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SINGLE CNS NEURONS LINK BOTH CENTRAL MOTOR AND CARDIOSYMPATHETIC SYSTEMS: A DOUBLE-VIRUS TRACING STUDY

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Abstract—Two anatomical experiments were performed to test the hypothesis that single CNS neurons link the central areas that regulate the somatomotor and sympathetic systems. First, the retrograde neuronal tracer cholera toxin β-subunit was injected into the lateral parafascicular thalamic nucleus, a region that projects to both the motor cortex and striatum. Several days later, a second injection of the retrograde transneuronal tracer, pseudorabies virus (PRV), was made in the same rats in the stellate ganglion, which provides the main sympathetic supply to the heart. Using immunohistochemical methods, we demonstrate that the cholinergic neurons of the pedunculopontine tegmental nucleus (PPN) are connected to both systems. The second experiment used two isogenic strains of Bartha PRV as double transneuronal tracers. One virus contained the unique gene for green fluorescent protein (GFP) and the other had the unique gene for β-galactosidase (β-gal). GFP-PRV was injected in the stellate ganglion and β-gal-PRV was injected into the primary motor cortex. Double-labeled neurons were found in the lateral hypothalamic area (50% contained orexin) and PPN (approximately 95% were cholinergic). Other double-labeled neurons were identified in the deep temporal lobe (viz., amygdalohippocampal zone and lateral entorhinal cortex), posterior hypothalamus, ventral tuberomammillary nucleus, locus coeruleus, laterodorsal tegmental nucleus, periaqueductal gray matter, dorsal raphe nucleus, and nucleus tractus solitarius. These results suggest these putative command neurons integrate the somatomotor and cardiosympathetic functions and may affect different behaviors (viz., arousal, sleep, and/or locomotion).

Key words: acetylcholine, cardiovascular, exercise, mesencephalic locomotor region, sympathetic nervous system.

Locomotive behaviors, such as exercise, are complex motor functions that involve coordinated skeletal muscle activity and concurrent cardiovascular adjustments. The CNS sites that generate and/or modulate these changes have been studied independently of each other. For example, motor responses can be elicited by stimulation of a variety of brainstem and forebrain regions (e.g. Garcia-Rill et al., 1985; Sinnamon, 1993; Whelan, 1996). In particular, activation of the lateral hypothalamic area (LHA) or mesencephalic locomotor region, which includes the pedunculopontine nucleus (PPN), produces stepping activity (e.g. Skinner and Garcia-Rill, 1984; Sinnamon and Stopford, 1987; Parada et al., 1988b; De Parada et al., 2000). Other studies have shown that stimulation in these same regions elicits cardiovascular changes (e.g. Spencer et al., 1989; Allen and Cechetto, 1992; Kubo et al., 1999).

Despite being largely investigated as mutually exclusive functional systems, an integration of somatomotor and cardiovascular systems occurs during locomotion (Kramer et al., 2000). For example, a coupling of locomotion and cardiorespiratory responses is seen during exercise (e.g. DiMarco et al., 1983; Eldridge et al., 1985; Kramer et al., 2000). Similar coordinated locomotor and cardiovascular responses are evoked by stimulation of the LHA or mesencephalic locomotor region (e.g. Eldridge et al., 1981; Motekaitis and Kaufman, 1996; Chong and Bedford, 1997; Beyaert et al., 1998). Cardiorespiratory changes are likely to be regulated by central command mechanisms and not by muscle or metabolic feedback systems since they can be produced by hypothalamic stimulation in animals that had neuromuscular blockade (Eldridge et al., 1981; Eldridge et al., 1985; Kramer et al., 2000). It is not clear from these findings whether the motor and supportive cardiovascular functions are controlled by independent neuronal systems or by single neurons.

In the present study, the entire brain was examined for single neurons that could be the anatomical substrate for integrating the somatomotor and cardiosympathetic systems. In preliminary experiments, we used a library of over 400 rat brains that had small injections of the retrograde tracer cholera toxin β-subunit (CTb) in individual midline and intralaminar thalamic nuclei (Krout and Loewy, 2000a,b; Krout et al., 2001, 2002). These same animals had also received an injection of the transneuronal tracer Bartha pseudorabies virus (PRV) into the main sympathetic ganglion of the heart, the stellate ganglion (Fig. 1A). This allowed us to systematically examine CNS sites that provide a dual innervation of the central cardiosympathetic system and also innervate individual thalamic nuclei. A unique population of double-labeled neurons was found in the PPN in cases with CTb injections in the lateral parafascicular thalamic nucleus. Because the lateral parafascicular thalamic nucleus projects to both the motor-related stri-

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Abbreviations: β-gal, β-galactosidase; ChAT, choline acetyltransferase; CTb, cholera toxin β-subunit; GFP, green fluorescent protein; KPBS, potassium phosphate buffer solution; LHA, lateral hypothalamic area; PnO, oral pontine reticular area; PPN, pedunculopontine tegmental nucleus; PRV, pseudorabies virus; REM, rapid eye movement.

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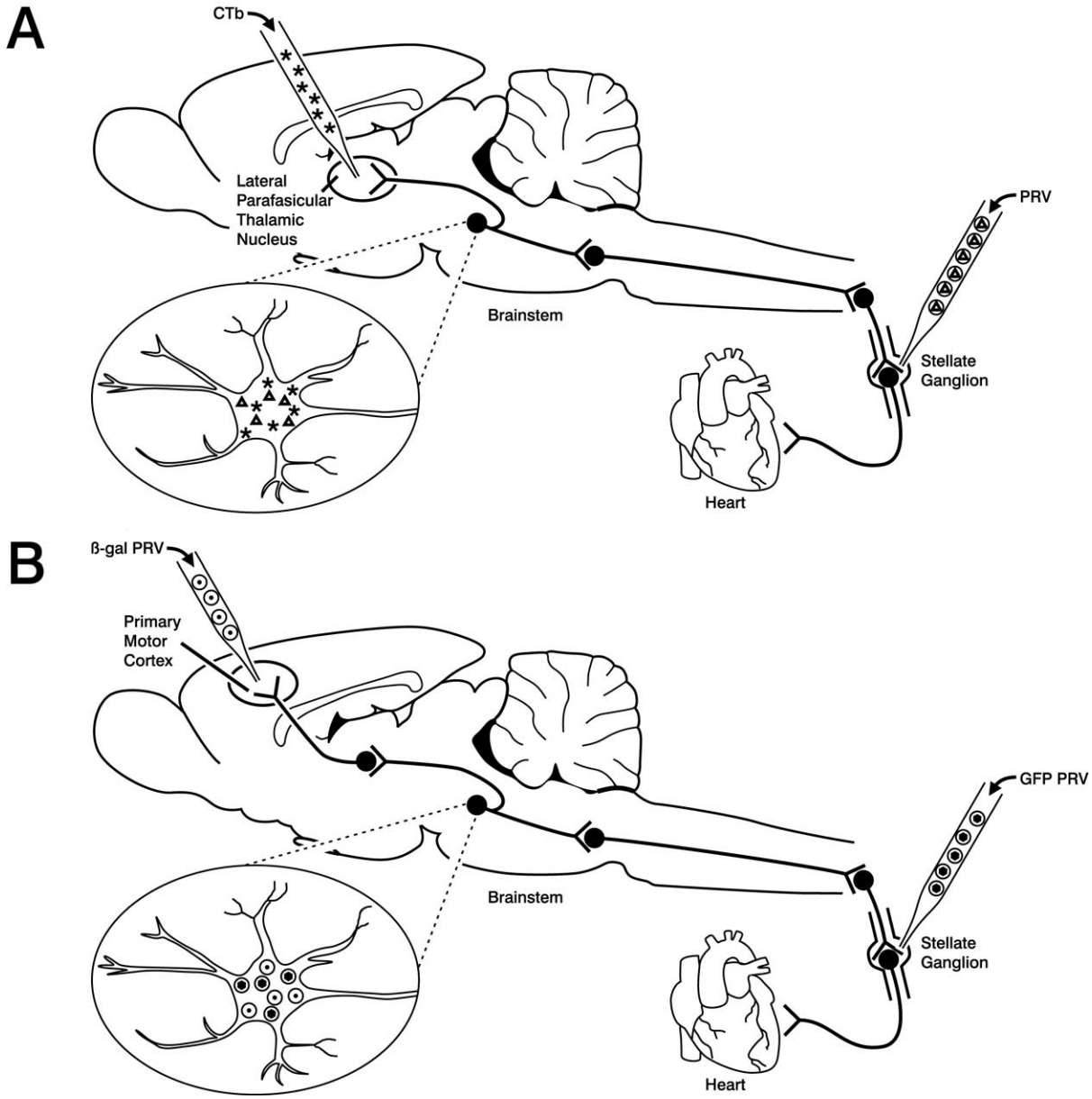

Fig. 1. Experimental design showing two different neuroanatomical tracing experiments. (A) Experiment 1: CTb was injected into the lateral parafascicular thalamic nucleus. After 5–9 days, PRV was injected into the ipsilateral stellate ganglion. As shown in the insert, the brains were examined with immunohistochemical methods for double-labeled neurons. (B) Experiment 2: GFP Bartha PRV was injected into the stellate ganglion. Two days later, β-gal Bartha PRV was injected into the ipsilateral primary motor cortex. Brain sections were examined for the co-localization of GFP and β-gal in single neurons.

atum and motor cortex, a second experiment was performed to determine whether single neurons in the PPN or other areas that project to the primary motor cortex are also linked to the cardiosympathetic system. In this second experiment, two genetically matched strains of Bartha PRV were used to identify single neurons capable of co-modulating the primary motor cortex and sympathetic preganglionic neurons. One Bartha PRV strain with a unique marker (β-galactosidase, β-gal) was injected into the motor cortex and another strain of Bartha PRV with a different unique marker (green fluorescent protein, GFP) was injected into the stellate ganglion (Fig. 1B). Single neurons that co-contained both strains of Bartha PRV were identified, suggesting that they modulate both motor-related forebrain circuits and cardiac-related components of the sympathetic nervous system.

EXPERIMENTAL PROCEDURES

CTb+PRV experiments

Sprague–Dawley rats ($n=9$; male; 200–400 g; Simonsen Laboratory, Gilroy, CA, USA) were anesthetized with sodium pentobar-

bital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. Injections of a 1% CTb solution made in distilled water (List Biological, Inc., Campbell, CA, USA) were delivered from glass micropipettes in the lateral parafascicular thalamic nucleus using coordinates from Paxinos and Watson (1997). Iontophoretic ejections were made using 7-μA on/off positive pulses from a Midguard precision current source (Stoelting, Wood Dale, IL, USA) for 15 min. Five to 9 days later, these rats were re-anesthetized and Bartha PRV (30 nL; 10^8 plaque-forming units/ml; K. Platt, Iowa State University, Ames, IA, USA) was injected into the right stellate ganglion, and then, after 5 additional days, the animals were re-anesthetized and perfused through the heart with 300 ml saline followed by 500 ml of 4% paraformaldehyde in 0.1-M sodium phosphate buffer (pH=7.4).

Cases were selected on the basis that the injection sites were limited to the lateral parafascicular thalamic nucleus, with only slight involvement of adjacent nuclei (Fig. 2A). Some of this material has been used in previous studies (Krout and Loewy, 2000a,b; Krout et al., 2001, 2002).

The brain was sectioned in the transverse plane at 50 μm on a freezing microtome and one 1-in-5 series was immunostained by a double avidin-biotin fluorescence method (Ferri et al., 1999) for CTb and PRV. A second 1-in-5 series was processed for CTb, PRV and choline acetyltransferase (ChAT) reactivity. For both groups of tissues (CTb+PRV and CTb+PRV+ChAT), sections were preincubated in a free avidin solution (100 μg/ml; Sigma, St. Louis, MO, USA), washed in potassium phosphate buffer solution (KPBS), placed a free biotin solution (20 μg/ml; Sigma) for 15 min, washed again, and reacted overnight with a 1:10,000 goat anti-CTb solution (List) made in 5% buffered donkey serum (pH=7.4). Sections were washed in KPBS, incubated in biotinylated rabbit anti-goat (1:100; Sigma) for 3 h, washed, moved into ABC solution (Vector, Burlingame, CA, USA) for 1 h, washed, and then placed in Cy3 streptavidin (1:200; Jackson ImmunoResearch, West Grove, PA, USA) solution for 2 h. They were then transferred directly into pig anti-PRV (1:25,000, K. Platt, Iowa State University) overnight.

To eliminate potential cross-reaction between streptavidin conjugates, the sections went through a blocking procedure (Ferri et al., 1999). First, the sections were washed in KPBS and then placed into the free avidin solution (100 g avidin/ml KPBS) for 40 min, washed, incubated in 10% formaldehyde for 40 min, washed again, and put in a free biotin solution (20 μg biotin/ml KPBS) for 40 min. Without washing, they were transferred to biotinylated rabbit anti-pig (1:100; Sigma) for 3 h, washed, placed in ABC solution for 1 h, washed, and moved to Alexa-488 streptavidin solution (1:75; Molecular Probes, Eugene, OR, USA). At this point, the first 1-in-5 series (for CTb and PRV) was washed, mounted onto slides, and coverslipped with a solution made with 90 ml glycerol, 10 ml 0.1-M Na_2HPO_4 buffer with 0.1% sodium azide (pH 7.4), and 0.1 g *n*-propyl gallate.

The second series of sections (CTb+PRV+ChAT) was not mounted but was processed for ChAT immunostaining. After the Alexa-488 streptavidin (see above), these sections were washed and then transferred to 5% donkey serum buffer containing sheep anti-ChAT (1:1000; Chemicon, Temecula, CA, USA) overnight. After another series of avidin/formaldehyde/biotin blocking steps as described above, the sections were washed, placed in a 1:100 biotinylated rabbit anti-sheep (Jackson) solution made in 5% donkey serum buffer for 3 h, washed, incubated in ABC (Vector) for 2 h, washed, and put into Marina Blue streptavidin (1:100; Molecular Probes) for 2 h. The sections were then washed, mounted onto slides, and coverslipped as above.

The location of each multi-labeled neuron (i.e. CTb+PRV or CTb+PRV+ChAT) was mapped by fluorescence microscopy using an X-Y plotting system (MDplot version 3.3, AccuStage, St. Paul, MN, USA). Cytoarchitectonic divisions followed the rat atlas of Paxinos and Watson (1997, 1999) and were determined with the aid of darkfield microscopy as well as an adjacent 1-in-5 series of sections counterstained with 0.1% Thionin (see Krout and Loewy, 2000a for details). The nomenclature for the PPN followed that described by Rye et al. (1987), except both cholinergic and non-cholinergic neurons within the cholinergic zone were included as part of the PPN. The number of double-

Abbreviations used in the figures

AcbC	accumbens nucleus, core
AcbSh	accumbens nucleus, shell
AHN	anterior hypothalamic nucleus
Ahz	amygdalohippocampal zone
APir	amygdalopiriform cortex
Bar	Barrington's nucleus
Cl	claustrum
CnF	cuneiform nucleus
CPu	caudate-putamen
CTF	central tegmental field
CVLM	caudal ventrolateral medulla
DMH	dorsomedial hypothalamic nucleus
DpMe	deep mesencephalic nucleus
DR	dorsal raphe nucleus
DRd	dorsal raphe nucleus, dorsal part
ENTl	lateral entorhinal cortex
F	Fornix
fr	fasciculus retroflexus
GiV	gigantocellular reticular nucleus, ventral part
Ic	internal capsule
IC	inferior colliculus
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LHA	lateral hypothalamic area
LL	lateral lemniscus
LPAG	periaqueductal gray matter, lateral column
lPF	lateral parafascicular thalamic nucleus
LV	lateral ventricle
MPO	medial preoptic nucleus
Mt	mamillothalamic tract
NTS	nucleus tractus solitarius
OPC	oval paracentral thalamic nucleus
Opt	optic tract
PAG	periaqueductal gray matter
PB	parabrachial nucleus
PH	posterior hypothalamic area
Pir	piriform cortex
Po	posterior thalamic nucleus
PPTg	pedunculopontine tegmental nucleus
PRC	precommissural nucleus
PVN	paraventricular hypothalamic nucleus
PVp	posterior periventricular hypothalamic nucleus
RCh	retrochiasmatic nucleus
RMg	nucleus raphe magnus
Rt	reticular thalamic nucleus
s.c.	superior colliculus
Scp	superior cerebellar peduncle
SN	substantia nigra
SNpc	substantia nigra pars compacta
SPTg	subpedunculopontine tegmental nucleus
SUBv	ventral subiculum
TMv	ventral tuberomammillary nucleus
VM	ventromeidal thalamic nucleus
VMc	caudal ventromedial thalamic nucleus
ZI	zona incerta

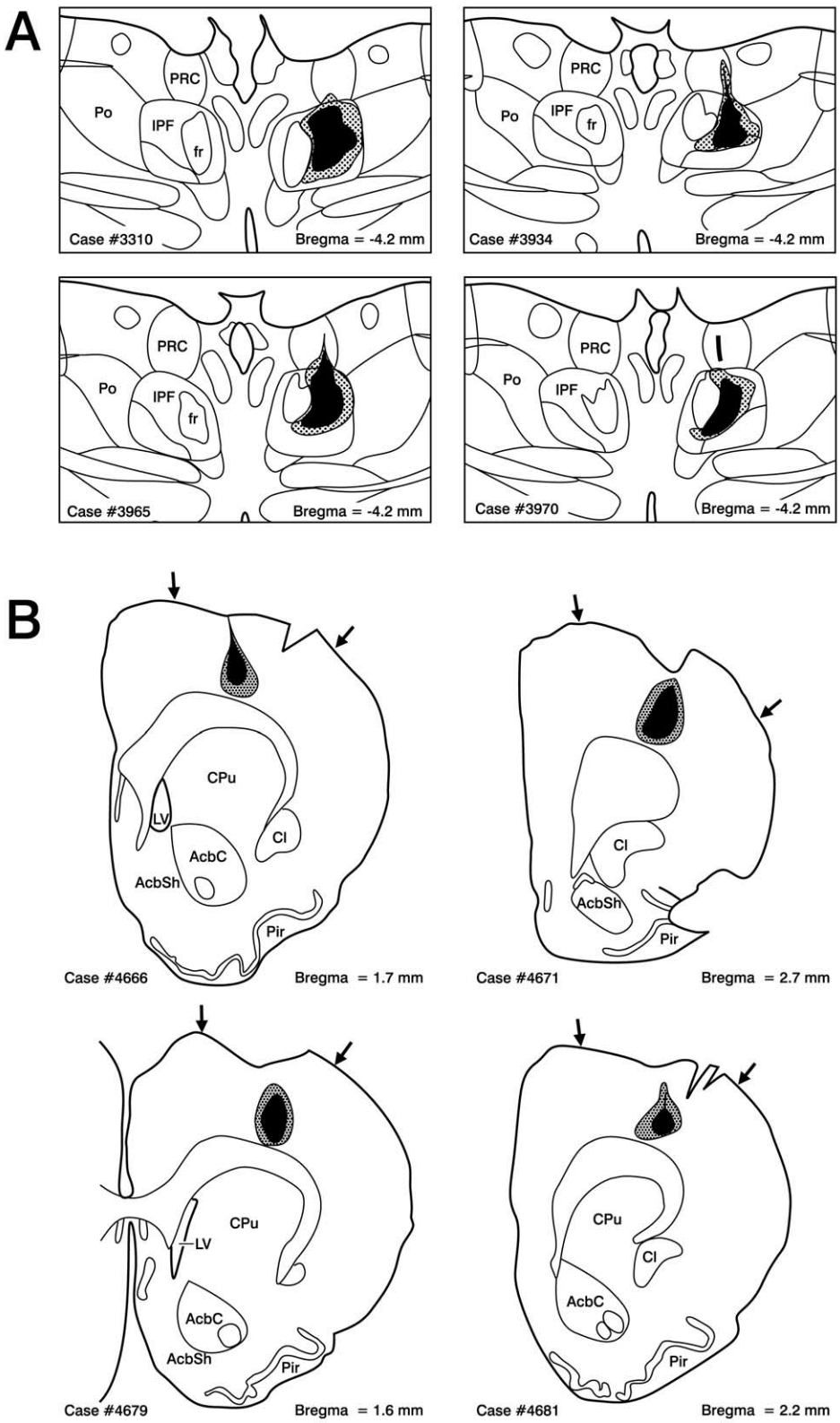

Fig. 2. Summary of injection sites. (A) Experiment 1 shows the location of CTb injections in the lateral parafascicular thalamic nucleus. (B) Experiment 2 illustrates the β-gal PRV injection sites in the motor cortex from the four cases used in the double-virus experiments. CTb was used as the injection site marker because PRV is taken up and rapidly transported from injection sites. The black region indicates the 'core' region of the injection site, whereas the stippled region indicates where CTb spread to the neuropil without cell-body labeling. The arrows in B indicate the cytoarchitectonic boundaries of the primary motor cortex.

(CTb+PRV) and triple (CTb+PRV+ChAT)-labeled cells in each brainstem nucleus was counted using analysis tools in the MDplot program. For each case, the percentage of CTb+PRV neurons in each nuclei that were also cholinergic was calculated [number of triple-labeled neurons/(number of double+ number of triple-labeled neurons)].

The location of the forebrain injection site for both the CTb experiments and the double-virus experiments were determined using the same procedure. A 1-in-5 series through the injected region (lateral parafascicular thalamic nucleus for CTb experiments and motor cortex for double-virus experiments) was placed into a 1:120,000 goat anti-CTb (List) solution made in the same 5% donkey serum buffer described above. After 2 days in this primary antisera, the sections were washed, placed into biotinylated donkey anti-goat (1:100; Jackson) for 3 h, washed again, put into ABC solution (Vector) for 1 h and then washed. The CTb injection sites were then visualized with DAB (1 Sigmafast tablet/15 ml distilled water; Sigma) for 2 to 5 minutes. Sections were mounted on gelatin-coated slides, allowed to dry, and counterstained with Thionin before being coverslipped with DPX (BDH Laboratories, Poole, UK). Injection sites were drawn using a camera lucida on a Leica Wild M8 stereoscope.

Double-virus transneuronal labeling experiments

Two isogenic Bartha strains of PRV were used in this experiment. Each contained a single unique heterologous gene inserted in the nonessential glycoprotein G locus of the unique short segment of the PRV genome: β-gal Bartha PRV or GFP Bartha PRV (Mettenleiter and Rauh, 1990; Jons and Mettenleiter, 1997).

Sprague–Dawley rats ($n=4$; female; 200–400 g; Harlan Laboratories, Indianapolis, IN, USA) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and GFP Bartha PRV was injected into the right stellate ganglion. All efforts were made to minimize the number of animals used and their suffering. The injectate was made as follows: 2 μl of 0.1% CTb were added to a 5 μl aliquot of GFP Bartha PRV (viral titer$=1\times10^8$ plaque forming units/ml). As measured by a graduated reticule, the volume delivered was 42 nl (i.e. approximately 2000 virions). After 2 days, β-gal Bartha PRV was injected stereotaxically into the motor cortex of the same rats (Fig. 2B) using coordinates from Paxinos and Watson (1997). This viral injection was prepared just like that for GFP Bartha PRV. After 3 additional days, the animals were anesthetized as before and received an injection of colchicine (100 μg/10 μl; Sigma) in the right lateral ventricle. After 1 more day, the animals were re-anesthetized and perfused as above.

The brain was sectioned as described above. A 1-in-5 series of sections was placed overnight in a mixture made in 5% buffered donkey serum (0.1-M Na_2HPO_4 buffer containing 0.1% sodium azide and 0.3% Triton X-100 (Sigma), pH=7.4) of goat anti-β gal (1:3000; Arnell Products, New York, NY, USA) and guinea-pig anti-GFP (1:2000; V. Karpitskiy, WUMS, St. Louis, MO, USA) along with one of four additional antibodies. Depending on the rostrocaudal level of the section, the tissue was reacted with either rabbit anti-tyrosine hyrdoxylase (1:1000, Chemicon; caudal medulla), rabbit anti-ChAT (1:5000; Chemicon; rostral medulla through the midbrain as well as the forebrain rostral of the optic chiasm), rabbit anti-orexin (1:5000; Phoenix, Belmont, CA, USA; rostral midbrain to hypothalamus caudal of the optic chiasm), or rabbit anti-arginine vasopressin (1:300,000; Phoenix; hypothalamus at the level of the optic chiasm). After washing in 0.02-M (pH 7.4), the sections were reacted in a mixture of biotinylated donkey anti-guinea-pig (1:150; Jackson), Cy3-donkey anti-rabbit (1:300; Jackson), and Cy2-donkey anti-goat (1:200; Jackson) made in 5% buffered donkey serum without azide for 3 h. Following a wash in KPBS, they were placed in peroxidase-conjugated streptavidin (1:500; Jackson; in donkey serum without azide) for 2 h, washed, reacted with biotinyl tyramide (1:300; NEN Life Science Products, Boston, MA, USA) for 10 min, washed again, and put in Marina Blue-conjugated streptavidin (1:100; Molecular Probes) for 2 h. After a final rinse in KPBS, the sections were mounted onto gelatin-coated slides, allowed to dry, and then coverslipped as above.

The location of each double-labeled (GFP+β-gal) and triple-labeled (GFP+β-gal+peptide) neuron was recorded using fluorescence microscopy. Cell group boundaries were determined according to Paxinos and Watson (1997) and Paxinos et al. (1999) with the aid of darkfield microscopy and relative to the distribution of peptide-containing regions.

The research described in this report was reviewed and approved by the WUMS Animal Care Committee and conforms to NIH guidelines.

RESULTS

Experiment 1: CTb injection in the lateral parafascicular thalamic nucleus and PRV injection in the stellate ganglion

Double-labeled (CTb+PRV) neurons were found in the brainstem and hypothalamus following CTb injections in the lateral parafascicular thalamic nucleus and PRV injections in the stellate ganglion. The main finding of this experiment was the presence of double-labeled neurons in the PPN with many fewer such neurons identified in other cell groups (Fig. 3A). The PPN was the only site that was labeled in every experiment ($n=9$), predominately in the caudal two-thirds of the nucleus. An average of 4.8±0.9 (mean±S.E.M.) double-labeled cells were found in this region, a significantly greater number than in any other brain region (one-way ANOVA, Tukey's pairwise comparison, $P<0.001$).

The other cell groups that contained double-labeled neurons were relatively large areas of the midbrain that extended for several millimeters rostrocaudally. These included the periaqueductal gray matter (viz., mainly lateral column, with lesser amounts in the ventrolateral and dorsolateral columns), substantia nigra, and deep mesencephalic reticular formation. Labeling was also observed along the dorsal boundary of the reticular portion of the substantia nigra. A occasional CTb+PRV neuron was observed in several other sites: LHA, ventrolateral subnucleus of the dorsal raphe nucleus, intermediate gray and white layers of the superior colliculus, parabrachial nucleus, Barrington's nucleus, locus coeruleus, ventral medullary reticular formation, subnucleus reticularis dorsalis, and the region immediately ventral to the nucleus ambiguus.

The laterodorsal, pedunculopontine, and subpeduncular tegmental nuclei accounted for over 40% of all the double-labeled neurons in the brainstem. Because many double-labeled neurons were found in these putative cholinergic cell groups, a second series of sections was processed to examine whether cholinergic PPN neurons project directly to the lateral parafascicular thalamic nucleus and are also multi-synaptically linked to the cardiosympathetic system. The number of double-labeled neurons (CTb+PRV) in the PPN in this series was not different from the original lateral parafascicular thalamic nucleus data (Student's paired t-test: $P<0.05$). In this second 1-in-5 series, 53 double-labeled neurons were found within the PPN and, of these, 50 were also cholinergic (approximately 95%). Fig. 4 shows an example

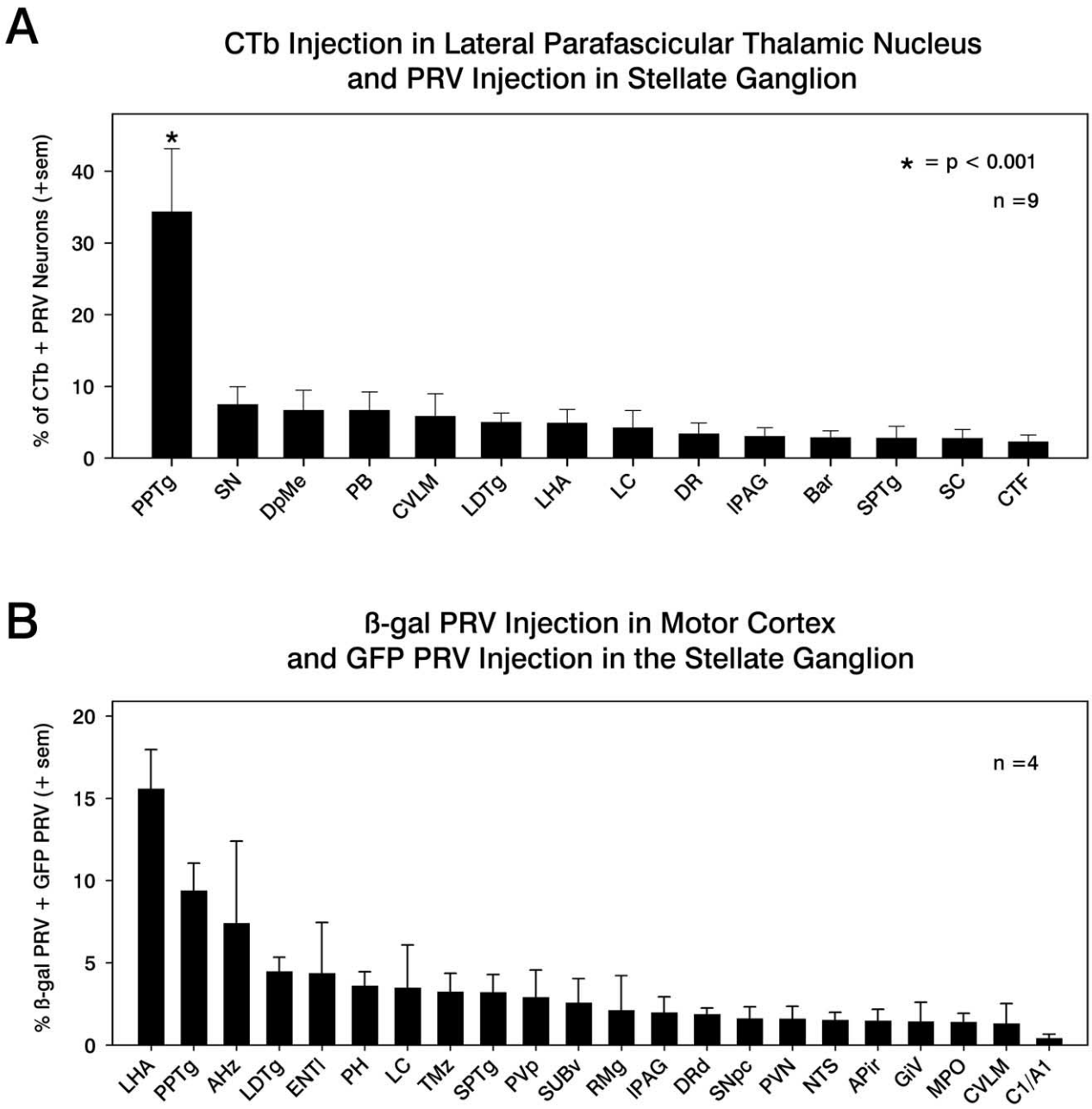

Fig. 3. Quantitative summary of double-labeled cells. Average percentage of double-labeled neurons found in individual nuclear groups in (A) experiment 1 and (B) experiment 2.

of triple-labeled neurons in the PPN. Most labeled neurons in the laterodorsal tegmental nucleus (24/26; approximately 90%) and subpeduncular pontine tegmental nucleus (18/22; approximately 80%) were also cholinergic.

Experiment 2: β-gal Bartha PRV injection in the primary motor cortex and GFP Bartha PRV-injection in the stellate ganglion

Cases with unilateral β-gal Bartha PRV injections in the right motor cortex and GFP Bartha PRV injections in the right stellate ganglion (n=4) were examined for double-labeled (β-gal+GFP) neurons. Overall, many more double-labeled neurons were found than in the CTb+PRV experiments. Most of these cells were found in the LHA and PPN (Fig. 3B and 5). In two cases with higher levels of GFP infection from the stellate ganglion, numerous double-labeled neurons were also found in two temporal lobe regions: the amygdalohippocampal transition zone and lateral entorhinal cortex. Several other regions contained a few β-gal+GFP neurons in every one of the four cases. These included the posterior hypothalamus, ventral tube-

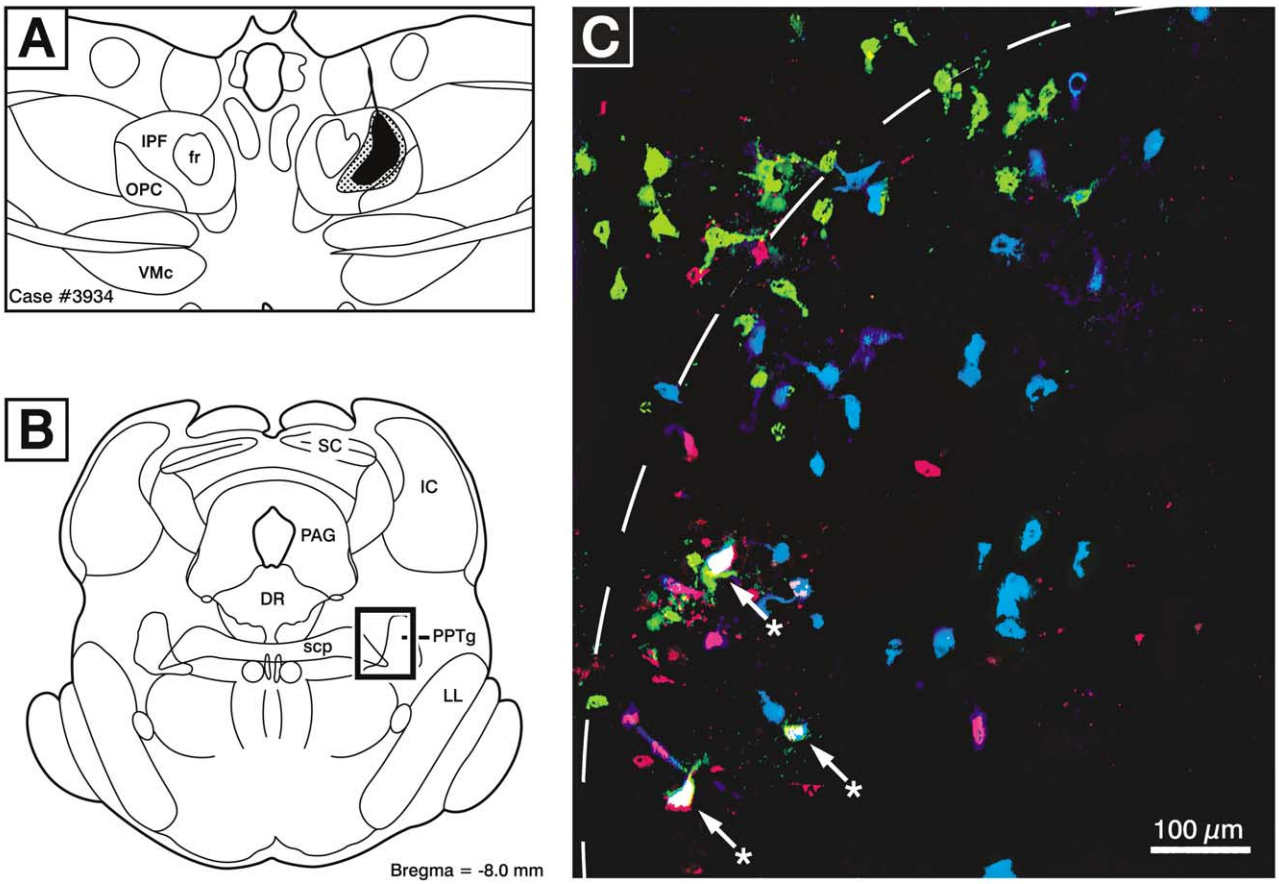

Fig. 4. Double-labeled neurons in the PPN found in experiment 1. (A) CTb was injected in the lateral parafascicular thalamic nucleus (case 3934) and PRV was injected in the ipsilateral stellate ganglion. (B) At the mesencephalic level, double-labeled cells were found in the PPN. The outlined area is illustrated in the photomicrograph (C). PRV-labeled neurons are green, CTb neurons are red, and ChAT-positive neurons are blue. Triple-labeled (CTb+PRV+ChAT) neurons appear white and are indicated by starred arrows.

romammillary nucleus, lateral column of the periaqueductal gray matter, subpeduncular pontine tegmental nucleus, dorsal part of the dorsal raphe, lateral dorsal tegmental nucleus, locus coeruleus, and medial portion of the nucleus of the solitary tract.

Similar to the data from the CTb+PRV experiments, a large percentage of triple-labeled neurons (β-gal+GFP+ChAT) were found in the laterodorsal, pedunculopontine, and subpeduncular tegmental nuclei (Fig. 3B). The PPN contained an average of 49.8±12.7 (mean±S.E.M.) double-labeled neurons and, of these, almost 95% were also cholinergic (189/199) (Fig. 5D, E). An example of these triple-labeled PPN neurons is shown in Fig. 4C. In addition, most laterodorsal tegmental nucleus (approximately 99%; 127/128) and subpeduncular tegmental nucleus (approximately 88%; 54/61) β-gal+GFP-labeled neurons were also ChAT positive (Fig. 5D, E).

The LHA had the most β-gal+GFP-labeled neurons in the double-virus experiments: 92.8±33.1 (mean±S.E.M.). As can be seen in Fig. 5A–C over half of these neurons also contained orexin immunoreactivity (approximately 57%; 211/371). An example of the triple-labeled LHA neurons is shown in Fig. 6A. A few neurons in the posterior hypothalamic area were also triple labeled with orexin (approximately 16%; 22/134).

DISCUSSION

This study demonstrates that single cholinergic PPN neurons (and other nearby neurons in the laterodorsal and subpeduncular nuclei) are multisynaptically linked to central motor areas (viz., motor-related portion of the thalamus and primary motor area of the cerebral cortex) as well as to central sympathetic circuits. In addition, orexin and non-orexin LHA neurons have branched, multisynaptic projections to the motor cortex and central sympathetic system.

The simplest interpretation of the PPN data is that double-labeled neurons possess branched axons, with one collateral innervating the lateral parafascicular thalamus nucleus and the other collateral is connected via multisynaptic connections to the central sympathetic system (Fig. 7). Because double-labeled neurons were found in the LHA in the double-virus experiments but not in the CTb+PRV experiments, it is likely that alternate pathways exist. For example, the LHA projects to the basal forebrain,

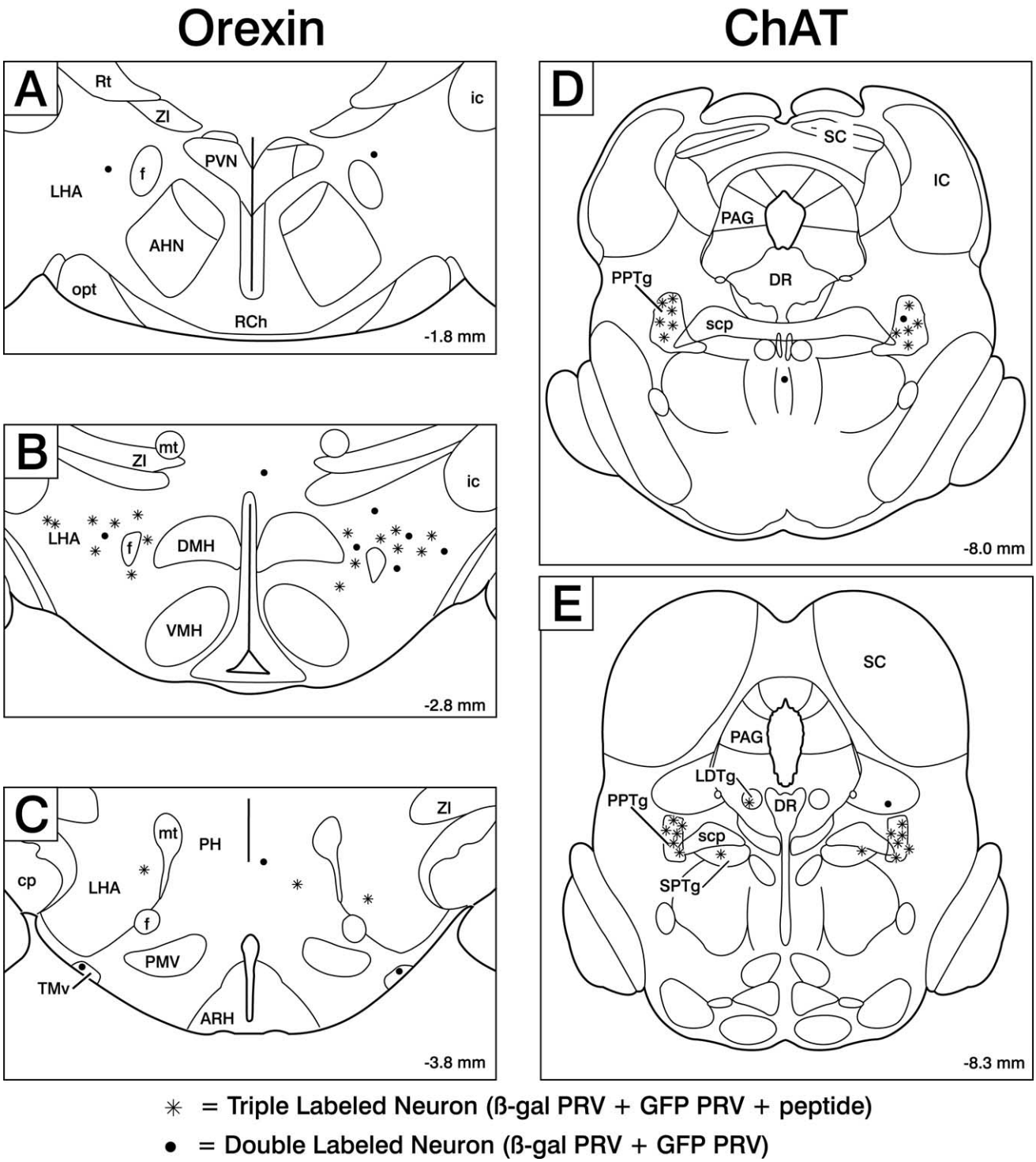

Fig. 5. Distribution of double-labeled neurons after double-virus transneuronal labeling experiment shown in schematic fashion in Fig. 1, experiment 2. Double- (β-gal PRV+GFP PRV) and triple- (β-gal PRV+GFP PRV+peptide) labeled neurons from case 4666 are transferred onto brain drawings from Paxinos and Watson (1997). Asterisks in panels A–C indicate neurons that contain β-gal PRV+GFP PRV+Orexin. Asterisks in panels D and E indicate neurons that contain β-gal PRV+GFP PRV+ChAT.

a region with widespread inputs to cerebral cortex, including the primary motor cortex (Saper, 1984; Cullinan and Zaborszky, 1991). It is possible that the LHA neurons are linked directly to the primary motor cortex (Saper, 2000), but when the LHA from each of the double-virus cases was examined for CTb+GFP Bartha PRV neurons (note: CTb served as a marker for determining the spread of the viral injection, but it is a standard retrograde tracer as well that

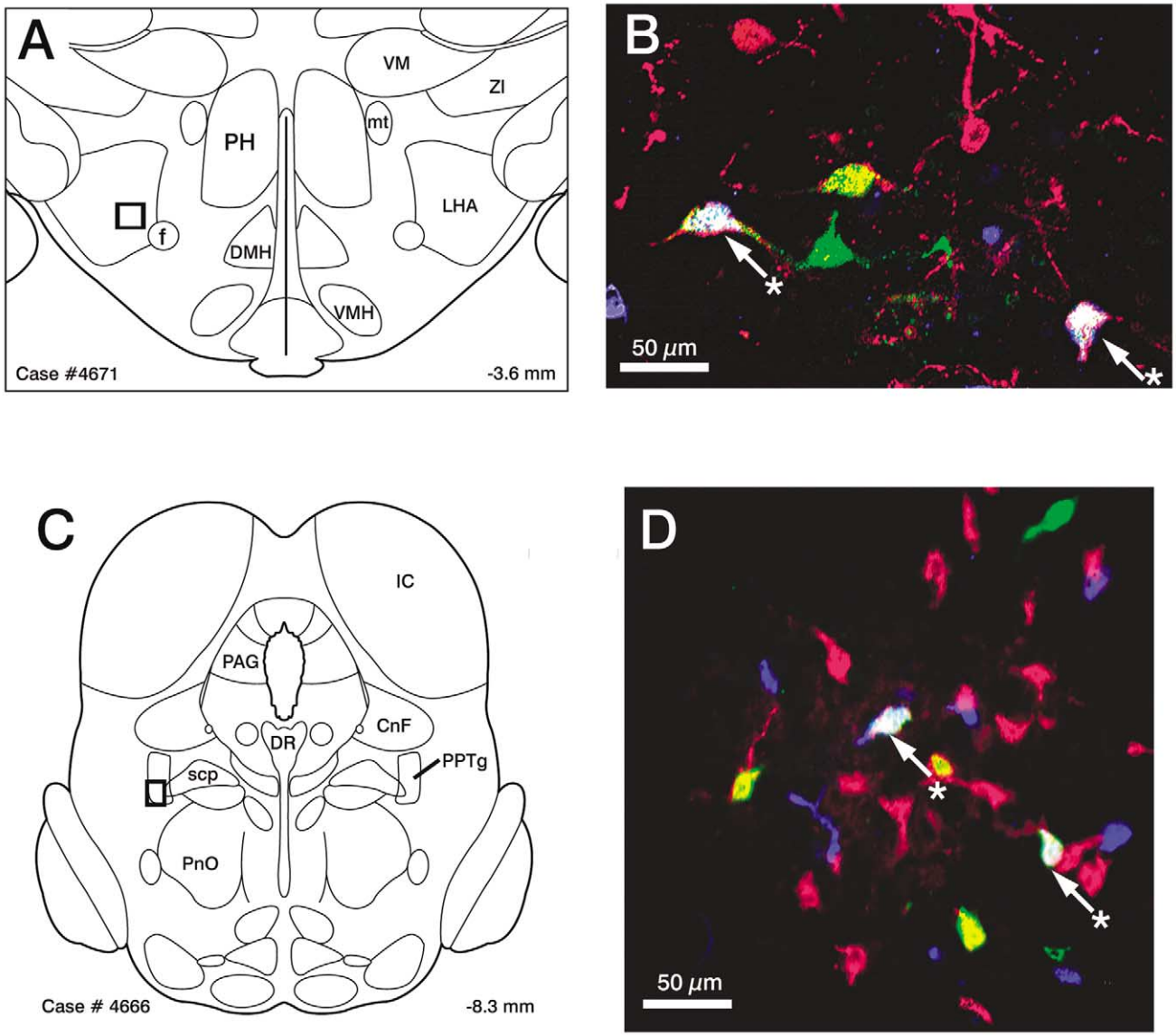

Fig. 6. Following separate injections of two different strains of Bartha PRV in motor cortex and stellate sympathetic ganglion (see Fig. 1, experiment 2), LHA and PPN neurons were transneuronally labeled with both viruses. (A) Hypothalamic sections showing the area of the photomicrograph presented in (B). (B) Orexin-LHA neurons appear red, β-gal PRV neurons are green, and GFP PRV neurons are blue. Double-labeled neurons (Orexin+β-gal PRV) appear yellow. Triple-labeled neurons (Orexin+β-gal+GFP) appear white and are indicated by arrows with asterisks; these neurons provide multisynaptic connections to the motor cortex and stellate sympathetic outflow. (C) Midbrain level showing the area of the photomicrograph presented in (D). (D) Cholinergic-PPN neurons appear red, β-gal PRV neurons are green, and GFP PRV neurons are blue. Double-labeled neurons (ChAT+β-gal PRV). Triple-labeled (β-gal+GFP+ChAT) neurons appear white and are indicated by arrows with asterisks; these neurons provide multisynaptic connections to the motor cortex and stellate sympathetic outflow.

permitted us to examine the direct inputs to the motor cortex), no double-labeled neurons were identified ($n=4$; data not presented), suggesting that the hypothalamocortical pathway is multisynaptic. In addition, the descending limb of this system probably depends upon a synaptic relay in the rostral ventrolateral medulla, since both the PPN and LHA innervate this region (Yasui et al., 1990; Allen and Cechetto, 1992). This area provides a dense input to the intermediolateral cell column, the primary site of origin of sympathetic outflow (Loewy et al., 1981; Helke et al., 1982; Ross et al., 1984).

Technical considerations

We have assumed that the CTb and PRV in single neurons were retrogradely transported by separate axonal branches (Fig. 7A), but it is possible that these neurons do not possess bifurcated axons, but rather were double-labeled as the result of co-transport of CTb and PRV (Fig. 7B). The lateral parafascicular thalamic nucleus contained a few PRV-labeled neurons after viral injections in the stellate ganglion at the 5-day survival periods used here (see below). While these thalamic neurons are likely third-

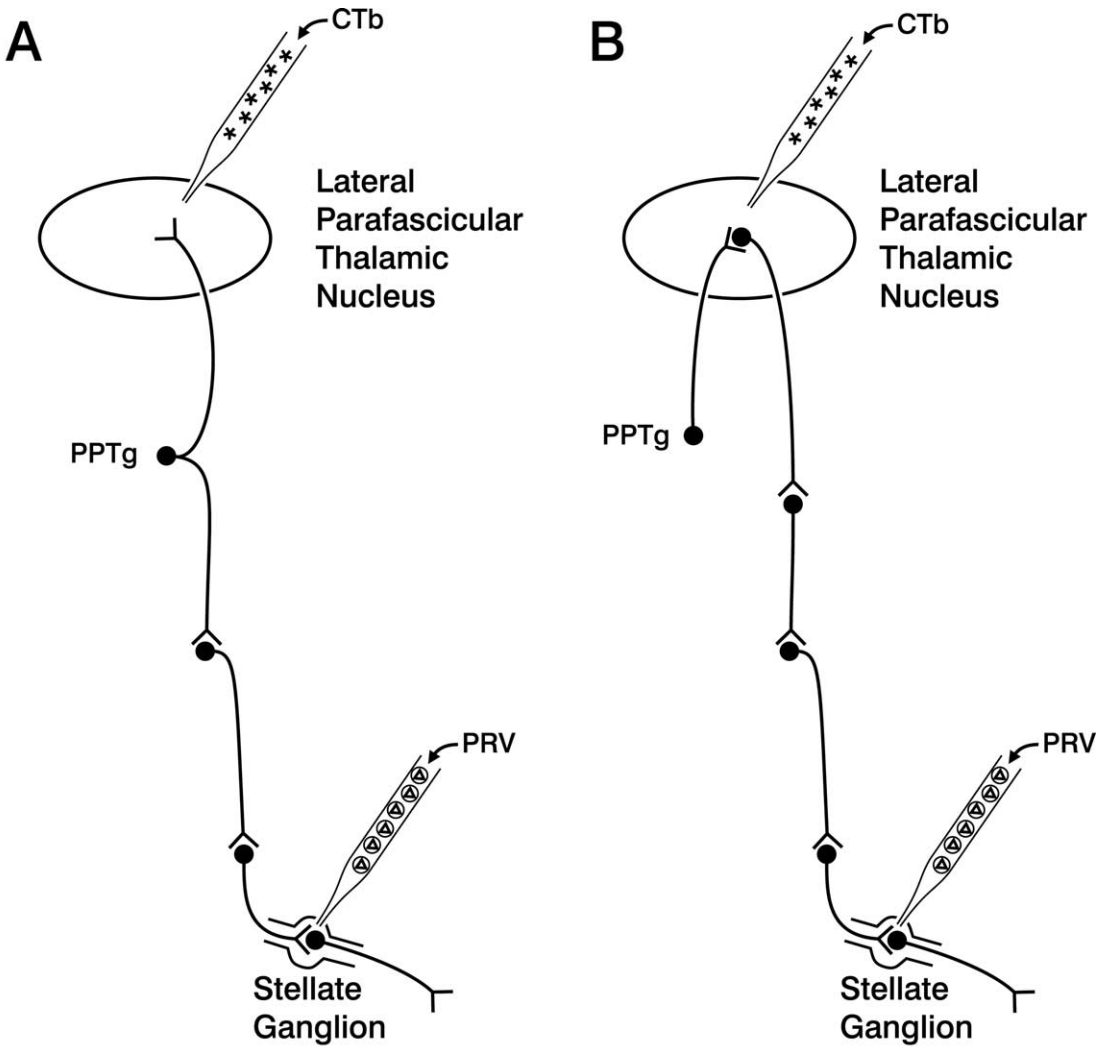

Fig. 7. Two alternative pathways that explain the presence of CTb+PRV labeling in the PPN. (A) PPN neurons may be double-labeled as the result of possessing bifurcated axons. (B) PPN neurons may be double-labeled as a result of co-transport of CTb along a chain of neurons that were infected with PRV.

or higher-order neurons labeled through a pathway that involves brainstem nuclei such as the PPN and rostral ventrolateral medulla, the lateral parafascicular thalamic nucleus also projects to other sympathetic premotor regions such as the nucleus raphe magnus (Hermann et al., 1997). If the viral labeling occurred through this latter pathway, then the CTb+PRV labeling in the brainstem could be the result of retrograde transport of both tracers from these thalamic cells. This is unlikely for three reasons. First, almost no PRV-labeled cells were seen in the lateral parafascicular thalamic nucleus at the 5-day survival period used in the present experiments (see below). In addition, other thalamic nuclei that never contain virally labeled neurons at this time point (e.g. central medial, medial parafascicular, reuniens, and rhomboid nuclei) consistently result in CTb+PRV brainstem neurons after being injected with CTb (unpublished observations). Second, the sequence of viral infection from the stellate ganglion does not support this possibility. At 3- and 4-day post-injection periods, no PRV-positive cells were seen in the thalamus, but they are present in most of the nuclei discussed here (Jansen et al., 1995). One extra day is an unlikely time period to allow PRV to be transported into thalamic cells, be replicated, cross a synapse, and be transported into the brainstem neurons. Third, in the double-virus experiments, no β-gal+GFP neurons were identified in the lateral parafascicular thalamic nucleus, suggesting that the brainstem labeling in these experiments was not due to co-transport of the viruses from lateral parafascicular thalamic nucleus neurons.

In order to further investigate the possibility that PRV-positive cells in the PPN are retrogradely labeled from the lateral parafascicular thalamic nucleus, all the remaining sections co-extensive with the CTb injection site were processed for PRV. Less than five PRV-positive neurons were identified in the region of the CTb injection in any of the nine cases. And, in one case (3307), no virally labeled cells were found in the thalamus, yet several CTb+PRV co-localized neurons

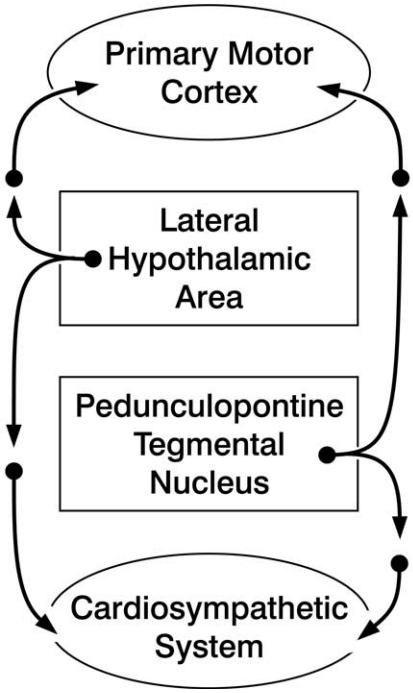

Fig. 8. Proposed neural circuits that send integrative signals to primary motor cortex and sympathetic system.

were observed in the PPN. Thus, the double-labeled neurons are not the result of co-transport of CTb and PRV from lateral parafascicular thalamic nucleus neurons, but are interpreted to have axons that send processes to both the thalamus/motor cortex and to sympathetic pre-motor systems.

Functional implications: pedunculopontine tegmental nucleus and LHA

The two most prominent sites containing double-labeled neurons were the PPN and LHA (Fig. 8). The PPN was the most common site of CTb+PRV neurons and the second most common site of β-gal+GFP neurons in the double-virus experiments. The lateral parafascicular thalamic nucleus projects to motor-related sites such as the motor cortex, dorsolateral striatum, and subthalamic nucleus (Berendse and Groenewegen, 1990, 1991). Additionally, the LHA had the most double-labeled neurons after viral injections in the motor cortex. Therefore, the discussion of these data will center on these putative forebrain pathways.

PPN

Two hypotheses can be put forward concerning the function of PPN neurons and their potential role in sympathetic regulation. First, these cells may modulate sympathetic functions that occur during rapid eye movement (REM) sleep and/or arousal. Second, the PPN is the site of integration of limbic and motor output systems (Winn et al., 1997), and during complex behavioral changes concomitant sympathetic adjustments are likely to occur.

PPN neurons initiate and maintain REM sleep and function in desynchronizing the cerebral cortex (Rasmusson, 1993; Rye, 1997). These neurons project to the oral pontine reticular area (PnO), which is regarded as the REM sleep induction zone (Mitani et al., 1988; Yamamoto et al., 1990; Leonard and Lydic, 1997). The PnO may also play a role in sympathetic modulation, since chemical stimulation of this region produces decreases in blood pressure (Shiromani et al., 1986; Dutschmann and Herbert, 1999). PnO neurons may be responsible for the stereotypical autonomic changes, such as sympathetically mediated transient increases in heart rate and blood pressure, that are associated with REM sleep (Mancia, 1993). We have noted transneuronal PRV labeling in the PnO at the 5-day survival period used here, indicating that these neurons are capable of regulating sympathetic functions (unpublished observations). While single-labeled PRV and CTb neurons were found throughout the PnO, no double-labeled neurons were identified in this region, raising the possibility that the PnO generates cardiovascular effects associated with REM sleep by parallel output systems. For example, some PnO neurons have efferents that innervate the lateral parafascicular thalamic nucleus (Krout et al., 2002) while other neurons provide information to central sympathetic circuits. Local interneurons could conceivably regulate the ascending and descending projections arising from the PnO. Alternatively, other sites, such as the PPN, that innervate the PnO may integrate the parallel outputs.

More direct evidence implicates the PPN in sleep and autonomic processes. For example, stimulation of the PPN produces changes similar to those seen after activation of the PnO, including REM-like sleep along with sympathetic changes (e.g. increased blood pressure and heart rate) and desynchronization of the EEG (Kawahara et al., 1993; Chong and Bedford, 1997; Rye, 1997; Kubo et al., 1999). These changes may be due to release of acetylcholine from terminals originating from the PPN and the laterodorsal tegmental nuclei in the PnO and thalamus, respectively (Glenn and Steriade, 1982; Steriade and Glenn, 1982; Hallanger et al., 1987; Levey et al., 1987; Lydic and Baghdoyan, 1993). Also, small chemical lesions of the PPN disturb REM sleep for several days and larger lesions produce long-term changes in REM sleep (viz., 28 days) suggesting that this nucleus subserves REM sleep and wakefulness functions (Webster and Jones, 1988; Inglis et al., 1995). Finally, the PPN has direct outputs to brainstem sympathetic premotor centers, including the ventral medulla (Yasui et al., 1990; Lai et al., 1999). It is possible, then, that the neurons shown here which co-innervate the thalamus and sympathetic premotor network have collaterals which also project to the REM sleep induction zone (i.e. PnO). No data, however, are currently available to support this hypothesis. At the least, the current experiments demonstrate that PPN neurons appear anatomically capable of desynchronizing the cortical EEG via the lateral parafascicular thalamic nucleus as well as modulating autonomic changes associated with the sleep/wake cycle, perhaps indirectly via the PnO or the rostral ventrolateral medulla.

The descending PPN efferent projections to brainstem nuclei (e.g. PnO) have received much attention, but PPN

neurons also modulate higher-brain regions since this area projects to the output nuclei of the basal ganglia (Saper and Loewy, 1982; Rye et al., 1987; Hallanger and Wainer, 1988; Gould et al., 1989; Inglis and Winn, 1995). One of the targets innervated by the PPN is the lateral parafascicular thalamic nucleus. This thalamic region innervates the dorsolateral striatum and motor cortex as well as the subthalamic nucleus and can also modulate the output of the basal ganglia (Berendse and Groenewegen, 1990, 1991; Mouroux and Feger, 1993; Gillies and Willshaw, 1998; Redgrave et al., 1999). These anatomical findings suggest that PPN neurons are capable of modulating both forebrain motor systems as well as simultaneously affect the sympathetic outflow to cardiovascular system, which could be quite important for somatomotor-sympathetic integration that occurs in different states ranging from heavy exercise to sleep.

LHA

It is well established that the LHA is an important center of autonomic regulation. For example, chemical stimulation of the LHA elicits a fall in blood pressure and heart rate (Spencer et al., 1989). In addition to this autonomic dimension, the LHA has also been implicated in the control of locomotion (see Sinnamon, 1993 for review). Chemical lesions of this hypothalamic area decrease motor activity, such as wheel-running (e.g. Blake and Gladfelter, 1986) and several studies have shown significant changes in locomotion following infusion of cholinergic or dopaminergic agonists or antagonists into the LHA (see below).

Many of these locomotor changes may be related to another aspect of LHA activity, viz., the control of food and water intake. For example, when dopamine was infused into the LHA of food- and water-deprived rats, the spontaneous activity of those rats was decreased compared with rats that had not been deprived (Parada et al., 1990). These findings suggest that the locomotor effects of dopaminergic input to the LHA are dependent on the rats' level of satiety. Because sulpiride, a dopaminergic receptor blocker, induces locomotion and feeding behaviors (Parada et al., 1988a,b) when infused in the LHA, the LHA motor system has been proposed to be involved in finding and acquiring food and water (De Parada et al., 2000).

LHA-driven locomotion is integrated with cardiosympathetic and respiratory responses (Eldridge et al., 1981, 1985; DiMarco et al., 1983; Waldrop et al., 1988), a feature that may be related to regulating energy resources during exercise (Iwamoto et al., 1996; Kramer et al., 2000). These responses may involve the recruitment of other autonomic systems since the LHA also regulates the release of adrenal catecholamines and pancreatic hormones (e.g. insulin). Future studies will be needed to test this hypothesis.

CONCLUSION

Cholinergic PPN and orexin as well as non-orexin LHA neurons send branched projections to CNS sites that regulate somatomotor and sympathetic functions. The ascending PPN projections are relayed in the lateral parafascicular thalamic nucleus, and ultimately reach the motor cortex. The LHA indirectly innervates the cerebral cortex via an undetermined pathway. Both PPN and LHA neurons provide descending multisynaptic projections to the stellate sympathetic outflow; this pathway is likely to use the rostral ventrolateral medulla as a relay site.

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