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# Fos Expression by Glutamatergic Neurons of the Solitary Tract Nucleus after Phenylephrine-Induced Hypertension in Rats

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ABSTRACT

The baroreflex pathway might include a glutamatergic connection between the nucleus of the solitary tract (NTS) and a segment of the ventrolateral medulla (VLM) called the caudal ventrolateral medulla. The main goal of this study was to seek direct evidence for such a connection. Awake rats were subjected to phenylephrine- (PE-) induced hypertension (N=5) or received saline (N=5). Neuronal activation was gauged by the presence of Fos-immunoreactive (Fos-ir) nuclei. Fos-ir neurons that contained vesicular glutamate transporter 2 mRNA (glutamatergic neurons) or glutamic acid decarboxylase mRNA (GABAergic neurons) were mapped throughout the medulla oblongata. Saline-treated rats had very few Fos-ir neurons. In PE-treated rats, Fos-ir neurons were detected in both NTS and VLM. In NTS, 72% of Fos-ir neurons were glutamatergic and 26% were GABAergic. In the VLM, 41% of Fos-ir neurons were glutamatergic and 56% were GABAergic. In VLM, Fos-ir glutamatergic neurons were evenly distributed and were often catecholaminergic, whereas Fos-ir GABAergic cells were clustered around Bregma −13.0 mm. This region of the VLM was injected with Fluoro-Gold (FG) in eight rats, four of which received PE and the rest saline. Fos-ir NTS neurons retrogradely labeled with FG were detected only in PE-treated rats. These cells were exclusively glutamatergic and were concentrated within the NTS subnuclei that receive the densest inputs from arterial baroreceptors. In conclusion, PE, presumably via baroreceptor stimulation, induces Fos in glutamatergic and GABAergic neurons in both NTS and VLM. At least 29% of the Fos-ir glutamatergic neurons of NTS project to the vicinity of the VLM GABAergic interneurons that are presumed to mediate the sympathetic baroreflex. J. Comp. Neurol. 460:525–541, 2003. 

**Indexing terms: baroreflex; baroreceptor reflex; sympathetic tone; vesicular glutamate transporter; neural control of blood pressure; in situ hybridization; caudal ventrolateral medulla; CVL; CVLM; ventrolateral medullary depressor area; baroreceptors; NTS**

The central pathway of the sympathetic baroreflex probably consists of a chain of at least three neurons interposed between the baroreceptor afferents and the preganglionic neurons (for reviews see Dampney, 1994; Blessing, 1997; Guyenet, 2000). According to this hypothesis, the first central relay of the reflex consists of glutamatergic neurons located in the nucleus of the solitary tract (NTS), which receive monosynaptic inputs from baroreceptor afferents and project to a segment of the ventrolateral medulla (VLM) called the caudal ventrolateral medulla (CVLM) (Blessing, 1997; Sved et al., 2000; Schreihofer and Guyenet, 2002) or ventrolateral medullary depressor area (Willette et al., 1987). The next central relay of the reflex might consist of propriomedullary GABAergic neurons located in the CVLM (Jeske et al., 1993; Dampney, 1994;

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Jeske et al., 1995; Blessing, 1997; Guyenet, 2000; Schreihofer and Guyenet, 2002). The third relay is a group of rostral ventrolateral medulla (RVLM) neurons that innervate the preganglionic neurons involved in controlling the heart, the kidneys, and most vascular beds except the skin (Brown and Guyenet, 1985; McAllen, 1986; Morrison and Reis, 1989; Morrison et al., 1991; Dampney, 1994; Sun, 1996; Blessing, 1997; Guyenet, 2000). These RVLM neurons are glutamatergic and many also express the C1 adrenergic phenotype (Lipski et al., 1995; Schreihofer and Guyenet, 1997; Stornetta et al., 2002a).

Several aspects of the baroreflex circuitry remain tentative. For instance, though many NTS neurons are excited or inhibited by arterial baroreceptor stimulation (Rogers et al., 1996; Deuchars et al., 2000; Zhang et al., 2000; Seagard et al., 2001), it is most likely that only a subset of these neurons mediate the sympathetic or the cardiovagal baroreflexes. The notion that the baroreflex pathway includes a glutamatergic projection from the NTS to the CVLM rests on pharmacological and anatomical evidence that is less than definitive (Guyenet et al., 1987; Gordon, 1987; Somogyi et al., 1989; reviewed by Blessing, 1997). The subnuclear origin of the postulated glutamatergic NTS projection to the CVLM is also controversial (Chan and Sawchenko, 1998) and interneurons might be interposed between the aortic baroreceptor afferents and the NTS neurons that relay baroreceptor information to the CVLM (Spyer, 1994; Chan and Sawchenko, 1998). Finally, the existence of a second intramedullary baroreflex circuit organized around the activation of medullospinal inhibitory inputs to preganglionic neurons remains a viable hypothesis (Lewis and Coote, 1996; Barman and Gebber, 1997).

The present study seeks more definitive evidence that the NTS contains a population of glutamatergic neurons that are activated by baroreceptor stimulation and project to the CVLM. Baroreceptor stimulation was produced by intravenous administration of the vasoconstrictor agent phenylephrine to awake rats (Chan and Sawchenko, 1994, Dun et al., 1995; Graham et al., 1995; Minson et al., 1997; Chan and Sawchenko, 1998). NTS neurons with projections to the CVLM were identified by retrograde labeling, and the presence of Fos-immunoreactive (Fos-ir) nuclei was used to identify NTS and CVLM neurons excited by baroreceptor stimulation. Glutamatergic and GABAergic cell bodies were identified by detecting the mutually exclusive and diagnostic mRNAs that encode the vesicular glutamate transporter2 (VGLUT2) (Takamori et al., 2001; Stornetta et al., 2002a) or glutamic acid decarboxylase 67 (GAD-67) (Wuenschell et al., 1986; Chan and Sawchenko, 1998; Stornetta and Guyenet, 1999).

## MATERIALS AND METHODS

Thirty male Sprague-Dawley rats (300–350 g, Hilltop Laboratories, Scotsdale, PA) were used in this study. All experiments were performed in accordance with NIH and Institutional Animal Care and Use Guidelines. All procedures and protocols were approved by the University of Virginia's Animal Research Committee.

### Physiological experiments

The experimental design was modeled after Chan and Sawchenko (1998). A 25-minute i.v. infusion of a fixed dose of the vasoconstrictor phenylephrine (PE) was administered to awake rats to stimulate arterial baroreceptors. Control rats received physiological saline. Neuronal activation was gauged by the presence of the early gene product c-Fos 2 hours after the beginning of the drug infusion. One group of animals (Group 1) was instrumented with an arterial line (PE50; O.D. 0.965 mm; Intramedic®) and a venous catheter (medical vinyl tubing, size V-3; SCI Inc., AZ, USA). These animals were used to determine the effect of PE on blood pressure (BP) and heart rate (HR). The rest of the rats (Groups 2 and 3) received the same infusion of PE or of saline through a venous catheter but they did not have an arterial line inserted. All of the histological results presented in this paper are from Group 2 and 3 rats. Group 1 rats were not usable for Fos studies because femoral artery catheterization caused a high background of Fos expression in the CVLM 2 days later even in rats treated with saline. This might be due to the partial ischemia of the leg that results from the procedure. Although Chan and Sawchenko (1998) did not state their reasons for not using rats with arterial catheters for Fos studies, we assume that they also encountered a background problem similar to ours. Chronic catheterization of the femoral artery has been used by many authors including us to monitor the effect of vasoconstrictor or vasodilator agents on BP in awake rats (Chan and Sawchenko, 1994; Graham et al., 1995; Stornetta et al., 2001). We found that the Fos background can be drastically reduced by using rats instrumented with just one catheter placed into the jugular vein for i.v. drug infusion. The drawback of this method is that there might be a slight difference in the BP elevation caused by PE in rats with and without femoral artery catheter. However, it is unlikely that a slight difference in the magnitude of the pressor challenge would affect the conclusions of the present study.

Anesthesia was induced and maintained with a mixture of ketamine (75 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg) administered i.m.. Surgery used standard aseptic methods, and after surgery, the rats (male Sprague-Dawley, 300–350 g) were treated with the antibiotic ampicillin (100 mg/kg) and the analgesic ketorolac (0.6 mg/kg sc). Three experimental groups were used.

***Group 1.*** Six pairs of rats were instrumented with catheters in the femoral artery and in the femoral vein as described previously (Stornetta et al., 2001). The catheters were exteriorized at the back of the neck and the animals were placed on a tether in a recording chamber located in a quiet environment with free access to food and water and their normal 12-hour light-dark cycle. Two days after surgery the first rat of a pair received a 25-minute infusion of PE (0.6 $\mu$g/$\mu$l in sterile saline; 12 $\mu$l/minute; 18–22 $\mu g \times kg^{-1} \times min^{-1}$) and the second rat received sterile saline (25-minute, same total volume). BP was recorded by using conventional methods from 10 minutes before the start of the infusion to 2 hours after this point. The reported values for mean BP are true means obtained from the digitized pulsatile trace (Spike 2 software, Cambridge Electronic Design Ltd, Cambridge, UK). HR was obtained from the BP pulse by using the same software. Exactly 2 hours after the start of the i.v. infusion the rats were euthanized with urethane (Sigma, 1.8 g/kg) and perfused transcardially first with a 100 ml rinse of sodium phosphate buffered saline (PBS), pH 7.4, and then with 500 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were extracted and stored from 24

to 48 hours at 4°C in fixative. Next, the medulla oblongata was cut transversally in 30 μm coronal sections. The sections were placed in cryoprotectant and kept at −20°C awaiting histological processing. Saline infusion produced no change in BP (resting level: 126.2 ± 2.6 mmHg; mean level during saline infusion: 123.1 ± 2.7; NS) or HR (resting level: 367.9 ± 3.3 bpm; mean level during saline infusion: 360 ± 2.9; NS). During phenylephrine infusion BP increased by an average of 46.4 ± 2.2 mmHg from a baseline level of 118.3 ± 2.0 mmHg; $P < 0.05$). BP returned to control levels within 10 minutes following the end of the PE infusion. PE infusion caused a sustained decrease in HR of 118.6 ± 10.0 bpm (from a resting level of 378 ± 5 bpm). There was negligible recovery of the HR during the latter part of the PE infusion suggesting that little or no baroreceptor resetting occurred in these rats. HR returned to the control level within 5 minutes after the end of the PE infusion. Fos immunohistochemistry was performed in Group 1 rats and revealed the presence of a high background of Fos expression in the CVLM and NTS in each of the control saline-treated rats (up to 25 labeled nuclear profiles per section). In the VLM, background Fos expression was especially prominent on the side ipsilateral to the catheterized leg. This high background precluded the accurate study of the changes in Fos expression selectively induced by PE.

***Group 2.*** A second group of rats (5 pairs) was instrumented with a single catheter placed in the left jugular vein. The jugular catheter was exteriorized in the neck and the animals were kept on a tether as described above. Two days after surgery one rat of a pair received a phenylephrine infusion and the second rat received saline as described for Group 1. Two hours after the start of the intravenous infusion the rats were euthanized as described for Group 1. The brain was postfixed and coronal sections were cut and stored in cryoprotectant as described for Group 1. The BP increase caused by phenylephrine infusion in Group 2 rats was not determined and assumed to be similar to that found in Group 1 rats. Very few Fos-ir nuclei were observed in the NTS and VLM of the saline-treated rats in Group 2 rats. The brains of Group 2 rats were therefore used to study the specific pattern of Fos expression induced by PE.

***Group 3.*** A third group of rats (N=4 pairs) first received an iontophoretic injection of the retrograde tracer Fluoro-Gold (FG; Fluorochrome, Inc., Englewood, Co) into the left CVLM to label NTS neurons with projections to this level. FG was ejected through glass pipettes of 30–35 μm exterior diameter filled with a 3% solution of the dye in sterile saline (5 μA positive current for 5 seconds every 10 seconds for 10 minutes). The electrode was directed 4.3 mm caudal to the parieto-occipital suture, i.e., 1.3 mm caudal to the presumed caudal end of the facial motor nucleus based on our prior experience with rats of this size. This region was targeted because it contained the highest concentration of Fos-ir GABAergic neurons in rats from Group 2 treated with PE (see Results) and therefore seemed to contain the propriomedullary GABAergic relay of the baroreflex. This region lies in and below the rostral end of the rostral ventral respiratory group (rVRG) (Guyenet et al., 2002). Therefore, before placing the FG-filled pipette into the VLM, the location of the rVRG was first identified by recording neuronal activity synchronized with inspiration by using conventional unit recording. A few electrode penetrations were sufficient to find the optimal location based on the presence of a strong and consistent inspiratory unit activity. The recording electrode was withdrawn, the cerebellar tissue was allowed to stabilize, and the contact point of the recording electrode with the cerebellar surface was identified in relation to pial microvessels under high magnification. The tip of the FG-filled electrode was placed at this contact point and the electrode was lowered to the depth corresponding to the bottom of the respiratory column as defined by the recording electrode (typically 8.5–8.7 mm below the cerebellar surface and 1.7–1.9 mm lateral to the midline). A week later, the rats were instrumented in the manner described for Group 2. Two days after instrumentation, pairs of rats were treated as described for Group 2. One rat of a pair received a 25-minute infusion of PE and the other received a saline infusion. Two hours after the start of the intravenous infusion the rats were euthanized and perfused and coronal brain sections were collected as described for the two other groups.

## Histology

All histochemical procedures were done by using free-floating sections removed from the cryoprotectant mixture and rinsed three times in 1x Dulbecco's sterile PBS, pH 7.4. Due to their large number, tissue sections were processed in several batches. Each batch always contained an equal number of brain sections from control and PE-treated rats.

Digoxigenin-labeled cRNA probes were used for in situ hybridization. The riboprobe for in situ detection of Vesicular Glutamate Transporter2 (VGLUT2) mRNA was constructed as follows. Template DNA was amplified from rat brainstem poly A+ RNA by using a one step reverse transcription (RT) polymerase chain reaction (PCR) reaction (Titan One Tube RT-PCR System, Roche Molecular Biochemicals, Mannheim, Germany). The DNA for VGLUT2 was amplified by using the primers: forward 5'cggggaaagaggggataaag3' and reverse 5'acacaaagcagagagggac3' yielding a 3373 base pair product. The PCR product was subcloned into the plasmid vector, pCRII-TOPO (Invitrogen, Carlsbad, CA). After verifying the identity of the inserted DNA by sequencing, single stranded RNA was synthesized in an in vitro polymerization reaction by using either SP6 or T7 RNA polymerases in the presence of digoxigenin-11-UTP (Roche Molecular Biochemicals). This probe was longer than the one described in our previous publications (Guyenet et al., 2002; Stornetta et al., 2002a) and produced a much more intense signal with the exact same pattern of labeling previously described (Stornetta et al., 2002a). The riboprobe for GAD-67 mRNA was prepared as described previously (Stornetta and Guyenet, 1999). The plasmid containing a 3.2-kb GAD-67 insert was kindly supplied and previously characterized by A. Tobin (Wuenschell et al., 1986; Esclapez et al., 1993). Hybridization histochemistry was done as previously described (Stornetta et al., 2001, 2002a). Digoxigenin was revealed with a sheep polyclonal antidigoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals) and alkaline phosphatase was reacted with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP). Previous testing has established the specificity of our probes (Stornetta et al., 2001, 2002a). Absence of labeling in facial, hypoglossal, and ambigual motor neurons was taken as the quality standard because these cells are the most prone to

exhibit nonspecific NBT/BCIP reaction product under suboptimal conditions.

The in situ hybridization protocol was always carried out before any immunohistochemistry, i.e., before detection of Fos immunoreactivity and or FG (for details see Schreihofer and Guyenet, 1997; Guyenet et al., 2002). However, antibodies against digoxigenin and Fos (Group 2) or against digoxigenin, Fos, and FG (Group 3) were typically incubated together. Next, the alkaline phosphatase colorimetric reaction was done and, finally, secondary antibodies were applied. In all groups Fos immunoreactivity was detected by using a rabbit anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, 1:1500 dilution) followed by a Cy-3 conjugated goat anti- rabbit IgG (Jackson Immunoresearch, West Grove, PA, 1:200 dilution). For Group 3 a guinea pig anti-FG antibody (Protos Biotech, New York, NY, 1:500 dilution) was also used, followed by biotinylated goat anti-guinea pig IgG (Jackson 1:400, dilution) and streptavidin Alexa-488 (Molecular Probes, Eugene, OR, 1:400 dilution).

Finally, the sections were mounted in sequential rostrocaudal order onto slides, dried, and covered with Vectashield (Vector, Burlingame, CA). Coverslips were affixed with nail polish. No label was observed in the absence of any of the primary antibodies.

### Cell mapping, cell counting, and imaging

The sections were examined under dark field illumination to identify our reference section defined as the most caudal one containing an identifiable cluster of facial motor neurons. This section was assigned the level 11.6-mm caudal to Bregma (−11.6 mm) according to the atlas of Paxinos and Watson (1998). Levels caudal to this reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. The rostrocaudal levels reported in the present paper are therefore directly comparable to those reported in all our prior publications. They correspond closely to the Bregma levels reported in the atlas of Paxinos and Watson. The computer-assisted mapping technique made use of a motor-driven microscope stage controlled by the Neurolucida software as described previously (Stornetta and Guyenet, 1999). After drawing the outline of the section and major landmarks under a 10x objective, neurons of interest were plotted by using a 25x or 40x objective under fluorescence or brightfield illumination as required. The Neurolucida files were exported to the NeuroExplorer software (MicroBrightfield, Colchester, VT) to count the various types of neuronal profiles within a defined area of the reticular formation. The Neurolucida files were exported to the Canvas 6 software drawing program for final modifications. Photographs were taken with a 12-bit color CCD camera (CoolSnap, Roper Scientific, Tuscon, AZ; resolution 1392 X 1042 pixels) (for details see Stornetta et al., 2002a). The neuroanatomical nomenclature is after Paxinos and Watson (1998).

In some instances an attempt was made to estimate the actual number of Fos-ir nuclei, hence of activated neurons, present within defined regions of the medulla oblongata. To do so we used the profile counting method and the Abercrombie correction (N = n x T/T+h) (Guillery, 2002) where n is the profile count and N the corrected number of cells. T was the thickness of the original vibrating microtome section (30 μm). The minimum feret of the Fos-ir nuclei was used as an approximation of h, the mean diameter that the nuclei might have had in the plane perpendicular to the sections. The value of h was determined by drawing the circumference of 12-20 GABAergic and 12-20 glutamatergic Fos-ir nuclei in both NTS and in VLM under 400x magnification with the Neurolucida system. The minimum feret was computed by using the Neuroexplorer software and the mean value (7.3–8.3 μm depending on the cell group and brain region) was used for the Abercrombie correction. Because cell counts were made in sequential 1/6 series of sections, the total number of Fos-ir cells contained within the region encompassed by these sections was estimated by multiplying the corrected cell counts by six.

### Statistics

One-way or two-way ANOVA for repeated measures was used to determine whether neurons with a given phenotype (e.g., glutamatergic neurons expressing Fos) were significantly more numerous in PE-treated rats than in controls within specific brain regions such as the ventrolateral medulla or the nucleus of the solitary tract. Statistical significance was set at a $P$ value of $< 0.05$.

## RESULTS

### Phenotype and distribution of Fos-ir neurons in the medulla oblongata of rats treated with phenylephrine or saline

All of the results described in this section were obtained by using brain sections from Group 2 rats. Fos immunoreactivity and VGLUT2 mRNA reaction product were examined in a one-in-six series of coronal sections (180 μm apart) and a second series from the same brains was used to examine the location of neurons containing Fos immunoreactivity and GAD-67 mRNA. The brain of PE-treated rats contained numerous Fos-ir neurons in specific regions of the NTS and VLM whereas the corresponding regions of saline treated rats (N=6) contained very few Fos-ir neurons. The typical histological appearance of Fos-ir nuclei in the NTS of a PE-treated rat is shown in Fig. 1$A_1$,$D_1$. The region shown in Fig. 1$A_1$ is located dorsomedial to the solitary tract at mid-area postrema level (Bregma level −13.58 mm as depicted in Fig. 2C). Fig. 1$A_2$ illustrates the absence of Fos immunoreactivity in the same brain region of a saline-treated rat.

The specificity of the detection of VGLUT2 mRNA and the intensity of the reaction product are illustrated in Fig. 1B,C. Fig. 1B shows the large number of VGLUT2-mRNA containing neurons in the NTS and their absence in the two underlying cholinergic nuclei, the dorsal motor nucleus of the vagus (DMV) and the hypoglossal nucleus (XII). Fig. 1C illustrates the high level of VGLUT2 mRNA in the inferior olive, a classic source of glutamatergic innervation of the cerebellum, and its absence in a fiber tract, the pyramidal tract.

In PE-treated rats, a majority of the Fos-ir neurons present within the NTS contained VGLUT2 mRNA (Fig. 1$D_1$, $D_2$). The area depicted in these panels is the dorsomedial part of the NTS, also illustrated in Fig. 1$A_1$. As expected from the high prevalence of VGLUT2 mRNA in Fos-ir neurons of the NTS, only a minority of Fos-ir neurons in this region contained GAD-67 mRNA (Fig. 1$E_1$,$E_2$). The reverse was found in the VLM. A majority of the

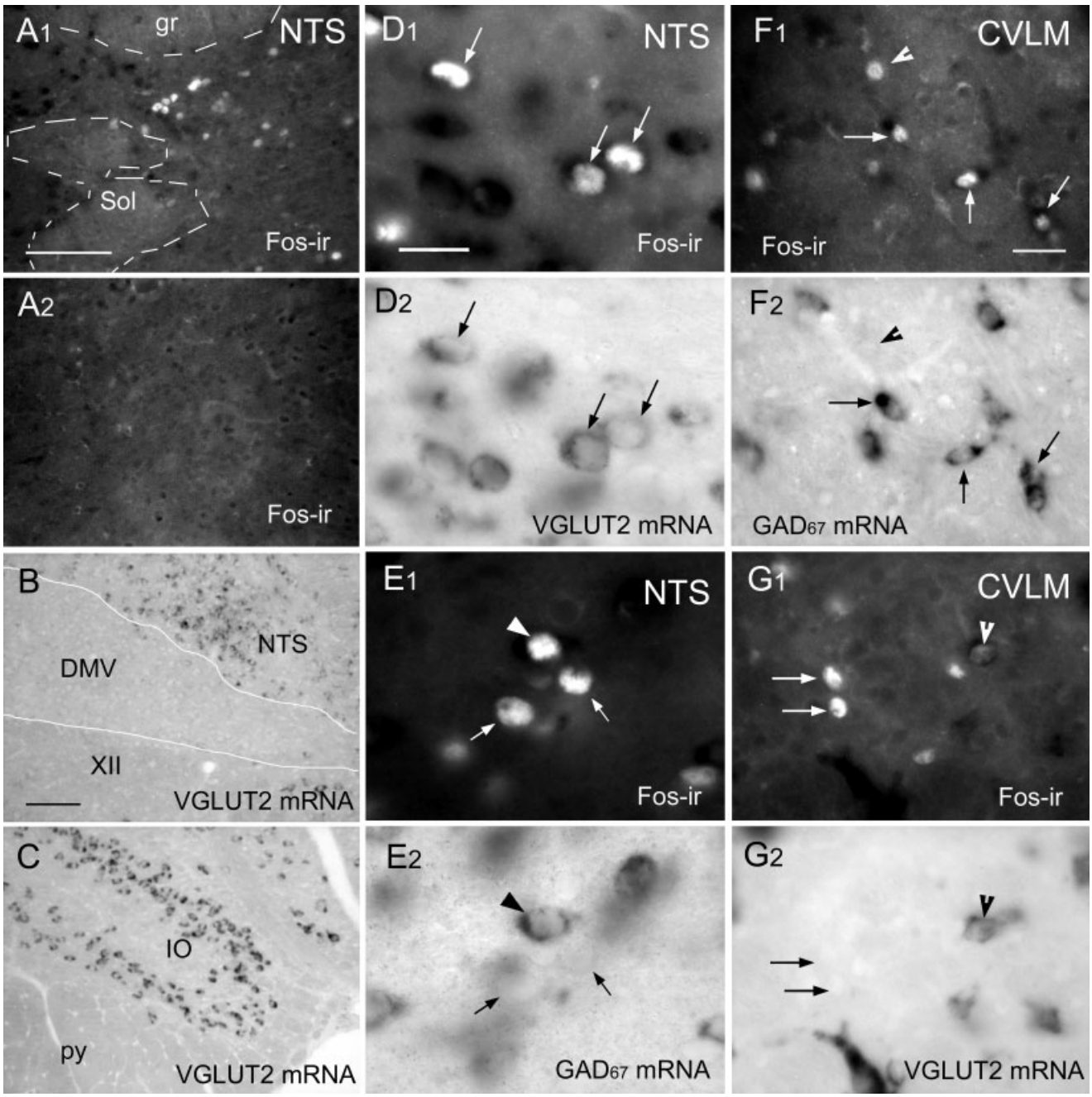


Fig. 1. Phenylephrine- (PE-) induced blood pressure elevation causes Fos expression in glutamatergic and GABAergic neurons of the medulla oblongata. **$A_1$,$A_2$**: Fos-immunoreactive (Fos-ir) nuclei located dorsomedial to the left solitary tract at area postrema level in a PE-treated rat ($A_1$) and in the same brain region of a saline-treated rat ($A_2$). This material was processed for Fos-immunoreactivity (Cy-3 immunofluorescence) and detection of vesicular glutamate transporter2 (VGLUT2) mRNA by in situ hybridization (in situ hybridization not shown). For orientation, the approximate boundaries of the fasciculus gracilis (gr) and the solitary tract (Sol) are drawn in $A_1$. **B**: VGLUT2 mRNA (alkaline phosphatase reaction product viewed under brightfield illumination) is present in nucleus of the solitary tract (NTS) neurons but absent from the cholinergic neurons located in the underlying dorsal motor nucleus of the vagus (DMV) and hypoglossal nucleus (XII). **C**: VGLUT2 mRNA labeling is intense in the inferior olive (IO) but is not found in the pyramidal tract (py). **$D_1$,$D_2$**: Most Fos-ir neurons present in the NTS of PE-injected rats are glutamatergic. Fos immunoreactivity is shown in $D_1$ (Cy-3, fluorescence illumination). The same field visualized under brightfield ($D_2$) shows that the three Fos-ir neurons (arrows) contain VGLUT2 mRNA. **$E_1$,$E_2$**: A minority of Fos-ir neurons present in the NTS of PE-injected rats ($E_1$, fluorescence illumination) contain GAD-67 mRNA ($E_2$, brightfield). The two arrows in $E_1$ and $E_2$ indicate Fos-ir cells devoid of GAD-67 mRNA. The arrowhead points to a Fos-ir neuron that contains GAD-67 mRNA. **$F_1$,$F_2$**: Most Fos-ir neurons present in the caudal ventrolateral medulla (CVLM) (Bregma −12.6 −13.4 mm) of PE-injected rats are GABAergic. Fos immunoreactivity is shown in $F_1$ (Cy-3, fluorescence). The same field visualized under brightfield ($F_2$) indicates that most of the Fos-ir neurons contained GAD-67 mRNA (arrows) save one (arrowhead). **$G_1$,$G_2$**: Most Fos-ir neurons present in the CVLM of PE-injected rats (Bregma −12.6 −13.4 mm) lack VGLUT2 mRNA. Fos immunoreactivity is shown in $G_1$ (Cy-3 fluorescence) and VGLUT2 mRNA in $G_2$ (brightfield). The majority of the Fos-ir neurons did not contain VGLUT2 mRNA (arrows). The arrowhead points to a weakly Fos-ir neuron that contained VGLUT2 mRNA. Scale bars: 100 μm in $A_1$ (applies to $A_1$,$A_2$); 100 μm in B (applies to B,C); 20 μm in $D_1$ (applies to $D_1$,$D_2$, $E_1$,$E_2$); 25 μm in F1 applies to $F_1$,$F_2$, $G_1$,$G_2$.

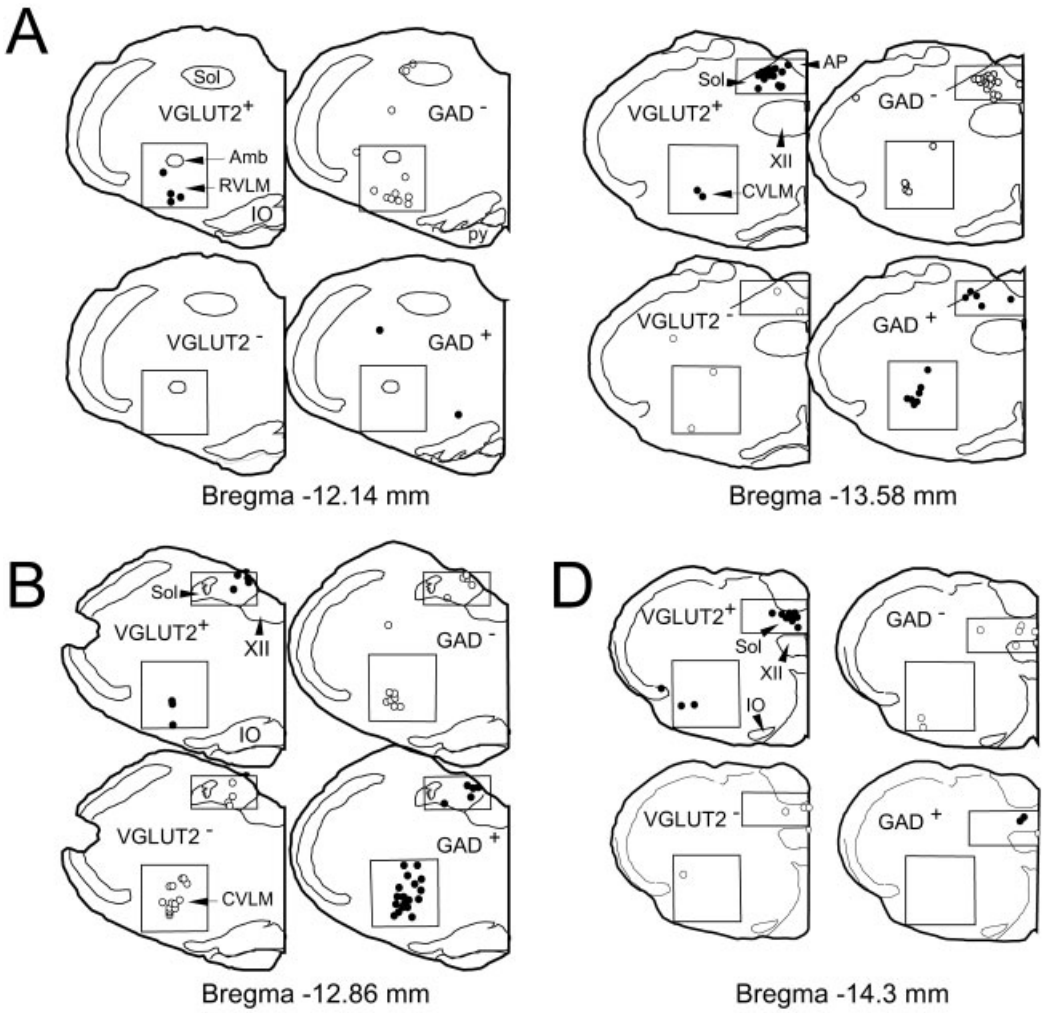


Fig. 2. Distribution of glutamatergic and GABAergic medullary neurons that express Fos after treatment with phenylephrine (PE). One series of sections was processed for detection of Fos immunoreactivity and vesicular glutamate transporter2 (VGLUT2) mRNA. An alternate series of sections was used to detect Fos immunoreactivity and GAD-67 mRNA. In each panel, the left side contains two plots from the Fos + VGLUT2 experiment depicting the location of Fos-immunoreactive (Fos-ir) neurons that contained (top, closed circles) or did not contain (bottom, open circles) VGLUT2 mRNA. The right side represents the corresponding plots for the Fos + GAD-67 experiment. The levels represented in **A–D** are 720 μm apart and illustrate the most salient features of the distribution, especially the striking complementarity of the VGLUT2 and GAD-67 mRNA patterns. The square or rectangular boxes drawn over the region of the nucleus of the solitary tract (NTS) and of the VLM delineate the areas where cell counts were made to obtain the quantitative data shown in Fig. 3. Abbreviations: Amb, nucleus ambiguus, pars compacta; AP, area postrema; CVLM, caudal ventrolateral medulla, a.k.a. depressor area of the VLM; IO, inferior olive; GAD, glutamic acid decarboxylase; py, pyramidal tract; RVLM, rostral ventrolateral medulla; Sol, nucleus of the solitary tract; XII, hypoglossal nucleus.

Fos-ir neurons present in the VLM of PE-treated rats contained GAD-67 mRNA (Fig. $1F_1$,$F_2$), whereas a minority contained VGLUT2 mRNA (Fig. $1G_1$,$G_2$).

The distribution of the Fos-ir neurons containing VGLUT2 or GAD-67 mRNA at four characteristic levels of the medulla oblongata of a representative PE-treated rat is illustrated in Fig. 2. Each panel contains two pairs of sections. The left pair is a plot of the Fos-ir neurons that did (top) or did not (bottom) contain VGLUT2 mRNA. The remaining pair represents a plot of the Fos-ir neurons from adjacent sections that did (bottom) or did not (top) contain GAD-67 mRNA.

In the NTS, Fos-ir neurons were found at area postrema level and in regions immediately adjacent, especially in the caudal direction. At and rostral to area postrema level most Fos-ir neurons were present in a narrow strip of cells hugging the mediodorsal edge of the solitary tract. Caudal to the area postrema, the Fos-ir cells tended to be preferentially concentrated in the mediodorsal part of the commissural nucleus. Within these NTS regions VGLUT2- or GAD-67-expressing Fos-ir cells were intermingled. In the VLM, Fos-ir neurons that contained GAD-67 mRNA were especially numerous at a level situated just rostral to the area postrema (Bregma level −12.86 mm; Fig. 2B), whereas the Fos-ir neurons that contained VGLUT2 mRNA were more evenly distributed. The medulla of saline-treated control rats had very few Fos-ir neurons (not illustrated). Note the striking complementarity of the

distribution of Fos-ir neurons that express VGLUT2 or GAD-67 mRNA. For example, at Bregma −12.86 mm a frank majority of the Fos-ir neurons of the VLM contained GAD-67 mRNA, whereas a minority contained VGLUT2 mRNA (Fig. 2B). At all levels, the number of Fos-ir neurons that contained VGLUT2 mRNA was similar to the number of cells that lacked GAD67 mRNA and vice versa.

The neuronal distribution described above was quantified by counting the number of Fos-ir nuclear profiles found at different levels of the VLM and NTS in three PE-treated and six control saline-treated rats. Counts were made by using a one in twelve series of sections (360 μm apart) spanning the region from just caudal to the facial motor nucleus (Bregma −11.8 mm) to the pyramidal decussation (Bregma −14.3 mm). In all these experiments, by convention, level Bregma −11.6 mm represents the caudal edge of the facial motor nucleus. The brain regions from where counts were made are outlined by boxes in Fig. 2. In the PE-treated rats, two series of adjacent sections were counted, one that was processed for detection of Fos immunoreactivity and VGLUT2 mRNA and the other that was processed for detection of Fos immunoreactivity and GAD-67 mRNA. Given the extremely small number of Fos-ir neurons present in the six saline-treated brains, we only counted the total number of Fos-ir nuclear profiles in these brains without regard to whether they might be glutamatergic or GABAergic. The results of these counting procedures are shown in Fig. 3. In the VLM, the Fos-ir neurons that contained GAD-67 mRNA were confined to a region situated rostral to the area postrema and their distribution exhibited a clear peak around Bregma −13.0 mm (1.3 mm caudal to the caudal edge of the facial motor nucleus, Fig. 3A). The Fos-ir neurons that contained VGLUT2 mRNA were more evenly distributed (Fig. 3B). In the NTS of PE-treated rats a clear majority of Fos-ir neurons contained VGLUT2 mRNA (Fig. 3D), whereas most of the remainder appeared to be cells containing GAD-67 mRNA (Fig. 3C). In every area investigated, the number of VGLUT2-expressing Fos-ir neurons present in PE-treated rats was significantly higher than the total number of Fos-ir neurons found in saline-treated rats and the same was true for GAD-67-expressing Fos-ir neurons. Glutamatergic neurons predominated over GABAergic neurons at all levels of the NTS.

Fig. 3E represents an estimate of the total number of Fos-ir neurons present in the NTS or the VLM of the rats illustrated in A–D. Counts were limited to the region indicated in A–D (Bregma −11.8 to Bregma −14.4 mm). The number of Fos-ir cells present in the NTS is somewhat underestimated because the caudal part of the commissural nucleus (caudal to Bregma −14.4 mm) was not included. The data indicates that in the NTS, 72 ± 1.1 % of the Fos-ir neurons were glutamatergic whereas 26.3 ± 2.3 % were GABAergic. The corresponding figures for the VLM were 40.7 ± 3.2 % glutamatergic cells and 55.9 ± 2.3 % GABAergic neurons (N=3). In each case the two percentages added up to close to 100% (94.9 ± 5 for VLM and 98.4 ± 3.2 for NTS; N=3) suggesting that virtually all of the medullary neurons that express Fos after PE-induced hypertension are either glutamatergic or GABAergic.

## Half of the Fos-ir glutamatergic neurons of the VLM are catecholaminergic

Given prior evidence that PE infusion activates tyrosine hydroxylase (TH)- immunoreactive neurons in the VLM (Li and Dampney, 1994; Minson et al., 1997; Chan and Sawchenko, 1998) and our finding that virtually all Fos-ir neurons that were not GABAergic contained VGLUT2 mRNA, we tested whether the Fos- and TH-ir neurons of the VLM are among the cells that contain VGLUT2 mRNA. Thus an additional series of sections from the same three PE-treated rats was processed for the detection of Fos and TH immunoreactivities and VGLUT2 mRNA. As shown in Fig. 4A, a large proportion of the TH-ir neurons of the VLM that contained Fos-ir nuclei also contained VGLUT2 mRNA. Furthermore, TH-ir cells constituted a large proportion of the glutamatergic neurons that had Fos-labeled nuclei throughout the medulla except caudal to the area postrema (Bregma levels −14.0 and −14.4 mm)(Fig. 4A). A TH-ir neuron of the VLM that contained VGLUT2 mRNA and a Fos-ir nucleus is shown in Fig. 5. Fig. 4B represents a breakdown of the various classes of Fos-ir neurons present in the VLM classified according to the presence or absence of TH immunoreactivity and VGLUT2 mRNA. These are Abercrombie-corrected estimates of the number of VLM cells located between levels Bregma −11.8 and −14.4 mm on both sides. TH-ir neurons made up 50.6 ± 4.6 % of all glutamatergic Fos-ir neurons present in the VLM. Only 9.3 ± 3% of the Fos-ir catecholaminergic cells did not contain detectable levels of VGLUT2 mRNA.

## VGLUT2 mRNA is present in the Fos-ir NTS neurons that project to the depressor region of the VLM (CVLM)

The GABAergic neurons of the VLM that express Fos after treatment with PE are thought to be propriomedullary interneurons that mediate the sympathetic baroreflex by inhibiting the presympathetic cells of the rostral VLM (Dampney, 1994; Guyenet, 2000). Having defined the exact location of these GABAergic neurons by the experiments described above, we injected FG in their midst to determine whether they or the region surrounding them receives projections from the NTS neurons that express Fos after PE infusion. Fig. 6A illustrates a typical FG injection site from a rat subjected to PE infusion. The center of the iontophoretic injections of FG produced a very small area of nonspecific damage that could be identified easily. Very little retrograde labeling was present in the NTS at the coronal level corresponding to the injection site. At this level (Bregma −13.0 mm), the most conspicuous areas of retrograde labeling were the region of the ventral respiratory column (rostral ventral respiratory group, rVRG, black arrow in Fig. 6A) and the inferior olive on the contralateral side (arrowheads in Fig. 6A). The labeling of the contralateral olive raises the possibility that FG might have labeled the contralateral olivary projection to the cerebellum via axons of passage. The other injection sites were of similar size and location. A plot of the center of each injection site (Fig. 6B) shows that the distribution of these sites corresponds to the region of the VLM that contains the highest density of Fos-ir GABAergic neurons (Figs. 2B, 3).

All of the results described in the remainder of this section were obtained by using brain sections from Group

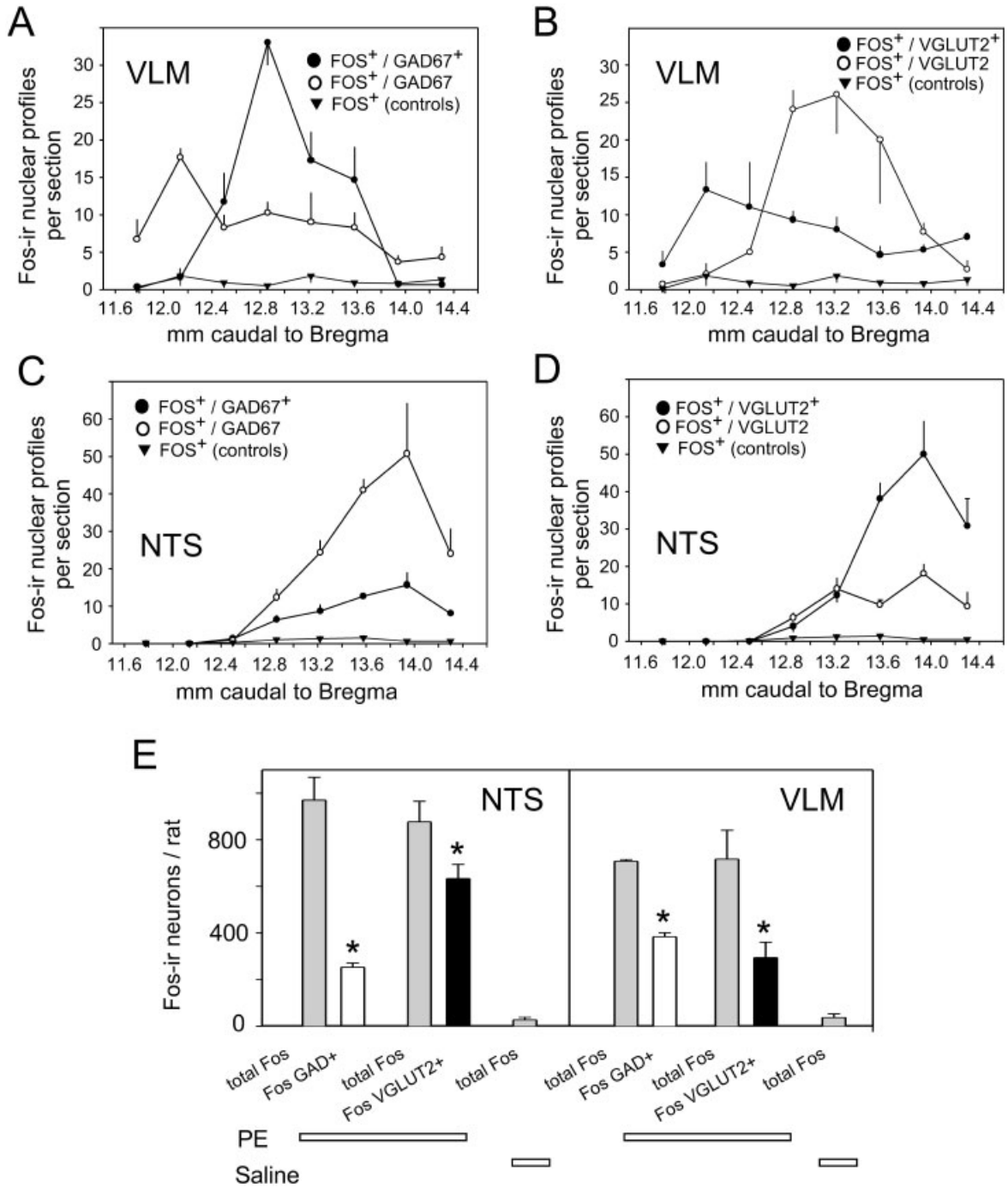


Fig. 3. Rostrocaudal distribution of glutamatergic and GABAergic medullary neurons that express Fos after infusion of phenylephrine (PE) or saline: group data. **A**,**B**: Distribution of Fos-immunoreactive (Fos-ir) ventrolateral medulla (VLM) neurons (bilateral counts) that contained GAD-67 mRNA (A) or vesicular glutamate transporter2 (VGLUT2) mRNA (B) in PE-treated rats (N=3). Because of their low numbers all the Fos-ir neurons of the saline-treated controls (N=6) were combined regardless of their mRNA content (A,B). **C**,**D**: Distribution of Fos-ir nucleus of the solitary tract (NTS) neurons that contained GAD-67 mRNA (C) or VGLUT2 mRNA (D) in PE-treated rats (N=3). Because of their low numbers all the Fos-ir neurons of the saline-treated controls (N=6) were combined (C,D). In all panels, Bregma −11.6 mm corresponds to the caudal most edge of the facial motor nucleus. Mid-area postrema level is at Bregma −13.58 mm. Each distribution from PE-treated rats in A–D is significantly different from that found in saline-treated rats by 2-Way ANOVA for repeated measures. **E**: Estimated total number of Fos-ir neurons that express GAD-67 or VGLUT2 mRNA in NTS or VLM (total of all sections from Bregma −11.6 to −14.4 mm) in rats treated with PE (N=3) or saline (N=6). For each structure, two "total Fos" columns are represented. The left one derives from the sections that were processed for detection of GAD-67 mRNA and the other from the alternate sections that were processed for detection of VGLUT2 mRNA. In the saline treated rats, only the total number of Fos-ir neurons was counted due to their low numbers. In this and the following figures, values are plotted as mean and standard error. *Significantly different from total Fos-ir cells in saline-treated animals by unpaired t-test.

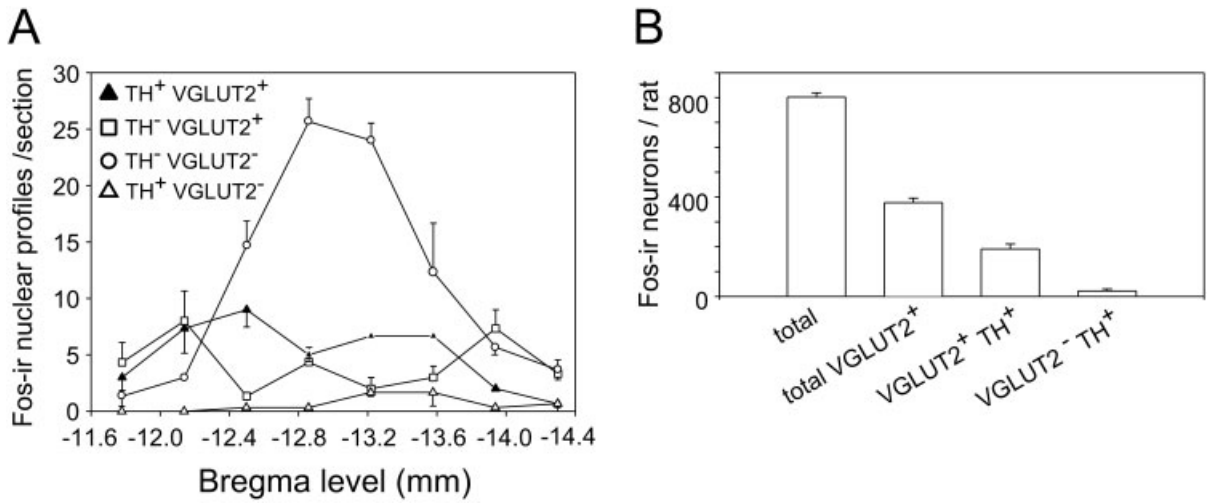


Fig. 4. A majority of the baroactivated glutamatergic neurons of the ventrolateral medulla (VLM) are catecholaminergic. **A**: Rostrocaudal distribution of Fos-immunoreactive (Fos-ir) nuclear profiles (bilateral counts within boxes defined in Fig. 2) classified according to the presence of vesicular glutamate transporter2 (VGLUT2) mRNA and or tyrosine hydroxylase (TH) immunoreactivity (N=3 rats treated with phenylephrine (PE)). **B**: Estimated total number of each class of neuron represented in A (N=3). TH-ir neurons represented 50.6% of all Fos-ir that contained VGLUT2 mRNA.

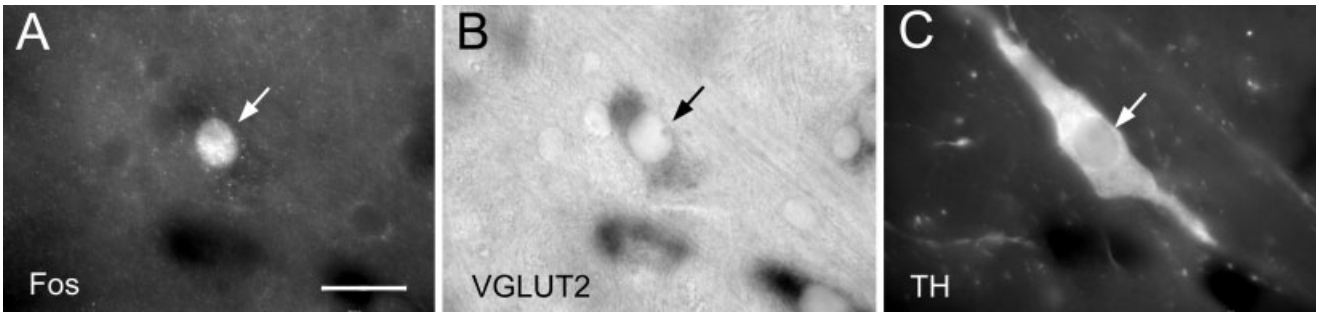


Fig. 5. Example of a Fos-immunoreactive glutamatergic and catecholaminergic neuron. **A**: Fos immunoreactivity (Cy-3; fluorescence). **B**: Vesicular glutamate transporter2 (VGLUT2) mRNA (alkaline phosphatase reaction; brightfield). **C**: Tyrosine hydroxylase (TH) immunoreactivity (Alexa 488; epifluorescence). Calibration: 20 μm.

3 rats (four 4 PE-treated rats and four saline-treated controls). A one in six series of coronal sections from each brain (180 μm apart) was reacted for detection of Fos, VGLUT2 mRNA, and FG immunoreactivity, and a series of adjacent sections from the same brains was reacted for detection of Fos and FG immunoreactivity and GAD-67 mRNA. Despite the double surgery and the introduction of FG into the VLM, the NTS region of Group 3 saline-treated rats contained no more Fos-ir neurons than the saline-treated rats of Group 2. Because the numbers of Fos-ir neurons present in the saline-treated Group 3 rats were again negligible, they were neither mapped nor counted.

Within the caudal NTS of PE-treated rats many Fos-ir cells contained both VGLUT2 mRNA and FG (Fig. 7A1–3). FG labeling was exclusively ipsilateral, though at the level of the commissural part of the NTS some of the labeled cells were located up to 150 μm beyond the midline. As illustrated in Fig. 7B1–3, Fos- and FG-ir neurons almost never contained GAD-67 mRNA. However, we did find GABAergic neurons without Fos-ir nuclei that contained FG and thus projected to the VLM (Fig. 7C1–3).

The anatomical location of the triple-labeled neurons detected in one Group 3 rat is shown in Fig. 8 at two representative levels: caudal end of area postrema in panel $A_1$ (Bregma −14.0 mm) and at the level of the commissural nucleus in panel $C_1$ (close to Bregma −14.4 mm). The triple labeled cells (stars) were only present on the side ipsilateral to the FG injection site (Fig. $8A_1$). The vast majority of these cells were located in the regions of the NTS known to receive the densest projections from arterial baroreceptors, namely a narrow strip of tissue located mediodorsal to the solitary tract at area postrema level (Fig. $8A_1$) and the mediodorsal aspect of the commissural NTS (Fig. $8C_1$). The very same areas also contained the bulk of the Fos-ir neurons that expressed VGLUT2 mRNA (Fig. $8A_2$,$C_2$). In contrast, the glutamatergic neurons that projected to the VLM (FG-labeled) but did not contain Fos-ir nuclei were more evenly scattered within the NTS (Fig. $8A_3$). Fig. $8B_1$–$_3$ represents a section adja-

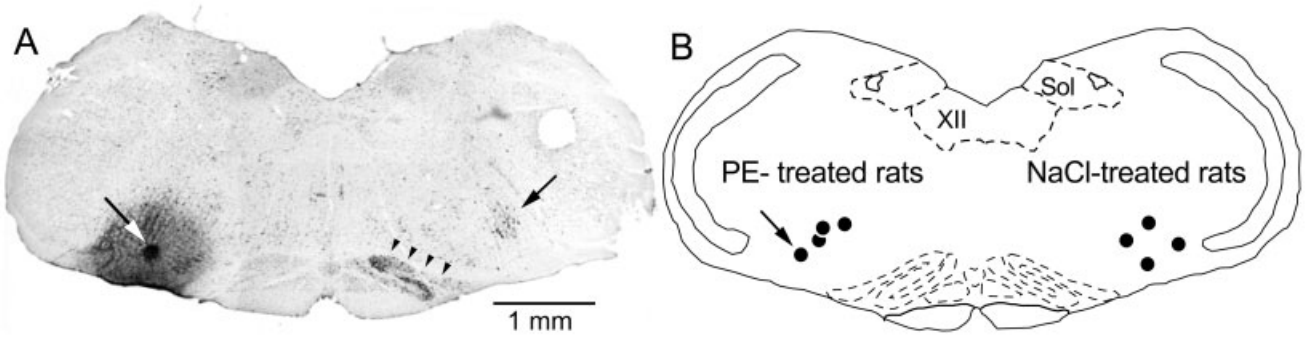


Fig. 6. Fluoro-Gold (FG) injection sites into the CVLM. **A**: Example of an FG injection centered in the caudal ventrolateral medulla (CVLM) at Bregma −12.8 mm. White arrow points to the center of the injection. Black arrowheads indicate retrogradely labeled cells within the contralateral inferior olive. Retrogradely labeled cells within the contralateral rVRG are indicated by a black arrow. **B**: Location of FG injection sites projected on a coronal section at Bregma level −13.0 mm. The injection site shown in A indicated by an arrow. Injection sites in the saline-treated rats were also on the left side but are plotted on the opposite side for clarity. Abbreviations: Sol, nucleus of solitary tract; XII, hypoglossal nucleus.

cent to that shown in Fig. $8A_1$–$_3$ that was processed for detection of FG and Fos immunoreactivity and GAD-67 mRNA. Most Fos-ir GABAergic neurons were present in the same areas as the Fos-ir glutamatergic neurons (compare Fig. $8A_2$,$B_2$). GABAergic neurons with axonal projections to the VLM were also found consistently but they tended to be clustered ventromedial to the solitary tract and they never contained Fos-ir nuclei (Fig. $8B_3$). In the other three PE-treated rats with FG injection into the VLM, the distribution of the various cell types was very similar.

In order to estimate the relative numbers of the various categories of neurons present in the NTS, profile counts of Fos-ir neurons or FG-ir neurons were made at three Bregma levels per rat (Bregma −13.86, −14.04, and −14.22 mm). Counts were made in three sections processed for detection of Fos and FG immunoreactivity and VGLUT2 mRNA and in three adjacent sections processed for detection of Fos and FG immunoreactivity and GAD-67 mRNA. The total number of each class of cell profile phenotype is represented in Fig. 9. Fig. $9A_1$,$A_2$ are profile counts from sections reacted for detection of GAD mRNA and Fig. $9B_1$,$B_2$ are profile counts from alternate sections reacted for detection of VGLUT2 mRNA. Though the majority of the FG-labeled neurons of the NTS lacked Fos immunoreactivity (87–88%; Fig. $9A_2$,$B_2$), a substantial minority of the Fos-ir and VGLUT2 mRNA-containing cells of the NTS contained FG (Fig. $9B_1$). This percentage, calculated by dividing the number of triple labeled neurons (positive for FG, Fos, and VGLUT2) by the total number of Fos-ir neurons that contained VGLUT2 mRNA was 23 ± 3.8 % (N=4) with a range of 14 to 29%. Finally, the NTS contains GABAergic neurons with projections to the CVLM (13% of the projection, Fig. $9A_2$) but virtually none of these cells (0.2% of the projection, Fig. $9A_2$) were Fos-ir in PE-treated rats.

## DISCUSSION

The present study reveals that a brief period of hypertension caused by injection of phenylephrine activates a glutamatergic projection from the caudal NTS to the region of the VLM that contains the postulated inhibitory GABAergic relay of the sympathetic baroreflex (CVLM). The somata of these NTS projection neurons are intermixed with GABAergic neurons that were also activated by PE but were not retrogradely labeled from the CVLM. The glutamatergic projection neurons are concentrated in the region of the NTS that receives the densest inputs from arterial baroreceptors, namely the dorsomedial NTS at area postrema level and the dorsal most layer of the commissural NTS.

The present study also confirms that the VLM neurons that express Fos following phenylephrine-induced hypertension are not exclusively GABAergic but include a significant proportion of glutamatergic neurons, many of which are C1 catecholaminergic cells.

### Technical considerations

Hypertension-induced Fos expression in the brainstem requires the integrity of arterial baroreceptor afferents because it is eliminated by sinoaortic denervation (Chan and Sawchenko, 1998; Potts et al., 1999). This evidence implies that cardiac mechanoreceptors, which send their axons through the vagus nerve, do not contribute significantly to PE-induced Fos expression. Because baroreceptors must be intact for PE to cause Fos expression, neuronal activation is most likely caused by the intense baroreceptor activation that occurs during the 25 minutes of increased blood pressure. Based on this likely assumption, the neurons that express Fos after PE infusion will henceforth be called baroactivated neurons to avoid lengthy periphrases. However, it is conceivable that some of the Fos-ir neurons could have been activated by the reduction of blood pressure that follows the end of the PE infusion rather than by baroreceptor activation during PE infusion. Additionally, there is no reason to assume that every neuron that expresses Fos following a hypertensive stimulus necessarily contributes to the cardiovagal or sympathetic baroreflex. Finally, given that many neurons do not express Fos when activated, the present experimental design may have missed baroactivated neurons that make important contributions to the baroreflex.

Three structurally related glutamate vesicular transporters have been described (Takamori et al., 2000; Her-

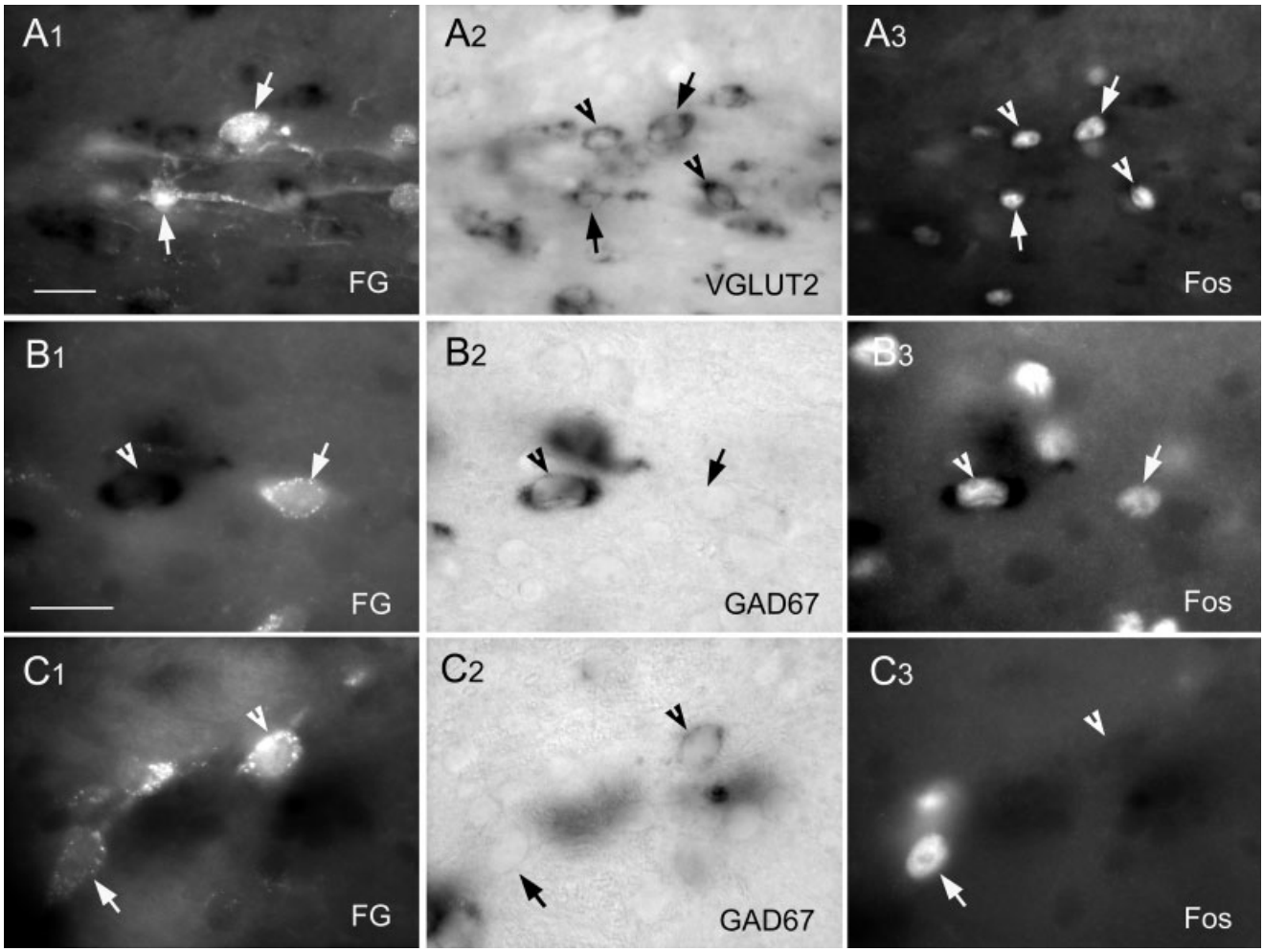


Fig. 7. Baroactivated glutamatergic neurons of the NTS project to the CVLM. **A1–3**: Baroactivated glutamatergic neurons of the nucleus of the solitary tract (NTS) that project to the caudal ventrolateral medulla (CVLM). A1: Fluoro-Gold (FG) immunoreactivity (Alexa 488; fluorescence). A2: Vesicular glutamate transporter2 (VGLUT2) mRNA (same field, alkaline phosphatase reaction product; brightfield). A3: Fos immunoreactivity (same field, Cy-3, fluorescence). Arrows: triple-labeled neurons. Arrowheads: glutamatergic Fos-immunoreactive (Fos-ir) neurons without FG. **B1–3**: Baroactivated GABAergic neurons of the NTS do not project to the CVLM. B1: FG immunoreactivity (Alexa 488; fluorescence). B2: GAD-67 mRNA (same field; alkaline phosphatase reaction product; brightfield). B3: Fos immunoreactivity (same field; Cy-3; fluorescence). Arrowhead: Fos-ir GABAergic neuron devoid of FG. Arrow: FG- and Fos-ir neuron devoid of GAD-67 mRNA. **C1–3**: Baroinsensitive GABAergic neuron of the NTS with projection to CVLM. C1: FG immunoreactivity (Alexa 488; fluorescence). C2: GAD-67 mRNA (same field; alkaline phosphatase reaction product; brightfield). C3: Fos immunoreactivity (same field; Cy-3; fluorescence). Arrowhead: FG-ir GABAergic neuron without Fos. Arrow: FG- and Fos-ir neuron devoid of GAD-67 mRNA. Scale bars: 25 μm in A1 (applies to A1–3; 20 μm in B1 (applies to B1–3 and C1–3.

zog et al., 2001; Takamori et al., 2001; Gras et al., 2002; Schäfer et al., 2002). VGLUT1 and VGLUT2 are diagnostic for glutamatergic neurons because their expression is necessary and sufficient for depolarization-mediated exocytosis of glutamate to occur (Takamori et al., 2000; Takamori et al., 2001). VGLUT2 is abundantly expressed in the NTS and in the VLM whereas VGLUT1 is absent from the NTS (Stornetta et al., 2002a). In the VLM, VGLUT1 mRNA is absent from the reticular formation areas involved in cardiorespiratory regulations (Stornetta et al., 2002b). VGLUT3 is expressed only by a very small minority of NTS or VLM neurons, many of which are serotonergic (Schäfer et al., 2002). In brief, within the NTS or VLM, the presence of VGLUT2 mRNA provides diagnostic identification for most glutamatergic neurons except for a small number that might be identifiable only by the presence of VGLUT3 or some yet to be discovered transporter. The latter type of glutamatergic neuron is unlikely to represent a sizable fraction of the neurons that express Fos after hypertension because virtually 100% of these neurons expressed either VGLUT2 or GAD-67 mRNA. GABAergic neurons can be identified by the presence of GAD-67 or GAD-65 mRNA. There is little evidence for differential distribution of the two GAD mRNAs in the medulla oblongata (Stornetta and Guyenet, 1999), particularly as regards the neurons that express Fos immunoreactivity after PE infusion (Chan and Sawchenko, 1998). GAD-67 probes provide a much stronger hybridization signal (Stornetta and Guyenet, 1999) and it is very likely that our probe identified most Fos-ir GABAergic neurons.

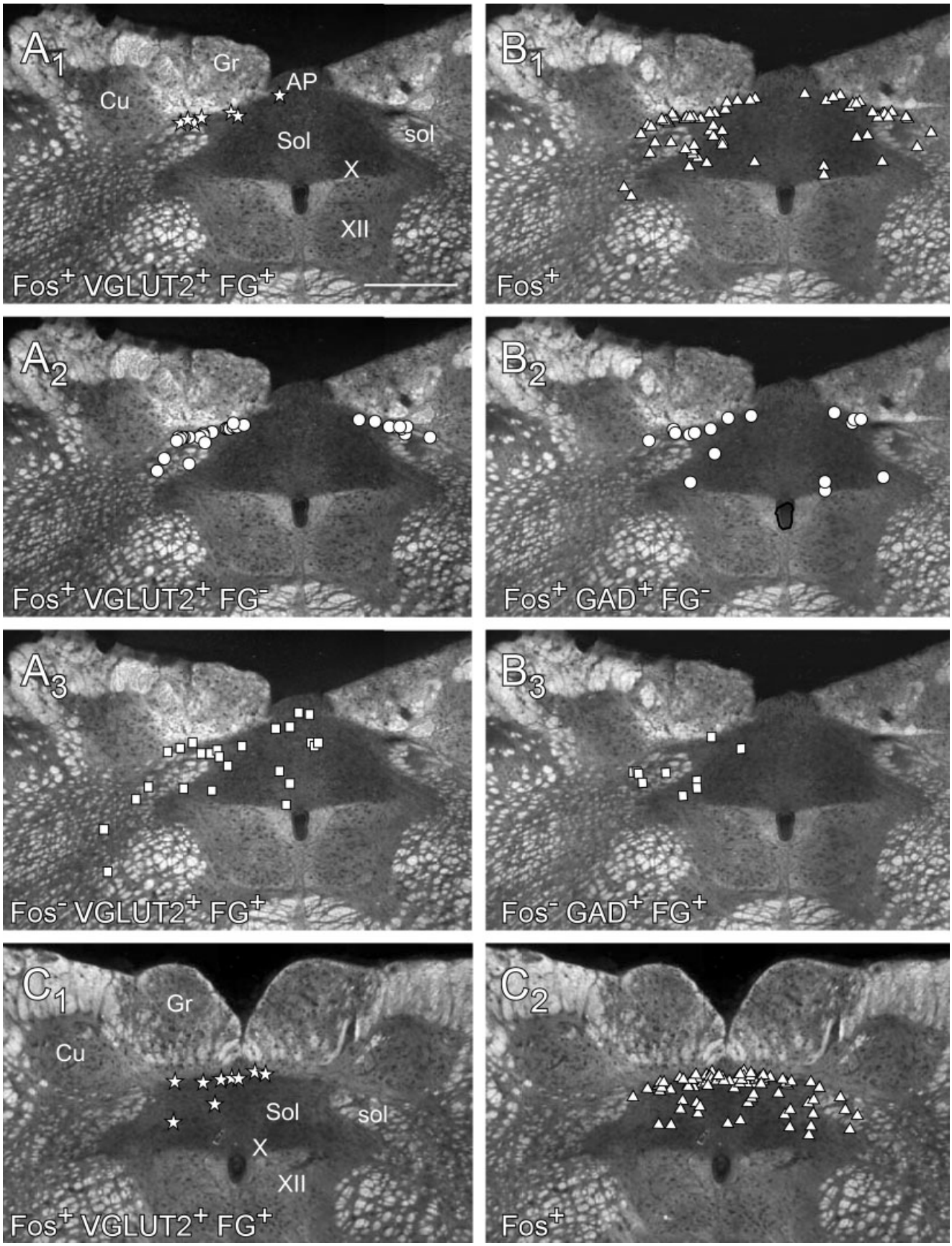
A1
Gr
AP
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Figure 8

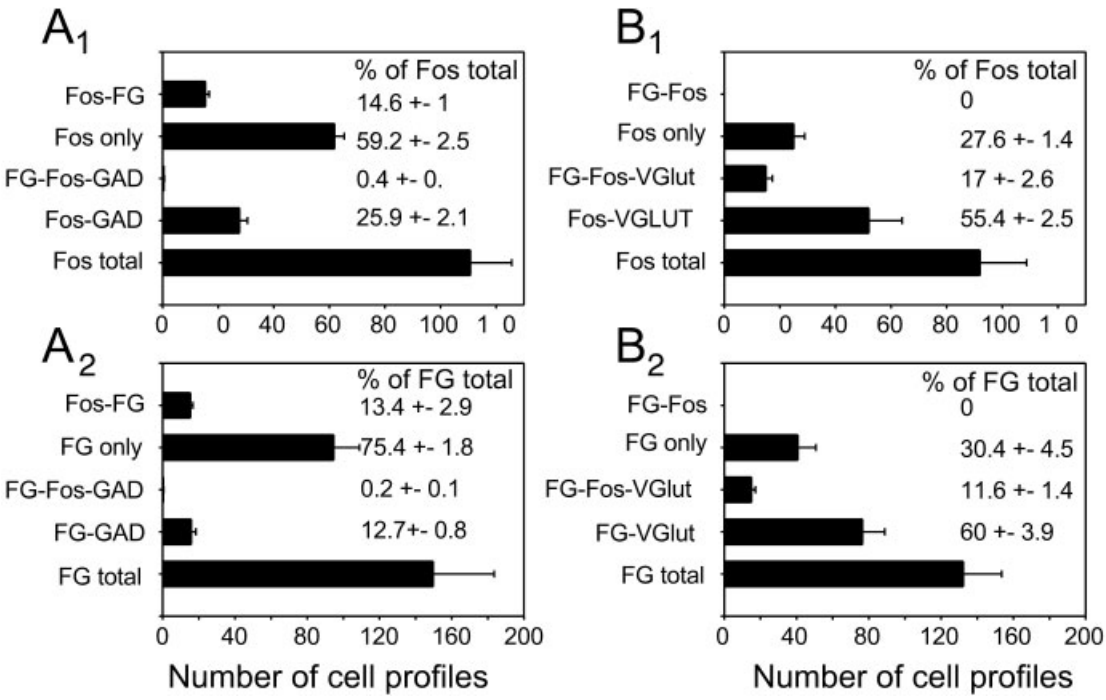


Fig. 9. Retrograde labeling of baroactivated NTS neurons: quantitative results. Sections from four phenylephrine- (PE)- treated rats were processed for detection of Fos and Fluoro-Gold (FG) immunoreactivities and GAD-67 mRNA. Alternate sections were processed for detection of Fos and FG immunoreactivities and vesicular glutamate transporter2 (VGLUT2) mRNA. Neuronal profiles were counted in the nucleus of the solitary tract (NTS) at three levels: mid area postrema, 360 μm and 720 μm caudal to that level. Cells' classes were defined according to the presence of specific combinations of the three markers. The total number of cell profiles of each class present at all three levels was added for each animal. Each bar represents the mean and standard error for the four rats. $\mathbf{A_1}$: Distribution of Fos-immunoreactive (Fos-ir) nuclear profiles in sections processed for GAD67 mRNA identification. $\mathbf{A_2}$: Distribution of FG-ir cell profiles in the same sections. $\mathbf{B_1}$: Distribution of Fos-ir nuclear profiles in sections processed for VGLUT2 mRNA identification. $\mathbf{B_2}$: Distribution of FG-ir cell profiles in the same sections.

The pattern of Fos immunoreactivity depends on the time between stimulus onset and euthanasia and the intensity of the stimulus. We chose an interval of 2 hours, judged optimal by Chan and Sawchenko (1998), and a dose of PE that was most probably very close to the lowest one used by this group. Though these authors report using a dose as low as 2.14 $\mu g \times kg^{-1} \times min^{-1}$, this figure is probably an error because a dose of around 20 $\mu g \times kg^{-1} \times min^{-1}$ can be calculated based on the rest of the information that they provided. Although this dose produced a higher BP increase in our case (46 ± 2 vs. 25 ± 1 mmHg), the ensuing pattern of Fos labeling that we obtained seemed similar to that reported by Chan and Sawchenko (1998). For instance, the estimated number of Fos-ir cells present in the NTS region was very close (about 850 in our case vs. 980 in theirs).

Fig. 8. Anatomical location of baroactivated NTS neurons with projections to the CVLM. Each panel is a darkfield photograph of a coronal section from a phenylephrine- (PE)- treated rat on which the exact location of specific cell types present in the section was digitally superimposed. $\mathbf{A_1}$–$\mathbf{_3}$: Section located at caudal end of area postrema and reacted for detection of Fluoro-Gold (FG) and Fos immunoreactivity and vesicular glutamate transporter2 (VGLUT2) mRNA. $A_1$: Triple-labeled cells, i.e., baroactivated glutamatergic cells that project to the caudal ventrolateral medulla (CVLM). $A_2$: Glutamatergic Fos-immunoreactive (Fos-ir) cells devoid of FG. $A_3$: Glutamatergic neurons with projection to CVLM but lacking Fos. $\mathbf{B_1}$–$\mathbf{_3}$: Section immediately adjacent to that shown in panels $A_1$–$_3$ and reacted for detection of FG and Fos immunoreactivity and GAD-67 mRNA. $B_1$: Fos-ir neurons (inclusive of all subtypes). $B_2$: Baroactivated GABAergic neurons (all were devoid of FG). $B_3$: GABAergic neurons with projection to CVLM but lacking Fos. $\mathbf{C_{1,2}}$: Section located 360 μm caudal to the area postrema and reacted for detection of FG and Fos immunoreactivity and VGLUT2 mRNA. $C_1$: Triple-labeled cells. $C_2$: Fos-ir cells inclusive of all subcategories. Abbreviations: AP, area postrema; Cu, cuneate nucleus; Gr, gracile nucleus; sol, solitary tract; Sol, nucleus of the solitary tract; X, dosal motor nucleus of the vagus; XII, hypoglossal nucleus. Scale bars: 500 μm in $A_1$ for all panels.

## Pattern of Fos expression induced by PE in the VLM

As previously described (Minson et al., 1997; Chan and Sawchenko, 1998), most of the VLM GABAergic neurons that express Fos following PE injection are located rostral to the calamus scriptorius with a peak as much as 1,200 μm rostral to that level. This peak corresponds to our estimate of 1,300 μm caudal to the facial motor nucleus (Fig. 10). Caudal to Bregma −12.8 mm, the region that contains the Fos-ir GABAergic neurons is virtually indistinguishable from the rVRG, which contains bulbospinal phrenic premotor neurons (Dobbins and Feldman, 1994; Guyenet et al., 2002). The GABAergic cells of the CVLM that express Fos after PE are probably propriomedullary interneurons that provide inhibitory inputs to one or more

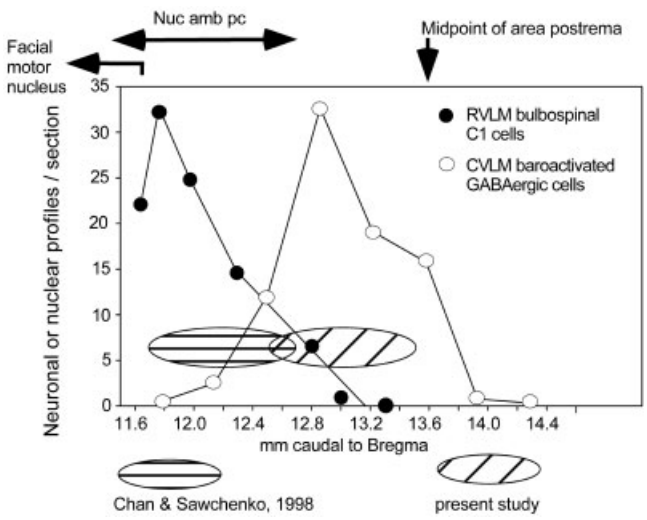


Fig. 10. Overlap between bulbospinal adrenergic neurons and the PE-activated GABAergic neurons of the VLM. The distribution of bulbospinal C1 adrenergic neurons is represented by using the same landmarks as in the present study (replot from Schreihofer and Guyenet, 2000). It is compared to that of the GABAergic neurons that express Fos immunoreactivity after phenylephrine- (PE)- induced hypertension (replot of data from Fig. 3 of the current study). The rostrocaudal spread of the Fluoro-Gold (FG) injections made by Chan and Sawchenko (1998) and in the present study is also represented. Nuc amb pc, nucleus ambiguus pars compacta.

classes of VLM neurons during activation of arterial baroreceptors and other cardiopulmonary afferents (Schreihofer and Guyenet, 2002). These GABAergic propriomedullary neurons lack projections to the spinal cord or the hypothalamus (Schreihofer and Guyenet, 2002) but they project to or through the RVLM (Jeske et al., 1993; Minson et al., 1997; Chan and Sawchenko, 1998), probably bilaterally (Li et al., 1991; Masuda et al., 1991; Minson et al., 1997). The presympathetic cells of the RVLM are among their likely targets (Jeske et al., 1993; Jeske et al., 1995; Blessing, 1997; Schreihofer and Guyenet, 2002). This connection may be monosynaptic (Jeske et al., 1995; Minson et al., 1997; Chan and Sawchenko, 1998) and may constitute the second intramedullary link of the sympathetic baroreflex (Sun and Guyenet, 1985; Gordon, 1987; Guyenet et al., 1987; for review see Dampney et al., 2002). Another likely target of the baroactivated GABAergic neurons of the VLM are the baroinhibited C1 adrenergic cells that project to the hypothalamus instead of the spinal cord (Verberne et al., 1999). Finally, it is conceivable that the baroactivated GABAergic neurons of the VLM also target selected respiratory neurons of the ventral respiratory group thereby contributing to the respiratory depression associated with the baroreflex.

Fig. 10 shows a replot of the location of the spinally-projecting C1 adrenergic neurons (from Schreihofer and Guyenet, 2000). The distribution of these cells closely approximates the rostrocaudal spread of the presympathetic neurons that control barosensitive sympathetic efferents (Verberne et al., 1999). Clearly, there is significant spatial overlap between the baroactivated GABAergic cells of the CVLM and the presympathetic vasomotor neurons of the RVLM. If the dendritic field of these two classes of neurons were taken into consideration (Lipski et al., 1995; Schreihofer and Guyenet, 1997) still greater overlap between the processes of these two cell populations would undoubtedly be found. Because of this overlap the hemodynamic effects produced by microinjecting chemicals into the VLM (increase or decrease in blood pressure depending on the level) (e.g., Willette et al., 1983) are probably always due to a balance between the activation of presympathic neurons and their inhibitory inputs from the CVLM.

According to the present data, most of the non-GABAergic VLM neurons that express Fos after PE infusion are glutamatergic because they contain VGLUT2 mRNA. In agreement, Fos is not expressed by glycinergic or cholinergic neurons in the VLM after PE-induced hypertension (Chan and Sawchenko, 1998). Our data are also congruent with the presence of VGLUT2 mRNA in most C1 cells (Stornetta et al., 2002a). Indeed, like others before us (Li and Dampney, 1994; Minson et al., 1997; Chan and Sawchenko, 1998), we found that infusion of PE activates some TH-ir neurons in the VLM. In our cases Fos/TH-ir cells were more numerous at mid-VLM level and absent caudal to the calamus scriptorius. This distribution profile and the fact that most Fos-ir catecholaminergic cells contained VGLUT2 mRNA suggest that these TH-ir cells are probably C1 adrenergic rather than A1 noradrenergic neurons (Stornetta et al., 2002a).

Chan and Sawchenko (1998) proposed two possible explanations for why some C1 cells might be activated by PE infusion. One is that the activation of these cells is involved in central resetting of the baroreceptor reflex. This interpretation cannot be excluded but it is not clearly supported by the present study and that of Li and Dampney (1994) in the rabbit because no recovery of heart rate was observed in these studies during the course of PE infusion. The second explanation proposed by Chan and Sawchenko (1998) is PE-induced stress. This hypothesis requires that activation by stress should override baroreceptor inhibition in some C1 cells or that some C1 cells are not inhibited by baroreceptor stimulation. This hypothesis is consistent with the most recent literature. Though many C1 cells are profoundly inhibited by stimulation of arterial baroreceptors (Schreihofer and Guyenet, 1997; Verberne et al., 1999), C1 cells that do not express Fos in response to hypotension or are not inhibited by blood pressure elevation have also been identified (Chan and Sawchenko, 1994; Li and Dampney, 1994; Madden et al., 1999; Verberne et al., 1999). Some of the C1 cells that are not under baroreceptor control may be presympathetic (bulbospinal) neurons that control the release of adrenaline from the adrenal medulla (Morrison, 2001). Others may control vigilance via projections to the locus coeruleus or the basal forebrain (Milner et al., 1989; Verberne et al., 1999; Hajszan and Zaborsky, 2002). A third potential explanation for the presence of Fos within C1 cells after PE infusion is that Fos is not induced by the rise in blood pressure in these cells but by the blood pressure drop that follows the administration of PE.

In any event, TH-ir cells accounted for only about half of the VLM glutamatergic neurons that expressed Fos after PE infusion. The function of the remaining glutamatergic cells remains unknown.

## Pattern of Fos expression induced in the NTS by PE

As shown before, Fos-ir neurons are confined to the caudal aspects of the NTS (Chan and Sawchenko, 1994; Li

and Dampney, 1994) and, in agreement with Chan and Sawchenko (1998), only a minority of them are GABAergic (26% in our case). The activation of GABAergic neurons in the NTS by baroreceptor stimulation is consistent with the EPSP/IPSP sequences commonly observed in this nucleus following baroreceptor stimulation (Housley et al., 1987; Paton et al., 1993; Spyer, 1994; Zhang and Mifflin, 2000). The present study is the first to positively identify the phenotype of the bulk of the Fos-ir neurons (72%) as glutamatergic based on their content of VGLUT2 mRNA. Glutamatergic and GABAergic neurons that expressed c-Fos-ir were intermingled in the NTS and together accounted for nearly 100% of all Fos-ir neurons.

## Baroactivated glutamatergic neurons of NTS project to the CVLM

Up to 29% of the NTS neurons that were glutamatergic and expressed c-Fos after PE infusion projected to or through the CVLM. Triple-labeled neurons were not found in saline-treated rats, therefore the presence of FG did not cause Fos expression in NTS neurons. The actual percentage of Fos-ir glutamatergic neurons that project to the CVLM is certainly higher than 29% because a single iontophoretic injection of FG cannot label an entire projection. These neurons projected to the region of the VLM identified as containing the highest density of GABAergic baroactivated neurons, the defining characteristic of the CVLM.

The baroactivated neurons with projections to the CVLM were largely confined to two longitudinally contiguous areas of the NTS, namely a small region located dorsomedial to the solitary tract at area postrema level (a.k.a. region I of Chan and Sawchenko (1998) and the dorsal most layer of the commissural nucleus between the two solitary tracts (a.k.a. region II of Chan and Sawchenko, 1998) (Fig. 8). These two areas, in both rat and rabbit, receive the densest projections from the aortic depressor nerve, which consists mostly ($> 85\%$ in rats) of arterial baroreceptor afferents (Wallach and Loewy, 1980; Ciriello, 1983; Cheng et al., 1997). The same two areas were also previously identified by Chan and Sawchenko (1998) as regions that contain especially high concentrations of Fos-ir neurons in PE-treated rats. Based on the results of FG injections placed into the rostral part of the VLM, these authors postulated that regions I and II contain baroactivated neurons that project to the RVLM rather than to the CVLM. Apparently without supporting evidence, Chan and Sawchenko (1998) also surmised that the NTS baroactivated neurons that project to the CVLM were located ventral to the solitary tract, just dorsal to the vagal motor nucleus (their region III). Because the latter region receives little or no input from baroreceptors (Ciriello, 1983; Housley et al., 1987), Chan and Sawchenko (1998) concluded that the baroactivated neurons that project to the CVLM are unlikely to receive monosynaptic inputs from baroreceptor afferents but, instead, are probably third or higher-order neurons in the baroreflex. The present data supports a different interpretation. Our FG injections, centered in the physiologically defined CVLM labeled very few Fos-ir neurons ventral or ventromedial to the solitary tract (Fig. 8). Instead, these injections labeled Fos-ir neurons predominantly in the dorsomedial strip that corresponds to Chan and Sawchenko's regions I and II. The simplest explanation for the discrepancy is that Chan and Sawchenko (1998) placed injections of FG into a region of the VLM that overlaps with the CVLM (an interpretation of their injection sites based on the written description is represented in Fig. 10). Alternatively, the Fos-ir glutamatergic neurons of the NTS may project to several areas of the brainstem including the caudal and rostral VLM, or possibly even further rostrally. Because the contralateral inferior olive was labeled after injection of FG into the CVLM (Fig. 6), it is probable that fibers of passage are labeled to some degree by iontophoretically applied FG.

Thus, according to our data, the regions that receive the bulk of the baroreceptor afferents and the regions that contain most of the Fos-expressing baroactivated neurons with projection to the VLM are indistinguishable. This colocalization does not demonstrate that the NTS baroactivated neurons with VLM projections receive monosynaptic inputs from arterial baroreceptors but it makes this classic though undemonstrated hypothesis more plausible. Except in one case (Deuchars et al., 2000), no attempt has been made to test whether the NTS neurons that receive monosynaptic inputs from baroreceptors actually project to the VLM. Deuchars et al. (2000) recorded from NTS projection neurons that were activated by elevating BP and they showed that some of them receive monosynaptic EPSPs during stimulation of the solitary tract region. However, they did not show that stimulation of baroreceptor afferents also elicits monosynaptic EPSPs in these cells. Nonetheless, this study, along with the slice work of Kawai and Senba (1996), reinforces the plausibility of a monosynaptic connection between arterial baroreceptor afferents and some of the NTS neurons that project directly to the CVLM.

The Fos-ir glutamatergic neurons of the NTS with projection to CVLM could be those that mediate the sympathetic baroreflex but they could also have other functions as well because baroreceptors also regulate cardiovagal motor neurons, respiration, and cerebral circulation (Talman and Dragon, 2002). Identifying which baroactivated NTS neurons are involved in the baroreflex has proven extremely difficult. In general, NTS neurons that receive baroreceptor inputs have discharge properties that do not seem to be appropriate to qualify as plausible components of the baroreflex. Indeed, with very rare exceptions most NTS baroactivated neurons do not encode mean blood pressure (Czachurski et al., 1988; Paton et al., 2001; Seagard et al., 2001). Instead, these cells respond to the rate of blood pressure change and they accommodate rapidly to sustained activation of baroreceptor afferents (Rogers et al., 1993; Rogers et al., 1996; Zhang and Mifflin, 2000; Paton et al., 2001; Seagard et al., 2001). These properties contrast sharply with those of the baroactivated GABAergic cells of the CVLM that are robustly pulse-modulated, encode mean pulsatile blood pressure faithfully, and do not accommodate noticeably to sustained rises in blood pressure (Jeske et al., 1993; Schreihofer and Guyenet, 2002, and unpublished results of Schreihofer and Guyenet). The properties of these CVLM GABAergic cells mirror the discharge characteristics of the sympathetic nerves and that of the presympathetic neurons of the RVLM (Brown and Guyenet, 1985) as expected from cells involved in the sympathetic baroreflex pathway. The discrepancy between the discharge characteristics of most NTS baroreceptive cells, including those with putative projections to the CVLM (Deuchars et al., 2000), and that of all three downstream stages of the

baroreflex pathway suggests to us that only a minority of the NTS neurons that receive arterial baroreceptor inputs may serve as a link in the baroreflex though other interpretations have been made (Rogers et al., 1993). Cardiovagal motor neurons receive a large fraction of their excitatory input from baroreceptors (McAllen and Spyer, 1978), they are at least partially intermingled with the GABAergic neurons of the CVLM (Nosaka et al., 1979) and they receive monosynaptic glutamatergic inputs from the region of the NTS (Neff et al., 1998). The possibility that the same NTS neurons might drive cardiovagal motor neurons and the GABAergic interneurons of the CVLM is plausible but untested.

In summary, the NTS glutamatergic neurons that project to the CVLM and express Fos after sustained hypertension are excellent candidates for mediating the sympathetic and cardiac baroreflex. The concentration of these neurons within the NTS region that receives the densest input from arterial baroreceptors also supports the possibility that these NTS projection neurons might receive monosynaptic inputs from these afferents. However, one should also consider the possibility that this population of NTS neurons could be heterogeneous and might serve other functions besides the baroreflex. For instance, PE infusion activates many neurons in the parabrachial nuclei and in the central gray matter (Li and Dampney, 1994). These regions receive direct projections from the NTS (Herbert et al., 1990; Beitz, 1995). Whether they receive baroreceptor information from neurons that are the same or different from those which project to the CVLM remains to be investigated.

## LITERATURE CITED


Barman SM, Gebber GL. 1997. Subgroups of rostral ventrolateral medullary and caudal medullary raphe neurons based on patterns of relationship to sympathetic nerve discharge and axonal projections. J Neurophys 77:65–75.

Beitz AJ. 1995. Periaqueductal gray. In: Paxinos G, editor. 2. The rat nervous system. New York: Academic Press, Inc., p 173–182.

Blessing WW. 1997. Arterial pressure and blood flow to the tissues. The lower brainstem and bodily homeostasis. New York: Oxford University Press. p 165–268.

Brown DL, Guyenet PG. 1985. Electrophysiological study of cardiovascular neurons in the rostral ventrolateral medulla in rats. Circ Res 56:359–369.

Chan RKW, Sawchenko PE. 1994. Spatially and temporally differentiated patterns of c-fos expression in the brainstem catecholaminergic cell groups induced by cardiovascular challenges in the rat. J Comp Neurol 348:433–460.

Chan RKW, Sawchenko PE. 1998. Organization and transmitter specificity of medullary neurons activated by sustained hypertension: implications for understanding baroreceptor reflex circuitry. J Neurosci 18: 371–387.

Cheng Z, Powley TL, Schwaber JS, Doyle FJ III. 1997. A laser confocal microscopic study of vagal afferent innervation of rat aortic arch: chemoreceptors as well as baroreceptors. J Auton Nerv Syst 67:1–14.

Ciriello J. 1983. Brainstem projections of aortic baroreceptor afferent fibers in the rat. Neurosci Lett 36:37–42.

Czachurski J, Dembowsky K, Seller H, Nobiling R, Taugner R. 1988. Morphology of electrophysiologically identified baroreceptor afferents and second order neurones in the brainstem of the cat. Arch Ital Biol 126:129–144.

Dampney RAL. 1994. Functional organization of central pathways regulating the cardiovascular system. Physiol Rev 74:323–364.

Dampney RAL, Coleman MJ, Fontes MAP, Hirooka Y, Horiuchi J, Li YW, Polson JW, Potts PD, Tagawa T. 2002. Central mechanisms underlying short- and long-term regulation of the cardiovascular system. Clin Exp Pharmacol Physiol 29:261–268.

Deuchars J, Li YW, Kasparov S, Paton JFR. 2000. Morphological and electrophysiological properties of neurones in the dorsal vagal complex of the rat activated by arterial baroreceptors. J Comp Neurol 417:233–249.

Dobbins EG, Feldman JL. 1994. Brainstem network controlling descending drive to phrenic motoneurons in rat. J Comp Neurol 347:64–86.

Dun NJ, Dun SL, Shen E, Tang H, Huang R, Chiu TH. 1995. C-Fos expression as a marker of central cardiovascular neurons. Biol Signals 4:117–123.

Esclapez M, Tillakaratne NJ, Tobin AJ, Houser CR. 1993. Comparative localization of mRNAs encoding two forms of glutamic acid decarboxylase with nonradioactive in situ hybridization methods. J Comp Neurol 331(3):339–362.

Gordon FJ. 1987. Aortic baroreceptor reflexes are mediated by NMDA receptors in caudal ventrolateral medulla. Am J Physiol 252:R628–R633.

Graham JC, Hoffman GE, Sved AF. 1995. c-Fos expression in brain in response to hypotension and hypertension in conscious rats. J Aut Nerv Syst 55:92–104.

Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S. 2002. A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J Neurosci 22:5442–5451.

Guillery RW. 2002. On counting and counting errors. J Comp Neurol 447:1–7.

Guyenet PG. 2000. Neural structures that mediate sympathoexcitation during hypoxia. Respir Physiol 121:147–162.

Guyenet PG, Filtz TM, Donaldson SR. 1987. Role of excitatory amino acids in rat vagal and sympathetic baroreflexes. Brain Res 407:272–284.

Guyenet PG, Sevigny CP, Weston MC, Stornetta RL. 2002. Neurokinin-1 receptor-expressing cells of the ventral respiratory group are functionally heterogeneous and predominantly glutamatergic. J Neurosci 22: 3806–3816.

Hajszan T, Zaborszky L. 2002. Direct catecholaminergic-cholinergic interactions in the basal forebrain. III. Adrenergic innervation of choline acetyltransferase-containing neurons in the rat. J Comp Neurol 449: 141–157.

Herbert H, Moga MM, Saper CB. 1990. Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. J Comp Neurol 293:540–580.

Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S. 2001. The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. J Neurosci 21:NIL1–NIL6.

Housley GD, Martin-Body RL, Dawson NJ, Sinclair JD. 1987. Brain stem projections of the glossopharyngeal nerve and its carotid sinus branch in the rat. Neurosci 22:237–250.

Jeske I, Morrison SF, Cravo SL, Reis DJ. 1993. Identification of baroreceptor reflex interneurons in the caudal ventrolateral medulla. Am J Physiol 264:R169–R178.

Jeske I, Reis DJ, Milner TA. 1995. Neurons in the barosensory area of the caudal ventrolateral medulla project monosynaptically on to sympathoexcitatory bulbospinal neurons in the rostral ventrolateral medulla. Neurosci 65:343–353.

Kawai Y, Senba E. 1996. Organization of excitatory and inhibitory local networks in the caudal nucleus of tractus solitarius of rats revealed in in vitro slice preparation. J Comp Neurol 373:309–321.

Lewis DI, Coote JH. 1996. Baroreceptor-induced inhibition of sympathetic neurons by GABA acting at a spinal site. Am J Physiol Heart Circ Physiol 270:H1885–H1892.

Li YW, Dampney RAL. 1994. Expression of Fos-like protein in the brain following sustained hypertension and hypotension in conscious rabbits. Neurosci 61:613–634.

Li YW, Gieroba ZJ, McAllen RM, Blessing WW. 1991. Neurons in rabbit caudal ventrolateral medulla inhibit bulbospinal barosensitive neurons in rostral medulla. Am J Physiol 261:R44–R51.

Lipski J, Kanjhan R, Kruszewska B, Smith M. 1995. Barosensitive neurons in the rostral ventrolateral medulla of the rat in vivo: morphological properties and relationship to C1 adrenergic neurons. Neurosci 69: 601–618.

Madden CJ, Ito S, Rinaman L, Wiley RG, Sved AF. 1999. Lesions of the C1 catecholaminergic neurons of the ventrolateral medulla in rats using anti-DBH-saporin. Am J Physiol Regul Integr Comp Physiol 277: R1063–R1075.

Masuda N, Terui N, Koshiya N, Kumada M. 1991. Neurons in the caudal ventrolateral medulla mediate the arterial baroreceptor reflex by in-

hibiting barosensitive reticulospinal neurons in the rostral ventrolateral medulla in rabbits. J Auton Nerv Syst 34:103–118.

McAllen RM. 1986. Identification and properties of sub-retrofacial bulbospinal neurones: a descending cardiovascular pathway in the cat. J Autonc Nerv Syst 17:151–164.

McAllen RM, Spyer KM. 1978. The baroreceptor input to cardiac vagal motoneurones. J Physiol (London) 282:365–374.

Milner TA, Abate C, Reis DJ, Pickel VM. 1989. Ultrastructural localization of phenylethanolamine N-methyltransferase-like immunoreactivity in the rat locus coeruleus. Brain Res 478:1–15.

Minson JB, Llewellyn-Smith IJ, Chalmers JP, Pilowsky PM, Arnolda LF. 1997. C-fos identifies GABA synthesizing barosensitive neurons in caudal ventrolateral medulla. Neuro Report 8:3015–3021.

Morrison SF. 2001. Differential control of sympathetic outflow. Am J Physiol Regul Integr Comp Physiol 281:R683–R698.

Morrison SF, Callaway J, Milner TA, Reis DJ. 1991. Rostral ventrolateral medulla—a source of the glutamatergic innervation of the sympathetic intermediolateral nucleus. Brain Res 562:126–135.

Morrison SF, Reis DJ. 1989. Reticulospinal vasomotor neurons in the RVL mediate the somatosympathetic reflex. Am J Physiol 256:R1084–R1097.

Neff RA, Mihalevich M, Mendelowitz D. 1998. Stimulation of NTS activates NMDA and non-NMDA receptors in rat cardiac vagal neurons in the nucleus ambiguus. Brain Res 792:277–282.

Nosaka S, Yamamoto T, Yasunaga K. 1979. Localization of vagal cardioinhibitory preganglionic neurons with rat brain stem. J Comp Neurol 186:79–92.

Paton JFR, Foster WR, Schwaber JS. 1993. Characteristic firing behavior of cell types in the cardiorespiratory region of the nucleus-tractus-solitarii of the rat. Brain Res 604:112–125.

Paton JFR, Li YW, Schwaber JS. 2001. Response properties of baroreceptive NTS neurons. Annals NY Acad Sci 940:157–168.

Paxinos G, Watson C. 1998. The rat brain in stereotaxic coordinates. San Diego: Academic Press.

Potts PD, Hirooka Y, Dampney RAL. 1999. Activation of brain neurons by circulating angiotensin II: direct effects and baroreceptor-mediated secondary effects. Neurosci 90:581–594.

Rogers RF, Paton JFR, Schwaber JS. 1993. NTS neuronal responses to arterial pressure and pressure changes in the rat. Am J Physiol Regul Integr & Comp Physiol 265:R1355–R1368.

Rogers RF, Rose WC, Schwaber JS. 1996. Simultaneous encoding of carotid sinus pressure and dP/dt by NTS target neurons of myelinated baroreceptors. J Neurophys 76:2644–2660.

Schreihofer AM, Guyenet PG. 1997. Identification of C1 presympathetic neurons in rat rostral ventrolateral medulla by juxtacellular labeling in vivo. J Comp Neurol 387:524–536.

Schreihofer AM, Guyenet PG. 2000. Sympathetic reflexes after depletion of bulbospinal catecholaminergic neurons with anti-DBH-saporin. Am J Physiol Regul Integr & Comp Physiol 279:R729–R742.

Schreihofer AM, Guyenet PG. 2002. The baroreflex and beyond: control of sympathetic vasomotor tone by GABAergic neurons in the ventrolateral medulla. Clin Exp Pharmacol Physiol 29:514–521.

Seagard JL, Dean C, Hopp FA. 2001. Properties of NTS neurons receiving input from barosensitive receptors. Annals NY Acad Sci 940:142–156.

Schäfer MKH, Varoqui H, Defamie N, Weihe E, Erickson JD. 2002. Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. J Biol Chem 277:50734–50748.

Somogyi P, Minson JB, Morilak D, Llewellyn-Smith I, McIlhinney JR, Chalmers J. 1989. Evidence for an excitatory amino acid pathway in the brainstem and for its involvement in cardiovascular control. Brain Res 496:401–407.

Spyer KM. 1994. Central nervous mechanisms contributing to cardiovascular control. J Physiol (Lond) 474:1–19.

Stornetta RL, Guyenet PG. 1999. Distribution of glutamic acid decarboxylase mRNA-containing neurons in rat medulla projecting to thoracic spinal cord in relation to monoaminergic brainstem neurons. J Comp Neurol 407:367–380.

Stornetta RL, Schreihofer AM, Pelaez NM, Sevigny CP, Guyenet PG. 2001. Preproenkephalin mRNA is expressed by C1 and non-C1 barosensitive bulbospinal neurons in the rostral ventrolateral medulla of the rat. J Comp Neurol 435:111–126.

Stornetta RL, Sevigny CP, Guyenet PG. 2002a. Vesicular glutamate transporter DNPI/VGLUT2 mRNA is present in C1 and several other groups of brainstem catecholaminergic neurons. J Comp Neurol 444:191–206.

Stornetta RL, Sevigny CP, Schreihofer AM, Rosin DL, Guyenet PG. 2002b. Vesicular glutamate transporter DNPI/GLUT2 is expressed by both C1 adrenergic and nonaminergic presympathetic vasomotor neurons of the rat medulla. J Comp Neurol 444:207–220.

Sun MK. 1996. Pharmacology of reticulospinal vasomotor neurons in cardiovascular regulation. Pharmacol Rev 48:465–494.

Sun MK, Guyenet PG. 1985. GABA-mediated baroreceptor inhibition of reticulospinal neurons. Am J Physiol 249:R672–R680.

Sved AF, Ito S, Madden CJ. 2000. Baroreflex dependent and independent roles of the caudal ventrolateral medulla in cardiovascular regulation. Brain Res Bull 51:129–133.

Takamori S, Rhee JS, Rosenmund C, Jahn R. 2000. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 407:189–194.

Takamori S, Rhee JS, Rosenmund C, Jahn R. 2001. Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter (VGLUT2). J Neurosci 21: NIL7–NIL12.

Talman WT, Dragon DN. 2002. Inhibiting the nucleus tractus solitarii extends cerebrovascular autoregulation during hypertension. Brain Res 931:92–95.

Verberne AJM, Stornetta RL, Guyenet PG. 1999. Properties of C1 and other ventrolateral medullary neurones with hypothalamic projections in the rat. J Physiol (Lond) 517:477–494.

Wallach JH, Loewy AD. 1980. Projections of the aortic nerve to the nucleus tractus solitarius in the rabbit. Brain Res 188:247–251.

Willette RN, Krieger AJ, Barcas PP, Sapru HN. 1983. Medullary gamma-aminobutyric acid (GABA) receptors and the regulation of blood pressure in the rat. J Pharmacol Exp Ther 226:893–899.

Willette RN, Punnen-Grandy S, Krieger AJ, Sapru HN. 1987. Differential regulation of regional vascular resistance by the rostral and caudal ventrolateral medulla in the rat. J Auton Nerv Syst18:143–151.

Wuenschell CW, Fisher RS, Kaufman DL, Tobin AJ. 1986. In situ hybridization to localize mRNA encoding the neurotransmitter synthetic enzyme glutamate decarboxylase in mouse cerebellum. Proc Natl Acad Sci 83:6193–6197.

Zhang J, Mifflin SW. 2000. Responses of aortic depressor nerve-evoked neurones in rat nucleus of the solitary tract to changes in blood pressure. J Physiol (Lond) 529:431–443.