of SpoIIAB, 14.1 μg of SpoIIAA, 100 μM ATP, and 16 μg of template in transcription buffer (800 μl) (*21*).

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Central Command Neurons of the Sympathetic Nervous System: Basis of the Fight-or-Flight Response

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During stress, the activity of the sympathetic nervous system is changed in a global fashion, leading to an increase in cardiovascular function and a release of adrenal catecholamines. This response is thought to be regulated by a common set of brain neurons that provide a dual input to the sympathetic preganglionic neurons regulating cardiac and adrenal medullary functions. By using a double-virus transneuronal labeling technique, the existence of such a set of central autonomic neurons in the hypothalamus and brainstem was demonstrated. These neurons innervate both of the sympathetic outflow systems and likely function in circumstances where parallel sympathetic processing occurs, such as in the fight-or-flight response.

The sympathetic nervous system regulates a broad range of visceral functions and, during extreme emotional or physical states, activates both the cardiovascular and adrenal catecholamine systems for homeostatic adjustments (*1*). The central nervous system (CNS) neurons responsible for coactivation of these autonomic changes are thought to be governed by a common set of central command neurons that provides dual projections to the sympathetic outflow systems that control the heart and adrenal gland. Although this biological idea was described in the late 1920s (*1*) and is taught as a basic principle of autonomic function, it has not been possible to define the command neurons and CNS circuits responsible for this response, because of the technical limitations. We have now developed a double-virus transneuronal labeling method to localize and to chemically characterize the central command neurons.

The general scheme of this study is presented in Fig. 1A. Two different genetically engineered forms of the Bartha strain of pseudorabies virus (PRV) were used as transneuronal tracers (*2*); each expressed a unique marker antigen in infected host cells (Fig. 1B). Both produce specific infections within functionally related chains of neurons. One virus was injected into the stellate ganglion—the major sympathetic ganglion that innervates the heart (*3*)—and the other virus was injected into the ipsilateral adrenal gland of anesthetized Sprague-Dawley rats or vice versa (*4*). After 4 days, rats were anesthetized and perfused with fixative, and their brains processed by a triple-antibody immunohistochemical procedure for the two unique virally induced cellular markers (gC viral glycoprotein and β-galactosidase) and also stained for a neurotransmitter enzyme or neurotransmitter (choline acetyltransferase, phenylethanolamine-*N*-methyltransferase, tyrosine hydroxylase, serotonin, or oxytocin) (*5*). A total of 20 rats contained double-virus infections; eight of these had CNS patterns of infection for both viruses that were similar to those found in earlier studies in which a single strain of PRV was injected into the adrenal gland (*6*) or the stellate ganglion (*7*).

The brain sites that were transneuronally labeled with the two different viruses that had been injected into the terminal fields of the sympathoadrenal and stellate sympathetic preganglionic neurons are illustrated in Fig. 2. Three areas of the medulla oblongata were labeled: (i) rostral ventrolateral medulla; (ii) rostral ventromedial medulla, which includes the lateral paragigantocellular reticular nucleus, parapyramidal nucleus, and ventral and pars alpha regions of the gigantocellular reticular nuclei; and (iii) caudal raphe nuclei (raphe magnus, raphe pallidus, and raphe obscurus). Monoaminergic medullary neurons contribute to this projection. C1 adrenergic neurons (Fig. 3), in both the rostral

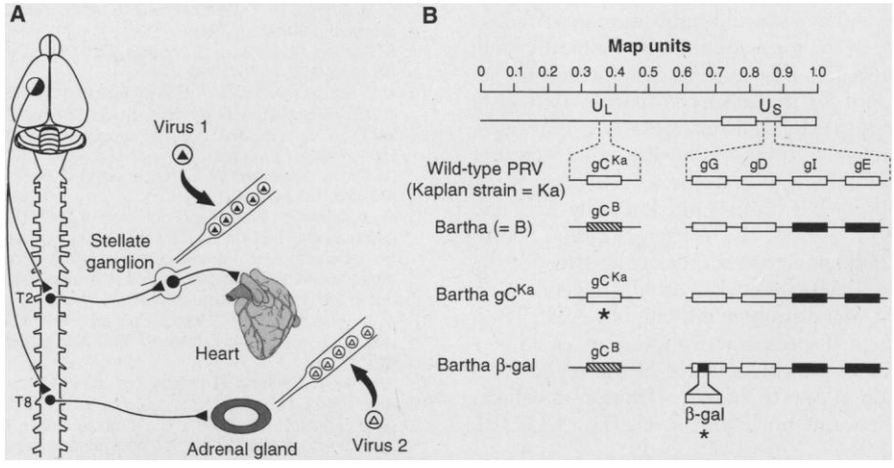

Fig. 1. (**A**) The CNS sites that regulate the sympathetic outflow of both the stellate ganglion and adrenal gland were identified by a double-virus transneuronal labeling method. In the same animal, one virus was injected into the stellate ganglion and the second virus into the ipsilateral adrenal gland. Each virus produced a unique intracellular marker in infected host neurons, and some neurons contained both markers, indicating that they regulate both sympathetic systems. (**B**) Two genetically modified forms of Bartha PRV used for transneuronal labeling of central sympathetic circuits (Bartha-gC^{Ka} PRV and Bartha β-galactosidase PRV). The genomes of these two viruses differ from the wild-type PRV and the original attenuated Bartha strain. Each modified virus contained a gene that produced a different intracellular antigen in the infected host neurons that could be detected by specific antibodies (asterisks). Bartha-gC^{Ka} PRV produced the wild-type form of the gC glycoprotein, which was detected by a mouse monoclonal antibody. Bartha β-galactosidase PRV was detected by a goat polyclonal antibody directed against β-galactosidase (*2*). U_L, unique long segment; U_S, unique short segment. Black boxes, deleted sequences; striped boxes, altered sequences.

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ventrolateral medulla and lateral paragigantocellular reticular nucleus, provided the greatest projection to the spinal cord and were most concentrated in the rostral ventrolateral medulla immediately caudal to the facial nucleus (Table 1). Double-labeled serotonergic neurons of the caudal raphe nuclei were found mainly in the parapyramidal and raphe magnus nuclei. In the pons, 25% of the infected A5 neurons were catecholaminergic, whereas none of the infected subcoeruleus neurons showed this property (Table 1). In three animals, the caudal ventrolateral periaqueductal gray matter (laterodorsal tegmental nucleus) contained double-labeled neurons, and most of these exhibited choline acetyltransferase immunoreactivity. In the hypothalamus, double-labeled neurons were found in the paraventricular and caudal lateral hypothalamic nuclei with additional neurons found in the perifornical area. Less than 10% of the double-infected neurons in the paraventricular hypothalamic nucleus contained oxytocin immunoreactivity. In the spinal cord, a small number of double-infected neurons were found in the dorsal horn (laminae I, II, and V) and intermediate gray matter (lamina VII) (approximately seven in an alternate series of sections through the T5 to T7 segments).

Bartha PRV and the two Bartha mutants used here produce highly specific transneuronal infections in the CNS (8, 9). However, a potential complicating factor could be that PRV caused local, nonspecific infec-

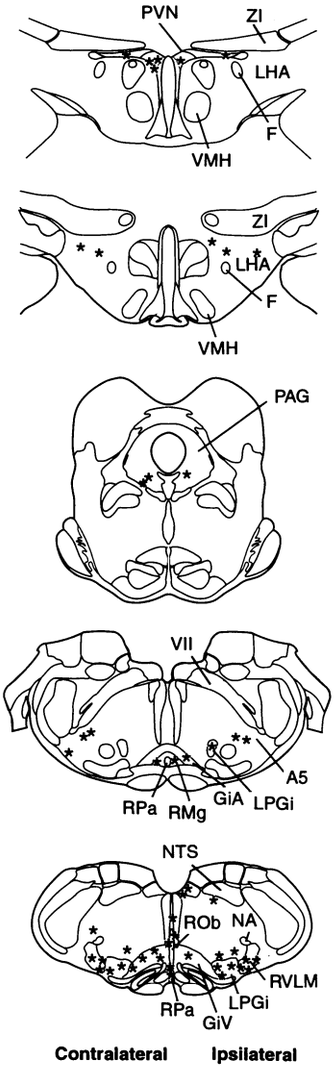

Fig. 2. CNS sites that project to both sympathetic preganglionic outflow systems that innervate stellate ganglion and adrenal gland. Double-labeled neurons are indicated by asterisks. Drawings were made from the computer graphics program Brain Maps (*14*). A5, A5 cell group; F, fornix; GiA, pars alpha region of the gigantocellular reticular nucleus; GiV, ventral region of the gigantocellular reticular nucleus; LHA, lateral hypothalamic area; PVN, paraventricular hypothalamic nucleus; LPGi, lateral paragigantocellular reticular nucleus; NA, nucleus ambiguus; NTS, nucleus tractus solitarius; PAG, periaqueductal gray matter; RMg, raphe magnus nucleus; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVLM, rostral ventrolateral medulla; VII, facial nerve; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta.

Table 1. Chemically defined CNS neurons that project to both the stellate and adrenal sympathetic preganglionic outflow systems. In experiment I, Bartha-gC^{Ka} PRV was injected into the stellate ganglion, and Bartha β-galactosidase PRV was injected into the adrenal gland of adult rats (n = 5). The column labeled "identified for chemical" gives the number of double-infected neurons identified for the indicated neuroenzyme or neurotransmitter in a one-in-five series of transverse sections. Data are expressed as mean ± SEM. In experiment II, the data from the converse experiment are presented. Bartha-gC^{Ka} PRV was injected into the adrenal gland, and Bartha β-galactosidase PRV was injected into the stellate ganglion (n = 3).

Brain structure	Number of double-infected neurons			
	Experiment I		Experiment II	
	Total	Identified for chemical	Total	Identified for chemical
		Adrenaline		*Adrenaline*
Rostral ventrolateral medulla	20.5 ± 4.3	11.5 ± 2.9	20.0 ± 7.1	13.7 ± 5.0
Lateral paragigantocellular reticular nucleus	12.0 ± 1.8	8.0 ± 1.5	12.7 ± 2.8	7.3 ± 1.8
Dorsal medulla	5.8 ± 1.9	5.0 ± 2.1	2.0 ± 1.2	1.0 ± 0.6
		Serotonin		*Serotonin*
Raphe obscurus	3.8 ± 1.9	1.4 ± 0.4	3.7 ± 1.9	1.7 ± 0.9
Raphe pallidus	1.8 ± 0.5	0.8 ± 0.2	2.0 ± 0.6	0.0 ± 0.0
Raphe magnus	7.0 ± 2.7	2.6 ± 0.9	8.7 ± 2.2	2.0 ± 0.6
Parapyramidal nucleus	3.0 ± 1.3	1.0 ± 0.3	8.0 ± 3.8	4.0 ± 2.3
		Noradrenaline		*Noradrenaline*
A5 area	14.2 ± 4.4	3.2 ± 1.0	12.3 ± 4.3	2.7 ± 0.9
		Oxytocin		*Oxytocin*
Paraventricular hypothalamic nucleus	8.0 ± 2.6	0.6 ± 0.4	9.7 ± 5.7	0.7 ± 0.7

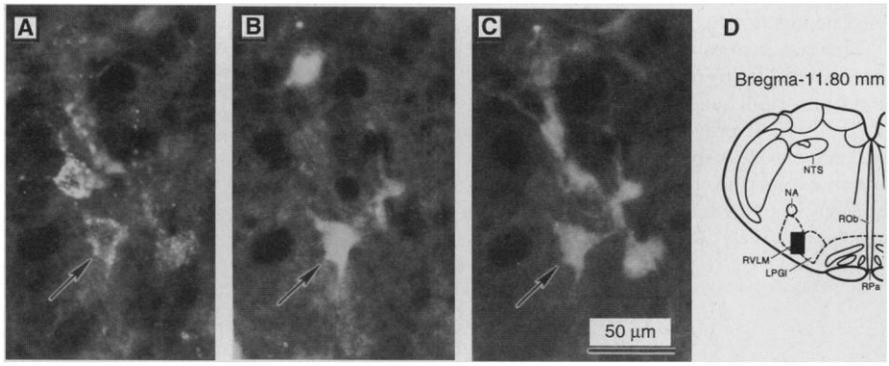

Fig. 3. C1 adrenergic neuron (arrow) that projects to both stellate and sympathoadrenal SPNs. (**A**) gC-positive immunoreactivity in a C1 transneuronally labeled neuron after an injection of Bartha-gC^{Ka} PRV into the stellate ganglion. (**B**) β-Galactosidase immunoreactivity in the same neuron as in (A) after an injection of Bartha β-galactosidase PRV into the adrenal gland. (**C**) Phenylethanolamine-*N*-methyltransferase immunoreactivity in the neuron shown in (A) and (B). (**D**) Line drawing indicating the region (rectangle) illustrated in the photomicrographs. Abbreviations are the same as in Fig. 2. Scale bar = 50 μm.

tions. For example, if PRV spread from one functional class of sympathetic preganglionic neurons (SPNs) to adjacent, functionally unrelated SPNs, this would produce false-positive double labeling in bulbo- and hypothalamospinal projection neurons. To control for this potential problem, we examined each mutant virus for nonspecific lateral spread of viral infection. Stellate SPNs were retrogradely labeled with a standard retrograde neuronal cell body marker, cholera toxin β-subunit, and the ipsilateral sympathoadrenal SPNs were infected with one of the two mutant viruses (*10*). The intermediolateral cell column was examined for double-labeled SPNs in the T5 to T7 spinal regions, which contain maximal overlapping populations of these two different functional classes of neurons. The amount of double labeling served as an index of false-positive labeling. Control experiments with two conventional retrograde neuronal markers established that 4 ± 1 double-labeled SPNs occur in a one-in-two series of longitudinal sections through these three spinal segments (*10*). In the control experiment with Bartha β-galactosidase PRV, 7.9 ± 2.2 (n = 8) SPNs were double labeled, whereas in the control experiment with Bartha gC^{Ka} PRV, 7.5 ± 1.3 SPNs were found (n = 4); neither value was statistically different (two-tailed Student's t test) from the earlier control data (*10*), suggesting that both of the PRV mutants used here produced specific infections in the SPN cell column. In addition, when both viruses were used together, the number of double-labeled SPNs was similar (experiment I, 7.7 ± 2.7 and experiment II, 7.3 ± 0.7). Together with earlier findings that indicate that during a viral infection in the CNS, microglia and macrophages seal off the infected area, enhancing the probability that infections spread in a transsynaptic fashion (*11*), these results indicate that Bartha PRV can be used to produce highly specific infections within functionally defined neural systems.

The two viruses used in this study had genomes similar to the attenuated live vaccine strain (Bartha PRV) that is used in the pig industry to prevent Aujeszky's disease, and each produces specific patterns of transneuronal infections in rats (*9*). Because Bartha PRV is an attenuated virus, the success rate is relatively low, approaching 20% when used at its optimal titer (*6*, *7*). Therefore, it was not surprising that, when two mutant Bartha variants were used simultaneously, the success rate was 3%. Although this may be regarded as a shortcoming of this method, the qualitative and quantitative results were highly reproducible (Table 1) and were in excellent agreement with earlier single-virus studies (*6*, *7*).

In summary, several CNS regions contain selective subsets of neurons capable of regulating both the cardiac and adrenal sympathetic outflow systems. Although each of these areas has been implicated in autonomic regulation (*12*), it is now clear that there are central neurons potentially capable of producing both sympathetically controlled neural and endocrine (adrenal catecholamine) responses. In all likelihood, these neurons function as command premotor neurons that direct multiple sympathetic responses in a simultaneous and parallel direction. As suggested by Cannon (*13*), they may affect the sympathetic outflow in general like the soft and loud pedals of a piano, by modulating all the notes being played at a given moment.

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4. Bartha gC^{Ka} PRV (100 nl) [(titer = $10^{4.5}$ plaque forming units per milliliter (pfu/ml)] was injected into the right stellate ganglion, and Bartha β-galactosidase PRV (100 nl) (titer = 1×10^7 to 2×10^7 pfu/ml) was injected into the ipsilateral adrenal gland of pentobarbital-anesthetized (50 mg per kilogram of body weight) adult Sprague-Dawley rats of either sex (Sasco, O'Fallon, MO) (175 to 200 g; n = 168); the converse experiment was also performed (n = 88). Out of a total of 256 rats, 30% showed no infection, 62% were infected with only one virus, and 8% exhibited double-virus infections. Within the latter category, 40% (8 rats) displayed highly specific infections for both viruses, whereas the remaining 60% (12 rats) were overinfected and, thus, discarded from our analysis.
5. The rats were reanesthetized with pentobarbital and perfused through the heart with 0.9% saline, followed by 2% paraformaldehyde–25% picric acid made in 0.1 M sodium phosphate buffer (pH 7.0). The brains were removed and processed for immunohistochemistry. Transverse sections of the brain were cut at 50 μm on a freezing microtome and incubated for 16 hours at room temperature in 5% normal donkey serum made in 0.3% Triton-X 100 and 0.02 M potassium phosphate–buffered saline (KPBS) containing three antibodies: goat to β-galactosidase (1:400 dilution) (Arnel Products, New York, NY), mouse monoclonal antibody to gC glycoprotein (ascites fluid) (1:400), and antibodies to one of the following proteins raised in rabbits: phenylethanolamine-*N*-methyltransferase (1:150) (Chemicon, Temecula, CA), tyrosine hydroxylase (1:500) (East-Acres Biologicals, Southbridge, MA), serotonin-bovine serum albumin (1:100) (Incstar, Stillwater, MN), oxytocin (1:500) (Chemicon), or choline acetyltransferase (1:500). The sections were washed twice in KPBS, incubated in 1:100 dilution of biotinylated donkey antibody to mouse immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA), washed, and then transferred to a solution of tetramethylrhodamine-conjugated streptavidin (1:100) (Jackson), fluorescein isothiocyanate–conjugated donkey antibody to goat IgG (1:50) (Jackson), and 7-amino-4-methylcoumarin-3-acetic acid–conjugated donkey antibody to rabbit IgG (1:50) (Jackson) for 4 hours. The sections were washed, mounted, and a cover slip placed over them with glycerol–phosphate-buffered saline mounting medium containing 0.1% *p*-phenylenediamine to prevent fading.
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