the mid septo-temporal portion of the hippocampus. Fibers which contained both SP and PHA-L immunoreactivity were a subset of both of these terminal fields, with most of the double labeled fibers being restricted to strata oriens and radiatum while apparently avoiding the pyramidal cell layer.

SP-ir cell bodies in the MS were restricted to the lateral border of the medial septum, similar to those described by Gall and Moore (1984). Following the WGA/HRP retrograde tracer injections in CA2/3 in the hippocampus some of the SP-ir cells were also labeled with WGA/HRP. The fact that all of the SP cells bodies in the area were not labeled with the WGA/HRP is probably due to incomplete labeling of the terminals in the CA2 region of the hippocampus.

Based on the results from the WGA/HRP retrograde and PHA-L anterograde tracing studies it can be concluded that the SP-ir cells along the border between medial and lateral septum are actually located in the medial septum. The study

done by Nyakas and co-workers (1987) using PHA-L injections into various areas of the MSDB also indicate that these cells project to various areas and layers of the hippocampus. Cell bodies in more medial aspects of the medial septum project to the more septal areas of the hippocampus and those in the more lateral area of the medial septum project more ventrally to the temporal pole. This is contradictory to that of Gall and Moore (1984) who suggested that these cells are located in the dorsolateral septum. If these cells were located in the lateral septum they would be expected to project to the medial septum and not the hippocampus (Swanson and Cowan, 1979).

The present anterograde and retrograde studies also provide confirmation of the projection which was suggested by the biochemical evidence of Vincent and McGeer (1981) and the degeneration studies of Peterson and co-workers (1987). The electrolytic lesions studies done by Vincent and McGeer (1981) in the septum produced a 40% reduction of SP levels in the hippocampus. The retrograde neuronal degeneration following transections of the fimbria-fornix, showed a loss of SP cell bodies along the lateral border of the medial septum, suggesting that these cells send their axons to the hippocampus via the fimbria-fornix.

Nyakas and co-workers (1987) described two fiber types that project from the MSDB to the hippocampus in their PHA-L labeling studies. Type I fibers are described as coarse thick axons and type II fibers are thin delicate and branching. Both types of fibers were observed in the present study, but the SP fibers located in the hippocampus were only of type II as they were always short, thin and heavily branched.

Previous morphological tracing studies have failed to determine the source of the SP fibers in CA2 of the hippocampus (Senut et al., 1989) or predict the

projection of SP cells in the medial septum of the rat (Davis and Köhler, 1985). The study by Senut and co-workers (1989) used injections of the retrograde tracer WGA-apohorseradish peroxidase conjugated to colloidal gold particles (WGA-apoHRP-gold) into the dorsal region of the hippocampus. They then used immunocytochemical procedures to detect the WGA-apoHRP-gold, as well as, SP and other peptides in the MSDB area. They did not locate any WGA-apoHRP-gold labeled SP cells in the medial septum, presumably because their hippocampal injections should have been more ventrally located.

The septohippocampal SP projection, like the cholinergic component, may be involved in learning and memory. Stäuble and Huston (1980) showed that intraseptal injections of SP facilitated passive avoidance learning. Related to this possible role in memory is the recent observation that SP is present in neuritic plaques in the hippocampus of Alzheimer's disease brains.

Having demonstrated that the SP neurons in the medial septum project to a restricted region of the hippocampal formation, it remains how to examine the terminal area more closely with the electron microscope. Using the techniques employed by Freund and Antal (1988) who described the connection of the GABAergic projection from medial septum the hippocampus, it should be possible to elucidate the types of synaptic contacts made by the SP terminals and by identity of their postsynaptic cells. Such an analysis would help to clarify the function of the septohippocampal SP projection.

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