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Descending projections from the hypothalamic paraventricular nucleus to the A5 area, including the superior salivatory nucleus, in the rat

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Summary. The descending projection of the hypothalamic paraventricular nucleus (PVN) to the A5 area was elucidated using a technique that combines retrograde labeling with horseradish peroxidase (HRP), anterograde labeling with PHA–L (*Phaseolus vulgaris* leucoagglutinin and immunohistochemistry for dopamine-β-hydroxylase (DBH). Following an iontophoretic injection of PHA–L into the PVN, HRP was applied to the greater petrosal nerve. Frozen sections of the hypothalamus and the caudal pons were first treated according to a protocol for HRP histochemistry using tetramethylbenzidine with cobalt-enhanced diaminobenzidine, and then they were processed for displaying PHA–L, and then for DBH immunohistochemistry. PHA–L labeled fibers from the PVN were observed in a ventrolateral part of the pontine reticular formation corresponding to the A5 area, where they give rise to a dense network around the cells of origin of the greater petrosal nerve (GPN cells) and DBH-positive cells. Terminals or varicosities labeled with PHA–L were preferentially observed around the somata of GPN cells, suggesting direct contact. However, apparent contact between both elements was hardly ever observed. On the other hand, terminals or varicosities were occasionally observed in close relation to DBH-positive cells. These results suggest that descending fibers of the PVN project more strongly to GPN cells than to DBH-positive cells. The relationship of this fiber pathway to control of the secretomotor or cardiovascular systems is discussed.

Key words: Hypothalamic paraventricular nucleus – Descending fibers – Superior salivatory nucleus – A5 neurons – PHA–L

Introduction

The hypothalamic paraventricular nucleus (PVN) is known to project directly to all the autonomic sympathetic and parasympathetic motoneurons in the spinal cord and to the motor nucleus of the vagus nerve (Saper et al. 1976; Armstrong et al. 1980; Swanson et al. 1980; Swanson and Kuypers 1980; Akmayev et al. 1981; Ter Horst et al. 1984; Luiten et al. 1985; Gray et al. 1986; Holstege 1987). However, direct morphological evidence has not been provided for a direct PVN input to the parasympathetic preganglionic nuclei such as the superior and inferior salivatory nuclei. These nuclei lie dispersed in the lateral reticular formation of the pons and medulla (Contreras et al. 1980). This general morphological characteristic makes it difficult to elucidate a direct neuronal connection between the PVN and the salivatory nuclei. The cells of origin of the greater petrosal nerve (GPN cells), which are the parasympathetic preganglionic neurons for the lacrimal, nasal, and palatine glands, are more readily distinguishable in the ventrolateral part of the pontine reticular formation at levels through the caudal tip of the superior olivary nucleus and the rostral tip of the facial nucleus (Contreras et al. 1980; Hosoya et al. 1984). The present study provides evidence for a direct projection of the PVN to GPN cells in the rat, combining the retrograde HRP and anterograde *Phaseolus vulgaris* leucoagglutinin (PHA–L) tracing techniques. Since the ventral cell group of the GPN is located close to the A5 catecholamine cell group, the immunohistochemical demonstration of dopamine-β-hydroxylase (DBH) was also carried out to discriminate GPN cells from A5 neurons.

Material and methods

Twenty five male Wistar rats (250 350 g body weight) were used in this study. They were anesthetized with pentobarbital and placed in a stereotaxic frame. A small hole was drilled in the skull and a glass micropipette was inserted to the desired depth with a stereotaxic guide. A glass micropipette was backfilled with 2.0% PHA–L in phospate buffered saline. Injection of PHA–L into the PVN was made iontophoretically for 20 min, according to a schedule in which a positive 7 μA current for 7 s was followed by negative 1 μA current for 5 s. After the PHA–L injection the pipette was left in place for 5 min. After 8 days, the animals were reanesthetized with pentobar-

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bital and 50% HRP (Sigma type VI) was applied to the greater petrosal nerve exposed in the tympanic cavity on the same side as the PHA–L injection. The technique used to apply HRP to the greater petrosal nerve has been described in a previous paper (Hosoya et al. 1984). After 2 days, the animals were deeply anesthetized and perfused through the left ventricle with Ringer's solution, then with 500 ml of a solution of 10% formalin and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The fixative was immediately followed by 100 ml of a solution of 5% glycerin in PB, and the brain was then removed and placed in a solution of 20% glycerin in PB.

The forebrain was sectioned on a freezing microtome at a thickness of 50 µm, and the rostral medulla and caudal pons were sectioned at 30 µm. The serial sections were divided into four groups. Sections in the first and third groups were stained for HRP with a protocol using tetramethylbenzidine and cobalt-enhanced diaminobenzidine (Rye et al. 1984), followed by immunohistochemical treatment for PHA–L and DBH. Sections in the second and fourth groups were stained only for PHA–L immunohistochemistry. At the beginning of the present study we used the original immunohistochemical staining technique for PHA–L described by Gerfan and Sawchenko (1984). This technique gave satisfactory results, but was later replaced by a simpler and less time consuming method, which gave excellent results. The immunohistochemical staining was carried out as follows. The sections were: 1) soaked in 0.1 M phosphate buffered saline (PBS) containing 0.16% bovine albumin and 0.3% triton for 30 min at 37° C; 2) incubated with a mixture of antibodies to PHA–L (× 4000, Vector Lab.) and DBH (× 2000, Eugene Tech. Inc.) for 3 h at 37° C, and then rinsed with PBS for 15 min; 3) incubated with biotinylated anti-goat IgG (Vector Lab.) for 2 h at 37° C and then rinsed thoroughly with PBS for 15 min; 4) immersed in avidin conjugated HRP (Vector Lab.) for 1 h at room temperature and then rinsed thoroughly with Tris-HCl buffer for 15 min; 5) immersed in a solution of 0.2% nickel ammonium sulfate, 0.05% diaminobenzidine (DAB), and 0.003% H_2O_2 in Tris-HCl buffer for 15 min at room temperature; 6) rinsed thoroughly with Tris-HCl buffer, followed by PBS; 7) incubated with anti-rabbit IgG (× 400, Pel-Freez) for 2 h at 37° C and then rinsed for 15 min with PBS; 8) incubated with peroxidase-antiperoxidase (× 4000, Pel-Freez) for 1 hour at room temperature; 9) thoroughly washed for 15 min with PBS and then immersed in 0.05% DAB and 0.003% H_2O_2 for 10 min. After immunostaining, the sections were mounted on gelatin-coated slides and coverslipped. The sections of the second and fourth group, which were treated only for PHA–L immunohistochemistry, were counterstained with borax-carmine and coverslipped.

Results

Of 25 animals, 15 received PHA–L injections outside the PVN; in these cases only a few labeled varicose fibers were observed in the A5 area. GPN cells in 4 animals had not been labeled thoroughly by HRP applied to the great petrosal nerve. Six remaining animals had PHA–L injections centered in the PVN (Fig. 1A), and descending labeled fibers were observed around GPN cells and DBH-positive neurons in the A5 area.

After HRP application to the greater petrosal nerve,

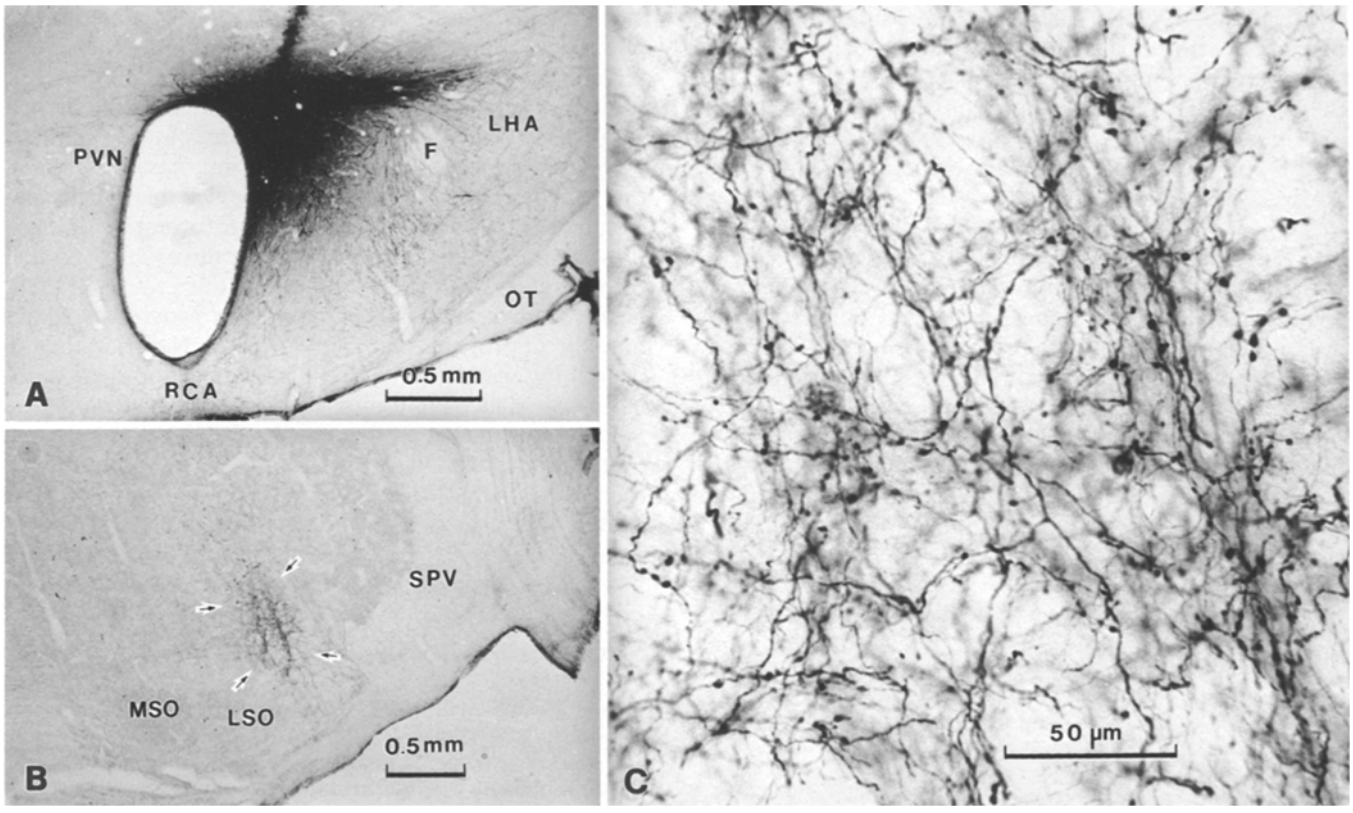

Fig. 1A–C. Photomicrographs showing the injection site of PHA–L into the PVN and anterogradely labeled fibers in the caudal pontine reticular formation (case 1537). **A** In this experiment, injected PHA–L was centered in the posterior part of the PVN. **B** Photomicrograph showing a dense network of PHA–L labeled fibers (arrows) in the pontine parvocellular reticular formation between the spinal tract of the trigeminal nerve (SPV) and the lateral nucleus of the superior olive (LSO). **C** A high-power photomicrograph of the same area in **B**. Note the PHA–L labeled terminal boutons and varicosities

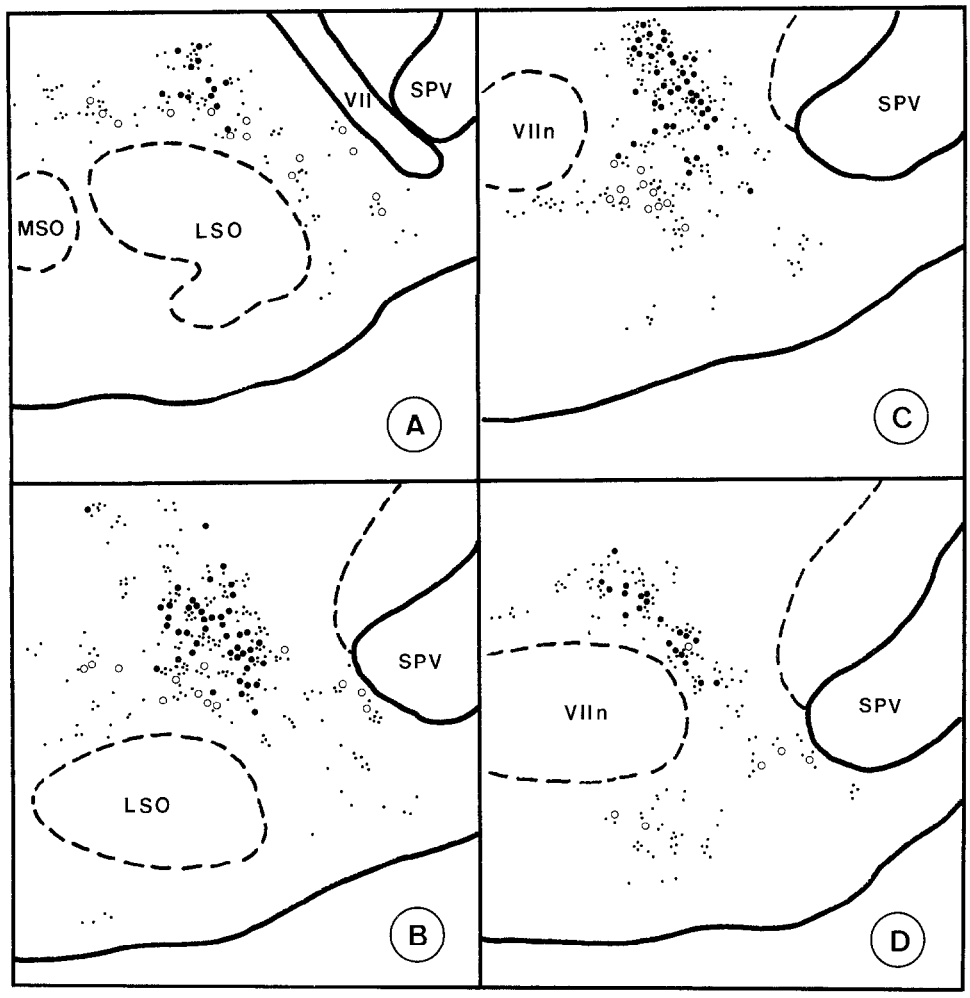

Fig. 2A–D. Camera lucida drawing of the ventrolateral part of the caudal pons, showing the location of HRP-labeled GPN cells (filled circle), DBH-immunoreactive neurons (open circle), and PHA-L labeled terminal boutons and varicosities (dot) originating from the PVN (case 1559). Sections are arranged from rostral to caudal **(A, D)**, and are separated by 90 μm. Note that terminal boutons and varicosities are seen around GPN cells and DBH-positive cells. LSO and MSO, lateral and medial nuclei of the superior olive; SPV, spinal tract of the trigeminal nerve; VII, facial nerve; VIIn, facial nucleus

retrogradely labeled GPN cells were seen mainly in the ventral half of the nucleus reticularis parvocellularis. HRP granules were distributed almost evenly within the cytoplasm, except for the nucleus. Since the proximal dendrites of labeled neurons had a few HRP granules, profiles of the proximal dendrites were faintly discerned. Labeled neurons were oval in shape with 2 or 3 proximal dendrites (Fig. 3B).

Labeled GPN cells were divided into dorsal and ventral groups, as reported by Contreras et al. (1980). Therefore, they will only be described briefly for comparison with DBH-positive neurons. Neurons of the ventral group were observed in the ventrolateral reticular formation just dorsolateral to the superior olivary complex and ventromedial to the descending intramedullary root of the facial nerve (Fig. 2A). The ventral group persisted caudal to the region just dorsolateral to the rostral third of the facial nucleus. At the level through the rostral tip of the facial nucleus, labeled neurons formed a compact cell group located just dorsolateral to the facial nucleus (Fig. 2C). In a given section, this compact cell group consisted of about 40 labeled neurons counted from a single section. The dorsal group located just ventral to the nucleus of the solitary tract, was much smaller than the ventral group and only three to five cells were found in each section. A few sporadic labeled neurons were also observed between dorsal and ventral groups, as well as in the regions just dorsal or ventral to the superior olivary complex.

Immunohistochemical staining for DBH revealed the location of A5 noradrenergic neurons in the ventrolateral region of the caudal pons, as described by Byrum et al. (1984) using histochemistry. At levels through the descending intramedullary root of the facial nerve, DBH-positive neurons were observed in restricted regions just dorsal and lateral or lateroventral to the superior olivary complex, corresponding to the dorsal and lateral groups of Byrum et al. (1984) (Fig. 2A and Fig. 3A). At levels through the rostral part of the facial nucleus, DBH-positive neurons were distributed sporadically in regions lateral or ventral to the facial nucleus (Fig. 2D). A few DBH-positive neurons were observed within the compact cell group consisting of GPN cells.

At the level through the caudal tip of the descending intramedullary root of the facial nerve, a small number of PHA-L labeled fibers with varicosities were found in the ventrolateral reticular formation, especially in the region between the descending intramedullary root of the facial nerve and the lateral superior olivary nucleus (Fig. 2A). At more caudal levels, PHA–L labeled fibers formed a dense network in a restricted region just dorsolateral to the superior olivary complex, through the rostral tip of

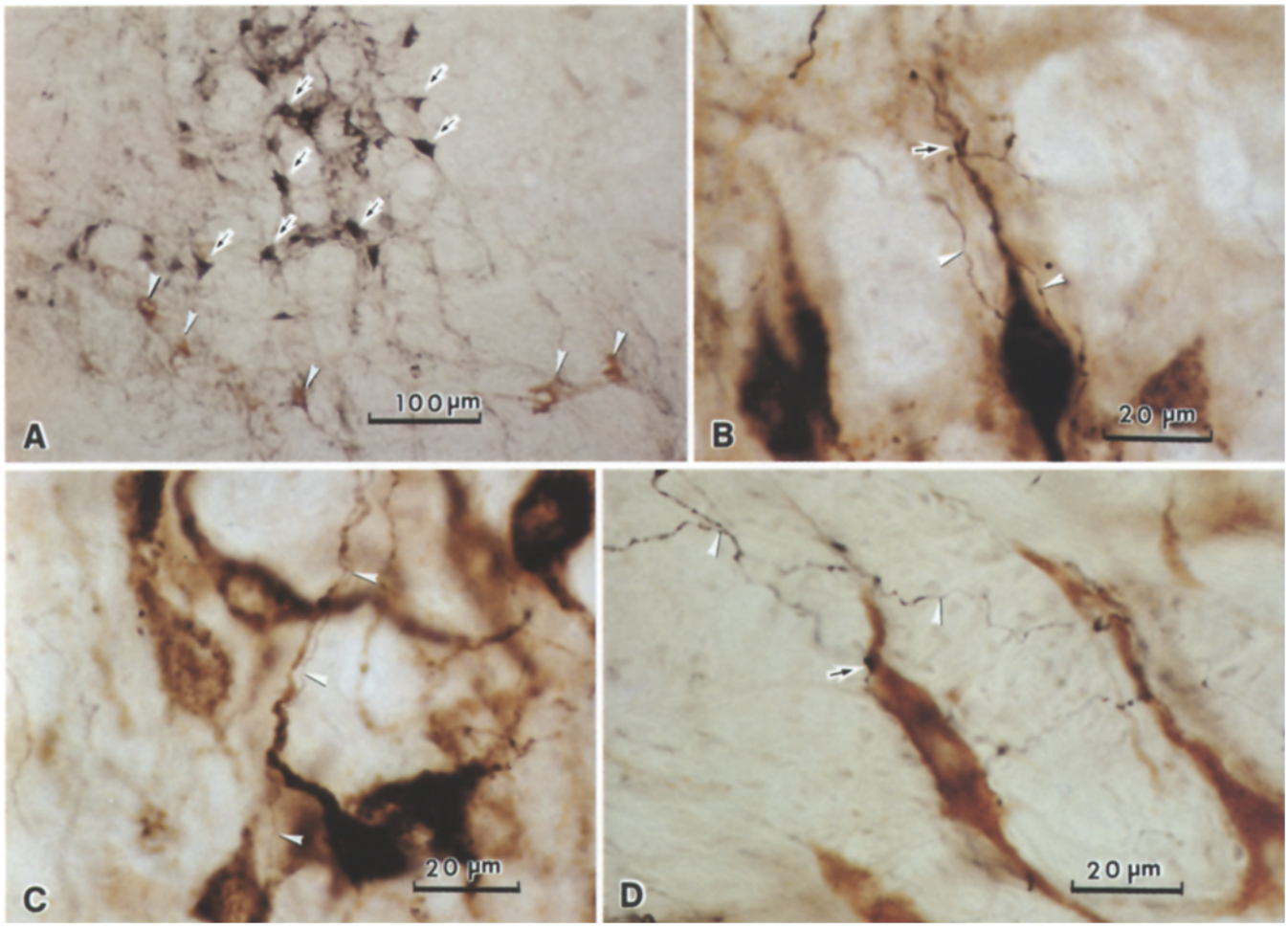

Fig. 3A–D. Color photomicrographs showing three neuronal elements, GPN cells labeled with HRP (black), DBH-positive cells (dark brown), and PHA–L labeled fibers (grayish black to balck). **A** Photomicrograph showing GPN cells (arrows) and DBH-positive cells (arrow-heads) within a network of PHA–L labeled fibers. **B, C** Higher magnification photomicrographs showing GPN cells and PHA–L labeled fibers. PHA–L labeled fibers (arrow-heads) are weaving around the dendrites of GPN cells. One of the varicosities of the PHA–L labeled fibers appears to contact the dendrite of a GPN cell (arrow). **D** higher magnification photomicrograph showing PHA–L labeled fibers and DBH-positive cells. Some PHA–L labeled fibers (arrow-heads) appear to terminate directly on DBH-positive cells (arrow)

the facial nucleus (Fig. 1B). From this dense network, branches consisting of labeled fibers extended laterally and ventrally to the regions dorsal or ventrolateral to the superior olivary complex. Each PHA–L labeled fiber showed varicosities and terminal boutons (Fig. 1C). In Fig. 2, varicosities and terminal boutons associated with PHA–L labeled fibers are plotted, in addition to GPN cells and DBH-positive neurons, to show their exact patterns of distribution. This figure clearly shows that varicosities and terminal boutons of PHA–L labeled fibers were distributed densely in the relatively restricted area with the GPN cells and A5 neurons. At more caudal levels, varicosities and terminal boutons of the labeled fibers persisted to the area just dorsolateral to the rostral tip of the facial nucleus (Fig. 2C, D). The GPN cells were mainly observed within the network of PHA–L labeled fibers. PHA–L labeled fibers with varicosities and terminal boutons were preferentially observed around the HRP-labeled GPN cells, but it was difficult to see direct contacts between PHA–L labeled terminal boutons or varicosities and HRP-labeled GPN cells (Fig. 3B, C). On the other hand, DBH-containing neurons were mainly found outside the network of PHA–L labeled fibers, except for a few DBH-positive neurons located inside the network. A small number of PHA–L labeled fibers with varicosities were occasionally observed in close proximity to DBH-containing neurons (Fig. 3D).

Discussion

Luiten et al. (1985) have demonstrated the course of descending fibers from the PVN to the brain stem using the PHA–L technique in the rat. Their description corresponds largely to those of Conrad and Pfaff (1976) in the rat and of Holstege (1987) in the cat, using autoradiography. Although these studies described projections to the A5 area, they did not relate this neuronal pathway

to GPN cells. The present study showed that descending fibers from the PVN project strongly to the ventrolateral region of the caudal pons where GPN cells are located. The descending PVN fibers, which display varicosities and terminal boutons, branch to form a dense network in this particular region, strongly suggesting that GPN cells receive a direct input from the PVN. Previous studies have demonstrated that the lateral hypothalamic area, including the perifornical region, projects to GPN cells, using a combination of anterograde and retrograde tracing methods (Hosoya et al. 1983; 1984). However, Byrum and Guyenet (1987), after injections of HRP into the A5 area of the rat, have shown that there are more labeled neurons in the PVN and the perifornical region than in the lateral hypothalamic area. These studies combined with the present results, suggest that GPN cells may receive descending afferents from the PVN and the perifornical region.

In immunohistochemically-stained sections, possible direct contacts between the soma of GPN cells and varicosities or terminal boutons of PHA–L labeled fibers were rarely observed. This does not mean that there are no direct contacts between both elements, because the retrograde HRP method used in the present study only labeled the cell soma and large primary dendrites. It is possible that descending PVN efferents make synaptic contact with more peripheral parts of the dendrites, such as second or third order branches. HRP-labeled neurons were preferentially located in the restricted region wherein dense varicosities or terminal boutons originating from the PVN were observed. This evidence strongly suggests that GPN cells receive a direct input from the PVN. However, it is necessary to show at the electron microscopical level whether descending fibers of the PVN actually terminate on GPN cells.

The superior salivatory nucleus consists of the parasympathetic preganglionic neurons that innervate the palatine, nasal, and lacrimal glands. The PVN has been implicated in autonomic and behavioral control, including feeding (Gold et al. 1977; Leibowitz 1978; Sawchenko et al. 1981) and has effects via the motor nucleus of the vagus nerve on the gastrointestinal system (see Zerihun and Harris 1983). In addition to these proposed functions of the PVN, the present study raises the possibility of a role for the PVN in controlling the secretomotor system, particularly in relation to salivation and lacrimation.

Although a few DBH-positive neurons were observed within the compact cell group consisting of GPN cells, a large number of DBH-positive neurons (A5 cells) were clearly separate from GPN cells. Both neuronal elements tend to form separate cell groups that lie near each other. Black-colored PHA–L labeled fibers were occasionally observed in close proximity to the brown-colored DBH-positive neurons. Neuronal connections between the hypothalamus and A5 cells had been suggested in several studies using autoradiography (Conrad and Pfaff 1976; Saper et al. 1976; Hosoya et al. 1983, 1984; Holstege 1987), where silver grain labelings originating from the PVN were observed in the ventrolateral part of reticular formation (the A5 area). However, in no previous study, both elements, A5 neurons and PVN descending fibers, were labeled simultaneously. Byrum and Guyenet (1987) demonstrated the retrograde labeled neurons in the PVN after WGA-HRP injections into the A5 area of the rat. The present study confirmed the PVN projection to the region of A5 cells. On the other hand, almost all (93%) of the A5 cells project to the spinal cord (Westlund et al. 1983; Byrum et al. 1984), especially to the intermediolateral cell column (Loewy et al. 1979; Loewy et al. 1986). This suggests that a large proportion of the DBH neurons receive descending inputs from the PVN and project to the spinal cord.

Electrical or chemical stimulation of the A5 area cause a decrease in heart rate and blood pressure (Neil and Loewy 1982). The A5 area projects directly to the intermediolateral cell column and also to the nucleus of the solitary tract (Loewy et al. 1986), and both connections are thought to be involved in cardiovascular control. Furthermore, electrical and chemical stimulation of the PVN produces cardiovascular changes (see Lovick and Coote 1988). In addition to its direct projection to the spinal cord, the PVN could influence the activity of sympathetic preganglionic neurons indirectly via projections to other cardiovascular nuclei such as the A5 nucleus (Lovick and Coote, 1988).

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