Altered Balance of γ -Aminobutyric Acidergic and Glutamatergic Afferent Inputs in Rostral Ventrolateral Medulla-Projecting Neurons in the Paraventricular Nucleus of the Hypothalamus of Renovascular Hypertensive Rats

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ABSTRACT

An imbalance of excitatory and inhibitory functions has been shown to contribute to numerous pathological disorders. Accumulating evidence supports the idea that a change in hypothalamic γ -aminobutyric acid (GABA)-ergic inhibitory and glutamatergic excitatory synaptic functions contributes to exacerbated neurohumoral drive in prevalent cardiovascular disorders, including hypertension. However, the precise underlying mechanisms and neuronal substrates are still not fully elucidated. In the present study, we combined quantitative immunohistochemistry with neuronal tract tracing to determine whether plastic remodeling of afferent GABAergic and glutamatergic inputs into identified RVLM-projecting neurons of the hypothalamic paraventricular nucleus (PVN-RVLM) contributes to an imbalanced excitatory/inhibitory function in renovascular hypertensive rats (RVH). Our results indicate that

both GABAergic and glutamatergic innervation densities increased in oxytocin-positive, PVN-RVLM (OT-PVN-RVLM) neurons in RVH rats. Despite this concomitant increase, time-dependent and compartment-specific differences in the reorganization of these inputs resulted in an altered balance of excitatory/inhibitory inputs in somatic and dendritic compartments. A net predominance of excitatory over inhibitory inputs was found in OT-PVN-RVLM proximal dendrites. Our results indicate that, along with previously described changes in neurotransmitter release probability and postsynaptic receptor function, remodeling of GABAergic and glutamatergic afferent inputs contributes as an underlying mechanism to the altered excitatory/inhibitory balance in the PVN of hypertensive rats. *J. Comp. Neurol.* 518:567–585, 2010.

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A proper balance of excitatory and inhibitory functions is required for nearly all brain functions, including cognitive, sensory, motor, and autonomic functions (Cline, 2005; Hensch and Fagioli, 2005). An imbalance of these opposing neurotransmitter systems, on the other hand, contributes to numerous neurological disorders, including epilepsy, autism, and schizophrenia (Cline, 2005; Dudek and Staley, 2007; Rubenstein and Merzenich, 2003; Wassef et al., 2003). A change in central nervous system (CNS) γ -aminobutyric acid (GABA)ergic inhibitory and glutamatergic excitatory synaptic functions has been shown to be involved in the exacerbated neurohumoral drive in prevalent cardiovascular disorders, including hypertension and heart failure (Bergamaschi et al., 1995; Haywood et al.,

2001; Hermes et al., 2008; Ito et al., 2001; Kleiber et al., 2008; Li and Pan, 2005, 2007b; Li and Patel, 2003; Mei et al., 2003). Despite this evidence, the precise underlying mechanisms and neuronal substrates involved are not fully elucidated.

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The hypothalamic paraventricular nucleus (PVN) plays an important role in the modulation of sympathetic vasomotor tone and blood pressure (Allen, 2002; Coote et al., 1998; Dampney et al., 2005; Martin and Haywood, 1992; Swanson and Sawchenko, 1983). In addition to the well-established projection to sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord, the rostral ventrolateral medulla (RVLM) is another major PVN target that mediates its influence on cardiovascular control (Allen, 2002; Ciriello et al., 1985; Coote et al., 1998; Dampney et al., 1987; Hardy, 2001; Kubo et al., 2000; Tagawa and Dampney, 1999; Yang and Coote, 1998). Activation of the PVN-RVLM pathway results in increased vasomotor RVLM neuronal activity (Yang et al., 2001; Yang and Coote, 1998) as well as pressor and sympathoexcitatory responses (Allen, 2002; Tagawa and Dampney, 1999; Yang and Coote, 1998).

Among the different peptides expressed in long-descending PVN neurons, oxytocin (OT) and vasopressin (VP) are the most prevalent (Swanson and Sawchenko 1982). A predominance of OT over VP expression in PVN-brainstem projections, including the RVLM, has been reported (Aguado et al., 2003; Gomez et al., 1993; Mack et al., 2002; Sawchenko and Swanson, 1982; Stocker et al., 2006). This is in agreement with other studies showing a predominance of OT over VP in the brainstem, with respect to both their levels and their immunoreactivities (Hancock and Nicholas, 1987; Lang et al., 1983; Nilaver et al., 1980). Microinjections of OT within the RVLM has been shown to induce an increase in mean arterial pressure and heart rate (Mack et al., 2002) as well as playing an important role in mediating cardiovascular responses to environmental stressors. Overall, these studies support the PVN-RVLM-OT pathway as playing an important role in mediating PVN influences on sympathetic and cardiovascular control.

The PVN-RVLM pathway has been implicated in enhanced sympathetic vasomotor tone in hypertensive rats. Lesions of the PVN attenuated the development of hypertension and lowered blood pressure in spontaneously hypertensive rats (SHRs) and RVH rats (Allen, 2002; Ciriello et al., 1984; Earle and Pittman, 1995; Goto et al., 1981; Herzig et al., 1991; Takeda et al., 1991). In addition, increased expression of FOS immunoreactivity was reported in the PVN of RVH rats (Jung et al., 2004), supporting an increased activation of PVN neurons. Importantly, an enhanced PVN excitatory drive to the RVLM was reported to mediate enhanced sympathetic vasomotor tone in hypertensive rats (Allen, 2002). Despite the evidence supporting a role for the PVN in enhanced sympathoexcitatory outflow during hypertension, the underlying cellular and synaptic mechanisms remain to be elucidated.

The excitability of PVN neurons, and consequently sympathetic outflow from this region, is highly dependent on a balanced activity between excitatory and inhibitory synaptic inputs. GABA, acting most predominantly on GABA_A receptors, is the major inhibitory neurotransmitter in the hypothalamus, restraining PVN neuronal activity and autonomic outflow (Chen et al., 2003; Decavel and Van den Pol, 1990; Tasker and Dudek, 1993; Zhang et al., 2002). Conversely, glutamate, acting both on AMPA and NMDA ionotropic receptors, is the major excitatory neurotransmitter (Boudaba et al., 1997; Decavel and van den Pol, 1992; Herman et al., 2000), stimulating PVN activity and autonomic outflow (Chen et al., 2003; Herman et al., 2002; Latchford and Ferguson, 2004; Li and Pan, 2006; Li et al., 2003).

An altered glutamatergic and GABAergic synaptic function was shown to contribute to exacerbated PVN neuronal activity and sympathetic outflow during hypertension. For example, an enhanced glutamatergic input into the PVN is required to maintain elevated sympathetic activity in hypertensive rats (Allen, 2002; Li and Pan, 2007b), a phenomenon that involves, at least in part, enhanced presynaptic glutamate release, as well as up-regulation of postsynaptic NMDA receptors (Li et al., 2008). The contribution of altered PVN GABAergic function to sympathoexcitation in hypertension is less clear. For example, a blunted PVN GABAergic function has been shown to play a role in exacerbated sympathoexcitation in chronically SHRs (Li and Pan, 2006, 2007). In RVH rats, however, a diminished and an enhanced PVN GABAergic inhibition was observed at early and late hypertensive stages, respectively (Haywood et al., 2001; Martin and Haywood, 1998). Thus, dynamic and time-dependent changes in GABAergic inhibitory function in renovascular hypertension could contribute to the onset of sympathoexcitation at early stages, while acting as a compensatory mechanism at late stages of hypertension.

Although these data support a role for altered glutamatergic/GABAergic balance as an important mechanism underlying altered PVN sympathetic control in hypertension, the precise underlying mechanisms are not fully delineated. Previous studies support both changes in neurotransmitter release probability and changes in numbers and types of postsynaptic receptors (Li et al., 2008a,b). However, whether remodeling of afferent synaptic inputs also occurs in hypertensive conditions is at present unknown. Given the growing evidence supporting an important role for the PVN-RVLM pathway in cardiovascular control, as well as its involvement in the pathophysiology of hypertension, we used in this study a combination of quantitative immunohistochemistry with neuronal tract tracing to investigate afferent synaptic remodeling in identified OT-PVN-RVLM neurons in RVH rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (150–180 g) were purchased from Harlan Laboratories (Indianapolis, IN). Rats were maintained under a 12-hour:12-hour light-dark cycle and given free access to food and water. All procedures were carried out in agreement with the Medical College of Georgia Institutional Animal Care and Use Committee guidelines.

Induction of two-kidney one-clip RVH rats

RVH rats were generated as previously described (Bergamaschi et al., 1995; Sonner et al., 2008). Briefly, rats were anesthetized with ketamine and xylazine (40 mg and 10 mg/kg, respectively, ip); the left renal artery was exposed through an abdominal incision, and the renal artery and renal vein were dissected free from the adherent tissues. The left renal artery was partially obstructed with a silver clip of 0.2-mm width. No clip obstruction was applied to the sham-operated group ($n = 15$). Animals were submitted to the final experimental procedures 3 or 6 weeks after the surgical procedure. These two groups were designated as early (3 weeks, $n = 3$) and late (6 weeks, $n = 12$) RVH rats (Carretero and Gulati, 1978; Martinez-Maldonado, 1991). Systolic blood pressure was measured in conscious rats using a pneumatic tail-cuff method (RTBP 2000; Kent Scientific). An average of three measurements was obtained per animal.

Retrograde labeling of RVLM-projecting PVN neurons (PVN-RVLM)

Labeling of PVN-RVLM neurons was done as previously described (Li et al., 2003; Sonner et al., 2008; Sonner and Stern, 2007). Briefly, rats were anesthetized (ketamine/xylazine mixture, 90 and 5 mg/kg, respectively, i.p.), and the head of the rat was placed in a stereotaxic frame. For injections in the RVLM, an ~ 4 -mm burr hole was made in the skull, and rhodamine-labeled microspheres (Lumaflo, Naples FL) were pressure injected unilaterally (400 nl) using the coordinates bregma -11.96 , lateral -2.0 , dorsal -8.0 . Animals were allowed to recover and were killed 5–7 days after surgery, and the brain was dissected for immunohistochemical experiments. The location of the injection sites were confirmed histologically. Injections in the RVLM usually extended from the caudal pole of the facial nucleus to ~ 1 mm caudally and were ventrally located with respect to the nucleus ambiguus (see Fig. 1; Li et al., 2003; Sonner et al., 2008; Sonner and Stern, 2007; Stern and Zhang, 2003). Cases showing a misplaced injection site were not included in the analysis. Hypothalamic sections were then processed for double immunohistochemistry.

Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardiacally with 0.01 M phosphate-buffered saline (PBS; pH 7.3–7.4; 150 ml) followed by 4% paraformaldehyde (350 ml). Brains were postfixed in 4% paraformaldehyde for 3 hours at 4°C. Fixed brains were cryoprotected at 4°C with 0.01 M PBS containing 30% sucrose for a minimum of 72 hours. Sequential 25- μ m sections through the hypothalamus, between bregma -1.80 and -2.12 (Paxinos et al., 1999), were cut with a cryostat, transferred into 0.01 M PBS, and incubated in a solution of 0.01 M PBS with 0.01% Triton X-100, 0.04% NaN_3 , and 5% normal horse serum for 1 hour. Hypothalamic sections were incubated for 24 hours in a cocktail of primary antibodies containing either a guinea pig antivesicular glutamate transporter 2 (VGLUT₂) polyclonal antibody (catalogue No. AB5907, lot No. 25020802; Chemicon, Temecula, CA; 1:10,000) or a mouse antiglutamic acid decarboxylase 67 (GAD₆₇) monoclonal antibody (catalogue No. MAB5406; Chemicon; 1:800), along with a rabbit antioxytocin (OT) polyclonal antibody (catalogue No. T-4084, Bachem, Torrance, CA; 1:15,000). Reactions with primary antibodies were followed by a 4-hour incubation in the presence of fluorescently labeled secondary antibody (anti-guinea pig or mouse Cy5-labeled antibody, 1:50; and donkey anti-rabbit FITC-labeled, 1:400). Rhodamine-labeled microsphere labeling was readily detected by fluorescence illumination. For the initial characterization of OT and VP immunoreactivity in retrogradely labeled PVN-RVLM neurons, a cocktail of primary antibodies containing the rabbit anti-OT antibody along with a guinea pig anti-VP polyclonal antibody (catalogue No. T-5048, lot No. 061305; Bachem; 1:15,000) was used, followed by an incubation in secondary antibodies (anti-guinea pig FITC-labeled, 1:400; and anti-rabbit Cy5-labeled, 1:50), as described above. All antibodies were diluted with PBS containing 0.01% Triton X-100 and 0.04% NaN_3 . All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Antibody characterization

In all cases, primary antibodies were titrated to determine the dilutions that produced the maximal number of immunoreactive elements with a minimal level of background staining. General control experiments included omission of primary or secondary antibodies and extensive tests of cross-reactivity of secondary antibodies. In all these cases, staining was completely absent. The guinea pig anti-VGLUT₂ antibody (Chemicon) was raised against a synthetic peptide corresponding to a region of the VGLUT₂ protein sequence (VQESAQDAYS YKDRDDYS). This sequence does not overlap with that of VGLUT₁ or VGLUT₃. According to Chemicon, preabsorption of the VGLUT₂ an-

tiserum with immunogen peptide eliminated all immunostaining. The specificity of this antibody has been previously determined by others using immunofluorescence protocols similar to those described here (Hrabovszky et al., 2004; Stocker et al., 2006). In both studies, preabsorption of the antibody with the synthetic peptide used as immunogen resulted in complete lack of immunolabeling. In our hands, the staining pattern obtained with the VGLUT₂ antibody corresponds to the patterns previously described by others using the same antibody in the hypothalamus (Castelli et al., 2007; Hrabovszky et al., 2004, 2005; Kiss et al., 2007; Stocker et al., 2006; Wittmann et al., 2005) as well as the pattern obtained using a different antibody (Fremeau et al., 2001; Fujiyama et al., 2001; Kaneko et al., 2002; Sakata-Haga et al., 2001; Tong et al., 2001; Varoqui et al., 2002).

The mouse monoclonal anti-GAD₆₇ antibody (Chemicon) was raised against a recombinant fusion protein containing the unique N-terminal regions of GAD₆₇ not shared by GAD₆₅ protein. According to Chemicon, this antibody reacts with the 67-kDa isoform of glutamate decarboxylase (GAD₆₇) of rat, mouse, and human origin and possesses no detectable cross-reactivity with GAD₆₅ by Western blot on rat brain lysate compared with a blot probed with AB1511, which reacts with both GAD₆₅ and GAD₆₇. The specificity of this antibody has been previously determined by others using immunofluorescence protocols similar to those described here (Benagiano et al., 2005; Sweatt et al., 2004). In both of those studies, preabsorption of the antibody with the synthetic peptide used as immunogen resulted in complete lack of immunolabeling. In our hands, and as we previously described (Park et al., 2006; Richards et al., 2005), the staining pattern obtained with the GAD₆₇ antibody corresponds to the patterns previously described by others using the same antibody in the hypothalamus (Al-Noori et al., 2008; Castelli et al., 2007) and other brain regions (Singec et al., 2004; Sweatt et al., 2004).

The rabbit anti-OT antibody (Bachem) was raised against the antigen sequence H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂. According to Bachem, this antibody has no cross-reactivity with other peptides typically found in the hypothalamus, including arg-vasopressin, α -ANP, CRF, or GFR. The specificity of this antibody was tested in our laboratory using OT-knockout mice generously provided by Dr. Mariana Morris (Wright State University, Dayton, Ohio), in which labeling was completely absent. In rats, the appropriate cellular distribution pattern was observed, with strong somatic immunofluorescence staining found in the supraoptic and paraventricular nucleus as well as fiber staining in the median eminence and posterior pituitary. This staining pattern overlapped with that obtained using a rabbit OT antiserum raised against the C terminus of OT

(also kindly provided by Dr. Mariana Morris) as well as with a mouse anti-OT-neurophysin (PS38) antibody (generously provided by Dr. Hal Gainer, NIH; Richards et al., 2005) raised against rat OT-neurophysin. The specificity of PS38 has been extensively characterized previously (Ben-Barak et al., 1985; Whitnall et al., 1985).

The guinea pig anti-VP antibody (Bachem) was raised against the antigen sequence H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂. According to Bachem, this antibody has no cross-reactivity with other peptides typically found in the hypothalamus, including OT, Met-enkephalin, PACAP38, or desamino-(D-Arg⁸)-VP. In our hands, this antibody gave the appropriate cellular distribution pattern expected for VP, with strong somatic immunofluorescence staining found in the supraoptic nucleus, PVN, and supra-chiasmatic nucleus, along with fiber staining in the median eminence and posterior pituitary. This staining pattern overlapped with that obtained using a mouse anti-VP-neurophysin (PS46) antibody (also generously provided by Dr. Hal Gainer; Richards et al., 2005) raised against rat VP-neurophysin. The specificity of PS46 has been extensively characterized previously (Ben-Barak et al., 1985; Whitnall et al., 1985).

Confocal imaging acquisition and analysis

Acquisition and quantification of immunoreactive signals were performed as previously described (Higata-niguchi et al., 2007). Stained sections were examined with a Leica TCS SL (Leica Microsystems, Mannheim, Germany) confocal microscope system. A helium-neon laser was used to excite fluorescein isothiocyanate (FITC) and rhodamine fluorochromes at 488 and 543 nm, respectively, and an argon-krypton laser was used to excite Cy5 fluorochrome at 633 nm. Fluorescent signal cross-talk among the channels was avoided by setting image-acquisition parameters with individually labeled sections. All images from the different experimental groups were digitized with identical acquisition settings.

Quantification of GAD₆₇ and VGLUT₂ immunoreactivities within specific PVN subnuclei

Stacks of six consecutive optical focal planes (z-step 1 μ m, image size 1,024 $\times$ 1,024 pixels, $\times$ 20 objective) were obtained from three to five sections/rat at two rostrocaudal levels of the PVN, according to Swanson and Kuypers (1980). These included 1) the middle one-third of the PVN, containing the lateral magnocellular (LM), dorsal cap (DC), and ventromedial (VM) subnuclei and 2) the most caudal one-third of the PVN, containing the posterior parvocellular (PaPo) subnucleus (Armstrong et al., 1980; Swanson and Kuypers, 1980). As previously reported (Li et al., 2003; Pyner and Coote, 2000; Stocker et al., 2004, 2006), these

rostrocaudal regions contain the highest density of PVN-RVLM projecting neurons.

Imaging analysis was performed in Metamorph software (Universal Imaging Corporation, Downingtown, PA). An automated tracing procedure incorporating a threshold paradigm (Alvarez et al., 2004) was applied to the channel containing the VGLUT₂ or the GAD₆₇ immunoreactivity information. Background intensity was calculated from random adjacent areas in the neuropil, and the threshold was set to pass intensities 1.5 times above background immunofluorescence. Regions of interest (ROIs; $n = 3$) of predetermined sizes were drawn within the LM, VM, DC, and PaPo PVN subnuclei. The distributions of retrogradely labeled and OT-ir neurons were used as landmarks to delineate the various subnuclei (see Fig. 1). The density of VGLUT₂ and GAD₆₇ thresholded signal within each ROI (expressed as percentage threshold area = area occupied by thresholded signal/total ROI area $\cdot 100$) was calculated, and a mean value for each sampled section was obtained. Then, a mean value of all the sampled sections/animal was calculated, resulting in mean VGLUT₂ and GAD₆₇ density values per PVN subnuclei per animal (Higa-Taniguchi et al., 2007; Jansen et al., 2003). Mean VGLUT₂ and GAD₆₇ density values were then obtained for each experimental condition/group. For control purposes, similar measurements and analyses were obtained from a region ventrally located to the main PVN as well as from the suprachiasmatic nucleus (SCN).

Quantification of VGLUT₂ and GAD₆₇ immunoreactivities in identified OTergic, PVN-RVLM-projecting neurons

Double-labeled OT-PVN-RVLM neurons were identified by merging confocal images obtained from the FITC and rhodamine fluorophores. A positive colocalization was considered by the appearance of yellow (red + green) profiles in the merged image (see Fig. 1). Stacks of confocal images containing the three channels (FITC, rhodamine, and Cy5) and spanning the whole extension of double-labeled neurons (~50 sections, z-step 0.5 μm , image size $1,024 \times 1,024$ pixels, $\times 63$ oil immersion lense, $\times 3$ digital zoom) were then obtained. Only double-labeled neurons whose somata were fully contained within the z-stack were selected for quantification. In most cases, segments of proximal dendrites were also included, and comparisons between somatic and these proximal dendrites were performed. All neurons contained within the sampled sections fulfilling these criteria were used for quantification purposes. These image stacks were imported into Volocity (Improvision, Lexington, MA), and neurons, along with associated VGLUT₂ir or GAD₆₇ir boutons, were reconstructed in three dimensions. Because the retrograde tracer used to identify PVN-RVLM neurons results in a punctate staining, the OT signal, which provides a diffuse,

homogenous staining of the neuron, and the VGLUT₂ and/or GAD₆₇ channels were classified to establish an intensity threshold for neuronal and bouton signals. Threshold was set to pass intensities five times above background immunofluorescence, as determined from the frequency distribution of voxel intensities calculated for each channel. The surface area of positive elements in the neuronal and bouton channels was measured independently. Volocity software algorithms allow colocalization analysis, discriminating between boutons that overlap with the neuron from those that do not. The density of overlapping boutons was calculated by dividing the number of overlapping boutons by the respective neuronal surface area, also provided by Volocity. To calculate and compare dendrites/somata immunoreactive ratios among groups, ratios were determined on the same neuronal profile and then averaged. These approaches have been recently validated in our laboratory (Higa-Taniguchi et al., 2007) to study changes in (nor)adrenergic synaptic innervation density under physiological and pathological conditions, as well as by others (see, e.g., Sykes and Condrón, 2005; Wimmer et al., 2006). All quantitative analyses were carried out blindly. To enhance contrasts and reduce noise, all photographs shown were adjusted for contrast and brightness and frequently filtered with a Gaussian filter (Adobe Photoshop version 10.0.1). Final composition was performed in CorelDraw 12.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Results were analyzed using a one-way or two-way ANOVA as indicated, followed by Bonferroni's post hoc tests. Differences were considered significant at $P < 0.05$. To assess differences in the incidence of OT and VP immunoreactivity in PVN-RVLM neurons, χ^2 tests were used.

RESULTS

Blood pressure measurements

Most of our studies involved sham and late-stage (6 weeks) RVH rats. However, as indicated below, a subset of experiments was also performed on early-stage (3 weeks) RVH rats. Systolic blood pressure values from sham, early-stage RVH, and late-stage RVH rats were 121 ± 6 mmHg, 152 ± 5 mmHg ($P < 0.0004$ vs. sham), and 208 ± 14 mmHg ($P < 0.0001$ vs. sham and $P < 0.0020$ vs. early-stage RVH), respectively. No differences in body weight were observed among experimental groups (results not shown).

Distribution of GABAergic and glutamatergic terminals in identified PVN subnuclei

To identify autonomically related and magnocellular neuroendocrine PVN subnuclei, we retrogradely labeled PVN neurons with rhodamine beads injected in the RVLM (as described in Materials and Methods) and immunohis-

tochemically identified OT- and VP-containing neurons. As previously described (Armstrong et al., 1980; Stern, 2001; Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983), PVN-RVLM neurons were found at two major rostrocaudal levels of the PVN: medial (~ 1.8 – 2.1 mm caudal to bregma) and caudal (~ 2.1 – 2.3 mm caudal to bregma). The PVN-RVLM neurons were concentrated within three major subnuclei: the dorsal cap (DC), the parvocellular ventromedial (VM) subnuclei (both located within the medial PVN), and the parvocellular posterior (PaPo) subnucleus (located in the caudal PVN). Magnocellular OT and VP neurons, on the other hand, were concentrated in the lateral magnocellular subnucleus (LM; Fig. 1). In several instances, double-labeled OT-PVN-RVLM and VP-PVN-RVLM neurons were observed throughout the PVN regions sampled (Fig. 1). An initial colocalization analysis in control rats revealed a significantly higher predominance of OT over VP staining in PVN-RVLM neurons. Among a total of 686 PVN-RVLM sampled neurons, 139 ($\sim 20\%$) and 53 ($\sim 7\%$) were found to be OT- and VP-ir, respectively ($P < 0.0001$, χ^2 test). Given the low incidence of VP colocalization with PVN-RVLM neurons, the remainder of the study was focused on OT-ir PVN-RVLM neurons. A more detailed analysis in this population indicated that the incidence of colocalization was similar between sham normotensive and late-stage RVH rats and did not vary between medial (DC and VM subnuclei) and caudal (PaPo subnucleus) aspects of the PVN: sham-caudal = $\sim 22\%$ colocalization (100/466), RVH-caudal = $\sim 21\%$ colocalization (85/408), sham-medial = $\sim 18\%$ colocalization (39/220), RVH-medial = $\sim 26\%$ colocalization (96/372; $P > 0.1$, χ^2).

GABAergic and glutamatergic terminals in the PVN were identified by their selective immunoreactivity to GAD₆₇ and VGLUT₂, respectively (Freneau et al., 2001). In both cases and as shown in the representative examples in Figure 2, dense immunoreactivity for both markers was found in the PVN, which displayed a characteristic neuropile punctate staining. No somatic staining was observed in either case.

Changes in GAD₆₇ and VGLUT₂ immunoreactivities within identified PVN subnuclei in late-stage RVH rats

To determine whether the density of PVN GAD₆₇ immunoreactivity changed in late-stage RVH rats and to assess whether this change was dependent on its topographical distribution within the PVN, we measured GAD₆₇ immunoreactivity within identified PVN subnuclei in sham and late-stage RVH rats ($n = 6$ in each group). Results are summarized in Figure 3. As shown, PVN GAD₆₇ immunoreactivity was significantly increased in RVH rats ($F = 34.7$, $P <$

0.0001 , two-way ANOVA), an effect that was observed in all the subnuclei sampled ($P < 0.001$ for DC, VM and PaPo subnuclei; $P < 0.05$ for LM; Bonferroni post hoc test). On the other hand, no differences in GAD₆₇ immunoreactivity between sham and late-stage RVH rats were observed in a region ventrally located to the main PVN (sham $19.7\% \pm 4.0\%$ density, RVH $23.8\% \pm 4.6\%$ density; $P > 0.5$) or in the suprachiasmatic nucleus (sham $10.3\% \pm 3.2\%$ density, RVH $14.3\% \pm 3.8\%$ density; $P > 0.5$).

A similar approach was used to measure changes in VGLUT₂ immunoreactivity in sham and late-stage RVH rats ($n = 6$ in each group). Results are summarized in Figure 4. Similarly to the case for GAD₆₇, we found PVN VGLUT₂ immunoreactivity to be significantly increased in RVH rats ($F = 15.6$, $P < 0.0001$, two-way ANOVA). Although a trend toward an increased VGLUT₂ immunoreactivity was observed in the DC, VM, and PaPo subnucleus, differences reached statistical significance only in the latter ($P < 0.01$, Bonferroni post hoc test). Unlike the case for the PVN, a small though significantly diminished VGLUT₂ immunoreactivity was found in a region located ventrally to the main PVN (sham $23.8\% \pm 1.6\%$ density, RVH $18.8\% \pm 1.4\%$ density; $P > 0.05$). No significant differences were observed, however, in the suprachiasmatic nucleus (sham $8.1\% \pm 2.3\%$ density, RVH $7.2\% \pm 3.8\%$ density; $P > 0.5$).

Measurements of GABAergic and glutamatergic boutons in close apposition to identified OT-positive PVN-RVLM neurons

Given the high neurochemical and functional cellular heterogeneity characteristic of the PVN, specific neuronal populations could be differentially affected despite an apparent global change in the density of immunoreactive terminals in RVH rats. Thus, to determine whether changes in GABAergic and glutamatergic PVN immunoreactive terminals were associated with OT-positive PVN-RVLM projecting neurons, the densities of GAD₆₇- and VGLUT₂-ir boutons in close apposition to these neurons were calculated. Representative examples of 3D-reconstructed, retrogradely labeled PVN-RVLM neurons and associated GAD₆₇ and VGLUT₂ immunoreactive boutons are shown in Figure 5. Despite the changes reported above in general GAD₆₇ immunoreactivity in middle regions of the PVN, containing the VM and DC subnuclei, the density of GAD₆₇- and VGLUT₂-ir boutons in close apposition with OT-PVN-RVLM neurons in the VM and DC subnuclei did not change in late-stage RVH rats (GAD₆₇ density: sham 0.012 ± 0.003 counts/ μm^2 , RVH 0.018 ± 0.004 counts/ μm^2 ; $P > 0.1$, $n = 7$; VGLUT₂: sham 0.021 ± 0.003 counts/ μm^2 , RVH 0.017 ± 0.005 counts/ μm^2 ; $P > 0.3$, $n = 14$). On the other hand, and as summarized

Figure 1. Identification of various PVN subnuclei, RVLM-projecting neurons, and OT- and VP-ir neurons in the PVN. **A:** Representative confocal photomicrographs obtained at the medial level of PVN (~1.8–2.1 mm caudal to bregma). Retrogradely labeled PVN-RVLM neurons (A1, red) and OT- and VP-ir neurons (A2, blue and green, respectively) are shown. In A3, both images were superimposed, and the areas contained within the white squares were reimaged at higher magnification using different thresholds and are displayed in A4 and A5. The numbered regions in A1 and A2 depict the dorsal cap (1, DC), ventromedial parvocellular (2, VM), and lateral magnocellular (3, LM) subnuclei. **B:** Representative confocal photomicrographs obtained at the caudal level of PVN (~2.1–2.3 mm caudal to bregma). Retrogradely labeled PVN-RVLM neurons (B1, red) and OT- and VP-ir neurons (B2, blue and green, respectively) are shown in B1 and B2. In B3, both images were superimposed, and the areas contained within the white squares were reimaged at higher magnification using different thresholds and are displayed in B4 and B5. Arrows point to examples of double-labeled OT-PVN-RVLM neurons. **C:** Representative example of a rhodamine bead injection site in the RVLM, displayed at various rostrocaudal levels in the ventral medulla (C1–C6). The arrows in C2,3 point to the center of the injection site. 3V, third ventricle; Py, pyramidal tract; IO, inferior olive; RO, nucleus raphe obscurus; LRt, lateral reticular nucleus. A magenta-green copy of this figure is available as Supporting Information Figure 1. Scale bars = 50 μm in A,B; 12 μm in insets; 100 μm in C.

Figure 2. Triple immunofluorescent staining for oxytocin (A; green), GAD₆₇ (B; red), and VGLUT₂ (C; white) in the caudal PVN. In D, all images are superimposed. In E, the region contained within the rectangular region in D is shown at higher magnification. Note the characteristic neuropile punctuate staining of GAD₆₇ and VGLUT₂. 3V, third ventricle. A blue-green copy of this figure is available as Supporting Information Figure 2. Scale bars = 50 μm in A; 50 μm in D (applies to B-D); 25 μm in E.

Figure 3. GAD₆₇ immunoreactivity in the PVN of normotensive sham and RVH rats. **A:** Representative confocal photomicrographs obtained at the medial level of PVN (1.8–2.1 mm caudal to bregma) in sham (A1) and late-stage RVH (A2) rats, showing retrogradely labeled PVN-RVLM neurons (red) and GAD₆₇ immunoreactivity (white). For better comparisons, representative regions of A1 and A2 are displayed at higher magnification in A3 and A4, respectively. **B:** Representative confocal photomicrographs obtained at the caudal level of PVN (2.1–2.3 mm caudal to bregma) in sham (B1) and RVH (B2) rats, showing retrogradely labeled PVN-RVLM neurons (red) and GAD₆₇ immunoreactivity (white). For better comparisons, representative regions of B1 and B2 are displayed at higher magnification in B3 and B4, respectively. **C:** Summary showing a significant increase in the relative GAD₆₇ immunoreactivity density in RVH rats in all PVN subnuclei analyzed. 3V, third ventricle. **P* < 0.05 vs. sham, *n* = 6 in each group. Scale bars = 50 μ m in A (applies to A,B); 10 μ m in insets.

below, significant changes in the densities and distributions of these inputs were observed in neurons located in the most caudal aspects of the PVN. Thus, the remainder of our work is focused on OT-PVN-RVLM neurons located in the PaPo subnucleus. A detailed analysis of the distribution of GAD₆₇- and VGLUT₂-ir boutons apposing somatic and proximal dendritic compartments of these neurons in sham and two developmental stages of RVH [i.e., early (3 weeks) and late (6 weeks)] is summarized below. Because no differences in any of the parameters evaluated were observed between the sham

Figure 4. VGLUT₂ immunoreactivity in the PVN of normotensive sham and RVH rats. **A:** **P* < 0.05 vs. sham, *n* = 6 in each group. Representative confocal photomicrographs obtained at the medial level of PVN (1.8–2.1 mm caudal to bregma) in sham (A1) and RVH (A2) rats, showing retrogradely labeled PVN-RVLM neurons (red) and VGLUT₂ immunoreactivity (white). For better comparisons, representative regions of A1 and A2 are displayed at higher magnification in A3 and A4, respectively. **B:** Representative confocal photomicrographs obtained at the caudal level of PVN (2.1–2.3 mm caudal to bregma) in sham (B1) and RVH (B2) rats, showing retrogradely labeled PVN-RVLM neurons (red) and VGLUT₂ immunoreactivity (white). For better comparisons, representative regions of B1 and B2 are displayed at higher magnification in B3 and B4, respectively. **C:** Summary showing a significant increase in the relative VGLUT₂ immunoreactivity density in the PaPo subnucleus. 3V, third ventricle. **P* < 0.05 vs. sham, *n* = 6 in each group. Scale bars = 50 μ m in A (applies to A,B); 10 μ m in insets.

groups at the two time periods (not shown), data from these control groups were pooled together for further analysis.

Changes in the density of GAD₆₇-ir boutons overlapping OT-PVN-RVLM neurons in the PaPo subnucleus

The surface area of reconstructed neurons was calculated, and results are summarized in Table 1. No differences in overall neuronal surface area were observed

Figure 5. Three-dimensional rendered images of OT-positive, retrogradely labeled PVN-RVLM neurons and associated GAD₆₇- and VGLUT₂-ir boutons. **A:** Representative example of an OT-ir (A1, green), PVN-RVLM retrogradely labeled (rhodamine beads, white, A2) neuron located in the PaPo subnucleus. In A3, the two images were superimposed. **B:** Example of a PVN-RVLM retrogradely labeled, OT-positive neuron located in the PaPo subnucleus and associated GAD₆₇-ir boutons, shown in two different spatial orientations. For better display, only the OT staining (green) is shown. Boutons in red and white colors represent those overlapping and those that are close to, but not overlapping, the OT-positive PVN-RVLM neuron, respectively. **C:** Example of a different PVN-RVLM retrogradely labeled, OT-positive neuron located in the PaPo subnucleus and associated VGLUT₂-ir boutons, shown in two different spatial orientations. For better display, only the OT staining is shown. Boutons in red and white colors represent those overlapping and those that are close to, but not overlapping, the OT-positive PVN-RVLM neuron, respectively. **Insets** show representative overlapping GAD₆₇ and VGLUT₂ boutons, respectively (white boxes), at a higher magnification. A magenta-green copy of this figure is available as Supporting Information Figure 3. Scale bars = 10 μ m in A; 10 μ m in B (applies to B,C); 1.5 μ m in insets.

among groups ($F = 0.4$, $P > 0.1$, one-way ANOVA). Similarly, no differences in somatic or proximal dendritic surface areas were observed among groups ($F = 1.7$ and 0.6 , respectively, $P > 0.2$, one-way ANOVA). Finally, no correlations between neuronal surface area and density of GAD₆₇- or VGLUT₂-ir boutons were observed ($R^2 = 0.13$ and 0.33 , respectively).

The overall density of GAD₆₇-ir boutons overlapping OT-PVN-RVLM neurons increased significantly in neurons from RVH compared with those of sham rats ($F = 19.1$, respectively, $P < 0.0001$, one-way ANOVA) and were observed both at early and late stages of hyper-

TABLE 1.

Whole-Cell and Somatic and Proximal Dendritic Surface Area of PVN-RVLM Neurons in Sham and Early-Stage and Late-Stage RVH Rats¹

	Sham (n = 37; μ m ²)	Early-stage RV (n = 13; μ m ²)	Late-stage RV (n = 26; μ m ²)
Whole cell	7,733 $\pm$ 546.9	7,624 $\pm$ 528.8	6,983 $\pm$ 724.4
Soma	5,819 $\pm$ 497.5	6,964 $\pm$ 626.3	4,797 $\pm$ 630.8
Dendrites	1,652 $\pm$ 182.8	1,270 $\pm$ 286.3	2,024 $\pm$ 496.5

¹Values are means $\pm$ SE for n neurons. No significant differences were observed among groups ($F = 0.4$, $P > 0.1$, one-way ANOVA), and no differences in somatic or proximal dendritic surface were observed ($F = 1.7$ and 0.6 , respectively, $P > 0.2$, one-way ANOVA).

tension ($P < 0.0001$ in both cases, Bonferroni post hoc test; Fig. 6A).

Differential changes in somatic and proximal dendritic compartments were observed. In OT-PVN-RVLM cell bodies, the density of GAD₆₇-ir boutons increased significantly in RVH rats ($F = 18.5$, $P < 0.0001$, one-way ANOVA), both at early and at late stages (see Fig. 6B). On the other hand, no significant differences were observed in the density of GAD₆₇ immunoreactivity in proximal dendrites ($F = 3.1$, $P > 0.05$, one-way ANOVA).

To examine further the differences and changes in GAD₆₇ innervation between cell bodies and proximal dendrites, the relative density of GAD₆₇ immunoreactivity between these two neuronal compartments was calculated in each group. In all groups, GAD₆₇-ir bouton density was more prominent in proximal dendrites vs. cell bodies (i.e., dendritic/somatic ratio > 1 ; see Fig. 8A). Even though no significant differences were observed among groups ($F = 2.3$, $P > 0.1$, one-way ANOVA), a tendency for a diminished dendritic/somatic ratio was observed in RVH rats.

Changes in the density of VGLUT₂-ir boutons overlapping OT-PVN-RVLM neurons in the PaPo subnucleus

The overall density of VGLUT₂-ir boutons overlapping OT-PVN-RVLM neurons increased significantly in neurons from RVH rats compared with those from sham rats ($F = 9.7$, $P < 0.0001$, one-way ANOVA), and changes were observed both at early and at late RVH stages ($P < 0.1$, Bonferroni post hoc test; see Fig. 7A).

Similarly to GAD₆₇-ir boutons, somatic and dendritic compartments were differentially affected. The density of VGLUT₂-immunoreactive boutons in OT-PVN-RVLM cell bodies increased significantly in RVH rats ($F = 16.7$, $P < 0.0001$, one-way ANOVA), both at early and at late stages (see Fig. 7B). In contrast to GAD₆₇ immunoreactivity, VGLUT₂-ir bouton dendritic density also increased signifi-

Figure 6. Summary data showing differences in GAD₆₇-ir bouton densities in OT-positive, retrogradely labeled PVN-RVLM neurons in the PaPo subnucleus from sham rats ($n = 18$ neurons) and early-stage (e-RVH, $n = 6$ neurons) and late-stage (I-RVH, $n = 13$ neurons) hypertensive rats. Differences were quantified at the whole-cell level (A1) and somatic (A2) and proximal dendritic (A3) compartments. * $P < 0.05$, ** $P < 0.0001$, Bonferroni post hoc test.

cantly in RVH rats ($F = 4.5$, $P < 0.05$, one-way ANOVA), both at early and at late stages (see Fig. 7C).

As in the case of GAD₆₇ immunoreactivity, VGLUT₂-ir bouton density in PVN-RVLM neurons was more prominent

Figure 7. Summary data showing differences in VGLUT₂-ir bouton densities in OT-positive, retrogradely labeled PVN-RVLM neurons in the PaPo subnucleus from sham rats ($n = 19$ neurons) and early-stage (e-RVH, $n = 7$ neurons) and late-stage (I-RVH, $n = 13$ neurons) hypertensive rats. Differences were quantified at the whole-cell level (A1) and somatic (A2) and proximal dendritic (A3) compartments. * $P < 0.05$, ** $P < 0.0001$, Bonferroni post hoc test.

in proximal dendrites over cell bodies (i.e., dendritic/somatic ratio > 1 ; Fig. 8B), and no significant differences were observed among groups ($F = 0.3$, $P > 0.5$, one-way ANOVA).

Figure 8. Summary data showing the proximal dendritic/somatic relative GAD₆₇ (A) and VGLUT₂ (B) immunoreactivity densities in OT-positive, retrogradely labeled PVN-RVLM neurons in the PaPo subnucleus from sham ($n = 18$ and 19 for GAD₆₇ and VGLUT₂, respectively) and early-stage (e-RVH, $n = 6$ and 7 for GAD₆₇ and VGLUT₂, respectively) and late-stage (I-RVH, $n = 13$ for both GAD₆₇ and VGLUT₂) hypertensive rats. Note that, in all groups, GAD₆₇- and VGLUT₂-ir bouton densities were more prominent in proximal dendrites vs. cell bodies (i.e., proximal dendritic/somatic ratio > 1), although a tendency for a diminished dendritic/somatic GAD₆₇ ratio was observed in RVH rats.

Shift in the balance of GAD₆₇/VGLUT₂ innervation of proximal dendrites in OT-PVN-RVLM neurons in the PaPo subnucleus in hypertensive rats

An imbalance in glutamatergic/GABAergic functions has been shown to contribute to numerous neurological disorders (Cline, 2005). Therefore, we also sought to determine whether the balance of VGLUT₂/GAD₆₇ inputs at somatic and proximal dendritic compartments was changed in RVH rats. For these comparisons, we replotted the data presented in Figures 6 and 7 into Figure 9, to compare better the relative predominance of these two inputs in each experimental group. The density of VGLUT₂ immunoreactivity in OT-PVN-RVLM somata from sham rats was more prominent than that of GAD₆₇ immunoreactivity ($P < 0.01$, mean VGLUT₂/mean GAD₆₇ immunoreactivity

Figure 9. Summary data comparing VGLUT₂- and GAD₆₇-ir boutons in somatic (A) and proximal dendritic (B) compartments in OT-positive, retrogradely labeled PVN-RVLM neurons in the PaPo subnucleus from sham ($n = 18$ and 19 for GAD₆₇ and VGLUT₂, respectively) and early-stage (e-RVH, $n = 6$ and 7 for GAD₆₇ and VGLUT₂, respectively) and late-stage (I-RVH, $n = 13$ for both GAD₆₇ and VGLUT₂) hypertensive rats. * $P < 0.05$, ** $P < 0.01$ compared with GAD₆₇ within the same experimental group, Bonferroni post hoc test.

ratio = 1.91). On the other hand, similar densities of these two types of inputs were observed in proximal dendrites ($P > 0.5$). A similar pattern was observed in OT-PVN-RVLM neurons from early-stage RVH rats: the density of VGLUT₂ immunoreactivity in cell bodies was significantly more prominent than the density of GAD₆₇ immunoreactivity ($P < 0.05$, mean VGLUT₂/mean GAD₆₇ immunoreactivity ratio = 1.85), whereas no differences were observed in proximal dendrites ($P > 0.5$). Finally, in OT-PVN-RVLM neurons of late-stage RVH rats, the somatic predominance of VGLUT₂ over GAD₆₇ was no longer present ($P > 0.5$), whereas the density of VGLUT₂ immunoreactivity became more prominent than GAD₆₇ immunoreactivity in proximal dendritic compartments ($P < 0.05$, mean VGLUT₂/mean GAD₆₇ ratio = 1.90).

DISCUSSION

The PVN-RVLM pathway is a major neuronal substrate underlying the hypothalamic control of autonomic outflow

to the cardiovascular system (Allen, 2002; Ciriello et al., 1985; Coote et al., 1998; Dampney et al., 1987; Hardy, 2001; Kubo et al., 2000; Tagawa and Dampney, 1999; Yang et al., 2001; Yang and Coote, 1998). Although PVN neurons giving rise to this pathway (as well as other medullary targets) are neurochemically heterogeneous, both anatomical and functional studies support OT as the predominant neuropeptide expressed in PVN neurons innervating the ventrolateral medulla (Gomez et al., 1993; Hancock and Nicholas, 1987; Lang et al., 1983; Mack et al., 2002; Nilaver et al., 1980; Sawchenko and Swanson, 1982; Stocker et al., 2006). In this study, we found that ~25% of the retrogradely labeled PVN-RVLM neurons expressed OT. Given recent studies supporting the involvement of PVN-RVLM neurons in elevated sympathoexcitation in hypertensive conditions (Allen, 2002), our goal was to determine whether a reorganization of afferent GABAergic and glutamatergic synaptic inputs in OT-PVN-RVLM neurons constitutes a potential mechanism contributing to the imbalance of inhibitory/excitatory functions known to occur in hypertensive conditions, including renovascular hypertension (Allen, 2002; Bergamaschi et al., 1995; Haywood et al., 2001; Horn et al., 1998; Ito et al., 2000; Li and Pan, 2006, 2007a,b; Li et al., 2008; Martin and Haywood, 1998; Sonner et al., 2008). The main results of this study can be summarized as follows: 1) GABAergic and glutamatergic innervation densities increased in RVH rats in various PVN subnuclei, most prominently in caudal aspects of the PVN; 2) changes in excitatory and inhibitory afferent input densities differentially affected neuronal somatic and proximal dendritic compartments of identified OT-positive PVN-RVLM neurons located in caudal aspects of the PVN, whereas an increase in glutamatergic innervation density was observed both in somata and in proximal dendrites, and GABAergic afferent inputs were selectively increased in somatic compartments; and 3) remodeling of GABAergic and glutamatergic afferent inputs was observed both at early and at late hypertensive stages.

Remodeling of GABAergic and glutamatergic afferent inputs during hypertension

In this study, we used two complementary approaches to determine whether changes in the distribution and density of GABAergic and glutamatergic inputs occurred in the PVN of hypertensive rats. First, GAD₆₇ and VGLUT₂ immunoreactivities were quantified within specific PVN subnuclei at two rostrocaudal levels of the PVN. Our results support an overall increase of both GABAergic and glutamatergic immunoreactivities in the PVN of hypertensive rats. A more detailed analysis, however, indicated that, whereas VGLUT₂ immunoreactivity was significantly increased only in the most caudal aspects of the PVN (PaPo subnucleus), GAD₆₇ immunoreactivity increased

both at the medial and at the caudal PVN levels. Taken together, these results could then suggest an increased predominance of inhibitory inputs in the medial aspects of the PVN, whereas a balanced excitatory/inhibitory innervation state is maintained in the caudal regions of the PVN of hypertensive rats. Care should be taken, however, when drawing such conclusions, given the high degree of cellular heterogeneity within the PVN. Thus, to circumvent this potential problem, we performed a quantitative analysis of GAD₆₇- and VGLUT₂-ir boutons in close apposition with somata and proximal dendrites of identified OT-PVN-RVLM projecting neurons at two different stages during the development of the hypertensive condition. Our results show that, despite concomitant increases in both GABAergic and glutamatergic inputs in OT-PVN-RVLM neurons located in the PaPo, their differential and compartment-specific reorganization resulted in a time-dependent, altered balance of excitatory/inhibitory inputs. Thus, in sham and early-stage RVH rats, a predominance of glutamatergic over GABAergic inputs was found in OT-PVN-RVLM somata, whereas equal densities were observed in proximal dendrites. Conversely, at late hypertensive stages, the glutamatergic innervation predominance shifted from the somata to proximal dendrites. Similarly, concomitant changes in both excitatory and inhibitory synapses, as well as compartment-specific changes in the balance between excitatory and inhibitory inputs, were previously described in cortical and hippocampal neurons (Cossart et al., 2001; Knott et al., 2002). Interestingly, despite the overall increase in GAD₆₇ immunoreactivity found in PVN subnuclei located in medial regions of the PVN, we found no changes in the density of GAD₆₇-ir (and VGLUT₂-ir) boutons in close apposition with OT-PVN-RVLM neurons in the VM and DC subnuclei in RVH rats. Whether other PVN neuronal populations located in these subnuclei reflected changes in GAD₆₇ apposing boutons remains to be determined. The significance of the subnucleus-dependent reorganization of afferent inputs to OT-PVN-RVLM neurons during hypertension is at present unknown. Nonetheless, our results underscore the importance of performing such studies within specifically identified neuronal populations, particularly in a highly heterogeneous nucleus such as the PVN.

Multiple mechanisms could account for changes in synaptic function and/or efficacy, including altered postsynaptic (e.g., changes in numbers and/or types of postsynaptic receptors) and presynaptic (e.g., changes in neurotransmitter release probability) mechanisms, as well as changes in the number and/or distribution of synaptic contacts (Conti et al., 1994; Mody et al., 1994). Results from the present study, along with those from previous studies, support the view that multiple mechanisms acting in concert, including changes in neurotransmitter release

probability, postsynaptic receptor densities (Li and Pan, 2006, 2007b), and structural remodeling of afferent inputs (this study), contribute to altered PVN inhibitory GABAergic and excitatory glutamatergic synaptic functions in hypertensive disorders (Allen, 2002; Haywood et al., 2001; Horn et al., 1998; Li and Pan, 2006, 2007a,b; Li et al., 2006, 2008a,b). Thus, in addition to contributing to network adaptation and optimization in response to physiological challenges such as lactation (Hatton, 1997; Theodosis and Poulain, 2001) and changes in circulating levels of estrogen, leptin, and ghrelin (Neal-Perry et al., 2008; Pinto et al., 2004) synaptic remodeling in hypothalamic circuits could also constitute an underlying pathophysiological mechanism, as previously described for other regions of the CNS (Dudek and Staley, 2007; Rubenstein and Merzenich, 2003; Wassef et al., 2003).

Potential mechanisms underlying afferent input remodeling during hypertension

A dynamic reorganization of afferent inputs in the PVN during hypertension could be mediated by a variety of potential underlying mechanisms. A common mechanism that operates within hypothalamic networks in response to physiological challenges involves the active removal of neuronal-glial wrapping processes, allowing new synaptic contacts to be formed on neuronal surfaces free of astrocytic coverage (Tweedle and Hatton, 1984). A well-established example is the activity-dependent removal of astrocytic coverage of OT magnocellular neurosecretory neurons in the supraoptic nucleus of lactating rats, resulting in increased GABAergic, glutamatergic, and noradrenergic innervation densities (Hatton, 2004; Theodosis et al., 2006). A similar relationship between concomitant changes in the degree of astrocytic coverage and density of synaptic inputs has been described in the arcuate nucleus during fluctuations in sex steroid levels (Garcia-Segura et al., 1994a,b). Although astrocytic structural and functional changes have been reported in different animal models of hypertension (Ishida et al., 2006; Pietranera et al., 2006; Tomassoni et al., 2004; Yamagata et al., 2006, 2008), a mechanistic relationship between synaptic and glial remodeling in the PVN of hypertensive rats remains to be established.

Activation of angiotensin II (AngII) receptors in brain astrocytes was shown to induce changes in intracellular Ca^{2+} levels (Gebke et al., 1998; Wang et al., 1996) and to regulate astrocyte growth and proliferation (Clark and Gonzalez, 2007; Clark et al., 2008). Thus, given the well-established role of AngII in the pathophysiology of renovascular hypertension and sympathoexcitation (Fink, 1997), future studies are warranted to evaluate thoroughly the role of AngII-mediated mechanisms on the altered

excitatory/inhibitory afferent balance described in these studies.

Functional implications of excitatory/inhibitory afferent remodeling during hypertension

Neuronal firing activity is strongly influenced by an interplay between excitatory and inhibitory inputs, and their balanced organization and activity are necessary for proper neuronal network functionality. The functional consequences of the excitatory/inhibitory afferent remodeling reported here in OT-PVN-RVLM neurons during hypertension are at present unknown. Nonetheless, there are some important aspects that should be discussed.

First, an increased density of both excitatory and inhibitory terminals in PVN-RVLM neurons during hypertension could be a mechanism to compensate for an increased neuronal size, as previously observed in hypothalamic magnocellular neurosecretory neurons during lactation (El Majdoubi et al., 1997; Theodosis and Poulain, 2001). The lack of significant changes in PVN-RVLM neuronal surface area during hypertension and the lack of correlation between densities of overlapping boutons and neuronal surface area, however, argue against this possibility.

Second, somatic and dendritic synaptic inputs serve different functional roles. Whereas somatic-projecting inputs influence the generation of Na^+ spikes and thus neuronal output, dendritic-projecting inputs, in particular the balance of excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs, respectively), influence the availability and activation of dendritic low-threshold voltage-gated Ca^{2+} currents and somatodendritic propagation of Ca^{2+} and Na^+ spikes (Egger et al., 2005; Llinas and Nicholson, 1971; Magee and Johnston, 1995; Miles et al., 1996; Spruston et al., 1995). We have recently shown that low-threshold Ca^{2+} channels, located predominantly in dendritic compartments, play a major role in controlling PVN-RVLM membrane excitability (Li et al., 2008; Sonner and Stern, 2007; Stern, 2001), in part by influencing somatodendritic propagation of Ca^{2+} signals, which we found to be exacerbated in RVH rats (Sonner et al., 2007, 2008). Thus, it is possible that a shift in the innervation pattern observed in hypertensive rats, leading to a net predominance of excitatory over inhibitory inputs in proximal dendrites of PVN-RVLM neurons, could facilitate the generation and propagation of larger dendritic EPSPs and consequently a more efficient activation of dendritic low-threshold Ca^{2+} channels.

Finally, it is also important to consider that even a balanced, concomitant increase in excitatory and inhibitory synaptic inputs could result in altered postsynaptic neuronal function. This has been shown in magnocellular neurosecretory neurons, in which a balanced increase in excita-

tory and inhibitory inputs resulted in an elevated firing rate and linearization of their input/output function (Leng et al., 2001). This could be due in part to an overall increase in background synaptic noise, recently shown to strongly influence the firing discharge probability and firing pattern in these neurons (Li et al., 2007).

We acknowledge that, despite the fact that several of the hypertensive-related changes in PVN GAD₆₇ and VGLUT₂ immunoreactivities reported here were observed at early stages of this condition, our experimental approach precluded us from determining whether the structural remodeling of GABAergic and glutamatergic inputs is causative or reactive to the elevated blood pressure in this animal model. Future studies addressing the time course relationship between the development of hypertension and the immunoreactive changes reported herein are warranted to address this issue precisely, as well as the functional consequences of the afferent synaptic remodeling in PVN neurons of hypertensive rats.

Methodological considerations and limitations

To identify PVN-RVLM neurones, rhodamine-labeled fluorescent latex microspheres were injected into the RVLM. This retrograde tracer results in highly restricted and well-defined injection sites (Katz et al., 1984) and is commonly used to trace CNS pathways, including PVN projections to brainstem nuclei (Cato and Toney, 2005; Cham et al., 2006; Li and Pan, 2005, 2006; Zahner et al., 2007). Although this tracer is not taken up by fibers in passage (Katz et al., 1984; Katz and Iarovici, 1990; Krug et al., 1998; Sasek and Helke, 1989), we cannot rule out potential labeling of severed axons in the area of the injection. Similarly, whereas for the most part the tracer was localized within the RVLM, we cannot rule out potential leakage of tracer into the caudal ventrolateral medulla (CVLM) or the nucleus ambiguus. This is, however, unlikely, in part because injections that were misplaced rostrally, caudally, or laterally to the RVLM [areas containing PVN descending axons running toward the dorsal brainstem or the spinal cord (Luiten et al., 1985)] failed to label neurons in the PVN retrogradely.

Confocal microscopy and 3D reconstructions were used to quantify immunoreactive boutons overlapping target-specific, sympathetic-related PVN neurons. We have recently used a similar approach to assess exercise-induced reorganization of (nor)adrenergic afferent inputs in the PVN (Higa-Taniguchi et al., 2007). Moreover, a similar approach was recently validated as an efficient tool to assess the plasticity of neurotransmitter innervation in identified neurons after prolonged stimulation (Mueller et al., 2005). Thus, although our approach likely reflects reorganization of putative synaptic contacts in PVN-RVLM neurons, elec-

tron microscopy studies will be needed to verify more conclusively the synaptic nature of the overlapping boutons quantified in our studies.

Another caveat of our studies is that labeled dendrites could be followed only within the thickness of a given section and, in many instances, were obscured by labeled neighboring neurons or dendrites. As a result, quantification was limited to somata and proximal dendrites (see Materials and Methods). Thus, whether changes similar to those reported in proximal dendrites also occur in more distal dendritic segments is at present unknown.

Finally, based on the evidence supporting a major contribution of the PVN-RVLM pathway to enhanced sympathoexcitation during hypertension (Allen, 2002) and OT, one of the most predominant neurochemical phenotypes of this neuronal population, our studies were focused on OT-PVN-RVLM neurons. It is well-established, however, that other neurochemically and target diverse descending pathways are involved in mediating PVN influences on autonomic and cardiovascular control. This includes a direct PVN projection to preganglionic sympathetic neurons in the spinal cord. In fact, a small proportion of presympathetic PVN neurons has been reported to project to both the RVLM and the spinal cord (Pyner and Coote, 2000; Shafton et al., 1998), a subpopulation that could have been included within the sampled OT-PVN-RVLM group in our study. Whether the changes reported in our studies are cell specific or, conversely, whether they affect other PVN neuronal populations (e.g., PVN-dorsal vagal complex projecting, magnocellular neuroendocrine) remains to be determined.

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