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Localization of excitatory amino acid and neuropeptide markers in neurons of the subicular complex projecting to the retrosplenial granular cortex of the rat

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SUMMARY

Retrograde labelling was combined with immunohistochemistry to localize neurons containing glutamate and different neuropeptides such as neurotensin, leu-enkephalin, and substance P-like immunoreactivity in the projection pathways from the presubiculum and subiculum to the retrosplenial granular cortex of the rat. Injections of horseradish peroxidase conjugated to subunit B of cholera toxin (CT-HRP) into the retrosplenial granular cortex labelled large numbers of neurons in the presubiculum. A significant number of retrogradely-labelled neurons was seen in the dorsal subiculum, whereas small numbers of CT-HRP-labelled neurons were also found in the ventral subiculum. In the presubiculum, 90-95% of the CT-HRP-labelled neurons (30-32 per section) were also immunoreactive for glutamate, and small numbers of retrogradely-labelled neurons also displayed neurotensin–, leu-enkephalin– or substance P-immunoreactivity. In the subiculum, approximately 90-95% of the CT-HRP-labelled neurons (19-20 per section) were also immunoreactive for glutamate, and a significant number of retrogradely-labelled neurons (70-75%, 14-15 per section) also displayed neurotensin immunoreactivity. In addition, small numbers of CT-HRP-labelled neurons in the subiculum were immunoreactive for leu-enkephalin or substance P. These results suggest that the complexity of the neurotransmitter(s)/neuromodulator(s) of the subicular projections to the retrosplenial granular cortex of the rat should be taken into account when considering the mechanisms of the retrosplenial cortical neurons thought to play a role in memory.

Key Words: Cingulate cortex – Hippocampus – Limbic system – Glutamate – GABA

Abbreviations: CA1, field CA1 of Ammon's horn; cc, corpus callosum; DG, dentate gyrus; PrS, presubiculum; RSa, retrosplenial agranular cortex; RSg, retrosplenial granular cortex; S, subiculum.

INTRODUCTION

The retrosplenial cortex (caudal cingulate cortex) has been recognized as a nodal point for the transfer of information between the hippocampal formation, many neocortical regions, and the thalamus. Several studies have demonstrated the cortical and thalamic connections of the retrosplenial cortex, especially the connections of the retrosplenial granular cortex (RSg) (Domesick, 1972; Vogt et al., 1981; Seki and Zyo, 1984; Sripanidkulchai and Wyss, 1986; Shibata, 1993; Price, 1995; Gonzalo-Ruiz et al., 1997a,b; Wang et al., 1999) as well as the reciprocal connections of the RSg and subicular cortices (Meibach and Siegel, 1977; Rosene and Van Hoesen, 1977; Sorensen, 1980; Vogt and Miller, 1983; Finch et al., 1984; Vogt et al., 1986; Witter et al., 1989, 1990; Van Groen and Wyss, 1990; Wyss and Van Groen, 1992).

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In the past two decades, several studies have implicated the RSg in learning and memory (Gabriel et al., 1983; Berger et al., 1986; Valenstein et al., 1987; Markowska et al., 1989; Matsunami et al., 1989; Sif et al., 1989; Sutherland and Hoesing, 1993; Cooper and Mizumori, 2000). In part, this probably depends on the presence of intact connections from the hippocampal formation to the RSg (Gabriel et al., 1980, 1989; Gabriel and Sparenborg, 1986, 1987; Smith et al., 2000). Memory impairment is an early and prominent manifestation of Alzheimer's disease (AD), and the cerebral cortex, including the RSg, hippocampal formation and parahippocampal gyrus, are among the brain areas most consistently and strongly involved in this patholgy (Hyman et al., 1987; Braak and Braak, 1991; Thangavel and Van Hoesen, 2000). Since the cholinergic system has long been implicated in cognitive function in aging and AD, many studies have focused on manipulating cholinergic function in experimental animals in an effort to elucidate the role of the cholinergic system in leaming and memory (for reviews, see Bartus et al., 1982; Olton and Wenk, 1987; Smith, 1988). Although the evidence offered in these studies is consistent with the view that cholinergic dysfunction may partly underlie the cognitive deficits associated with aging and AD, it has become increasingly evident that cholinergic dysfunction cannot provide a complete picture to account for these deficits (for reviews, see Dunnett et al., 1987; Robbins et al., 1989; Wenk et al., 1989a,b). Thus, alterations in cholinergic function might well interact in tandem with other neurotransmitter systems to produce additive or even synergistic effects on the cognitive deficits related to aging and AD.

Immunohistochemical studies have shown that subicular neurons contain glutamic acid decarboxylase (GAD)/γ-aminobutyric acid (GABA) (Mugnaini and Oertel, 1985; Ottersen and Storm-Mathisen, 1984), glutamate (Glu) (Cotman and Nadler, 1981; Storm-Mathisen and Ottersen, 1983; Ottersen et al., 1990; Ottersen, 1991), substance P (SP) (Roberts et al., 1984; Borhegyi and Leranth, 1997), neurotensin (NT) (Roberts et al., 1984), and enkephalin (Enk) (Finley et al., 1981; Gall et al., 1981; Petrusz et al., 1985). However, apart from general interest in glutamate/aspartate as a transmitter(s) in hippocampal formation (Collingridge et al., 1983; Wierasko, 1983; Nadler et al., 1990; Gundersen et al., 1991; Fleck et al., 1993), little is known about the precise contributions of these immunoreactivities in projections from the subicular complex to the RSg. Since delineation of the neurotransmitter systems involved in learning and memory is of considerable interest, the present study was carried out to define the transmitter-related characteristics of the projections from the presubiculum and subiculum to the RSg by retrograde pathway tracing combined with immunohistochemical detection of excitatory and inhibitory amino acids and several neuropeptides (NT, SP, Enk). A preliminary report of the findings has appeared in abstract form (Gonzalo-Ruiz et al., 2000).

Materials and Methods

Experimental animals and anaesthesia

Eight adult rats of both sexes (Wistar, 250-300 g body weight) were used. The animals were kept under standard laboratory conditions (20º C ambient temperature, 12h light/dark cycle, tap water and regular rat chow ad libitum). The rats were anaesthetized with Nembutal (45mg/kg, injected intraperitoneally) for the surgical procedure (tracer injection). Prior to perfusion with fixative, the animals were reanaesthetized in the same manner but with up to double the dose used for the surgical procedure. All animals were housed and handled in all respects according to national legislation and the guideliness approved by the Animal Care Committee of the University of Valladolid, which comply with or are even more stringent than EEC Directive 86/609.

Surgical Procedures

Anaesthetized animals were placed in a stereotaxic frame and a hole was made in the left parietal bone with a dental drill. The dura was opened with a fine hypodermic needle and a Hamilton microsyringe (10 µl) containing HRP conjugated to subunit B of cholera toxin (CT-HRP; List Biological Laboratories, Campbell, CA, USA) was positioned in different parts of the left RSg using stereotaxic coordinates taken from the atlas of Paxinos and Watson (1986). Between 0.5 and 0.6µl of a 2% solution of CT-HRP (in sterile distilled water) was slowly pressure-injected over a 10 minute period, after which the needle was left in place for a further 10 minutes before being withdrawn. The scalp incision was sutured and the animal was allowed to recover from the anaesthetic. After a postinjection survival period of 1-2 days, the rats were reanaesthetized and sacrified by transcardiac perfusion of 60 ml of 0.9% saline at 20ºC containing heparin (1000 IU) to flush blood from the vascular system, followed by ca 500 ml of fixative solution containing 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.2 (also perfused at 20º C).

Histochemical and Immunohistochemical Procedures

Immediately after perfusion, the brain was removed, trimmed to a block of convenient size, incorporating both the RSg and the subicular

complex, and sectioned on the coronal plane at 50 µm using a Vibratome. Adjacent sections were collected as six series. Each series was made up of six or seven coronal sections incorporating the subicular complex. One series of sections was processed for CT-HRP histochemistry with tetramethylbenzidine (TMB) as chromogen, according to the method of Mesulam (1978). These sections were mounted on gelatinized slides, counterstained with 0.1% neutral red, dehydrated, covered with Permount, and examined and photographed under brightfield and darkfield illumination.

The other five series of sections were processed first for the histochemical demonstration of CT-HRP by incubation for 10-15 min in 0.005% 3'3'–diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in 0.1 M PB, pH 7.2, containing 0.005% cobalt acetate/nickel chloride (this chromogen produces a black granular reaction product). After washing in several changes of PB, the sections from these five series were processed for the immunohistochemical localization of Glu, GABA, NT, Enk or SP, respectively. Each neurochemical was analyzed in six or seven sections from each of the eight animals included in the study. The sections were first immersed for 1 h in 10% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 0.1 M lysine, and were then incubated in a solution containing a polyclonal rabbit antibody against Glu (1:1000 dilution; Arnel Products Co., New York, NY,USA), GABA (1:2000 dilution; Sigma Chemical Co., St. Louis, Mo, USA), NT (1:2000 dilution; Eugene Tech. Inter, Ridgfield Park, NJ, USA), Enk (1:5000 dilution; INCSTAR, Stillwater, MN, USA), or SP (1:2000 dilution; Eugene Tech. Inter) for 18-24 h at 4º C. After incubation in the primary antibody, sections were washed in 1% NGS and then incubated in biotinylated goat anti-rabbit IgG (Vector; 1:200 in PBS with 1% NGS). They were then washed in PBS and immersed in an avidin-biotin-HRP complex (Vector 1:100) for 60 min. The immunoreaction product was demonstrated using 0.005% DAB and 0.01% hydrogen peroxide in PB (this chromogen produces a diffuse brown reaction product). Sections were rinsed in several changes of PB, mounted on gelatinized microscope slides, dehydrated, covered with Permount, and examined and photographed under brightfield illumination [at low (objective x 2) at high magnification (objective x 100)].

As controls, some sections from each series were incubated as described above but without the addition of primary antibody or after replacing the rabbit primary antibody with normal rabbit serum. No Glu, GABA, NT, Enk or SP-immunoreactive neurons or neuropil were observed in these control sections.

Analysis of material and presentation of results

All of the sections through the subicular complex (each separated by approximately 300 µm) in each of the six series were examined systematically. The specificity of the immunoreaction was checked by comparing sections stained with Glu, GABA, NT, Enk or SP antiserum and control material, respectively. Structures immunostained by antibodies but not visualized in the control slides were considered to be specifically immunolabelled and are designated here as Glu–, GABA–, NT–, Enk–, or SP-immunoreactive. All CT-HRP single-labelled cell bodies and all double-labelled cell bodies (CT-HRP/Glu, CT-HRP/NT, CT-HRP/Enk, CT-HRP/SP) were scored at 20x magnification using an ocular grid, and were mapped onto drawings of frontal, cresyl violet-stained sections of the subicular complex, taken from the atlas of Paxinos and Watson (1986). In sections reacted with DAB, relatively few CT-HRP-labelled cells (300-350 per animal) were identified. CT-HRP-labelled cells and double-labelled cells were counted to obtain a rough estimate of the relative proportions of each and are depicted on schematic drawings by different types of symbols (Fig. 2A, B).

RESULTS

Nomenclature

Following the criteria, terminology and mapping of Vogt and Peters (1981), the retrosplenial cortex of the posterior cingulate gyrus was subdivided into two parts: retrosplenial agranular cortex (RSa) and the RSg (e.g. Fig. 1A-E). The term "subicular complex" has been used by many authors to indicate a conglomerate of cytoarchitectonically different, small cortical areas that included the prosubiculum, the subiculum, the presubiculum, the postsubiculum, and the parasubiculum (Rose, 1926; Lorente de Nó, 1934). However, based on cytoarchitectonic premises, connectivity criteria and the histochemical data, for the purpose of the present description the terms prosubiculum, postsubiculum and parasubiculum will not be used and our results will therefore refer only to the subiculum and presubiculum under the conceptual name of "subicular complex" (e.g. Fig. 2A,B).

Localization and extent of CT-HRP injections

The findings in this study are based on eight animals (some of which were also used in the study by Gonzalo-Ruiz and Morte, 2000) that received a single injection of CT-HRP into various parts of the left RSg. Examples of the injection sites, drawn and photographed from TMB-reacted sections, are shown in Fig. 1A-E and Fig. 2A,B. In four of these animals, the injections

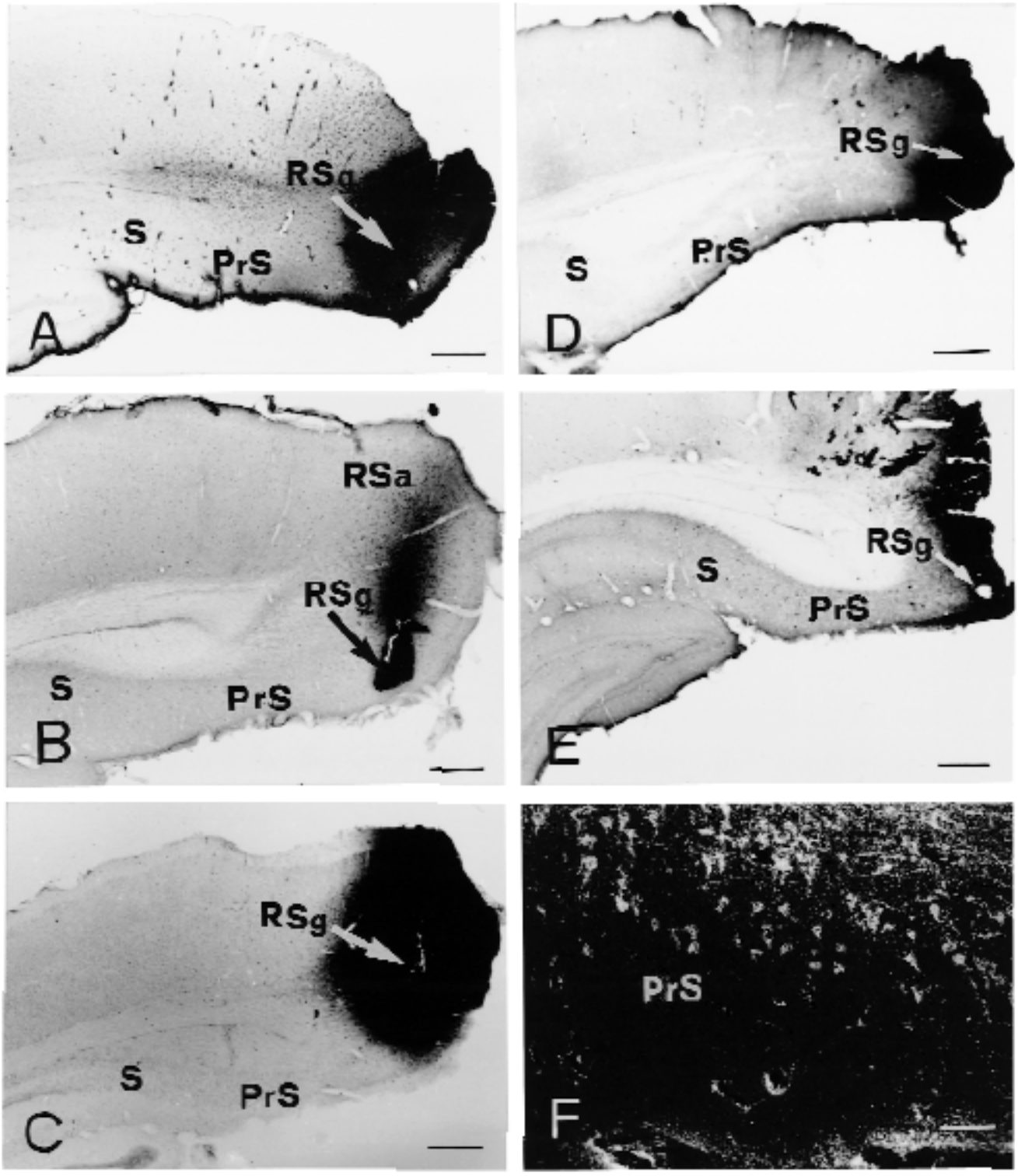

Fig. 1.- **A-E:** Low-magnification photomicrographs of coronal sections through the retrosplenial cortex from tetramethylbenzidine-reacted sections, showing representative injections of CT-HRP centred on the retrosplenial granular cortex (RSg, arrows). **F:** Darkfield photomicrograph of coronal section through the presubiculum (PrS), showing retrogradely labelled cells and anterogradely labelled terminals following the injection shown in Figs. 1C, 2B. Scale bars: 200 µm (A-E), 100 µm (F).

involved layers I-VI of either the rostral or caudal RSg (e.g. Figs. 1A,C-E,2A,B). In one of these animals, the injection of CT-HRP was centred on deep layers (V-VI) of the dorsal region of the caudal RSg and extended to the superficial layers (I-III) of the ventral regions of the RSg (e.g. Fig. 1B). In the other three animals, the injections were centred on superficial layers (I-III) of either the rostral or caudal RSg. The localization and extent of CT-HRP injections in the RSg were confirmed by the distribution of retrogradely labelled thalamo-cortical and cortico-cortical afferent neurons as established in previous studies (Sripanidkulchai and Wyss, 1986; Vogt et al., 1981, 1986; Shibata, 1993; Gonzalo-Ruiz et al., 1997a,b).

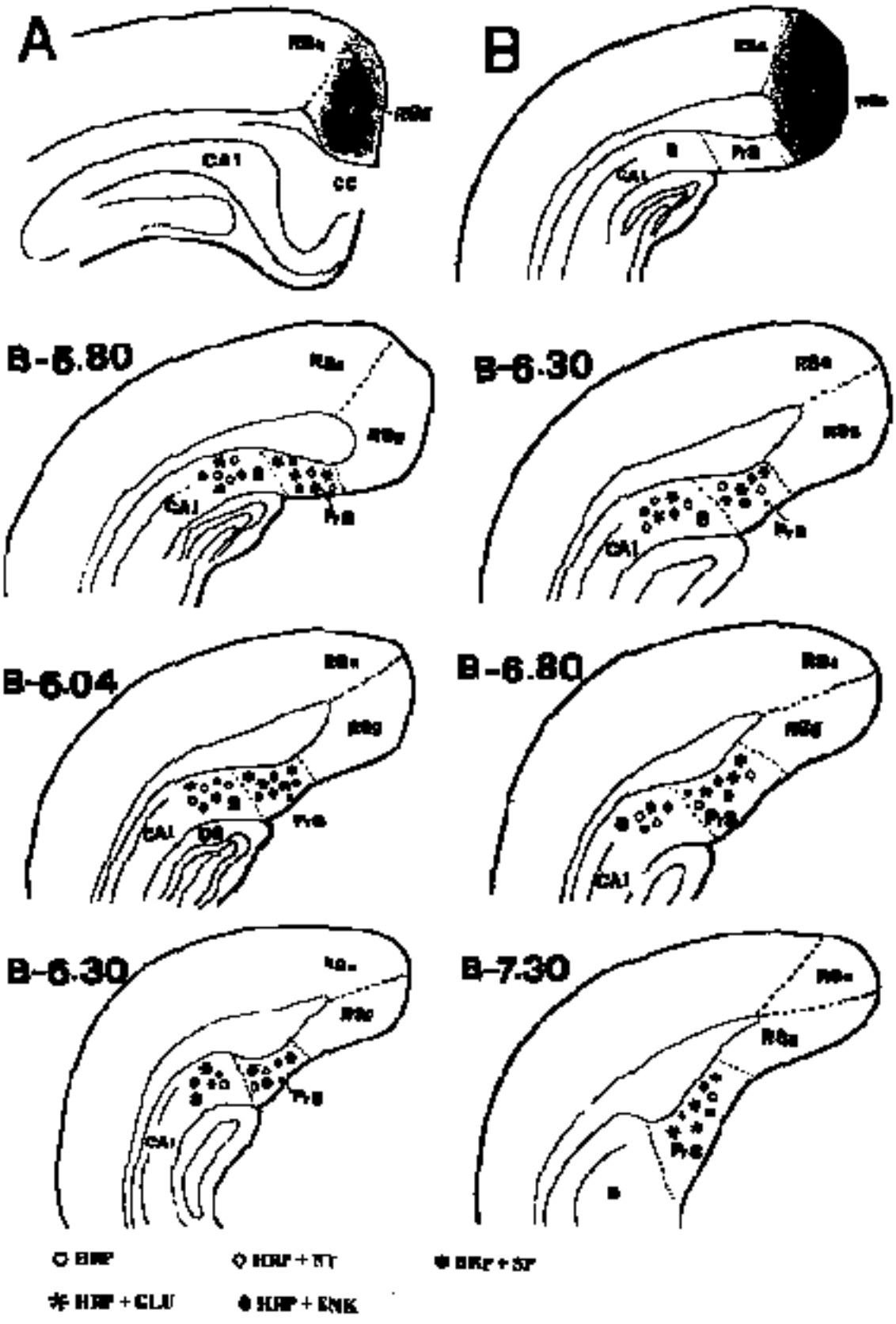

Fig. 2.- **A, B:** Schematic summaries of the distribution and relative numbers of retrogradely single-labelled or retrogradely labelled glutamate (Glu)–, neurotensin (NT)–, enkephalin (Enk)–, or substance P (SP)-immunoreactive neurons in the subicular complex following injections of CT-HRP into either the rostral **(A)** or caudal RSg **(B)**. The number of cells that each symbol represents can be summarized as follows: CT-HRP/Glu: 8-10 cells, CT-HRP/NT: 6-8 cells, CT-HRP, CT-HRP/Enk, CT-HRP/SP: 1-6 cells.

General observations

The distribution of Glu, GABA, Enk, NT and SP-immunoreactive neurons in the subicular complex was consistent in all animals (e.g. Figs. 3A, D, 4A, D) and was largely in agreement with previous descriptions (e.g. Finley et al., 1981; Gall et al., 1981; Storm-Mathisen and Ottersen, 1983; Storm-Mathisen et al., 1983; Ottersen and

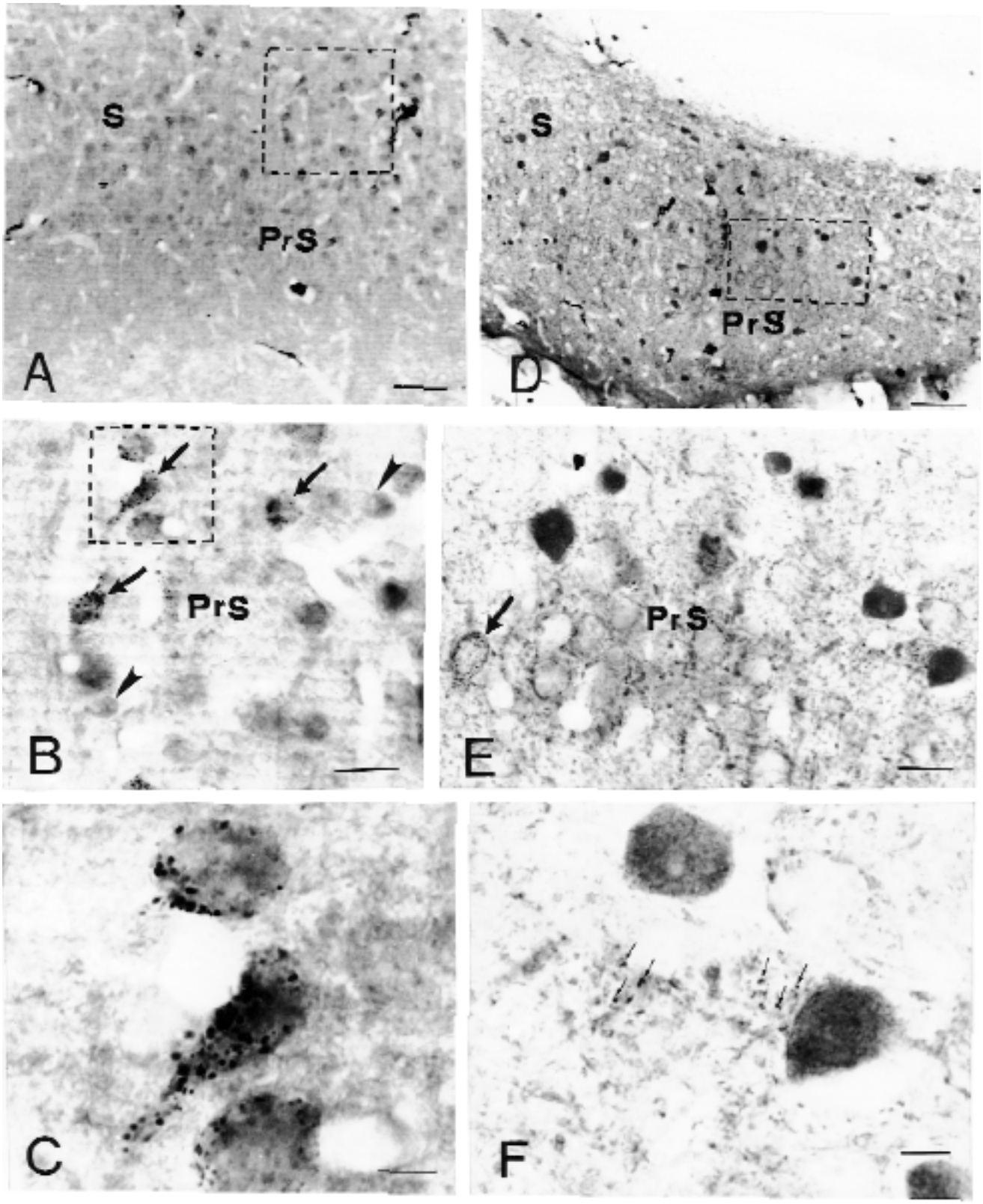

Fig. 3.- **A:** Low-magnification photomicrograph through the presubiculum (PrS) following the injection shown in Figs. 1C, 2B. The section was processed for the visualization of horseradish peroxidase conjugated to subunit B of cholera toxin (CT-HRP) and glutamate (Glu). Differences between double- and single-labelled neurons are not detectable at this magnification. The boxed area is enlarged in B. **B:** High magnification of boxed area in A, showing double-labelled neurons (arrows) and Glu-single-labelled neurons (arrowheads). **C:** High magnification of boxed area in B showing double-labelled neurons. **D:** Photomicrograph through the presubiculum (PrS) and subiculum (S) following the injection shown in Figs. 1C, 2B. The section was processed for CT-HRP and γ-aminobutyric acid (GABA). The boxed area is enlarged in E. **E:** High magnification of boxed area in D, showing a CT-HRP-single-labelled neuron (arrow) and GABA-labelled neurons. **F:** High magnification of part of E, showing GABA-labelled neurons and CT-HRP-labelled fibres and terminal-like structures in the neuropil (small arrows). Scale bars: 100 μm (A,D), 50 μm (B), 25 μm (E), 10 μm (C,F).

Storm-Mathisen, 1984; Roberts et al., 1984; Petrusz et al., 1985; Borhegyi and Leranth, 1997). However, the distribution and the number of retrogradely labelled cells and of double-labelled cells differed, depending on both the size of the CT-HRP injection and the method used for the visualization of CT-HRP. Sections processed for CT-HRP histochemistry using TMB as the chromogen displayed larger numbers of retrogradely labelled neurons (Fig. 1F) than those processed using DAB as the chromogen in combination with immunohistochemistry (e.g. Fig. 3B). In sections processed sequentially for the visualization of CT-HRP and Glu, GABA, Enk, NT or SP, double-labelled neurons were identified by the distribution, throughout their somata and proximal dendrites, of distinct granules attributable to the retrograde transport of CT-HRP, superimposed over a diffuse light brown immunoreaction product (e.g. Figs. 3C, 4C). Single retrogradely labelled neurons displayed only black granular deposits of transported CT-HRP (e.g. Fig. 3E) and immunoreactive neurons devoid of CT-HRP labelling were identified by a diffuse light-brown immunoreaction product throughout their somata and dendrites (e.g. Fig. 4C).

Transmitter-related immunoreactivity in identified projection neurons from the subicular complex to the retrosplenial granular cortex

In all animals (e.g. Fig. 1A-E) injections of CT-HRP confined to the RSg resulted in numerous labelled neurons in the presubiculum and subiculum. The highest numbers of retrogradely labelled cells were found in the presubiculum (Fig. 1F), while a significant number was seen in the dorsal subiculum. In addition, following injections involving the ventral region of the RSg, a few retrogradely labelled neurons were also found in the ventral subiculum.

In the presubiculum approximately 90-95% of the CT-HRP-labelled neurons (30-32 per section) were also Glu-immunoreactive (Figs. 2A,B, 3A,B). However, up to 10% of the neurons projecting to the RSg were not immunoreactive for Glu and were codistributed (1-3 per section) with Glu-immunoreactive projection cells throughout the presubiculum (Fig. 2A,B). In addition, a few sparsely scattered retrogradely labelled neurons in the presubiculum (1 to 3-4 per section) also displayed NT–, Enk– or SP-immunoreactivity (Fig. 2A,B). In the presubiculum, most of the neurons projecting to the RSg were seen ipsilateral to the injection site and a few retrogradely labelled neurons were also observed contralaterally. Retrogradely labelled neurons and double-labelled neurons were mainly found in layers V and VI of the presubiculum (Figs. 2A,B, 3A,B). These projections exhibited a rostrocaudal organization such that rostral parts of the presubiculum projected to rostral regions of the RSg (Fig. 2A, level B-5.80 to B-6.30), whereas caudal parts of the presubiculum projected to caudal parts of the RSg (Fig. 2B, level B-6.30 to B-7.30).

In addition to the pattern of labelling described above, injections of CT-HRP into the RSg also revealed the presence of CT-HRP-containing fibres or terminal-like structures, which appeared to display irregularly distributed black granules of what was apparently the HRP reaction product, in the neuropil and close to neuronal cell bodies of the presubiculum (e.g. Figs. 1F, 3E). Within areas displaying heavy anterograde labelling, this sometimes made it difficult to differentiate neurons displaying immunoreactivity for GABA that were overlain by CT-HRP-containing fibres (e.g. Fig. 3E,F).

In the presubiculum, most of the CT-HRP-labelled, double-labelled (CT-HRP/Glu, CT-HRP/NT, CT-HRP/Enk or CT-HRP/SP), and Glu+, GABA+, NT+, Enk+, or SP+ single-labelled neurons were of medium size (20-24 µm), with darkly stained perikarya (e.g. Fig. 3B,C,F) whereas a few CT-HRP-labelled, double-labelled and single-labelled neurons were generally small (10-12 µm), with somata ranging in shape from ovoid to multipolar (e.g. Fig. 3C,E).

In the subiculum most of the CT-HRP-labelled neurons (90-95%, 19-20 per section) were also Glu-immunoreactive (Fig. 2A,B) and a significant number (70-75%, 14-15 per section) also displayed immunoreactivity for NT (Figs. 2A,B, 4D-F). However, up to 10-25% of neurons projecting from the subiculum to the RSg were not immunoreactive for Glu or NT and these neurons were codistributed with Glu-immunoreactive or NT-immunoreactive projection cells (Figs. 2A,B, 4F). In addition, a few retrogradely labelled neurons (1 to 4-5 per section) in the subiculum were also immunoreactive for Enk, or SP– (Figs. 2A,B, 4A-C), whereas none of the CT-HRP-labelled neurons in the subiculum displayed immunoreactivity for GABA. Most of the double-labelled and CT-HRP single-labelled neurons were mainly located in the dorsal two-thirds of the subiculum (e.g. Figs. 2A,B, 4D). However, in some animals a few labelled cells were also found scattered in the ventral subicular region (not shown). All neurons projecting to the RSg were seen ipsilateral to the injection site (Figs. 2A,B, 4A-F) and appeared as a dense cluster of cells in deep pyramidal layers of the subiculum (e.g. Fig. 4D). Most of the retrogradely labelled, double-labelled (CT-HRP/Glu, CT-HRP/NT, CT-HRP/Enk or CT-HRP/SP) and Glu+, NT+, Enk+, or SP+ single-labelled neurons in the subiculum were of medium (20-24 µm) to relatively large size (30-35 µm), with pyramidal somata (e.g. Fig. 4E,F). A few scattered and weakly labelled neurons with ovoid or multipolar somata were also present in the subiculum (e.g., Fig. 4B,C).

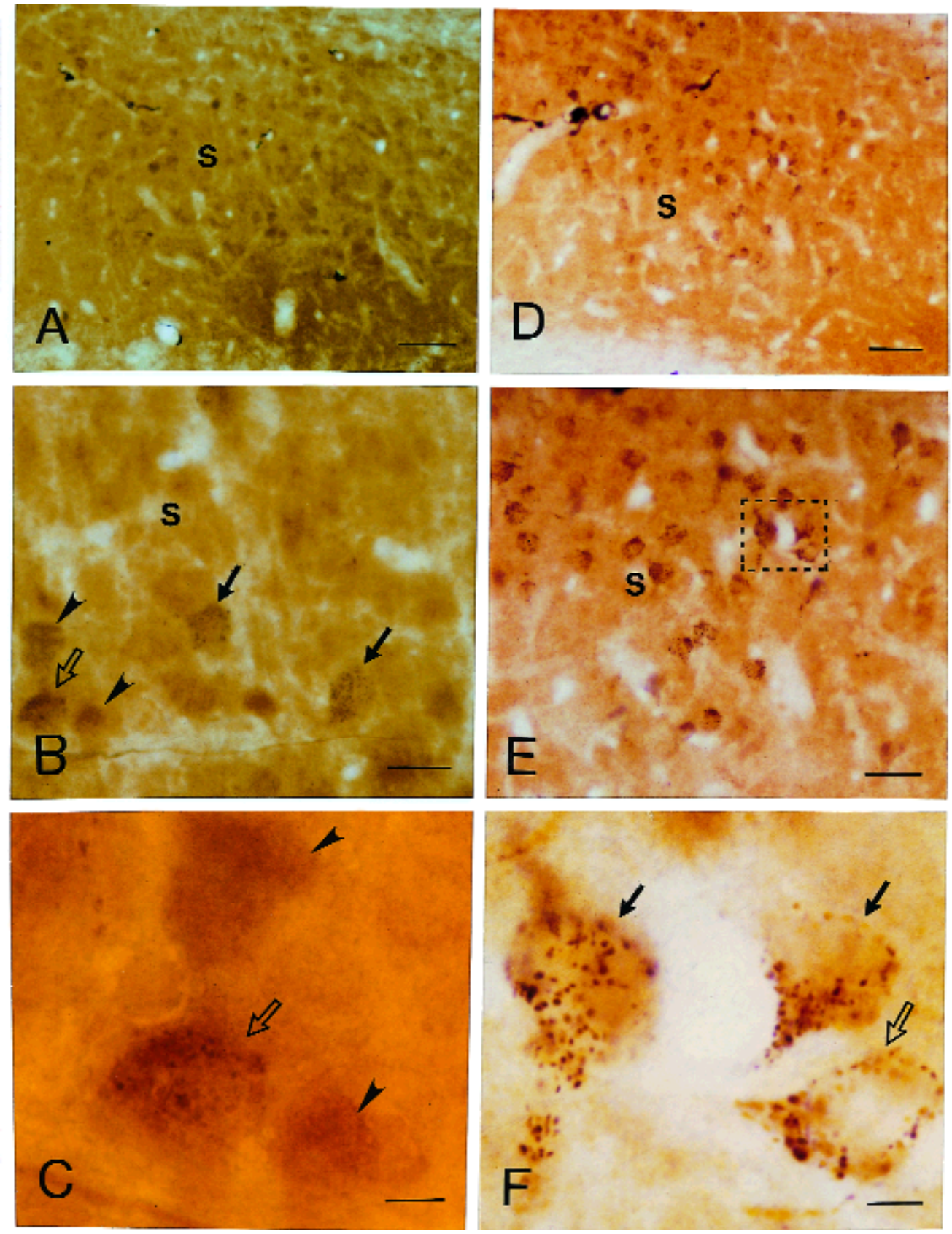

Fig. 4.- **A:** Low-magnification photomicrograph through the subiculum (S) following the injection shown in Figs. 1C, 2B. The section was processed for horseradish peroxidase conjugated to subunit B of cholera toxin (CT-HRP) and enkephalin (Enk). Differences between double- and sinle-labelled neurons are not detectable at this magnification. **B:** High magnification of part of A, showing double-labelled neurons (arrows) and cells that display only Enk-immunoreactivity (arrowheads). The double-labelled neuron indicated by an open arrow is shown in C. **C:** High magnification of part B, showing a double-labelled neuron (open arrow) and the Enk-single-labelled neurons (arrowheads) indicated by arrowheads in B. **D:** Photomicrograph through the subiculum (S) following the injection shown in Figs. 1C, 2B. The section was processed for CT-HRP and neurotensin (NT). **E:** High magnification of part of D. Differences between double- and single-labelled neurons are not detectable at this magnification. The boxed area is enlarged in F. **F:** High magnification of boxed area in E, showing double-labelled neurons (solid arrows) and a CT-HRP-single-labelled neuron (open arrow). Scale bars: 100 µm (A,D), 50 µm (B,E), 10 µm (C,F).

The above results can be summarized as follows:

1. Injections of CT-HRP centered in the RSg labelled many neurons within the subicular complex, mainly in the presubiculum, although with a minor proportion in the subiculum.
2. In the presubiculum, 90-95% of the retrogradely labelled neurons displayed immunoreactivity for Glu, whereas small numbers of the CT-HRP-labelled neurons also displayed Enk-, NT- or SP-immunoreactivity.
3. In the subiculum, 90-95% of the retrogradely labelled neurons were also immunoreactive for Glu and a significant number (70-75%) of the CT-HRP-labelled neurons also displayed NT-immunoreactivity. In addition, small numbers of the retrogradely labelled neurons in the subiculum were immunoreactive either for Enk or SP.
4. In the subiculum, retrogradely labelled and double-labelled neurons were concentrated mostly in its dorsal two-thirds, whereas in the presubiculum CT-HRP-labelled and double-labelled neurons exhibited a rostro-caudal organization. Thus, rostral parts of the presubiculum project to more rostral regions of the RSg and caudal parts of the presubiculum project to more caudal regions of the RSg.
5. Although injections centered in RSg resulted in bilateral retrograde labelling of small numbers of Glu-immunoreactive neurons in the presubiculum, the projections to the RSg from subicular cortices were predominantly ipsilateral.
6. In all animals, CT-HRP-containing fibres or terminal-like structures were found in the neuropil of the presubiculum, whereas non-anterogradely labelled terminal-like structures were seen in the neuropil of the subiculum.

In conclusion, the main findings are (i) that most of the CT-HRP-labelled neurons in presubiculum and subiculum are Glu-immunoreactive and, (ii) that a very large fraction of retrogradely labelled neurons in subiculum are also positive for NT.

DISCUSSION

Methodological Considerations

CT-HRP as a retrograde axonal tracer

It is generally agreed that HRP is a sensitive tracer, and it is well known that the TMB method is consistently more sensitive than the DAB method used in our double-labelling studies. Thus, the small numbers of both single CT-HRP and double-labelled cells found in the double-labelled preparations could be due to the limited sensitivity of the DAB method and also, to some extent, to the short survival times used in this study. However, even with these methodological limitations, this study did allow us to detect the strongest projections from the presubiculum and subiculum to the RSg, even though we failed to detect some neurons with only a small axonal branch projecting to the RSg.

Sensitivity of the immunohistochemical method

The immunocytochemical technique of Hsu et al. (1981) is generally considered to be a sensitive method. However, it should be noted that a small number of neurons projecting to the RSg from the subicular complex were immunonegative. This finding could be interpreted in terms of "false negatives", implying that there are projection neurons in which no immunoreactivity was detected because of a technical problem, such as poor antibody penetration due to the thickness of the sections or because no colchicine pretreatments were used, or because of a loss of antigen during tissue processing, or as a result of the fixative used in the present study. An alternative possibility is that immunonegative neurons projecting to the RSg could have a different phenotype or phenotypes. Because the immunoreactions were carried out on different sections, we do not know whether CT-HRP-single-labelled neurons were immunonegative for the neurochemicals investigated or whether they contained one of the other transmitter markers not labelled in that particular section.

Specificity of the immunoreaction

Several issues arise in relation to the procedures used in this study. The first concerns the presence of Glu-immunoreactivity in the cell bodies of the subicular complex neurons projecting to the RSg. Here, we show that 90-95% of the neurons projecting to the RSg from the presubiculum and subiculum contain immunohistochemically detectable levels of Glu. The significance of high cell body levels of Glu with respect to the transmitter phenotype of neurons has been questioned and extensively discussed in the literature (Fonnum, 1984; Ottersen et al., 1990). Ottersen and Storm-Mathisen (1984) suggested that large amounts of Glu in the cell bodies may correlate with a metabolic role not necessarily concerned with neurotransmission. It should be pointed out that several features differentiate Ottersen and Storm-Mathisen's study from ours, including the fixatives used in both studies. Although Conti et al. (1987) indicated that the use of different fixatives did not appear to change the specificity of the staining, fixatives containing glutaraldehyde, the only kind employed by Ottersen and Storm-Mathisen (1984), gave a higher background staining than

that seen in the present study. Also, the antibodies used may differ in their specificities. The Glu antibody used in the present study is well characterized (Arnel Products, Catalogue Nº 58A, 1992) and has been widely used (Phend et al., 1992), and immunoabsorption tests based on procedures described by Hepler et al. (1988) show that the Glu antiserum does not cross-react with aspartate, GABA, B-alanine, glycine or other amino acids. The fact that in our material the maps of immunostaining with Glu antibodies were consistent with the extensive data in the literature (Storm-Mathisen and Ottersen, 1983; Ottersen and Stomm-Mathisen, 1984) together with the results of the control experiments, suggest that subiculo-retrosplenial projection neurons with high levels of Glu could use this amino acid as an excitatory neurotransmitter.

With respect to the peptide antibodies, the rabbit anti-Enk antibody used here has been well characterized and the specificity of this antiserum has been established by abolition of immunostaining when the antiserum was absorbed with 50 µg/ml Leu-enk (INCSTAR, catalogue Nº 10411, 1993), as was the similarly produced NT antibody (Eugene Tech. International, Catalogue Nº NT113), and the immunostaining with the SP antibody was completely blocked by absorption with 10µg/ml SP (Eugene Tech. International, Catalogue Nº NT106). In addition, in our material the patterns of immunostaining with Enk, NT and SP antibodies were consistent with those described in previous studies (Finley et al., 1981; Gall et al., 1981; Roberts et al., 1984; Petrusz et al., 1985; Borhegyi and Leranth, 1997). These observations, together with the results of the control experiments, make it unlikely that our findings would be reflecting non-specific immunolabelling. However, with the immunohistochemical approach it is difficult to exclude the possibility that an antiserum raised against a given peptide might also react with other structurally related peptides.

Identification of neurochemicals in neurons projecting to the RSg

The present report describes the localization of neurotransmitter –related molecules in the projection neurons from the subicular complex to the RSg. To the best of our knowledge, this study is the first to map a variety of neurochemicals in identified projection neurons. The essential features concerning each neurochemical are described and discussed below.

Amino acids in identified neurons projecting to the RSg from the subicular complex

Here we show that most of the cell bodies of the neurons projecting to the RSg from the presubiculum and subiculum contain immunohistochemically detectable levels of Glu. There is also previous evidence of the presence of Glu in the cell bodies of the hippocampus, including subicular pyramidal cells (Storm-Mathisen and Ottersen, 1983; Storm-Mathisen et al., 1983; Ottersen and Storm-Mathisen, 1984; Ottersen et al., 1990; Ottersen, 1991). Furthermore, previous immunocytochemical studies have reported that projection neurons from the hippocampus use excitatory amino acids as transmitters (Cotman and Nadler, 1981; Collingridge et al., 1983; Wierasko ,1983; Nadler et al., 1990; Gundersen et al., 1991; Fleck et al., 1993). Despite this, none of those studies provided documentation on putative glutamatergic projections from the subicular complex to the RSg. Since Glu has been proposed to be the major excitatory transmitter in the CNS, including the cerebral cortex (Krnjevic, 1974; Fonnum, 1984; Dori et al., 1989; Fleck et al., 1993; Riedel et al., 1995; Riedel and Reymann, 1996), the present findings strongly support the notion that the RSg may receive excitatory inputs from Glu-immunoreactive neurons of the subicular complex. The presence of Glu in the terminals of identified subiculo-cortical axons in the RSg, however, would be a more reliable indicator.

The results of the present study also support the concept that the projections from the subicular complex to the RSg appear to be organized topographically. The anterior regions of the dorsal subiculum and presubiculum project to anterior parts of the RSg, whereas the posteroventral portion of the subiculum projects to the posterior region of the RSg. This observation is consistent with the findings of earlier studies, which also emphasized the differential distribution of afferents to the RSg from the subiculum and presubiculum (Meibach and Siegel., 1977; Sorensen, 1980; Finch et al., 1984; Witter et al., 1989, 1990; Van Groen and Wyss, 1990; Wyss and Van Groen, 1992; Amaral and Witter, 1995). Furthermore, although the highest proportion of double-labelled neurons was found in the presubiculum, moderate numbers of Glu-immunoreactive cells in the subiculum were also labelled by restricted RSg injections. These findings confirm and extend the results of previous studies that demonstrate low-to-moderate projections from subiculum to the cingulate cortex as compared to the significantly denser projections from the presubiculum (Meibach and Siegel, 1977; Sorensen, 1980; Finch et al., 1984; Witter et al., 1989, 1990; Van Groen and Wyss, 1990; Wyss and Van Groen, 1992; Amaral and Witter, 1995).

It is known from previous studies that the RSg projects to and receives a significant putative glutamatergic projections from the anterior thalamic nuclei (ATN) (Gonzalo-Ruiz et al., 1997a,b, 1998; Wang et al., 1999), and several lines of evidence indicate that the termination of afferent fibres from the ATN, like those from subicular projections to the RSg, are highly laminar.

Subiculo-retrosplenial projections mainly terminate in layers II and III, with a modest innervation of layer IV (Finch et al., 1984), whereas afferent fibres from the ATN terminate most heavily in layer I of the RSg and, to a lesser extent, in layer IV (Domesick, 1972; Berger et al., 1980; Vogt et al., 1981). The significance of this highly selective laminar distribution in the RSg is unknown. However, this anatomical organization is ideally suited for the integration of putative glutamatergic inputs from the ATN (Gonzalo-Ruiz et al., 1997a,b, 1998) and from the subicular complex (present findings) by retrosplenial pyramidal neurons. This suggests that projections to the posterior cingulate cortex from the ATN and from the subicular complex establish excitatory synaptic contact with different regions of the apical dendrites of the same pyramidal neurons, and that a spatial summation integrates the input from both sources.

We also show here that cell bodies of the subicular complex contain GABA, in keeping with previous evidence that subicular neurons contain GAD/GABA (Mugnaini and Oertel, 1985; Ottersen and Storm-Mathisen, 1984). Because CT-HRP-containing fibres or terminal-like structures were present in the neuropil and overlaid the soma and proximal dendrites of GABA-immunoreactive neurons of the presubiculum, we do not know whether the cell bodies of neurons projecting from the subicular complex to the RSg might contain GABA. It is unfortunate that limitations inherent to the technique make it difficult to confirm the presence of such cells projecting to the RSg. Further work will be necessary to demonstrate the existence of a GABAergic projection to the RSg.

Neuropeptides in neurons projecting to the RSg from the subicular complex

We have also shown that a significant number of neurons projecting to the RSg from the subiculum contain NT, whereas moderate-to-small numbers of neurons projecting to the RSg from the subiculum and presubiculum contain Enk and SP, in keeping with previous studies reporting that a population of neurons within the subicular complex display NT–, SP–, or Enk-like immunoreactivity (Finley et al., 1981; Gall et al., 1981; Roberts et al., 1984; Petrusz et al., 1985; Borhegyi and Leranth, 1997). Furthermore, previous studies have also demonstrated peptidergic projections from the hippocampus and subiculum to the cortex (Roberts et al., 1981, 1982; Vincent et al., 1981). Thus, the present findings indicate that NT–, SP–, or Enk-immunoreactive neurons in the presubiculum and subiculum may have a neurotransmitter/neuromodulator role in subiculo-retrosplenial projections.

Because our immunoreactions were carried out on different sections, we do not know whether some of the neurotransmitters studied coexist in neurons projecting to the RSg. The colocalization of NT, Enk or SP with classical neurotransmitters (Chan-Palay et al., 1978; Sutin and Jacobowitz, 1988) and with several other neuropeptide transmitters (Erichsen et al., 1982; Pickel et al., 1980) has been found in several nuclei of the CNS. While double/triple-immunocolocalization studies in combination with retrograde tracing are needed to clarify the specific neurotransmitter combination of neurons projecting from the subicular complex to the RSg, the fact that a very large fraction of projecting neurons in the subiculum contain NT suggests that most of these NT-positive neurons must also be immunoreactive for Glu and that Glu and NT might work synergistically in regulating the activity of cortical neurons.

Possible functional implications of the subiculo-retrosplenial projection

Several studies have demonstrated that the retrosplenial cortex contributes to the role of the hippocampus in memory and leaming (Gabriel and Sparenborg, 1986, 1987; Gabriel et al., 1980, 1989; Valenstein et al., 1987; Markowska et al., 1989; Matsunami et al., 1989; Sif et al., 1989). Although much of the research on the participation of neurotransmitter systems in cognitive decline associated with aging and AD has concentrated on the cholinergic hypothesis of geriatric cognitive deficits (Bartus et al., 1982), there is a fair amount of evidence to indicate that several other neurotransmitters, such as Glu (Danysz et al., 1995; Riedel et al., 1995; Riedel and Reymann, 1996; Ungerer et al., 1998) and several neuropeptides (Roberts et al., 1984; Schlesinger et al., 1986, Wenk et al., 1989a,b; Decker and McGaugh, 1991), appear to play a role in memory processes and interact with the cholinergic system. In the light of these findings, we therefore suggest that Glu and several unrelated neuropeptides, such as NT, SP, or Enk, may be utilized as a neurotransmitter(s)/neuromodulator(s) in projections from the subicular complex to the RSg and may thus play a role in cognitive functions.

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