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# Neurochemical organization of projections from nuclei of the pontomesencephalic tegmentum to the retrosplenial granular cortex of the rat

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## Summary

Retrograde labelling was combined with immunohistochemistry to localize neurons containing serotonin, dopamine-β-hydroxylase, choline acetyltransferase, glutamate, leu-enkephalin, neurotensin, and substance P-like immunoreactivity in the projections from nuclei of the mesencephalic tegmentum to the retrosplenial granular cortex in the rat. Injections of horseradish peroxidase conjugated to subunit B of cholera toxin (CT-HRP) into the retrosplenial granular cortex resulted in large numbers of CT-HRP-labelled neurons in the dorsal raphe nucleus and in the locus coeruleus, whereas small numbers were found in the median raphe nucleus. In addition, following injections into the retrosplenial cortex which also involved the adjacent anterior cingulate cortex, small numbers of CT-HRP-labelled neurons were found in the laterodorsal tegmental nucleus. In the dorsal and median raphe nuclei, most of the CT-HRP-labelled neurons (80-85% in the dorsal and 90-95% in the median raphe nuclei) were immunoreactive for serotonin and small numbers (1 to 4-5 per section) of retrogradely labelled neurons were immunoreactive for glutamate, neurotensin, leu-enkephalin or substance P. In the locus coeruleus, 90-95% of the CT-HRP labelled neurons were immunoreactive for dopamine-β-hydroxylase, whereas a few neurons (1-3 per section) also displayed neurotensin —and substance P— immunoreactivity. In the laterodorsal tegmental nucleus, most of the CT-HRP-labelled neurons contained choline acetyltransferase and small numbers were immunoreactive for substance P. These results suggest that serotonergic and noradrenergic fibres represent the main ascending projections from the mesencephalic tegmentum to the retrosplenial granular cortex.

**Key Words**: Raphe nuclei – Locus coeruleus – Laterodorsal tegmental nucleus – Serotonin – Dopamine-β-hydroxylase – Choline acetyltransferase – Amino acids – Neuropeptides

## Introduction

The retrosplenial cortex (caudal cingulate cortex) has been recognized as a nodal point for the transfer of information between the hippocampal formation, many neocortex regions, and the thalamus (Vogt et al., 1986; Sripanidkulchai and Wyss, 1986, 1987; Thompson and Robertson, 1987; Van Groen and Wyss 1990; Wyss and Van Groen, 1992; Shibata, 1993). In addition to the well- known thalamocortical and corticothalamic projections, in recent years several neurochemically identifiable afferents entering into the cerebral cortex, including the retrosplenial granular cortex (RSg), have been described. Thus, several lines of evidence indicate that cholinergic input arises from the basal forebrain (Mesulam et al., 1983a; Eckenstein et al., 1988; Gonzalo-Ruiz and Morte, 2000), a putative glutamatergic projection from the thalamus (Gigg et al., 1992; Gonzalo-Ruiz et al., 1997a,b; Wang et al., 1999) and from the basal forebrain (Gritti et al., 1997; Gonzalo-Ruiz and Morte, 2000), and an extrinsic GABAer-

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gic input, also from the basal forebrain (Fisher et al., 1988; Gritti et al., 1997; Gonzalo-Ruiz and Morte, 2000). Recent studies have also shown that the RSg is intimately involved in higher cognitive functions such as learning and memory (Gabriel et al., 1983; Gabriel and Sparenborg, 1987; Valenstein et al., 1987; Gabriel, 1993; Sutherland and Hoesing, 1993). Since the cholinergic (Drachman and Leavit, 1974; Bartus et al., 1982; Wenk et al., 1984) and the glutamatergic (Danysz et al., 1995; Riedel et al., 1995; Riedel and Reymann, 1996; Ungerer et al., 1998) systems have been implicated in these cognitive functions, the cholinergic and glutamatergic inputs to the cerebral cortex, including the RSg, have received much attention (Mesulam et al., 1983a; Woolf et al., 1983; Senut et al., 1989; Gigg et al., 1992; Gage et al., 1994; Gonzalo-Ruiz et al., 1997a,b; Wang et al., 1999). However, despite this wealth of information there are still significant gaps in our knowledge of the role of the projections to the RSg and their chemical anatomy, especially in relation to the inputs from the pontomesencephalic tegmentum, such as from the dorsal (DR) and median raphe nuclei (MnR), from the locus coeruleus (LC) and from the laterodorsal tegmental nucleus (LDTg).

Immunohistochemical studies have shown that the DR and MnR neurons contain indoleamine 5-hydroxytryptamine (serotonin, 5-HT; Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983), gamma-aminobutyric acid (GABA; Nanapoulos et al., 1982; Belin et al., 1983), glutamate (Glu; Storm-Mathisen and Ottersen, 1983; Ottersen and Storm-Mathisen, 1984), glutaminase (Kaneko et al., 1989) and several peptides, such as substance P (SP; Chan-Palay et al., 1978; Ljungdahl et al., 1978; Sutin and Jacobowitz, 1988), neurotensin (NT; Emson et al., 1985) and enkephalin (Enk, Petrusz et al., 1985), whereas neuronal somata in the LC contain noradrenalin (Dahlström and Fuxe, 1964; Fuxe, 1965), Glu (Ottersen and Storm-Mathisen, 1984), SP (Ljungdahl et al., 1978; Sutin and Jacobowitz, 1988), NT (Uhl et al., 1979; Emson et al., 1985), Enk (Finley et al., 1981; Petrusz et al., 1985) and neuropeptide Y (Sutin and Jacobowitz, 1988). Furthermore, there is strong evidence to suggest that the LDTg contains many cholinergic neurons (Armstrong et al., 1983; Satoh and Fibiger, 1986) and several neuropeptides (Sutin and Jacobowitz, 1988). However, the precise contributions of these immunoreactivities to projections from the mesencephalic tegmentum to the RSg remain to be determined. Thus, the present study was carried out to define the transmitter-related characteristics of the main ascending projections from nuclei of the pontomesencephalic tegmentum to the RSg. A preliminary report of the major findings has already appeared in abstract form (Gonzalo-Ruiz and Sanz, 1999).

## Materials and Methods

### *Experimental animals and anaesthesia*

Eight Wistar albino rats of both sexes (250-300 g body weight) were used and were kept under standard laboratory conditions (20°C ambient temperature, 12 h light/dark cycle, tap water and regular rat chow ad libitum). The animals were anaesthetized with Nembutal (45mg/kg, injected intraperitoneally) for the surgical procedure (injection of tracer). 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. In all respects, the animals were housed and handled according to national legislation and the guidelines approved by the Animal Care Committee of the University of Valladolid, which conform to or are even more demanding than EU Directive 86/609.

### *Tracer injection*

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 (10µl) containing HRP conjugated to subunit B of cholera toxin (CT-HRP; List Biological Laboratories, California) was positioned in different parts of the left RSg using stereotaxic coordinates 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 minutes period, following which the needle was left in place for a further 10 minutes before being withdrawn. The scalp was satured and the animal was allowed to recover from the anaesthetic.

### *Fixation*

Following postinjection survival periods of 1-2 days, the rats were reanaesthetized and killed by transcardiac perfusion of 60 ml of 0.9% saline at 20°C containing heparin (1.000 IU) to flush blood from vascular system, followed by ca 500 ml of fixative solution containing 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 (also perfused at 20°C).

### *Histochemical and immunohistochemical procedures*

Immediately after perfusion, the brain was removed, trimmed to a block of convinient size incorporating the RSg and the mesencephalic tegmentum, and sectioned on the coronal plane at 50 µm using a Vibratome. The sections were collected as eight series. 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 bright-field and dark-field illumination. The other seven series 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 phosphate buffer, pH 7.2 (PB) containing 0.005% cobalt acetate/nickel chloride (this chromogen produced a black granular reaction product). After washing in several changes of PB, the sections in these seven series were processed for the immunohistochemical localization of ChAT, 5-HT, DβH, Glu, NT, Enk, and SP respectively. Sections processed for ChAT were first immersed for 1 h in 10% normal rabbit serum (NRS) whereas the series of sections processed for 5-HT, DβH, Glu, NT, Enk and SP 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. Sections processed for the immunohistochemical localization of ChAT were then incubated in a solution containing a rat monoclonal antibody (1:4 dilution; Boehringer-Mannheim) for 18-24 h at 4°C, while those for 5-HT, DβH, Glu, NT, Enk and SP were incubated in a solution containing a polyclonal rabbit antobody against 5-HT (1:5000 dilution; Eugene Tech. Inter), DβH (1:500 dilution; Protos Biotech Corporation), Glu (1:1000 dilution; Arnel), NT (1:2000 dilution; Eugene Tech. Inter), Enk (1:5000 dilution; INC-STAR), or SP (1:2000 dilution; Eugene Tech. Inter) for 18-24 h at 4°C. After incubation in the primary antibody, sections were washed in either 1% NRS (for ChAT) or in 1% NGS (for 5-HT, DβH, Glu, NT, Enk or SP) and then incubated in biotinylated anti-rat IgG (Vector; 1:100 in PBS with 1% NRS) for ChAT immunocytochemistry and in biotinylated goat anti-rabbit IgG (Vector; 1:200 in PBS with 1% NGS) for the immunohistochemical localization of 5-HT, DβH, Glu, NT, Enk and SP. Sections were then washed in PBS and immersed in an avidin-biotin-HRP complex (Vector 1:100) for 60 min. Immunoreaction product was demonstrated using 0.005% DAB and 0.01% hydrogen peroxide in PB (this chromogen produced 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 bright-field illumination at both low and high magnification.

As a control, some sections from each series were incubated as described above but without the addition of primary antibody or after replacing the rat primary antibody with normal rat serum and the rabbit primary antibody with NRS. There was a complete absence of 5-HT, DβH, ChAT, Glu, NT, Enk or SP-immunoreactive neurons or neuropil in such control sections.

*Analysis of material and presentation of results*

In each of the seven series, all of the sections through the mesencephalic tegmentum (each separated by approximately 350 μm) were examined systematically. The specificity of the immunoreaction was checked by comparing sections stained either with 5-HT, DβH, ChAT, Glu, NT, EnK or SP antiserum and control material respectively. Structures immunostained by antibodies but not seen in the control slides were considered to be specifically immunolabelled and are designated here as 5-HT-, DβH-, ChAT-, Glu-, NT-, Enk-, or SP-immunoreactive. All CT-HRP single-labelled cell bodies and all double-labelled cell bodies (CT-HRP/5-HT, CT-HRP-DβH, CT-HRP/ChAT, CT-HRP/Glu, CT-HRP/NT, CT-HRP/Enk, CT-HRP/SP) were identified, coded and mapped, using a camera lucida, onto drawings of frontal, cresyl violet-stained sections of the basal forebrain, derived from the atlas of Paxinos and Watson (1986). In sections reacted with DAB, relatively few CT-HRP-labelled cells (200-225 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 were represented on schematic drawings using different types of symbols (Fig. 2B-D).

## Results

*Localization and extent of CT-HRP injections*

According to the terminology and the 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., Figs.1A,2A). The findings in this study are based on eight animals 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-F and Fig. 2A. In five of these animals the injections involved layers I-VI of either the rostral (Figs.1A, 2A) or the caudal RSg (Fig.1B, D-F). In no case did the spread of CT-HRP to other regions occur except for a small amount, which spread to the adjacent anterior cingulate cortex (Figs.1A, 2A). In the other three animals, the injections were centred on deep layers (V-VI) and spread to the superficial layers (I-III) of either the rostral or the caudal RSg (e.g., Fig.1C). The localization and extent of CT-HRP injections in the RSg was confirmed by the distribution of retrogradely labelled thalamo-cortical and cortico-cortical afferent neurons as established in previous studies (Sripanidkulchai and Wyss, 1986; 1987; Vogt et al., 1986; Van Groen and Wyss, 1990; Shibata, 1993; Gonzalo-Ruiz et al., 1997a,b).

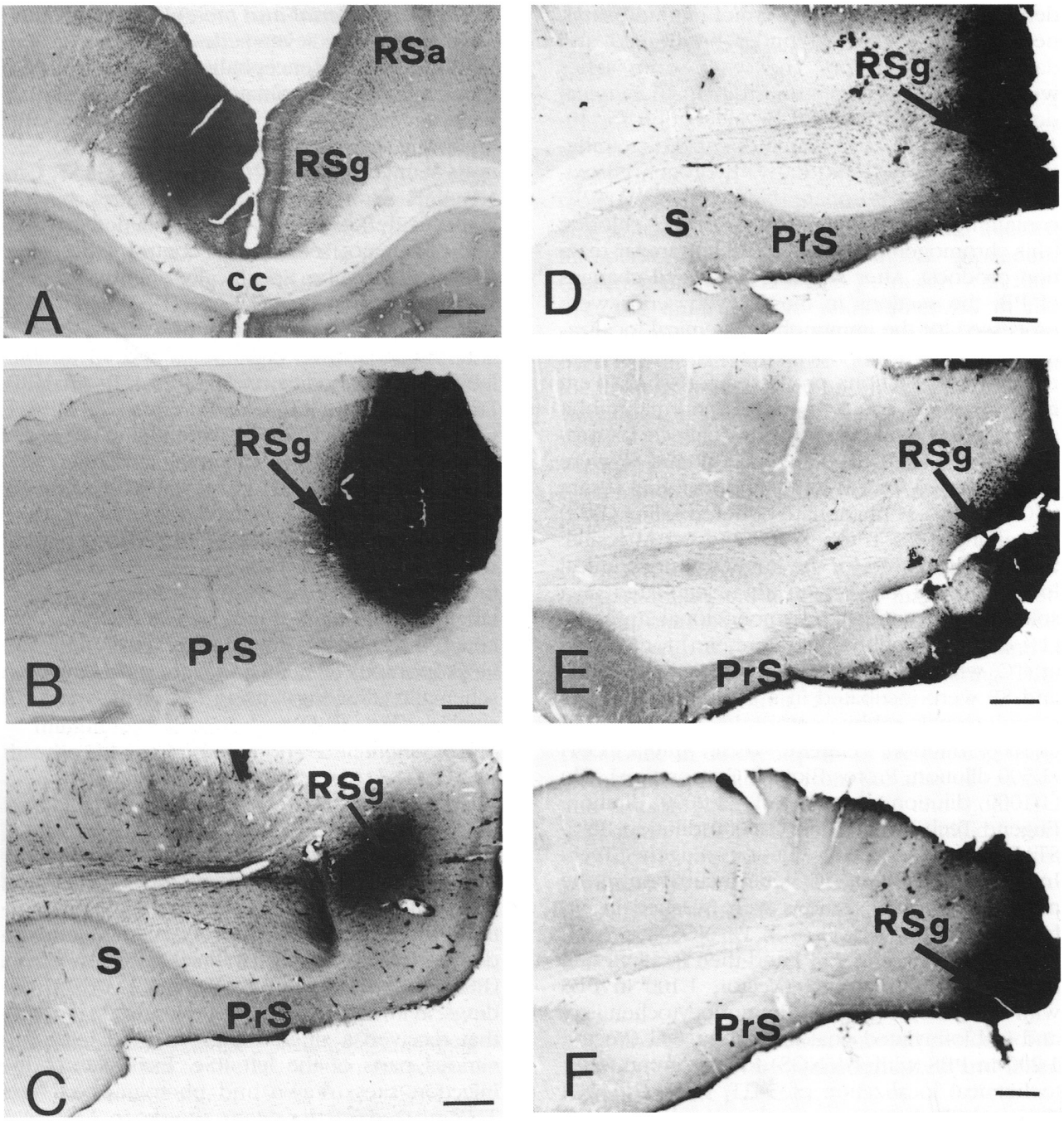


**Fig. 1.- A-F:** Low-magnification photomicrograph of coronal sections through the RSg from TMB-reacted sections, showing representative injections of CT-HRP centred on the RSg (arrows).Scale bars= 200 μm (A-F).

*General observations*

The distribution of 5-HT, DβH, ChAT, Glu, Enk, NT and SP-immunoreactive neurons in the various nuclei of the pontomesencephalic tegmentum analyzed was similar in all animals (e.g., Figs.3A,D, 4A,D, 5A,C, 6A, 7A,C, 8A,C) and largely in agreement with previous descriptions (Dahlström and Fuxe, 1964; Ljungdahl et al., 1978; Armstrong et al., 1983; Finley et al., 1981; Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983; Storm-Mathisen and Ottersen, 1983; Storm-Mathisen et al., 1983; Ottersen and Storm-Mathisen, 1984; Emson et al., 1985; Petrusz et al., 1985; Sutin and Jacobowitz, 1988). However, the distribution and the number of retrogradely labelled cells and of double-labelled cells differed according to both the size of the CT-HRP injection and in relation to 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 than those processed using DAB as the chromogen (e.g. compare Fig.2E-G with Figs. 3A,D, 4A, 7A). In sections processed sequentially for the visualization of CT-HRP and 5-HT, Glu, Enk, NT, SP, DβH or ChAT, double-labelled neurons were identified by the distribution, throughout their somata and proximal den-

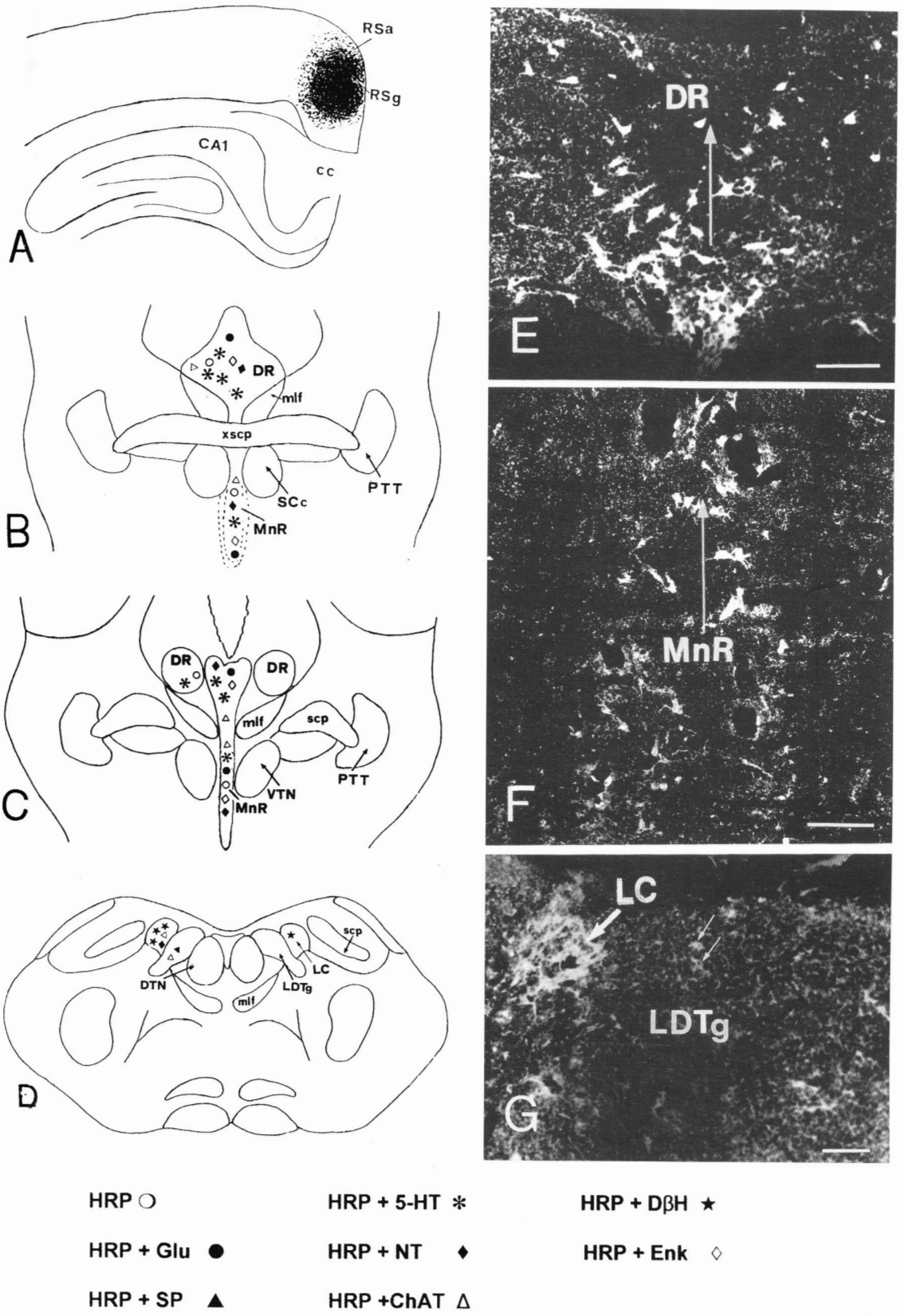


**Fig. 2.-** **A:** Schematic drawing from a section processed with TMB as chromogen showing the location and size of the centre of the CT-HRP injection into the RSg. **B-D:** Schematic summary of the distribution and relative number of retrogradely labelled 5-HT-, DβH-, ChAT-, Glu-, NT-, Enk- or SP-immunoreactive neurons projecting to the RSg, following the injection shown in Figs. 1A, 2A. The key to the symbols is on the figure and the key to the lettering is in the list of abbreviations. The number of cells that each symbol represents can be summarized as follows: CT-HRP/5-HT, CT-HRP/DβH: 8-10 cells; CT-HRP/ChAT, CT-HRP/Glu, CT-HRP/NT, CT-HRP/Enk, CT-HRP/SP: 1-5 cells. **E-G:** Low-magnification photomicrographs of coronal sections through the DR (E), the MnR (F) and LC (G), showing retrogradely labelled cells following the injections shown in Figs. 1A, 2A (straight white arrows in E and F indicate the midline). Scale bars= E,F: 100 μm; G: 200 μm.

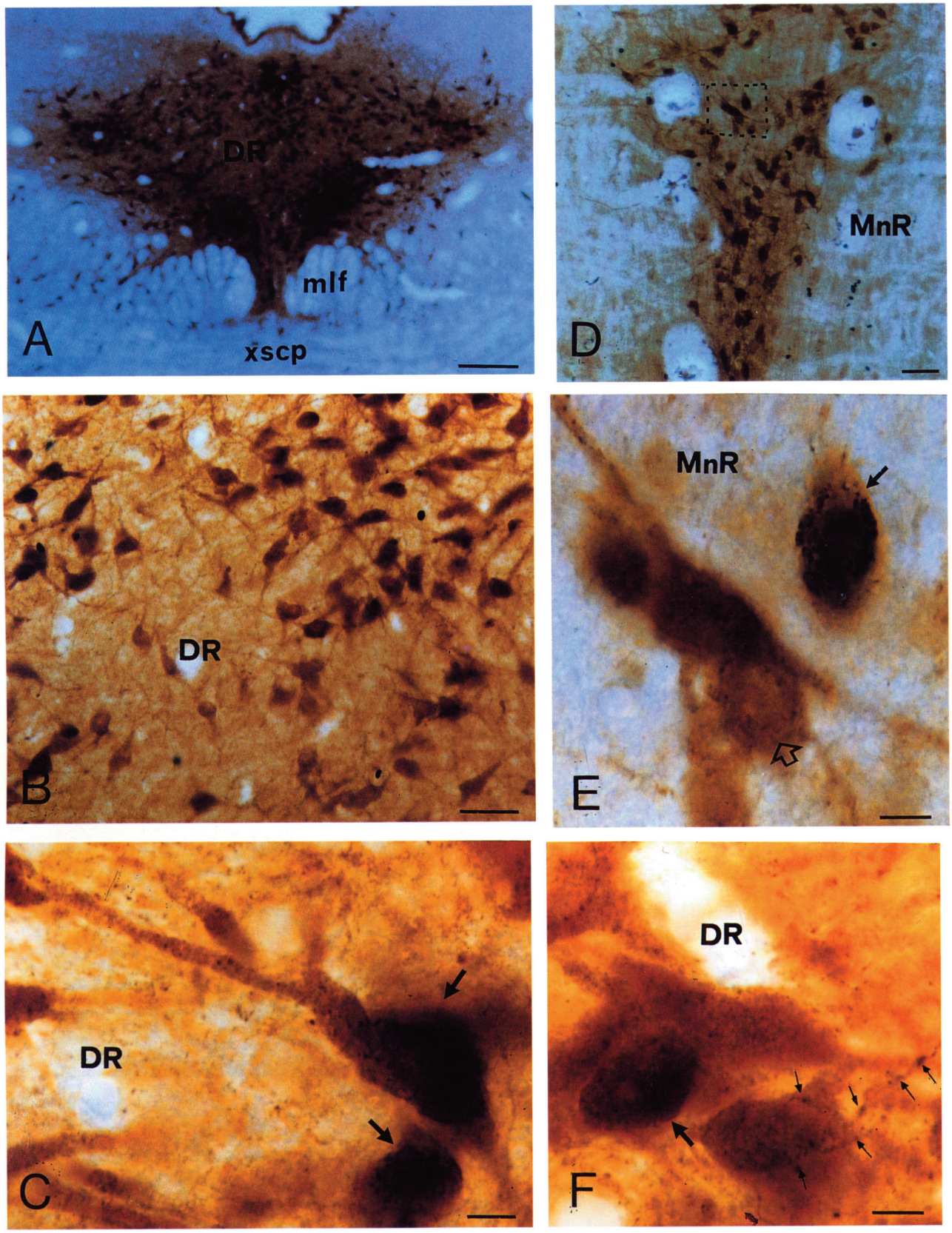


**Fig. 3.-** **A:** Photomicrograph through the DR following the injection shown in Figs. 1A, 2A. The section (level B of Fig. 2) was processed for CT-HRP and 5-HT. **B:** High magnification photomicrograph of part of Fig. 3A. Differences between double- and single-labelled neurons are not detectable at this magnification. **C:** High magnification of double-labelled neurons (CT-HRP/5-HT) in the DR (arrows). **D:** Photomicrograph through the MnR following the injection shown in Figs. 1A, 2A. The section (level B of Fig. 2) was processed for CT-HRP and 5-HT. The boxed area is enlarged in E. **E:** High magnification of boxed area in E, showing a double-labelled neuron (solid arrow) and a 5-HT-single-labelled neuron (open arrow). **F:** High magnification micrograph through another part of the DR following the injection shown in Figs. 1A, 2A. The field shows 5-HT-single-labelled neuron (large arrow) and CT-HRP anterogradely labelled fibres and terminal-like-structures in the neuropil (small arrows). Scale bars= A: 100 µm; B,D: 50 µm; C,E,F: 10 µm.

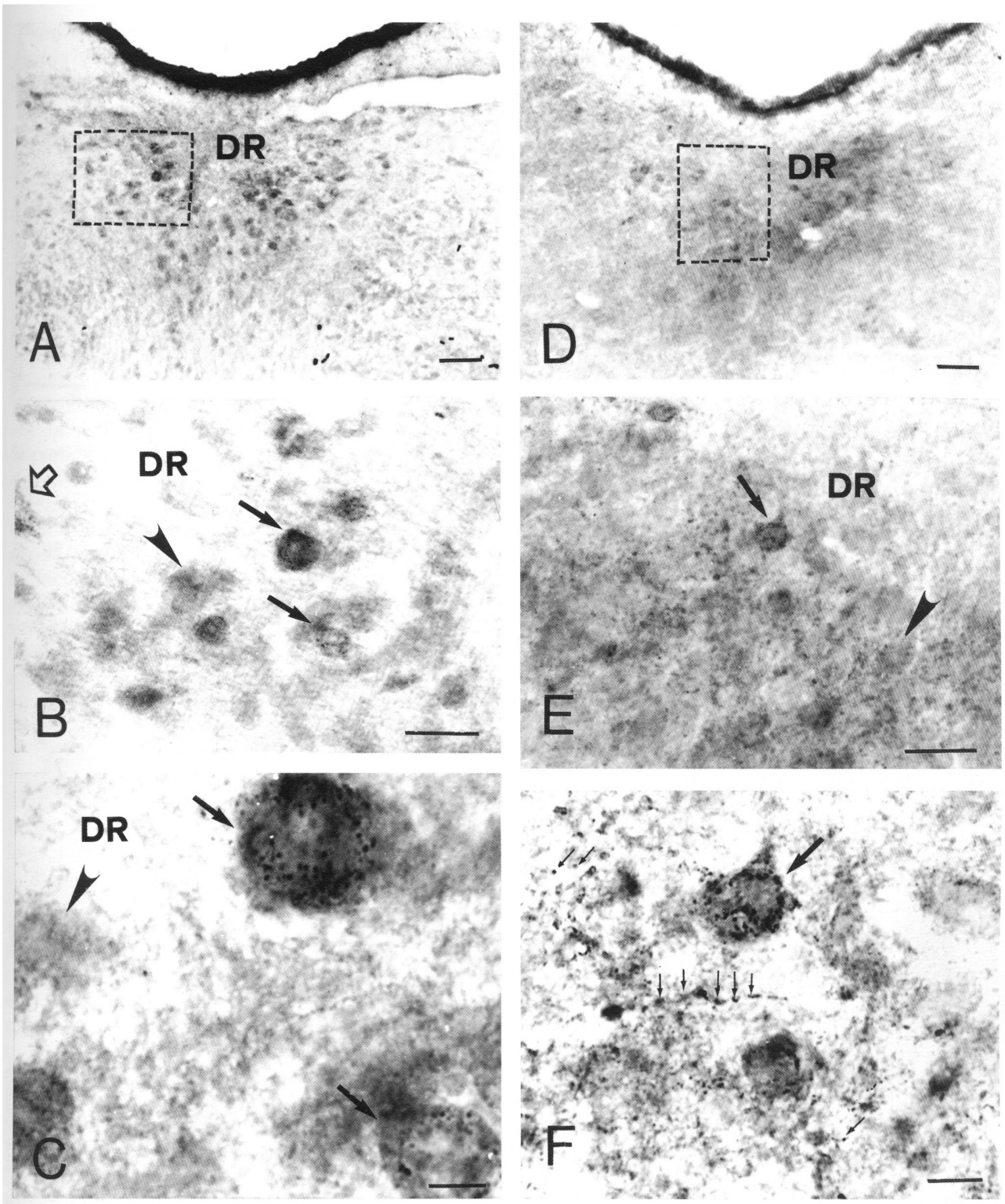


**Fig. 4.-** **A**: Photomicrograph through the DR following the injection shown in Figs. 1A, 2A. The section (level C of Fig. 2) was processed for the visualization of CT-HRP and Glu. Differences between double- and single-labelled neurons are not detectable at this magnification. The boxed area is enlarged in B. **B:** High magnification of boxed area in A, showing double-labelled neurons (solid arrows), a CT-HRP-single-labelled neuron (open arrow) and a Glu-single-labelled neuron (arrowhead). **C:** High magnification of the double- (arrows) and single-labelled neuron (arrowhead) indicated in Fig. 4B. **D:** Low-magnification photomicrograph through the DR following the injection shown in Figs. 1A, 2A. The section (level C of Fig. 2) was processed for CT-HRP and Enk. The boxed area is enlarged in E. **E:** Higher magnification of boxed area in D, showing a double-labelled neuron (solid arrow) and a Enk-single-labelled neuron (arrowhead). **F:** High magnification of the double-labelled neuron indicated by arrow in E. The field also shows terminal-like structures (small arrows). Scale bars= A,D: 100 μm; B,E: 50 μm; E,F: 10 μm.

drites, of distinct blue/black granules of CT-HRP reaction product superimposed over a diffuse light brown immunoreaction product (e.g., Figs. 3C,E, 7C, 8B,E). Single retrogradely labelled neurons displayed only blue/black granular deposits of transported CT-HRP (e.g., Fig.4B) and immunoreactive neurons devoid of CT-HRP labelling were identified by a diffuse light-brown immunoreaction product throughout their somata and dendrites (Figs. 3E, 7B).

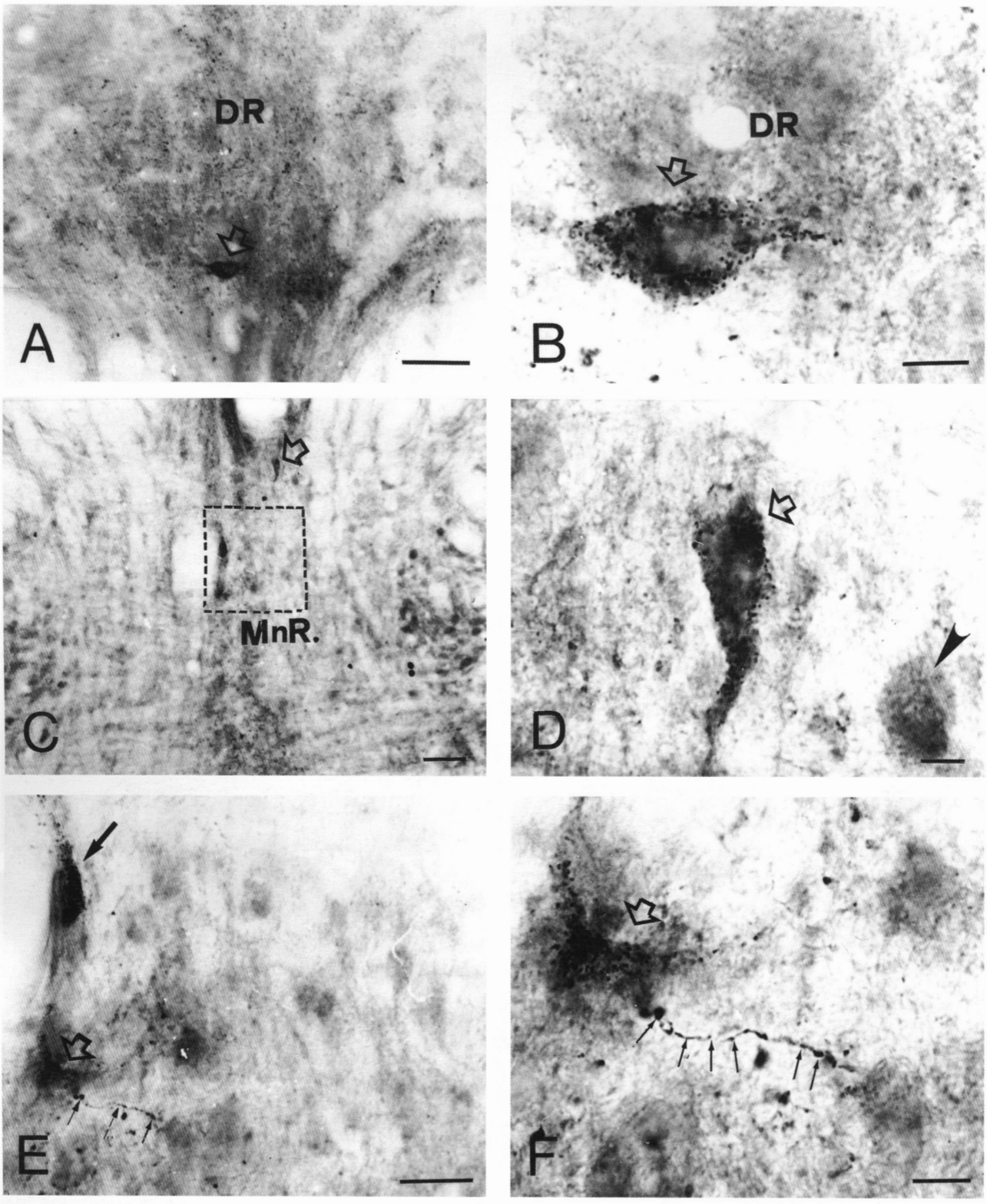


**Fig. 5.-** **A:** Low-magnification photomicrograph through the DR following the injection shown in Figs. 1A, 2A. The section was processed for the visualization of CT-HRP and NT. The cell indicated by open arrow is shown in B. **B:** High magnification of the double-labelled neuron indicated by open arrow in A. **C:** Photomicrograph through the MnR following the injection shown in Figs. 1A, 2A. The section was processed for the visualization of the CT-HRP and NT. The neuron indicated by open arrow is shown in D and the boxed area is enlarged in E. **D:** High magnification of the double-labelled neuron indicated by open arrow in C. The field also shows a NT-single-labelled neuron (arrowhead). **E:** Higher magnification of boxed area in C, showing double-labelled neurons (solid and open arrow) and terminal-like structures (small arrows). **F:** High magnification of the double-labelled neurons indicated by the open arrow in E. The field also shows anterogradely labelled fibres and terminal-like structures (small arrows). Scale bars= A,C: 100 μm; E: 50 μm; B,D,F: 10 μm.

*Transmitter-related immunoreactivity in identified projection neurons from the mesencephalic tegmentum to the retrosplenial granular cortex*

In all animals, injections in the RSg (Fig. 1) resulted in massive retrograde neuronal labelling in different nuclei of the pontomesencephalic tegmentum. The largest numbers of retrogradely labelled cells were found in DR and the LC (Fig. 2E,G). Numerous labelled neurons were also found in MnR (Fig. 2F) and fewer of them were found in the LDTg (Fig. 2G). The injections also resulted in the retrograde transport of CT-HRP in the dorsal tegmental nucleus of Gudden, and in other brain areas such as the basal forebrain and anterior thalamic nuclei (not illustrated). This pattern of projections to the RSg was largely consistent with previous descriptions (Saper, 1982; Sripanidkulchai and Wyss, 1986; 1987; Thompson and Robertson, 1987; Van Groen and Wyss, 1990; Vertes, 1991; Vertes et al., 1999). However, the transmitter-related characteristics of the *main ascending projections* from the mesencephalic tegmentum, such as from the raphe nuclei, LC and LDTg to the RSg have received comparatively little attention in previous studies. Thus, in this study our attention will focus on the neurotransmitter(s)/neuromodulator(s) associated with the projections from the nuclei of the pontomesencephalic tegmentum to the RSg.

*Projections from raphe nuclei*

In all animals, injections of CT-HRP confined to the RSg (e.g., Figs. 1A-F and 2A) produced large numbers of retrogradely labelled neurons in the DR and moderate to small numbers in the MnR. *In the DR*, approximately 80-85% of CT-HRP-labelled neurons were also 5-HT-immunoreactive (Figs. 2B,C, 3A,B). However, up to 20% of neurons projecting to the RSg were not immunoreactive for 5-HT and these neurons (1 to 4-5 per section) were codistributed with 5-HT-immunoreactive projection cells in all parts of the DR (Fig. 2B,C). In addition, a few retrogradely labelled neurons in the DR (1 to 4-5 per section) were also immunoreactive for Glu, Enk, NT or SP (Figs.2B,C, 4A,B,D,E, 5A, 6A). In DR, most of the double-labelled and and CT-HRP-labelled neurons were located in its ventromedial and ventrolateral regions (Figs. 2B,C, 3A,B), particularly in the area surrounding the dorsal aspect of the medial longitudinal fasciculus (Figs. 2B,3B). A few CT-HRP-labelled and double-labelled cells were also scattered in the dorsomedial and dorsolateral regions of the nucleus (Figs. 2B,C, 4A,B,D,E). Although 80-90% of the double-labelled and CT-HRP-labelled neurons were found in the DR ipsilateral to the injection site (Figs. 2B,C,E, 3A,B), a moderate to small number (10-20%) of neurons projecting to the RSg was also observed bilaterally in the ventromedial and dorsomedial parts of the nucleus (Figs. 2E, 4A,D). In

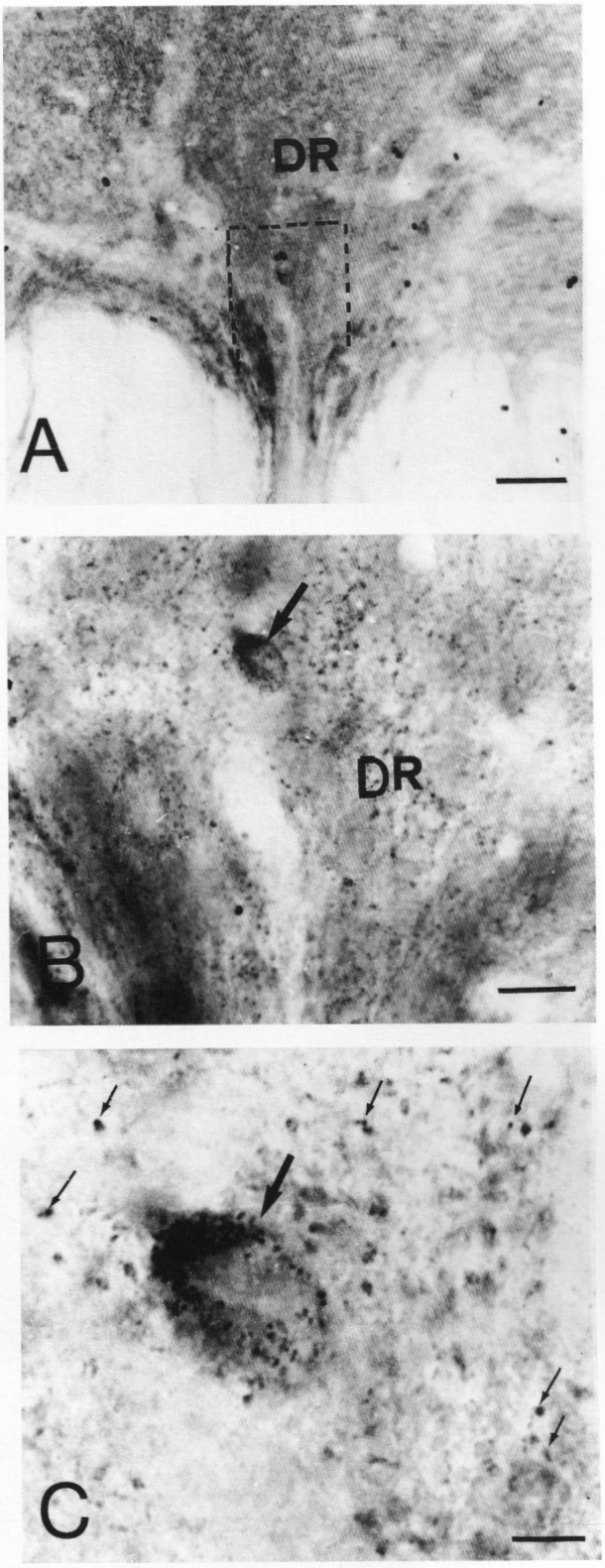


**Fig. 6.-** **A:** Low-magnification photomicrograph through the DR following the injection shown in Figs. 1A, 2A. The section was processed for CT-HRP and SP. The boxed area is enlarged in B. **B:** High magnification of boxed area in A, showing a double-labelled neuron (arrow). **C:** High magnification of the double-labelled neuron indicated by the arrow in B. The field also shows terminal-like structures (small arrows). Scale bars= A: 100 μm; B: 50 μm; C: 10 μm.

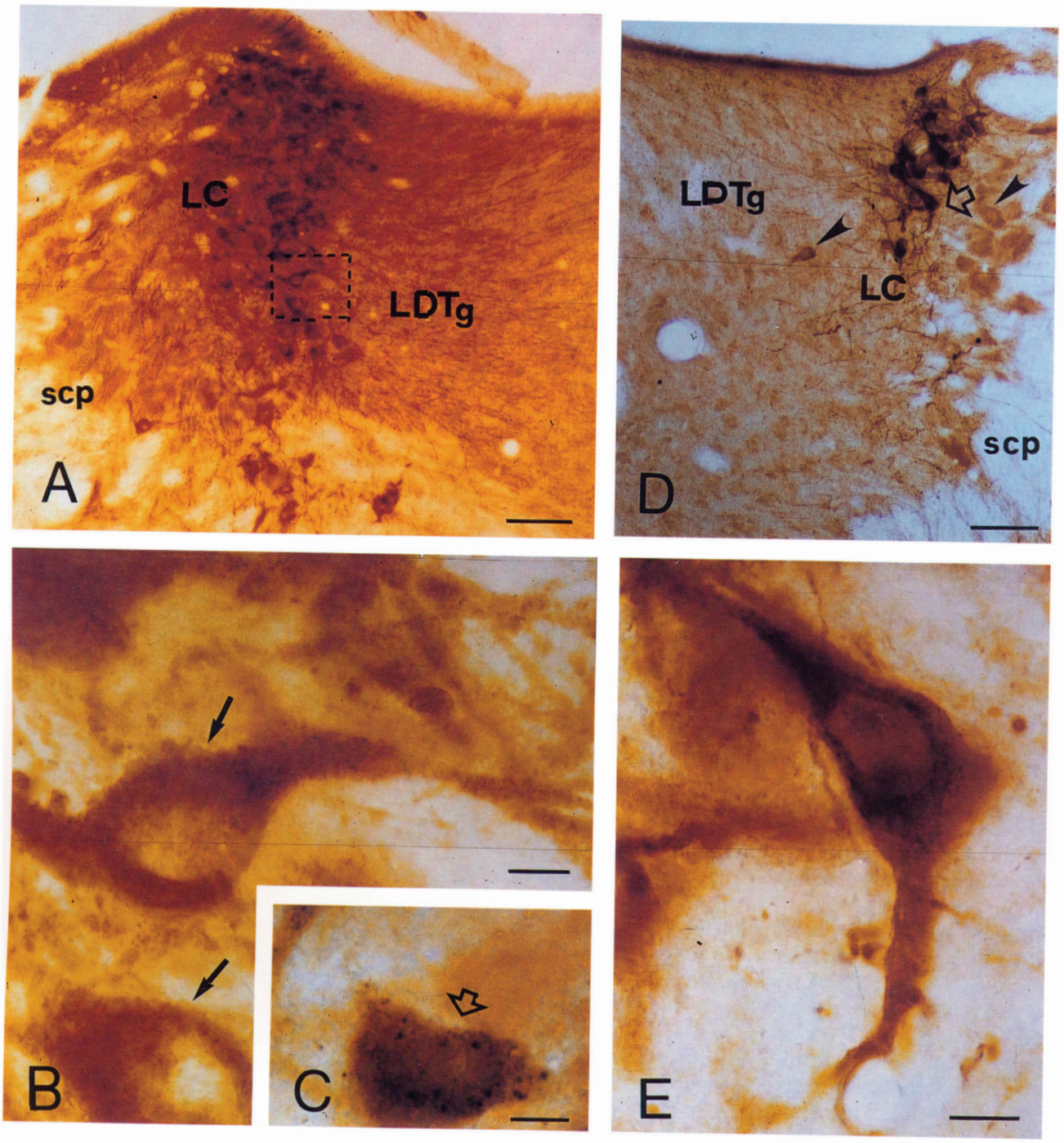


**Fig. 7.-** **A:** Photomicrograph through the ipsilateral LC following the injection shown in Figs. 1A, 2A. The section (level D of Fig. 2) was processed for CT-HRP and DβH. Differences between double- and single-labelled neuron are not detectable at this magnification. The boxed area is enlarged in B. **B:** Higher magnification of boxed area in A, showing DβH-single-labelled neurons (arrows). **C:** High magnification photomicrograph of another part of A, showing a double-labelled neuron (open arrow). **D:** Photomicrograph through the contralateral LC following the injections shown in Figs. 1A, 2A. The section (level D of Fig. 2) was processed for CT-HRP and DβH. Note DβH-single-labelled neurons (arrowheads). The double-labelled neuron indicated by the open arrow is enlarged in E. **E:** High magnification of the neuron indicated by the open arrow in D. Scale bars= A,D: 100 μm; B,C,E: 10 μm.

the DR, CT-HRP-labelled and double-labelled neurons were scattered throughout the rostro-caudal extent of the nucleus. However, they were concentrated predominantly in the rostral two-thirds of the nucleus (Figs. 2B,C, 3A,B). Retrogradely labelled, double-labelled neurons (HRP/5-HT, HRP/Glu, HRP/SP, HRP/EnK or HRP/NT) and 5-HT+, Glu+, SP+, Enk+, or NT+ single-labelled neurons in the DR were medium-(14-24μm) to relatively large-sized (30-40 μm), with somata that ranged in shape from ovoid to multipolar, often with several dendrites emerging from the soma (Figs. 3C, 4C,F, 5B, 6C). The long axes of these neurons were generally orientated along the coronal plane, parallel to the fibres of the medial longitudinal fasciculus (e.g. Fig. 3B,C).

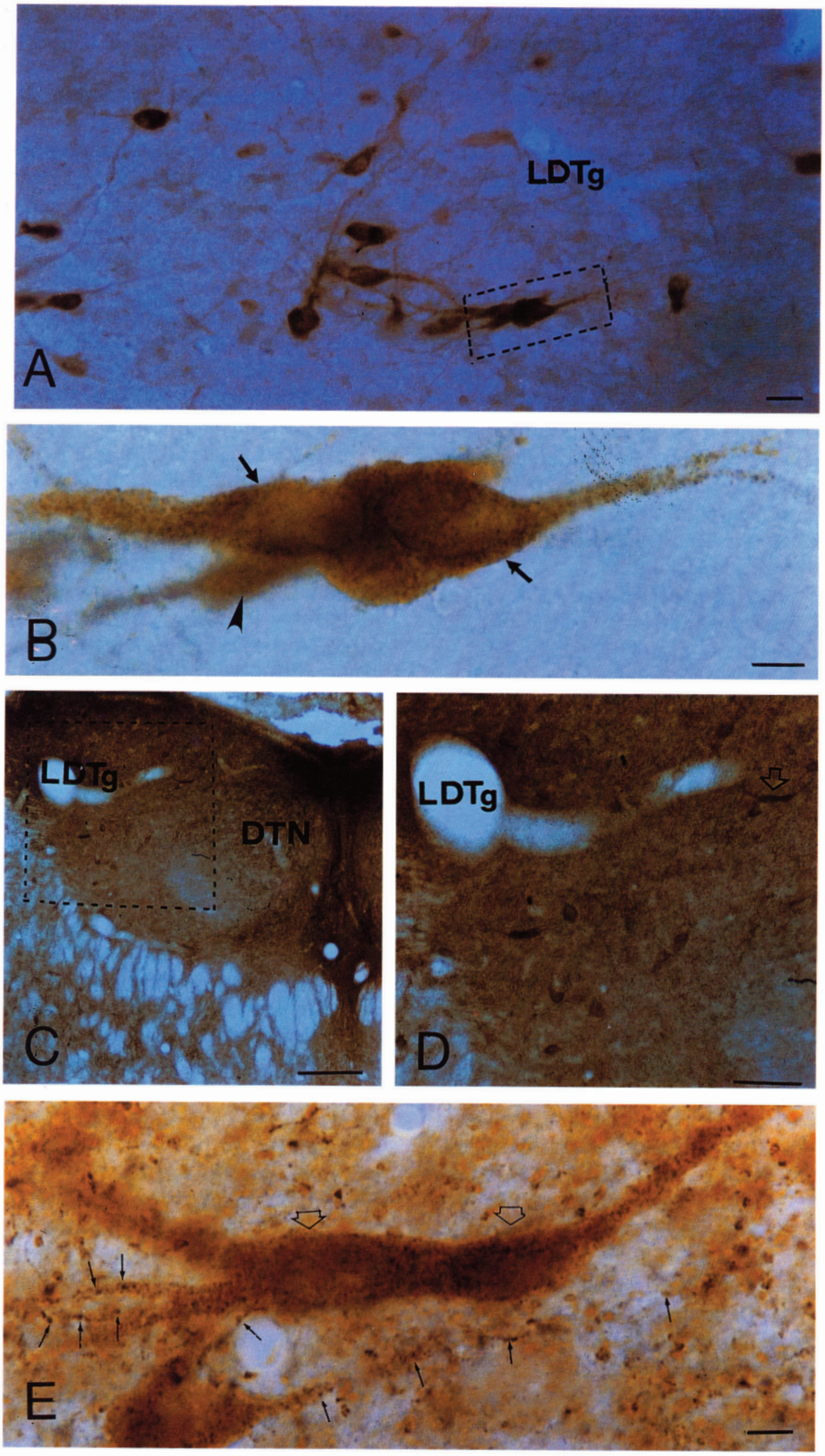


**Fig. 8.-** **A:** Photomicrograph through the caudal part of the LDTg following the injection shown in Figs. 1A, 2A. The section (level D of Fig. 2) was processed for CT-HRP and ChAT. Differences between double- and single-labelled neurons are not detectable at this magnification. The boxed area is enlarged in B. **B:** Higher magnification of the boxed area in A, showing double-labelled neurons (arrows) and a ChAT-single-labelled neuron (arrowhead). **C:** Photomicrograph through the caudal part of the LDTg following the injection shown in Figs. 1A, 2A. The section was processed for the visualization of CT-HRP and SP. The boxed area is enlarged in D. **D:** Higher magnification of boxed area in C. Double- and single-labelled neuron are not detectable at this magnification. The neuron indicated by open arrow is shown in E. **E:** High magnification photomicrograph of part of D, showing double-labelled neurons (open arrows) and anterogradely labelled fibres and terminal-like structures (small arrows). Scale bars= A: 25 μm; B,E: 10 μm; C: 100 μm; D: 50 μm.

*In the MnR*, approximately 90-95% of CT-HRP-labelled neurons were also 5-HT-immunoreactive (Figs. 2B,C, 3D,E). However, up to 10% of neurons projecting from the MnR to RSg were not immunoreactive for 5-HT and these neurons were codistributed with 5-HT+ projection cells (Fig. 2B,C). In addition, a few retrogradely labelled neurons (1-3 per section) in the MnR were also immunoreactive for Glu, Enk, NT or SP (Figs. 2B,C, 5C-F). Although double-labelled and CT-HRT single-labelled neurons were scattered throughout the rostrocaudal extent of the MnR, they were predominantly located in the rostral two-thirds of the nucleus (Figs. 2B,C, 3D). In the MnR, the labelled neurons were located along the midline, with their long axes predominently parallel to the dorsoventral axis of the nucleus (Figs. 3D, 5C-E). Retrogradely labelled, double-labelled neurons (HRP/5-HT, HRP/Glu, HRP/Enk, HRP/NT or HRP/SP) and 5-HT+, Glu+, Enk+, NT+ or SP+ single-labelled neurons in MnR were mostly of small to medium size (15-20μm), with round to ovoid somata (e.g., Figs. 3E, 5D,E). A few labelled neurons of relatively large size (30-40μm) with fusiform somata, often with several dendrites emerging from the soma, were also present throughout this nucleus (e.g., Fig. 3E).

*Projections from the locus coeruleus*

In all animals, injections of CT-HRP confined to the RSg produced large numbers of retrogradely labelled neurons in the LC (Fig. 2G). In the LC, most CT-HRP labelled neurons were found to be DβH-immunoreactive (Figs. 2D, 7A,D). A small number of the CT-HRP-labelled neurons in the LC (1 to 2-3 per section) were also immunoreactive for SP or NT (Fig. 2D). In the LC, CT-HRP labelled and double-labelled neurons were scattered throughout the nucleus. However, they were particularly concentrated in the area surrounding the superior cerebellar peduncle (Figs. 2D, 7A,D). Although retrogradely labelled and double-labelled neurons in the LC were almost entirely ipsilateral to the injection site (Figs. 2D, 7A), a few double-labelled neurons were occasionally seen contralaterally (Figs. 2D, 7D). Retrogradely labelled, double-labelled (HRP/DBH, HRP/SP or HRP/NT), and DBH+, SP+ or NT+ single-labelled neurons in LC were mostly medium sized, with round to ovoid somata (Fig. 7B,C), although a few of relatively large size, with somata that ranged in shape from ovoid to multipolar, often with several dendrites emerging from the soma, were also occasionally seen in the LC (e.g., Fig. 7E).

*Projections from the laterodorsal tegmental nucleus*

Injections of CT-HRP into the RSg which also involved the anterior cingulate cortex (e.g., Figs. 1A, 2A) resulted in small numbers of retrogradely labelled neurons in the LDTg (Fig. 2G). Most of the retrograde labelled neurons in the LDTg were seen ipsilateral to the injection site and a few neurons projecting to the RSg were also observed contralaterally. In the LDTg, 90% of the CT-HRP-labelled neurons were also ChAT-immunoreactive (Figs. 2D, 8A) and few retrogradely labelled cells (1 to 2-3 per section) were immunoreactive for SP (Figs. 2D, 8C,D). In the LDTg, CT-HRP and double-labelled neurons were concentrated at the caudal level of the nucleus (Figs. 2D, 8A,C). Retrogradely labelled, double-labelled (HRP/ChAT or HRP/SP) and ChAT+ or SP+ single-labelled neurons in LDTg were of relatively large size (40-50μm), with fusiform to multipolar somata (Fig. 8B,E).

In addition, in all animals CT-HRP-containing fibres or terminal-like structures, which appeared to display irregularly distributed blue/black granules, presumably of HRP reaction product, were present in the neuropil and close to neuronal cell bodies of the several structures analyzed, including the DR (Figs. 3F, 4F, 6C), MnR (Fig. 5E,F) and LDTg (Fig. 8E), indicating reciprocal connections with the RSg.

The above results can be summarized as follows:

1. Injections of CT-HRP centered in the RSg permitted recognition of many retrogradely labelled neurons within the raphe nuclei, predominantly in the DR although with a minor proportion in the MnR. Large numbers of retrogradely labelled neurons were also found in the LC and small numbers in the LDTg.
2. In the DR and MnR, large numbers (80-85% in DR and 90-95% in MnR) of retrogradely labelled neurons were immunoreactive for 5-HT, whereas small numbers (1 to 4-5 per section) of CT-HRP-labelled neurons displayed immunoreactivity for Glu, Enk, NT or SP.
3. Most of the retrogradely labelled and double-labelled neurons in the DR were located in the ventromedial and ventrolateral parts of the nucleus, particularly in the area surrounding the dorsal aspect of the medial longitudinal fasciculus. Small numbers of CT-HRP-labelled and double-labelled neurons were also found in the dorsomedial part of the DR. In the MnR, retrograde labelling and double-labelled neurons were located close to the midline.
4. Although injections centred in the RSg resulted in bilateral retrograde labelling of moderate to small numbers of 5-HT neurons in the dorsomedial and ventromedial regions of the DR, the raphe projections to the RSg were predominantly ipsilateral.
5. In the LC, most retrogradely labelled neurons were immunoreactive for DβH and

small numbers of CT-HRP-labelled neurons displayed NT- or SP-immunoreactivity.

6. In the LDTg, most of the retrogradely labelled neurons were immunoreactive for ChAT, whereas a few neurons also displayed SP-immunoreactivity.
8. In all animals HRP-containing fibres or terminal-like structures were found in the neuropil of the raphe nuclei, in the LC and in the LDTg. Thus, mesencephalic tegmentum neurons probably receive inputs from the same cortical areas which they innervate.

## Discussion

The pattern of afferents to the RSg observed in this study is largely in agreement with previous analyses of the projections from the nuclei of the pontomesencephalic tegmentum to the cortex, including the RSg (Loughlin et al., 1982; Saper, 1982; Groenewegen and Van Dijk 1984; Thompson and Robertson, 1987; Van Groen and Wyss, 1990; Vertes, 1991; Conde et al., 1995; Vertes et al., 1999). However, to the best of our knowledge this study is the first to extensively map a variety of neurochemical markers in afferents from the raphe nuclei, the LC and LDTg to the RSg. Thus, our discussion will focus on the possible neurotransmitter(s)/neuromodulator (s) used by these projection neurons to the RSg.

### *Methodological Considerations*

#### *CT-HRP as a retrograde axonal tracer*

The use of HRP as a retrograde axonal tracer in combination with transmitter immunohistochemistry is now routine. 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 either single CT-HRP- or double-labelled cells found in the double-label preparations could be due to the limited sensitivity of the DAB method and to some extent, also, to the short survival times used in this study. However, even with these methodological limitations, this study allowed us to detect the strongest projections from the pontomesencephalic tegmentum to the RSg even though we failed to detect some neurons with only a small axonal branch projecting to the RSg.

#### *Specificity of the immunoreaction*

Several issues arise in regard to the procedures used in this study. The first concerns the presence of 5-HT-immunoreactivity in the cell bodies of neurons projecting from raphe nuclei to the RSg. Here we show that 80-85% of the neurons projecting from the DR to the RSg and 90-95% of those projecting from the MnR are immunoreactive for serotonin. The 5-HT antibody used in this study was raised in rabbits, against Limulus hemocyanin conjugate, and is well characterized and very widely used (Eugene Tech. International, Catalogue N° NT102). The maps of 5-HT immunostaining in this study are in agreement with previous reports (Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983). Thus, the strong 5-HT-immunoreactivity observed in the DR and MnR suggests that our findings reflect specific immunolabelling and that serotonin is a significant neurotransmitter in projections from the raphe nuclei to the RSg. Another important issue concerns the presence of dopamine-β-hydroxylase in the cell bodies of the LC neurons projecting to the RSg. Here we show that 90-95% of the neurons projecting from the LC to the RSg are immunoreactive for DβH. The DβH antibody used in the present study is well characterized (Protos Biotech Corporation); its specificity has been described previously (Joh and Ross, 1983) and it is very widely used (Asanuma, 1992). The fact that in our material the maps of immunostaining with DβH antibody were consistent with the data in the literature (Dahlström and Fuxe, 1964; Fuxe, 1965), together with the results of the control experiments, suggest that noradrenalin is an important neurotransmitter in projections from the LC to the RSg. Yet another issue concerns the presence of ChAT-immunoreactivity in the cell bodies of neurons projecting from LDTg to the RSg. The ChAT antiserum used in this study was a monoclonal antibody (Boehringer -Mannheim) prepared from a rat-mouse hybridoma. The specificity of the ChAT antibody has been well established (Eckenstein and Thoenen, 1982) and in our material the pattern of immunostaining with ChAT antibody is similar to that described in previous studies (Armstrong et al., 1983; Mesulam et al., 1983b; Eckenstein et al., 1988). These observations, together with the results of control experiments, make it unlikely that our findings would reflecting non-specific immunolabelling.

We have also shown that a small number of neurons projecting to the RSg from the DR and and MnR contain immunohistochemically detectable levels of Glu. The evidence presented here relates primarily to the presence of this amino acid in cell bodies. 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; Storm-Mathisen and Ottersen, 1986; Ottersen et al., 1990). On the basis of observations of sections from the brains of glutaraldehyde-perfused mice and rats processed for Glu immunocytochemistry, 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. While the same conclusion may be derived from the present results, it should be pointed out that several features distinguish Ottersen and Storm-Mathisen´s study from ours, including the fixatives used in the two studies. Although, Conti et al. (1987) indicated that the use of different fixatives does not seem change the specificity of the staining, fixatives containing glutaraldehyde, the only kind employed by Ottersen and Storm-Mathisen, give a higher background staining than that seen in the present study. Thus, the small proportion of Glu-containing neurons detected in this study could be due to the low concentration of glutaraldehyde in the fixative solution. Also, the antibodies used may differ in their specificity. The Glu antibody used in the present study was raised in rabbits, against amino acids conjugated to haemocyanin with glutaraldehyde, and is well characterized (Arnel Products, Catalogue N° 58A, 1992), and immunoabsorption tests based on the 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. Although no techniques are available to resolve the issue of whether immunohistochemically detected Glu in cell bodies is related to metabolic or neurotransmitter functions, 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; Storm-Mathisen et al., 1983; Ottersen and Storm-Mathisen, 1984), in conjunction with the results of the control experiments, suggest that it is at least possible that 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 by us is well characterized and the specificity of this antiserum has been established by the abolition of immunostaining when the antiserum is absorbed with 50 µg/ml Leu-Enk (INCSTAR, catalogue N° 20066, 1983) as is the similarly produced NT antibody (Eugene Tech. International, Catalogue N° NT113), and immunostaining with the SP antibody is completely blocked by absorption with 10µg/ml SP (Eugene Tech. International, Catalogue N° NT106). In addition, in our material the patterns of immunostaining with Enk, NT and SP antibodies were consistent with those described in previous studies (Uhl et al., 1979; Finley et al., 1981; Emson et al.1985; Petrusz et al. 1985; Sutin and Jacobowitz, 1988). These observations, in conjunction with the results of the control experiments, make it unlikely that our findings would be reflecting non-specific immunolabelling. However, with the immunohistochemical approach used it is difficult to exclude the possibility that an antiserum raised against to a certain peptide might also react with other, structurally related peptides.

*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 pontomesencephalic tegmentum were immunonegative. This finding might be explained in terms of "false negatives", implying that there are projection neurons in which no immunoreactivity was detected owing to a technical problem (such as poor antibody penetration or loss of antigen during tissue processing). 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 might contain one of the transmitter markers not labelled for in that particular section. It is unfortunate that the limitations inherent to the technique make it difficult to estimate the proportion of cells projecting to the RSg that do not express one of the transmitter markers studied.

*Identification of neurochemicals in neurons projecting to the RSg*

The present report describes the localization of neurotransmitter -related molecules in the main projection neurons from the nuclei of the pontomesencephalic tegmentun to the RSg. The essential features concerning each neurochemical are described and discussed below.

*Serotoninergic pathways from the brainstem raphe nuclei to the RSg*

We observed that 80-85% of the cell bodies of neurons projecting from the DR and 90-95% of those projecting from the MnR to the RSg are immunoreactive for serotonin, in keeping with evidence that both nuclei contain most of the 5-HT-immunoreactive neurons of the CNS (Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983). There is evidence from previous studies that the raphe nuclei project to the cortex (Jacobs et al., 1978; Moore et al., 1978; Parent et al., 1981; Vertes, 1991; Waterhouse et al., 1993; Vertes et al., 1999). Furthermore, a serotoninergic input to the cortex from the midbrain raphe nuclei has been suggested on the basis of immunohistochemical studies (Wilson and Molliver, 1991; Jacobs and Azmitia, 1992; Datiche et al., 1995). However, none of these studies provided documentation on the serotoninergic innervation of RSg.

The present study confirms the existence of such projections and provides a detailed analysis showing a serotoninergic input from the DR and MnR to the RSg. Our results show that most of the retrogradely labelled and double-labelled neurons were located in the ventromedial and ventrolateral regions of the DR and close to the midline in the MnR. The finding of a difference in the pattern of retrograde labelling in the DR following injections into the RSg is in keeping with previous studies suggesting that the DR can be divided into subnuclei projecting to different specific targets (Jacobs et al., 1978; Waterhouse et al., 1986, 1993; Kazakov et al., 1993; Gonzalo-Ruiz et al., 1995a). Based on these results and on those of others (Imai et al., 1986; Vertes, 1991; Waterhouse et al., 1993; Vertes et al., 1999), it could be suggested that DR cells projecting to cortical and subcortical structures could be co-localized within the nucleus and could innervate multiple, functionally related targets via axon collaterals. Since the ventromedial and ventrolateral parts of the DR and the midline region of the MnR also project to the anterior thalamic nuclei (ATN) (Gonzalo-Ruiz et al., 1995a), it seems a reasonable assumption that the serotoninergic projection neurons in the DR and MnR would send braches to both and that the serotonin might influence limbic activity at the level of the RSg as well as the ATN.

The results of the present study also support the notion that the projections from the raphe nuclei to RSg appear to be organized topographically. This observation is consistent with the findings of earlier studies indicating that nearly all neocortical regions receive significantly denser projections from the rostral than from the caudal part of the DR (O´Hearn and Molliver, 1984; Waterhouse et al., 1986; Vertes, 1991). Furthermore, although the greatest proportion of double-labelled neurons was found in the DR, a small number of 5-HT-immunoreactive cells in the MnR was labelled by restricted RSg injections. These findings confirm and extend the results of previous studies that demonstrated low-to-moderate numbers of MnR projections to the cingulate cortex as compared with the significantly denser projections from the DR (O´Hearn and Molliver, 1984; Waterhouse et al., 1986; Datiche et al., 1995; Vertes et al., 1999).

On the other hand, it is known from previous studies that all cortical areas receive significant 5-HT innervation and that the pattern of termination varies from one area to another and that cortical 5-HT-positive axons are not of uniform morphology (Lidov et al., 1980; Steinbusch, 1981; Kosofsky and Molliver, 1987). Lidov et al. (1980) demonstrated that the RSg contains a moderately dense labelling of 5-HT-immunoreactive fibres and terminals that are largely confined to layer I and III. The significance of this highly selective laminar distribution of serotonin-containing axons in a single cytoarchitectural area of the RSg is unknown. However, the discrete laminar distribution of 5-HT axon terminals in the RSg suggests that the effects of serotonin projections would be restricted to particular neuronal elements in the RSg. The present findings raise the possibility that the two distinct sets of raphe projection neurons, which have different patterns of termination (Korsofsky and Molliver, 1987), could affect different sets of cortical neurons and that they may have different functional effects.

*Noradrenergic pathway to the RSg*

We observed that most of the cell bodies of neurons projecting from the LC to the RSg contain DβH. There is evidence from previous studies that the LC neurons project to the cortex (Jones and Moore, 1977; Morrison et al., 1979; Audet et al., 1988; Lewis and Morrison, 1989). There is also very strong evidence that the LC contains a large population of DβH-immunoreactive neurons and that this nucleus is the major source of noradrenergic projection in the CNS (Dahlström and Fuxe, 1964; Fuxe, 1965). Thus, the strong degree of DβH-immunoreactivity observed in the LC suggests that noradrenergic cells within this nucleus would give rise to the major noradrenergic projection to the RSg. Furthermore, several immunohistochemical studies have also shown that in the cerebral cortex, noradrenergic axons show considerable regional, as well as laminar, specificity (Morrison et al., 1978, 1979; Audet et al., 1988; Lewis and Morrison, 1989; Parnavelas and Papadopoulos, 1989; Papadopoulos and Parnavelas, 1991), and that large numbers of DβH-labelled axon terminals in the cortex form conventional synapses (Papadopoulos et al., 1989; Séguéla et al., 1990). Morrison et al. (1979) demonstrated that noradrenergic projections to area 29 (RSg) are more dense than to any other neocortical areas. On the other hand, early studies provided evidence that in the RSg there are distinct and clear laminar distributions of DβH-immunoreactive terminals. Thus, whereas DβH-positive axons are most dense in layers Ib and Ic, they are present at a moderate density in layers III and IV. Deep-layer terminations are less prominent (Jones and Moore, 1977). Furthermore, ultrastructural analysis has revealed that, similar to 5-HT, varicosities on noradrenergic terminals reflect points of synaptic contact and noradrenalin release. In layer I there is an increased density of both DβH axon terminals and varicosities (Audet et al., 1988; Papadopoulos et al., 1989). The significance of the highly selective laminar distribution of noradrenergic axons in the RSg is unknown. However, the discrete laminar distribution of DβH terminals probably reflects a key position

for the integration of noradrenergic input with arborizations of apical dendrites of deep-layer pyramidal neurons.

The present findings highlight the fact that in layers I-III of the RSg, both noradrenergic and serotonin-containing terminals are coextensive with terminals from the anterior thalamic nuclei (Van Groen and Wyss, 1990; Shibata, 1993; Gonzalo-Ruiz, et al., 1997a,b), each of which has a different and selective pattern of laminar distribution; namely, those from the anteroventral thalamic nucleus to layer I whereas those from the anterodorsal thalamic nucleus to layer III (Domesick, 1972). Thus, in this region of the cortex, the principal thalamic afferents and the afferents from brainstem noradrenergic and serotonin-containing neurons terminate in the same cortical layers, which show a high degree of specificity in the formation of these projections and are likely to reflect differences in their contributions to cortical circuitry. Further eludication of the significance of this relationship between noradrenergic, serotoninergic and anterior thalamic terminals will require an ultrastructural analysis of their precise mode of termination.

*Cholinergic pathway to the RSg*

We observed that small numbers of the cell bodies of neurons projecting from the LDTg contained ChAT. There is evidence from previous studies that the LDTg neurons project to the medial limbic cortex, including the cingulate cortex (Saper, 1982; Groenewegen and Van Dijc, 1984; Cornwall et al., 1990; Van Groen and Wyss, 1990; Condé et al., 1995). There is also very strong evidence that the LDTg contains a large population of ChAT-immunoreactive neurons and that this nucleus is a source of cholinergic projections in the CNS (Armstrong et al., 1983; Mesulam et al., 1983b). Furthermore, ChAT-immunocytochemistry and acetylcholinesterase (AChE) staining (Butcher and Woolf, 1984; Eckenstein et al., 1988; Lysakowski et al., 1989) have also revealed the presence of putative cholinergic fibres in the neuropil of the RSg. Thus, the ChAT-immunoreactivity observed in the LDTg suggests that cholinergic cells within this nucleus give rise to a small cholinergic projection to the RSg. These findings confirm and extend the results of previous studies that demonstrated cholinergic projection from the LDTg to the medial limbic cortex, including the cingulate cortex (Satoh and Fibiger, 1986; Woolf and Butcher, 1986; Eckenstein et al., 1988). By contrast, earlier studies provided evidence that the pontomesencephalic cholinergic cells give rise to important ascending projections to the forebrain, into both the thalamic and the extrathalamic cortically projecting "relay" systems (Mesulam et al., 1983b; Sofroniew et al., 1985; Satoh and Fibiger, 1986; Woolf and Butcher, 1986). From quantitative estimates, it apeared that most cholinergic cells in the LDTg project to the thalamus (Sofroniew et al., 1985; Woolf and Butcher, 1986; Gonzalo-Ruiz et al., 1995b; Oakman et al., 1999), whereas of the LDTg cells projecting to the basal forebrain cholinergic cortically "relay" neurons, approximately 15% or less were identified as ChAT+ (Hallanger and Wainer, 1988; Jones and Cuello, 1989).

In sum, the findings in this study are consistent with the view that the RSg has a dual cholinergic innervation: one arising from the basal forebrain (Gage et al., 1994; Gonzalo-Ruiz and Morte, 2000) and the other from the LDTg (Satoh and Fibiger, 1986; Eckenstein et al., 1988). The functional significance of this dual cholinergic projection to the RSg remains to be determined. However, it is of considerable interest that the region of the RSg receiving cholinergic afferents from the LDTg is also selectively innervated by monoaminergic-containing fibres originating in the midbrain tegmentum. In light of these findings, we suggest that the RSg may therefore be a substrate for interactions between serotoninergic, noradrenergic and cholinergic mechanisms, thought to be important in the control of complex functions such as learning and memory (Wenk et al., 1984, 1987; Vogt et al., 1991), by affecting specific, possibly quite localized cortical areas, where the output to subcortical areas by pyramidal cells in layer V is modulated.

*Glutamate-like immunoreactivity in identified neurons projecting to the RSg from the pontomesencephalic tegmentum*

The present study has also shown that a few cell bodies of the neurons projecting to the RSg from the DR and MnR contain immunohistochemically detectable levels of Glu. There is also previous evidence for Glu in the cell bodies of the DR and MnR (Storm-Mathisen and Ottersen, 1983; Ottersen and Storm-Mathisen, 1984). Since Glu is the major excitatory neurotransmitter in the CNS and plays an important role in a number of CNS functions (Fonnum, 1984; Fleck et al., 1993), the present findings support the notion that the RSg may receive excitatory inputs from Glu-immunoreactive neurons of the DR and MnR. The results reported here relate to the presence of Glu in cell bodies. Since high levels of Glu in neuronal cell bodies do not constitute, alone, satisfactory evidence for a Glu phenotype, the possibility that the presence of Glu in a few retrogradely labelled neurons in the DR and MnR might reflect a transmitter role, must remain hypothetical. However, the fact that Glu staining was present in only a small number of CT-HRP-labelled neurons suggests a remarkable selectivity, more likely to be related to a neurotransmitter pool of Glu, which is synthesized at a higher rate, than to a general metabolic pool of Glu

(Storm-Mathisen and Ottersen, 1986). Furthermore, previous studies have identified glutamate as the probable transmitter of cerebrocortical pyramidal neurons in their intracortical associational pathways and their projections to subcortical structures (Fonnum et al., 1981; Fonnum, 1984; Jones, 1986). Although these studies did not focus specifically on the cingulate cortex, it is likely that the findings can be applied to the cingulate region as well as to other cerebrocortical projection pathways. Thus, there is no reason to exclude this amino acid as a putative neurotransmitter of pontomesecephalic tegmentum projections to the RSg.

The functional significance of the possible glutamatergic cortically projecting cells (Gigg et al., 1992; Gonzalo-Ruiz et al., 1997a,b; Wang et al., 1999, present findings) remains to be determined. However, considerable evidence indicates that glutamatergic neurotransmission is involved in cognitive functions such as learning and memory (Danysz et al., 1995; Riedel et al., 1995; Riedel and Reymann, 1996; Ungerer et al., 1998). In light of these findings, we therefore suggest that Glu may be utilized as a neurotransmitter in projections to the RSg from the pontomesencephalic tegmentum nuclei and thus may play a role in cognitive functions.

*Neuropeptides in neurons projecting to the RSg from nuclei of the pontomesencephalic tegmentum*

We have also shown that small numbers of neurons projecting to the RSg from several nuclei of the pontomesencephalic tegmentum contain Enk, NT or SP, in keeping with previous studies reporting that a population of neurons within the midbrain tegmentum, including the DR, MnR, LC and LDTg, display NT-, SP- or Enk-like immunoreactivity (Ljungdahl et al., 1978; Uhl et al., 1979; Finley et al., 1981; Emson et al., 1985; Sutin and Jacobowitz, 1988). Thus, the present findings indicate that NT-, SP- or EnK-immunoreactive neurons in the pontomesencephalic tegmentum nuclei may have a neurotransmitter/neuromodulator role in projections to the RSg.

Because our immunoreactions were carried out on different sections, we do not know whether some of the neurotransmitters we studied coexist in neurons projecting to the RSg. The colocalization of NT, Enk or SP with classical neurotransmitters (Chan-Palay et al., 1978; Aronin et al., 1984; Sutin and Jacobowitz, 1988; Van Bockstaele and Chan, 1997) and with several other neuropeptide transmitters (Erichsen et al., 1982; Pickel et al., 1980) has been reported for 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 nuclei of the pontomesencephalic tegmentum to the RSg, these anatomical data suggest that these several unrelated neuropeptides transmitter candidates in tegmento-cortical neurons might represent loci for interactions between these neurochemical systems and might work synergistically in regulating the activity of cortical neurons.

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## ABBREVIATIONS

| | |
|---|---|
| CA1 | field CA1 of Ammon´s horn |
| cc | corpus callosum |
| DR | dorsal raphe nucleus |
| DTN | dorsal tegmental nucleus of Gudden |
| LC | locus coeruleus |
| LDTg | laterodorsal tegmental nucleus |
| mlf | medial longitudinal fasciculus |
| MnR | median raphé nucleus |
| PPT | pedunculopontine tegmental nucleus |
| PrS | presubiculum |
| Rsa | restrosplenial agranular cortex |
| RSg | retrosplenial granular cortex |
| S | subiculum |
| SCc | compact subnucleus of the central superior nucleus |
| scp | superior cerebellar peduncle |
| VTN | ventral tegmental nucleus of Gudden |
| xscp | decussation of superior cerebellar peduncle |