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Water deprivation activates a glutamatergic projection from the hypothalamic paraventricular nucleus to the rostral ventrolateral medulla

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Abstract

Elevated sympathetic outflow contributes to the maintenance of blood pressure in water-deprived rats. The neural circuitry underlying this response may involve activation of a pathway from the hypothalamic paraventricular nucleus (PVH) to the rostral ventrolateral medulla (RVLM). We sought to determine whether the PVH-RVLM projection activated by water deprivation is glutamatergic and/or contains vasopressin- or oxytocin-neurophysins. Vesicular glutamate transporter2 (VGLUT2) mRNA was detected by *in situ* hybridization in the majority of PVH neurons retrogradely labeled from the ipsilateral RVLM with cholera-toxin subunit B (CTB; 85% on average with regional differences). Very few RVLM-projecting PVH neurons were immunoreactive for oxytocin- or vasopressin-associated neurophysin. Injection of biotinylated dextran amine (BDA) into the PVH produced clusters of BDA-positive nerve terminals within the ipsilateral RVLM that were immunoreactive (ir) for the VGLUT2 protein. Some of these terminals made close appositions with tyrosine-hydroxylase-ir dendrites (presumptive C1 cells). In water-deprived rats (n=4), numerous VGLUT2 mRNA-positive PVH neurons retrogradely labeled from the ipsilateral RVLM with CTB were c-Fos-ir (16–40% depending on PVH region). In marked contrast, few glutamatergic, RVLM-projecting PVH neurons were c-Fos-ir in control rats (n=3; 0–3% depending on PVH region). Most (94 ± 4%) RVLM-projecting PVH neurons activated by water deprivation contained VGLUT2 mRNA. In summary, the majority of PVH neurons that innervate the RVLM are glutamatergic and this population includes the neurons that are activated by water deprivation. One mechanism by which water deprivation may increase the sympathetic outflow is the activation of a glutamatergic pathway from the PVH to the RVLM.

Keywords

VGLUT2; c-Fos; autonomic; tyrosine hydroxylase; oxytocin; vasopressin

INTRODUCTION

Water deprivation modifies ingestive behavior, the activity of hypothalamic neuroendocrine systems, and sympathetic outflow (Thrasher et al., 1987; Kiss et al., 1994; Scroggin et al.,

1999; Stricker et al., 2002; Stocker et al., 2004b; 2005). Activation of the sympathoadrenal system by water deprivation contributes to the maintenance of arterial blood pressure and is reflected by increased levels of circulating catecholamines (Thornton and Proppe, 1988; Kiss et al., 1994), elevated heart rate (Scroggin et al., 1999; Scroggin et al., 2002), increased lumbar sympathetic nerve activity (Scroggin et al., 1999), and an exaggerated depressor response to ganglionic blockade (Stocker et al., 2004b; 2005). The elevated sympathetic tone caused by water deprivation depends upon neurons located within the hypothalamic paraventricular nucleus (PVH) (Stocker et al., 2004a, b; 2005). For example, bilateral inhibition of the PVH with microinjection of the GABA_A receptor agonist muscimol decreases renal and lumbar sympathetic nerve activity and arterial blood pressure in 48-h water-deprived rats (Stocker et al., 2004b; 2005). While the PVH can theoretically increase sympathetic outflow through mono- or poly-synaptic pathways to sympathetic preganglionic neurons, a pathway through the rostral ventrolateral medulla (RVLM) is likely to play a central role. This region of the medulla oblongata contains the largest collection of bulbospinal, blood-pressure regulating sympathoexcitatory neurons (Schreihofer and Guyenet, 1997; Guyenet et al., 1998; Schreihofer and Guyenet, 2000; Stornetta et al., 2002b), and water deprivation increases c-Fos immunoreactivity in both these neurons and in the PVH neurons that innervate the RVLM (Stocker et al., 2004a; 2005).

The neurotransmitters used by the PVH neurons that innervate the RVLM are not known precisely. Previous reports suggest that some parvocellular PVH neurons with descending projections to the hindbrain may be oxytocinergic or vasopressinergic (Sawchenko and Swanson, 1982; Hallbeck and Blomqvist, 1999; Hallbeck et al., 2001; Mack et al., 2002; Kc et al., 2002). In this regard, microinjection of vasopressin or oxytocin into the RVLM increases arterial blood pressure (Andreata-Van Leyen et al., 1990; Gomez et al., 1993; Mack et al., 2002; Kc et al., 2002) and vasopressin- and oxytocin-immunoreactive fibers have been found in close proximity to spinally-projecting RVLM neurons (Hancock and Nicholas, 1987; Gomez et al., 1993). Moreover, blockade of vasopressin-type 1 receptors attenuates PVH-evoked increases in RVLM unit discharge (Yang et al., 2001; Yang and Coote, 2003). Another likely possibility is glutamate since many recent reports indicate the presence of vesicular glutamate transporter2 (VGLUT2) mRNA in both the magnocellular and parvocellular regions of the PVH (Ziegler et al., 2002; Rosin et al., 2003; Lin et al., 2003; Hur and Zaborszky, 2005; Hrabovszky et al., 2005). In support of the glutamate hypothesis, iontophoretic application of the excitatory amino acid receptor antagonist kynurenic acid attenuates the increase in RVLM unit discharge to chemical and electrical stimulation of the PVH (Yang et al., 2001). In addition, bilateral microinjection of kynurenic acid into the RVLM of water-deprived rats significantly decreased arterial blood pressure (Brooks et al., 2004a). Altogether, these observations suggest that water deprivation may increase sympathetic outflow by recruiting a glutamatergic pathway from the PVH to the sympathoexcitatory neurons of the RVLM.

In the present study, we sought more definitive evidence that RVLM-projecting PVH neurons are glutamatergic and that water deprivation selectively activates this pathway. PVH neurons were retrogradely labeled from the ipsilateral RVLM with cholera toxin subunit B (CTB) and glutamatergic neurons were identified by *in situ* hybridization for VGLUT2 mRNA. We also examined whether these RVLM-projecting PVH neurons were oxytocinergic and/or vasopressinergic by using antibodies directed against the respective neuropeptides. In addition, the RVLM was examined using confocal microscopy for PVH axon terminals that were anterogradely labeled with biotinylated dextran amine (BDA) and immunoreactive for VGLUT2 protein. In a final set of experiments, we determined whether the RVLM-projecting PVH neurons that are activated by water deprivation express VGLUT2 mRNA. In these experiments, c-Fos expression was used as an index of neuronal activation.

MATERIALS AND METHODS

Animals

All experiments were performed in Adult male Sprague-Dawley rats (Charles River Laboratories) weighing 300–350 g housed in a temperature-controlled room (22–23°C) with a 14:10 light-dark cycle (lights on at 7 AM). Tap water and laboratory chow (Harlan Teklad LM-485, 0.3% NaCl) were available *ad libitum* except where noted. All experimental and surgical procedures conformed with the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

Surgery and injections

Cholera toxin subunit B (CTB) injections into the RVLM—PVH neurons with projections to the ipsilateral RVLM were identified by microinjecting the retrograde tracer CTB (0.25% in isotonic saline, 15–20 nl; List Biological Laboratories, Campbell, CA) into the RVLM as described previously (Stocker et al., 2005). Briefly, rats (n=7) were anesthetized with sodium pentobarbital (60 mg/kg, i.p), instrumented with a catheter in the femoral artery to record arterial blood pressure, and placed into a stereotaxic frame with the incisor bar positioned 11 mm below the interaural line. A small portion of the occipital bone was removed, and the area postrema was visualized. A glass micropipette was angled 20° rostrally and lowered into the RVLM at the following coordinates in reference to the dorsal surface and caudal tip of area postrema: 1.8 mm lateral, 1.6–2.0 mm rostral, and 2.8 mm ventral. Initially, the RVLM was located functionally by a pressor response (>30 mmHg) to microinjection of L-glutamate (2 nmol in 20 nl isotonic saline). Then, the pipette was removed from the brain, emptied, filled with CTB and returned to the same position. All microinjections were performed over 30 s with glass micropipettes (O.D. 20–40 µm) connected to a pneumatic picopump (Dagan), and micropipettes were left in place for a minimum of 5 min after injection of CTB. Once the overlying musculature and skin was sutured and the femoral arterial catheter was removed, each rat was given ampicillin (100 mg/kg, i.m) and returned to its home cage.

Biotinylated Dextran Amine (BDA) injections into the PVH—To identify terminal fields within the RVLM that originate from the ipsilateral PVH, an additional group of rats (n=7) received an iontophoretic injection of the anterograde marker BDA (MW 10,000; Molecular Probes, Eugene, OR) targeted at the PVH. Animals were anesthetized with 3% isoflurane (in 100% O₂) and placed into a stereotaxic head frame with the skull level between bregma and lambda. A small craniotomy was performed to remove bone overlying the cortex to allow a glass micropipette to be lowered into the PVH. BDA (10% dissolved in 0.1 M phosphate buffer, pH 7.4) was iontophoretically injected into the PVH using glass micropipettes (O.D. 20–30 µm) at the following coordinates in reference to bregma: 1.8 mm caudal, 0.5–0.7 mm lateral, and 7.7 mm ventral to the dorsal surface of the skull. The iontophoretic injection parameters were 5 second pulses, 50% duty cycle, 5 µA positive current, 10 minutes.

Water Deprivation and Perfusions

Approximately 2 wks after microinjection of CTB into the RVLM, rats were randomly assigned to one of two groups. One group was deprived of water but not food for 48 hr (n=4), whereas control rats (n=3) had continuous access to both food and water. Then, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p), and blood (0.4 ml) was collected from the left ventricle into microcentrifuge tubes containing heparin (10 units) by using a 23 gauge needle and 1 cc syringe. Samples were used for determination of hematocrit levels, P_{osmol}, and plasma protein levels as described previously (Stocker et al.,

2005). Immediately after the blood sample was collected, rats were perfused transcardially with 50 ml of 0.1 M phosphate-buffered saline (PBS) followed by 300 ml of 4% paraformaldehyde (4°C) dissolved in 0.1 M PBS. Brains were removed and post-fixed in 4% paraformaldehyde at 4°C for 1–3 days. The medulla and hypothalamus were cut at 30 µm using a vibrating microtome (Leica, Nüssloch, Germany). Sections from all experiments were collected into 6 serially adjacent sets and stored in cryoprotectant solution at –20° C for up to 2 weeks (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) awaiting histological processing. Brains used for the confocal analysis were cryoprotected by immersion in 30% sucrose for 48–72 hours at 4° C, then frozen on dry ice and sectioned on a cryostat (Leica) at 15 µm and stored at –20° C for up to 2 weeks in the cryoprotectant solution.

Histology

In situ hybridization—Sections were removed from cryoprotectant solution, rinsed in sterile saline and placed into pre-hybridization mixture at room temperature for 30 minutes, then at 37° C for 1 hour. Pre-hybridization mixture consisted of 0.6 M NaCl, 0.002 M EDTA, 0.05% NaPP_i, 0.5 mg/ml yeast total RNA, 0.05 mg/ml yeast tRNA, 1X Denhardt's BSA, 50% formamide, 10% dextran sulfate, 0.05 mg/ml oligo A, 10 mM of the four deoxynucleoside triphosphates, 0.5 mg/ml herring sperm DNA and 10 mM dithiothreitol in 0.1 M Tris-Cl pH 7.5.

Anti-sense riboprobes for VGLUT2 and GAD-67 were transcribed from a 3.3 kb VGLUT2 template generated from a VGLUT2 plasmid from our laboratory or from GAD-67 template from a plasmid provided by A. Tobin (UCLA) using SP6 polymerase or T7 polymerases in the presence of digoxigenin-11-UTP (Roche) as described previously (Stornetta et al., 2003). The riboprobes were column purified with ProbeQuant G-50 Micro Columns (Pharmacia Biotech, Piscataway, NJ). The amount of digoxigenin-11-UTP incorporation was estimated from a dot blot with a dilution series of riboprobe and control RNA (Roche). Riboprobes were used that were similar in spot density to the control RNA (~20–100 ng/µl). The riboprobes were added directly to the prehybridization solution at a concentration of 50–100 pg/µl; the sense probe was added to some sections at a matched concentration as a background control. After addition of the probe, the sections were placed at 55–60° C for 18 to 56 hours. Sections were then rinsed through decreasing concentrations of salt solutions, treated with a solution of RNase A at 37° C and also rinsed in the lowest salt concentration at 55° C for 30 minutes.

Immunocytochemical detection of digoxigenin, CTB, oxytocin- or vasopressin-associated neurophysins, and c-Fos in cell bodies of the PVH—

Sections were rinsed in 0.1 M Tris buffered saline pH 7.4 (TBS) and blocked for non-specific immunoreactivity by 30 minute incubation with 10% horse serum and 0.1% Triton-X-100 in TBS and then incubated with antibodies as per experimental design. All primary antibodies were incubated simultaneously. Sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000, Roche), rabbit anti-c-Fos antibody (1:1000, Calbiochem, San Diego, CA) and a goat anti-CTB antibody (1:2000, List Biological, Campbell, CA) were incubated with the tissue in the same blocking solution for 24–48 hours at 4° C. After rinsing in TBS, sections were incubated for 10 minutes in a solution of 0.1 M NaCl, 50 mM MgCl₂, and 0.1 M Tris, pH 9.5 (NMT). Detection of the digoxigenin-labeled VGLUT2 riboprobe was revealed by production of a blue/brown alkaline phosphatase reaction product following incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche) in NMT (2–3 hours in the dark, shaking at room temperature). Intermittent microscopic observation was used to determine optimal signal to noise (i.e. strong signal in subthalamic nucleus of thalamus, no signal in zona incerta). The reaction

was quenched with 3×10 -minute rinses in 0.1 M Tris/1 mM EDTA (pH 8.5). The sections were rinsed in TBS and incubated simultaneously in appropriate secondary antibodies for 45 minutes at room temperature. Secondary antibodies included Alexa 488 donkey anti-goat IgG to reveal the CTB (1:250, Molecular Probes, Eugene, OR) and Cy3 donkey anti-rabbit (1:250, Jackson, West Grove, PA) to reveal the c-Fos. Sections were further rinsed in TBS, then in 100 mM phosphate buffer, pH 7.4 and mounted onto slides. Sections were air dried and covered with VectaShield (Vector, Burlingame, CA), and coverslips were attached with nail polish.

For detection of oxytocin- (OT-NP) or vasopressin-associated (VP-NP) neurophysins, some sections were processed as described above except a mouse anti-OT-NP (PS38, 1:25) or mouse anti-VP-NP (PS41, 1:25) antibody was used along with the anti-CTB antibody. The neurophysin antibodies were generously provided by Dr. Hal Gainer (NIH).

Immunocytochemical detection of BDA, VGLUT2 and/or tyrosine hydroxylase (TH) in terminals and dendrites in brainstem sections—To determine whether terminal fields within the RVLM that originated from ipsilateral PVH neurons were positive for VGLUT2 and made close contacts with TH neurons in the RVLM (i.e. C1 cells), brainstems were cut horizontally at 15 μ m and processed for the simultaneous detection of BDA, VGLUT2 and TH (n=3). Sections were first blocked with 10% horse serum in TBS and 0.1% Triton X-100 for 30 minutes. Then, sections were incubated with mouse anti-TH antibody (1:2000; Chemicon, Temecula, CA) and guinea pig anti-VGLUT2 antibody (1:2500; Chemicon) for 18–24 hours at 4° C. Sections were rinsed in TBS and incubated for 45 minutes with Cy3-goat anti-guinea pig IgG (1:250, Jackson, West Grove, PA), Cy5 donkey anti-mouse IgG (1:250, Jackson) and streptavidin Alexa 488 (1:250, Molecular Probes). Sections were then rinsed, mounted on gelatin coated slides, dehydrated through a series of graded alcohols and xylene and covered with Krystalon (Harleco, EM Science) followed by a coverslip. Sections were examined and photographed with a Zeiss LM510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY.)

Histological processing of injection sites in RVLM and PVH—The brainstem from CTB-injected rats was cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 48–72 hours at 4°C, frozen on dry ice, and then cut at 15 μ m using a cryostat. Sections were rinsed in TBS, blocked in 10% horse serum and 0.1% Triton-X-100 in TBS and incubated in goat anti-CTB antibody (1:2000, List Biological) – 48 hours at 4° C. Then, these sections were incubated in Alexa 488 donkey anti-goat IgG (1:250, Molecular Probes) 45 minutes at room temperature, rinsed, mounted, dehydrated and covered as described above.

The hypothalamus from BDA-injected rats was sectioned at 30 μ m using a vibrating microtome and stored in cryoprotectant. Hypothalamic sections were incubated in mouse anti-OT-NP antibody (PS38, 1:25) overnight at 4 °C to provide anatomical landmarks of the PVH. Hypothalamic sections were then rinsed and further incubated for 45 minutes with Cy3 donkey anti-rabbit IgG (1:250, Jackson) and streptavidin Alexa 488 (1:250, Molecular Probes). Sections were rinsed, mounted, dehydrated and covered as described above.

Sections adjacent to the PVH injection sites were reacted with the AB solution from the ABC kit (Vector) according to the manufacturer's instructions, rinsed and reacted for 5–10 minutes with 0.05% 3,3'-diaminobenzidine and 0.005% hydrogen peroxide, rinsed and mounted. Sections adjacent to the RVLM injection sites were rinsed and mounted. Both sets of adjacent sections were counterstained with 0.25% thionin acetate, dehydrated through a graded series of alcohols and xylenes and covered with DPX mountant (Sigma).

Antibody characterizations

Rabbit anti-c-Fos antibody: (Calbiochem) was raised against a synthetic peptide (SGFNADYEASSRC) corresponding to amino acids 4–17 of human c-Fos and recognizes the ~55 kDa c-Fos protein on Western blots. The pattern of labeling in the current experiment match that expected with label restricted exclusively to the nucleus of cells with many more cells showing the label in experimental than in control animals.

Mouse anti-TH monoclonal antibody: (Chemicon) was raised against tyrosine hydroxylase purified from PC12 cells and recognizes an epitope on the outside of the regulatory N-terminus of tyrosine hydroxylase. As reported by Chemicon, this antibody recognizes a protein of approximately 59–63 kDa by Western blot and does not cross-react with dopamine-beta-hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase or phenyl ethanolamine-N-methyl transferase on Western Blots. The pattern of labeling produced in the current study is seen in cell soma and dendrites as well as putative terminals and was restricted to known catecholamine cell groups.

Goat anti-CTB antibody: (List Biological) was raised against purified cholera toxin B subunit (choleragenoid). In our laboratory, preabsorption of the antibody with 3 fold molar excess concentration of cholera toxin B subunit (choleragenoid) (List Biological) resulted in a lack of any labeling in tissue sections adjacent to sections where clear labeling was seen in cell soma and dendrites tested with the same antibody concentration with no preabsorption.

Sheep anti-digoxigenin antibody conjugated to peroxidase: (Roche) was raised against digoxigenin in sheep and purified Fab fragments from the immunized sheep's serum were conjugated with horseradish peroxidase. According to Roche, preabsorption of the antibody with digoxigenin resulted in a complete lack of immunostaining. In our hands, the labeling was restricted to cytoplasm surrounding the nucleus and extending a short way into primary dendrites, as expected for a label tagging mRNA.

Guinea pig anti-VGLUT2 antibody: (Chemicon) was raised against a synthetic peptide (AG 209, Chemicon) corresponding to a region of the VGLUT2 protein sequence (VQESAQDAYSYKDRDDYS) that does not overlap with the sequence of VGLUT1 or VGLUT3. In our laboratory, preabsorption of the antibody with 3 fold molar excess of the AG209 immunogen resulted in a complete lack of immunolabeling.

Mouse anti-OT-NP (PS38) antibody: (Dr. Hal Gainer) was raised against rat OT-NP and recognizes mature OT-NP and the respective prohormone (Ben-Barak et al., 1985). The specificity has been extensively characterized previously and does not cross react with a number of peptides found in the hypothalamus including VP-NP or the vasopressin peptide (Ben-Barak et al., 1985; Whitnall et al., 1985). In our laboratory, OT-NP-immunoreactivity (but not VP-NP) was absent in oxytocin-knockout mice generously provided by Dr. Linda Rinaman (University of Pittsburgh).

Mouse anti-VP-NP (PS41) antibody: (Dr. Hal Gainer) was raised against rat neurophysin and recognizes VP-NP; however, the antibody does not distinguish between the mature neurophysin versus the prohormone (Ben-Barak et al., 1985; Whitnall et al., 1985). The specificity has been extensively characterized elsewhere and this antibody does not cross react with OT-NP or the oxytocin peptide (Ben-Barak et al., 1985; Whitnall et al., 1985). Immunoreactivity with this antibody is absent in the homozygous Brattleboro rat which lacks the peptide vasopressin and VP-NP.

Mapping & Statistical Analysis—Sections were examined under bright field and epifluorescence using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Microimaging). Maps of 1) CTB-positive neurons in PVH containing one or more of the additional markers (VGLUT2 mRNA, c-Fos, OT-NP, and/or VP-NP immunoreactivity) and 2) injection sites in RVLM and PVH were constructed with the Neurolucida software (Micro Brightfield, Colchester, VT) utilizing a Ludl motor driven microscope stage and the Lucivid camera, as previously described (Stornetta et al., 2003). Only cell profiles that included a nucleus were counted. The superimposed Neurolucida files were exported into the Canvas drawing software (Version 9, Deneba Systems Inc., Miami, FL) for text labeling and final presentation. The neuroanatomical nomenclature is after Paxinos and Watson (Paxinos and Watson, 1986). Photographs were taken with a 12-bit color CCD camera (Regina 1300; resolution 1392 X 1042 pixels, Q Imaging, Burnaby, B.C., Canada) and the resulting TIFF files were imported into Adobe Photoshop (Version 7; Adobe Systems, Mountain View, CA). Output levels were adjusted to include all information-containing pixels. Balance and contrast was adjusted to reflect true rendering as much as possible. No other “photo-retouching” was done. Figures were assembled and labeled within the Photoshop software.

The PVH was sampled at two rostral-caudal levels. Level 2 was the middle third of the PVH and contained a lateral and prominent posterior magnocellular subnucleus. Level 3 was the most caudal and consisted of the medial parvocellular (MP) and lateral parvocellular (LP) divisions. Based on previous anatomical studies in our laboratory (Stocker et al., 2004a) and others (Pyner and Coote, 2000), these levels of the PVH contain the highest density of neurons with projections to the RVLM. Counts of labeled neurons at each level and subnucleus were performed in one section per animal. Counts and percentages of labeled cells were compared between regions of the PVH (Level 2, Level 3-LP, and Level 3-MP) using a one-way ANOVA followed by a Bonferroni t-test when significant F values were obtained (SigmaStat version 3). Plasma osmolality, plasma protein concentration, and hematocrit were compared between control and water-deprived rats by a t-test (Systat 10.2, Systat Software, Inc., Richmond, CA, USA).

RESULTS

VGLUT2 mRNA and VGLUT2 immunoreactivity are present in PVH neurons with axonal projections to the RVLM

As noted by previous authors (Ziegler et al., 2002; Rosin et al., 2003; Lin et al., 2003; Hur and Zaborszky, 2005; Hrabovszky et al., 2005), VGLUT2 mRNA was detectable by *in situ* hybridization in a large number of PVH neurons (Fig. 1A, B). As expected, VGLUT2 mRNA *in situ* hybridization reaction product was located exclusively within the cytoplasm of neurons (Fig. 1A). The distribution pattern of VGLUT2 mRNA and GAD67 mRNA detected with the same histological technique were complementary to each other in and around the PVH (Fig. 1C). That is, the PVH contained a large number of neurons positive for VGLUT2 mRNA but merely devoid of GAD67 mRNA expressing neurons with the exception of the lower half of the periventricular zone. These complementary labeling patterns were also readily apparent in the thalamus, the anterior nucleus of the hypothalamus, and the zona incerta (Fig. 1B, C).

A major goal of the present study was to determine whether PVH neurons with axonal projections to the ipsilateral RVLM are glutamatergic. PVH neurons were retrogradely labeled from the RVLM with CTB in 7 rats. Ten days later, sections were processed for simultaneous detection of CTB immunoreactivity and VGLUT2 mRNA to test for the coexistence of these markers in PVH neurons ipsilateral to the injection site. All the CTB injections (n = 7; Figs. 2A, B and 3A) were centered ventromedial to the compact division of nucleus ambiguus within 500 microns of the caudal end of the facial motor nucleus and

within 500 microns of the ventral medullary surface. This region of the medulla oblongata is known to contain the highest density of bulbospinal blood-pressure regulating sympathoexcitatory neurons many of which express tyrosine-hydroxylase (C1 cells) (Schreihöfer and Guyenet, 1997; Guyenet et al., 1998; Schreihöfer and Guyenet, 2000; Stornetta et al., 2002b). PVH contained numerous neurons positive for both CTB and VGLUT2 mRNA (Fig. 2C, D). CTB-immunoreactive neurons with or without VGLUT2 mRNA *in situ* hybridization reaction product were mapped in each of the 7 rats and their number was determined within selected subregions of the PVH. While the majority of CTB-labeled PVH neurons contained VGLUT2 mRNA *in situ* hybridization reaction product (representative example in Fig. 2D, 3B), there were regional differences in VGLUT2 mRNA expression. Most CTB-positive neurons contained VGLUT2 mRNA within the medial parvocellular PVH (MP) but neurons in the lateral parvocellular PVH (LP) contained a larger proportion of retrogradely labeled neurons apparently devoid of VGLUT2 mRNA. The dorsal parvocellular PVH (DP) may also have contained a higher proportion of CTB labeled neurons devoid of VGLUT2 mRNA (Fig. 3B) but this small structure was not reliably identified in our material and accurate counts could not be obtained. The main regional differences described above were reproducible across animals (Table 1). Overall 85% of all the CTB-labeled PVH neurons counted were positive for VGLUT2 mRNA but this percentage varied from 50% within the LP at level 3 to 94% within the MP at level 2.

The next experiment was designed to verify that PVH neurons make VGLUT2 protein and that the protein is appropriately exported to nerve terminals within the RVLM. The anterograde tracer BDA was successfully injected into the PVH in 3 of 7 rats. Success was defined by a deposit of adequate size (100–200 micron diameter) and an injection site that was entirely confined to the PVH. The second criterion was satisfied by showing that the BDA-immunoreactive injection sites were entirely surrounded by oxytocinergic neurons, a marker that objectively delineates the boundaries of the PVH (Fig. 4D). VGLUT2 immunoreactivity was undetectable in somata or dendrites. In RVLM and elsewhere, VGLUT2 immunoreactivity consisted exclusively of punctata reminiscent of synaptic terminals by shape and size (Fig. 4B, G). Examination of the RVLM by confocal microscopy revealed the presence of clusters of VGLUT2-immunoreactive nerve terminals labeled with BDA in each of the three rats (Fig. 4AC, F–H). Using tissue sections from the same three rats, we also examined whether the RVLM targets of PVH neurons include C1 neurons. This was done by searching for close appositions between TH-immunoreactive dendrites and terminals immunoreactive for both VGLUT2 and BDA. Several examples of such close appositions were found, one of which is shown in Fig. 4EH. Note that the BDA-immunoreactive fiber has three VGLUT2-immunoreactive varicosities that appear to be in the same focal plane (within 0.4 μ m) as the TH-immunoreactive dendrite.

We also determined whether some of the PVH neurons that project to the RVLM were oxytocinergic or vasopressinergic. Very few RVLM-projecting PVH (CTB-positive) neurons were immunoreactive for OT-NP (Level 2: 2 ± 1 cells per section; Level 3: 4 ± 2 ; total PVH: 6 ± 3 cells in three sections per rat; $n=5$ rats) though the sampled region contained many OT-NP-ir cells (75 ± 11 cells counted in three sections per rat). The diminutive number of OT-NP-ir neurons with demonstrable projections to RVLM is in stark contrast with the large number of VGLUT2 mRNA containing PVH neurons labeled with CTB in the same series of experiments (6 ± 3 vs. 121 ± 18). Moreover, we did not observe any RVLM-projecting PVH neurons immunoreactive for VP-NP even though the sampled region contained many such neurons (total of 49 ± 12 cells counted in three sections per rat; 5 rats).

Water deprivation activates VGLUT2 mRNA-expressing PVH neurons with axonal projections to the RVLM

Our previous studies have demonstrated that water deprivation increases c-Fos immunoreactivity in PVH neurons that project to RVLM (Stocker et al., 2004a; Stocker et al., 2005). A goal of the present study was to identify whether these neurons are glutamatergic. In agreement with previous reports (Stocker et al., 2004a; 2005), the PVH of water-deprived rats ($n = 4$) contained vastly more c-Fos-ir nuclei than the same structure in normally hydrated rats (Fig. 5A–C). To determine whether the PVH neurons activated by water deprivation include glutamatergic neurons that innervate RVLM, we searched the PVH for c-Fos and CTB positive neurons that contained VGLUT2 mRNA. As shown in Fig. 5D–F, the PVH of water-deprived rats contained large numbers of such triple-labeled neurons. The location of these triple-labeled neurons at two levels of the PVH in one rat is shown in Fig. 6 (stars). Forty percent of glutamatergic PVH neurons that project to RVLM (neurons positive for CTB and VGLUT2 mRNA) in the LP region (Level 3) were activated by water deprivation as judged by the presence of a c-Fos-immunoreactive nucleus (Fig. 6, Table 2). In other areas studied, the percentage was significantly lower (16% in Level 3 MP and 22% in Level 2, Table 2). In normally hydrated rats, a mere 1% of the CTB-labeled VGLUT2-positive PVH neurons contained detectable levels of c-Fos immunoreactivity (Table 2).

The vast majority of the RVLM-projecting PVH neurons activated by water deprivation (positive for both CTB and c-Fos) contained VGLUT2 mRNA *in situ* hybridization reaction product (Fig. 6). The actual percentage varied from 71 to 100% and was 94% for the PVH overall (Table 3). Only one OT-NP-ir neuron was found to be c-Fos-ir in the PVH of all 5 water-deprived rats examined and this cell was not CTB-ir. Given the lack of VP-NP-ir PVH neurons with RVLM projections, the effect of water deprivation on c-Fos expression in this neuronal subtype was not examined.

As previously reported (Stocker et al., 2004a; 2005), water deprivation significantly increased plasma osmolality (control: 294 ± 1 vs. water deprived 319 ± 2 , mOsm/kg H₂O, $P < 0.001$), plasma protein concentration (control: 6.3 ± 0.1 vs. water deprived 7.3 ± 0.1 g/dl, $P < 0.01$), and hematocrit (control: 41 ± 1 vs. water deprived 50 ± 1 %, $P < 0.01$).

DISCUSSION

PVH neuronal activation contributes to the changes in sympathetic tone associated with many physiological or pathophysiological conditions such as water deprivation, hypertension and heart failure (Martin and Haywood, 1998; Allen, 2002; Felder et al., 2003; Ito et al., 2003; Stocker et al., 2004a; 2005). The neural pathway from PVH to spinal sympathetic vasomotor efferents is likely to relay in the RVLM (Allen, 2002; Brooks et al., 2004a), a region of dense projection from the PVH which contains the bulk of the bulbospinal sympathoexcitatory blood-pressure regulating neurons (Schreihofer and Guyenet, 1997; Guyenet et al., 1998; Schreihofer and Guyenet, 2000; Stornetta et al., 2002b). However, little is known of the neurotransmitter(s) utilized by RVLM-projecting PVH neurons. The present study demonstrates that these neurons are almost all glutamatergic, including the subset that is activated by water deprivation. The study also provides evidence that these glutamatergic PVH neurons may monosynaptically innervate some of the sympathoexcitatory neurons of the RVLM.

Technical Considerations

Glutamate is the main excitatory neurotransmitter of the central nervous system. Three recently characterized genes have been shown to encode a vesicular glutamate transporter

that is highly selective for glutamate over other amino acids (VGLUT1, VGLUT2, and VGLUT3) (Ni et al., 1994; Bellocchio et al., 1998; Fremeau et al., 2001; Fujiyama et al., 2001; Hayashi et al., 2001). In the normal adult brain, the expression of VGLUT1 and VGLUT2 is mutually exclusive of markers of GABAergic or glycinergic neurons (GAD65 or GAD67 mRNA) (Ziegler et al., 2002; Stornetta et al., 2002a). VGLUT1 or VGLUT2 protein is located within terminals that form asymmetric synapses typical of classic ionotropic glutamatergic transmission (Fremeau et al., 2001; Herzog et al., 2001; Fujiyama et al., 2004; Hajszan et al., 2004). VGLUT1 is highly expressed in the neocortex, hippocampus and other cortical or limbic structures, whereas VGLUT2 is localized to the diencephalon, midbrain and brainstem (Hayashi et al., 1993; Aihara et al., 2000; Fremeau et al., 2001; Hisano et al., 2002; Ziegler et al., 2002; Stornetta et al., 2002a, b; Lin et al., 2003; Fujiyama et al., 2004). VGLUT2 is the predominant isoform found in the hypothalamus including the PVH (Ziegler et al., 2002; Lin et al., 2003). In summary, there is overwhelming evidence to date that the presence of VGLUT2 mRNA in cell bodies and that of the cognate protein in the terminals identifies CNS neurons that use glutamate as their ionotropic transmitter.

The immediate early gene *c-Fos* identifies neurons whose gene expression has been recently upregulated (Dragunow and Faull, 1989; Dampney et al., 2003). Strictly speaking, *c-Fos* expression indicates that a neuron was synaptically influenced by a stimulus and does not necessarily mean that its discharge was elevated by this stimulus although the latter interpretation is likely and commonly made (Luckman et al., 1994). The intensity of *c-Fos* expression depends on the stimulus strength, duration and time between the stimulus onset and tissue fixation. Water deprivation is a 48 hour-long perturbation that produces a gradual but not strictly synchronous increase in several stimuli susceptible to trigger *c-Fos*-expression in PVH neurons (plasma osmolality, intravascular volume, neurohumoral activation, etc). Given the relatively short half-life of the protein, it is likely that the presence of *c-Fos* protein in the present study reflects the activation of the gene by one or more of these stimuli during the last few hours before sacrifice. However, not all neurons express *c-Fos* (Dragunow and Faull, 1989) and the present findings may not have identified every RVLM-projecting PVH neuron affected by water deprivation.

Most RVLM-Projecting PVH Neurons are Glutamatergic

Anatomical considerations indicate that PVH neurons can influence sympathetic preganglionic neurons via either mono- or poly-synaptic pathways (Saper et al., 1976; Swanson and Kuypers, 1980; Strack et al., 1989; Shafton et al., 1999; Pyner and Coote, 2000; Cano et al., 2001; 2004; Stocker et al., 2004a). However, PVH is heterogeneous and controls many types of sympathetic efferents besides vasomotor. The projection from PVH to RVLM has a documented role in many conditions where the sympathetic vasomotor tone is up-regulated (Ito et al., 2003; Stocker et al., 2005). In the present study, we provide definitive evidence that the majority of RVLM-projecting PVH neurons are glutamatergic. First, approximately 85% of PVH neurons retrogradely-labeled from the ipsilateral RVLM expressed VGLUT2 mRNA. Second, PVH axon terminals in the ipsilateral RVLM contained the VGLUT2 protein. It is likely that these terminals targeted sympathetic-regulatory neurons within the RVLM as confocal microscopy revealed that these glutamatergic PVH terminals made close appositions to tyrosine hydroxylase-ir dendrites (presumptive C1 neurons). These rostrally-located C1 neurons project to the spinal cord and display electrophysiological properties characteristic of blood pressure-regulating sympathoexcitatory neurons (Schreihöfer and Guyenet, 1997; Guyenet et al., 1998; Schreihöfer and Guyenet, 2000; Stornetta et al., 2002b). The present evidence for monosynaptic input of PVH glutamatergic neurons onto sympathoexcitatory neurons relies on close appositions and is not quantitative, therefore we cannot assert that every PVH

neuron with an RVLM projection contacts sympathoexcitatory neurons monosynaptically, nor can we exclude PVH projections onto interneurons. Our interpretation that PVH contributes a direct glutamatergic input to sympathoexcitatory neurons is consistent with previous evidence that iontophoretic applications of the glutamate receptor antagonist kynurenic acid attenuates the increased firing of these RVLM neurons caused by PVH stimulation in rats (Yang et al., 2001). However, Tagawa and Dampney (1999) had previously reported that microinjection of kynurenic acid into the RVLM had no effect on the pressor and sympathoexcitatory responses to disinhibition of the PVH. The Yang et al. study (2001) provides some clue to these apparent discrepancies by noting that kynurenic acid attenuated the excitation of RVLM neurons only when specific PVH sites were activated whereas a vasopressin-1 (V1) receptor antagonist was a more effective blocker of the effect of PVH stimulation at other sites.

The PVH also contains a number of peptides including vasopressin and oxytocin (Sawchenko and Swanson, 1982; Lind et al., 1985; Hallbeck and Blomqvist, 1999; Hallbeck, 2000; Hallbeck et al., 2001). According to several studies, these peptidergic PVH neurons project to the spinal cord and/or to the dorsomedial medulla (Nilaver et al., 1980; Sawchenko and Swanson, 1982; Hallbeck and Blomqvist, 1999; Hallbeck et al., 2001) or the ventrolateral medulla (Hancock and Nicholas, 1987; Gomez et al., 1993; Mack et al., 2002). We found a few OT-NP-ir PVH neurons with projections to RVLM (2.2% of CTB-labeled neurons in PVH). This figure closely matches the percentage of oxytocin neurons projecting to the caudal ventrolateral medulla (3.3%) reported by Mack et al. (2002). Hancock and Nicholas (1987) also reported an oxytocinergic innervation of the ventrolateral medulla using immunohistochemical labeling of oxytocin fibers making close contacts with PNMT-ir neurons. However, we did not observe any RVLM-projecting neurons that were immunoreactive for VP-NP. This result is consistent with Gomez et al., (1993) who reported very few VP-ir neurons in PVH retrogradely labeled from RVLM injections of FluoroGold and observed that the VP-ir neurons were in a different compartment of PVH than the retrogradely labeled neurons. The predominance of OT-NP-ir neurons with RVLM projections in comparison to the lack of VP-NP-ir neurons projecting to RVLM is also in agreement with other evidence that oxytocin is overwhelmingly predominant over vasopressin in the brainstem (Nilaver et al., 1980; Lang et al., 1983). This finding is in contrast to Kc et al. (2002) who reported VP in 20% of neurons labeled in PVH from CTB injections in PreBötzing complex. This discrepancy could be due to a narrowly directed vasopressin projection specifically to the more caudally located PreBötzing complex or the difference could be explained by consideration of antibody specificity and sensitivity. Another caveat regarding the immunolabeling of the peptides is that some of the peptidergic neurons could be expressing their peptides in the cell bodies at a level undetectable by the antibody and thus we are underestimating the population of both VP-NP-ir and OT-NP-ir RVLM-projecting neurons.

The neurons activated by water deprivation as identified by c-Fos immunoreactivity did not contain oxytocin (1 c-Fos positive OT-NP-ir cell in 5 water-deprived rats). These observations suggest that RVLM-projecting PVH neurons do not use vasopressin and that a few may use oxytocin but that the latter are unlikely to be involved in the sympathetic changes elicited by water deprivation. These conclusions appear to contradict the above-mentioned study by Yang et al. (2001) but oxytocin, a weak V1a receptor agonist (Mouillac et al., 1995) rather than vasopressin could conceivably have been the endogenous ligand responsible for the V1-receptor antagonist-sensitive responses elicited by PVH stimulation in RVLM sympathoexcitatory neurons (Yang et al. 2001). Also, as mentioned above, our immunolabeling may underestimate the population of peptidergic PVH neurons projecting to the RVLM.

Another potential peptidergic neurotransmitter of RVLM-projecting PVH neurons is angiotensin II. Lind and colleagues (Lind et al., 1985) have reported that the PVH contains numerous angiotensin II-immunoreactive cell bodies and fibers. This notion is further supported by prior pharmacological evidence indicating that blockade of angiotensin II-type 1A receptors in the RVLM attenuated the pressor and sympathoexcitatory responses to disinhibition of the PVH (Tagawa and Dampney, 1999). However, it must be determined whether activation of angiotensin II-type 1A receptors results from a direct versus indirect pathway from the PVH to the RVLM or whether those RVLM-projecting PVH neurons utilize angiotensin II as a neurotransmitter. This latter possibility will likely remain unknown due to the lack of a specific marker for “angiotensinergic” neurons.

Water Deprivation Activates a Direct Glutamatergic Pathway from the PVH to the RVLM

Elevated sympathetic outflow contributes to the maintenance of arterial blood pressure in water-deprived rats (Kiss et al., 1994; Scrogin et al., 1999; Stocker et al., 2004b; 2005). The neural mechanisms underlying this response are unknown but are likely to depend on sympathetic-regulatory neurons within the PVH and RVLM. In this regard, water deprivation has been reported to increase c-Fos immunoreactivity in spinally-projecting neurons of the PVH and RVLM as well as RVLM-projecting PVH neurons (Stocker et al., 2004a; 2005). The present study confirms this latter finding. Moreover, bilateral inhibition of the PVN with microinjection of the GABA_A receptor agonist muscimol significantly decreases renal and lumbar sympathetic nerve activity and arterial blood pressure in water-deprived rats (Stocker et al., 2004b; 2005). Finally, Brooks and colleagues (Brooks et al., 2004a) reported that blockade of excitatory amino acid receptors within the RVLM decreases arterial blood pressure in water-deprived rats. Taken together, these observations raise the possibility that water deprivation activates a glutamatergic pathway from the PVH to the RVLM to increase sympathetic outflow. The present findings provide strong support for this model as virtually every RVLM-projecting PVH neuron that was c-Fos immunoreactive in water-deprived rats expressed VGLUT2 mRNA. However, the increased dependence of arterial blood pressure on excitatory amino acid receptor activation within the RVLM of water-deprived rats may not depend solely on the PVH and other nuclei may also contribute through an increased glutamatergic drive or withdrawal of inhibitory drive (e.g., GABA). Furthermore, the elevated sympathetic outflow in water-deprived rats may also rely on pathways independent of the RVLM, the most likely candidate being a direct pathway from PVH to sympathetic preganglionic neurons.

Water deprivation produces a number of changes in body fluid status including increased plasma osmolality, elevated circulating angiotensin II, and a decreased intravascular volume (Stocker et al., 2002; Brooks et al., 2004b; Stocker et al., 2005). Any one or a combination of these stimuli may contribute to the elevated sympathetic outflow and increased dependence on PVH or RVLM sympathetic-regulatory neurons. With regard to the PVH, each of the aforementioned signals has been reported to increase cell discharge of PVH neurons with descending projections to the hindbrain and/or spinal cord (Ferguson, 1988; Bains and Ferguson, 1995; Chen and Toney, 2000; Toney et al., 2003). Interestingly, Brooks and colleagues (2005) have shown an intracarotid infusion of a hypotonic solution decreased arterial blood pressure and lumbar sympathetic nerve activity in water-deprived rats but had no effect in control rats. Furthermore, intravenous infusion of 5% dextrose in water but not isotonic saline to normalize plasma osmolality and blood volume (versus blood volume only) attenuated the increased dependence of arterial blood pressure on excitatory amino acid receptor activation within the RVLM in water-deprived rats (Brooks et al., 2004b). Thus increased plasma osmolality in water-deprived rats is a major contributing factor to the activation of a glutamatergic pathway from PVH to the RVLM, at least under anesthesia. While the origin of this osmotically-driven circuit is not known, it seems reasonable that

neurons in the forebrain lamina terminalis are involved. First, the forebrain lamina terminalis including the organum vasculosum of the lamina terminalis and median preoptic nucleus densely innervate the PVH (Sawchenko and Swanson, 1983). Second, these forebrain lamina terminalis neurons are osmotically-responsive (Bourque et al., 1994; Stocker and Toney, in press), and lesion of the region attenuates many homeostatic responses to hyperosmolality (Bourque et al., 1994). The role of these forebrain structures in the increased sympathetic outflow in water-deprived rats is further supported by the observation that intracarotid infusion of hypotonic solution decreased arterial blood pressure in water-deprived rats (Brooks et al., 2005). Future studies are needed to elucidate the cellular mechanisms and neural circuitry by which hyperosmolality increases sympathetic outflow through the PVH.

In summary, the majority of PVH neurons innervating the RVLM expressed VGLUT2 mRNA. In water-deprived rats, an even greater proportion of RVLM-projecting PVH neurons activated by water deprivation contained VGLUT2 mRNA. These observations support the hypothesis that elevated sympathetic outflow in water-deprived rats depends upon activation of a glutamatergic pathway from the PVH to RVLM. An interesting question is whether the same or different sets of PVH neurons with RVLM projections contribute to other sympathoexcitatory states such as arterial hypertension and heart failure (Allen, 2002; Akine et al., 2003; Ito et al., 2003; Stocker et al., 2005). A more detailed knowledge of the co-transmitters and projection pattern of the PVH neurons that are activated under these various conditions may provide the answer.

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Figure 1. VGLUT2 mRNA and GAD67 mRNA in the hypothalamic paraventricular nucleus (PVH) and surrounding regions

A. Brightfield photomicrograph of VGLUT2mRNA *in situ* hybridization reaction product in the middle rostral-caudal level of the PVH (Level 2). Note the VGLUT2 mRNA reaction product was distributed in the magnocellular (PM) and parvocellular (MP) divisions of the PVH. B. Lower magnification image illustrating the large number of VGLUT2 mRNA-positive neurons located in the PVH and the overlying thalamus. C. GAD67 mRNA *in situ* hybridization reaction product in the same region of the brain. Note that the pattern of labeling for VGLUT2mRNA and GAD67 mRNA was complementary to each other in and around the PVH. Scale bar in C = 200 μ m in A, 800 μ m in B and C. Abbreviations: AH anterior hypothalamic nucleus, MP: parvocellular division of the PVH, PM: magnocellular division of the PVH, PVH: paraventricular nucleus of the hypothalamus, Th: thalamus, Xi, xiphoid nucleus, ZI, zona incerta.

Figure 2. Retrograde labeling of VGLUT2 mRNA-expressing PVH neurons following injection of cholera toxin B (CTB) into the rostral ventrolateral medulla (RVLM)

A: CTB injection site (immunofluorescence). B: lower-magnification photograph of an adjacent section stained with thionin highlighting the location of the nucleus ambiguus, pars compacta (Amb). The absence of a distinctive inferior olivary nucleus identifies this section at the rostral end of the RVLM immediately caudal to the facial motor nucleus (py, pyramidal tract). The box outlines the region shown in panel A. C, D: example of CTB-labeled PVH neuron (Alexa 488 immunofluorescence, panel C), containing VGLUT2 mRNA *in situ* hybridization reaction product (D, brightfield). Scale bar in A = 100 μ m for A, 300 μ m for B. Scale bar in C = 20 μ m for C and D.

Figure 3. Location of CTB injection sites in the RVLM and retrogradely-labeled PVH neurons expressing VGLUT2 mRNA

A. Plots of CTB injection sites. The largest cross sectional extension of the injection site is represented. Computer-assisted plots of PVH neurons containing CTB alone (filled squares) or CTB plus VGLUT2 mRNA *in situ* hybridization reaction product (open circles). B: Several PVH subnuclei are identified on the right hand side of the plots. Levels 2 and 3 (after Stocker et al.(2004a)) were selected for cell counting. The rostral end of the PVH (level 1 after Stocker et al. (2004a)) contained relatively few cells and was not systematically identified in all animals. Scale bar = 500 μ m.

Figure 4. VGLUT2 protein immunoreactivity is present in axonal terminals of PVH neurons that innervate RVLM

A: Biotinylated dextran amine (BDA)-immunoreactive cluster of nerve terminals within RVLM (confocal microscopy; Alexa 488 fluorescence). B: VGLUT2-immunoreactive terminals in the same field (confocal microscopy, Cy3 fluorescence). C: merged image showing that each of the BDA-labeled varicosities are VGLUT2-immunoreactive (yellow terminals show colocalization). Arrows in A–C point to individual nerve terminals. D: BDA injection site (conventional epifluorescence, Cy3) entirely confined to the PVH outlined by the presence of oxytocin-associated neurophysin (OT-NP) immunoreactive cell bodies (Alexa-488). Inset in D shows an adjacent section incubated with Vector ABC and reacted with di-aminobenzidine (dark brown reaction product) and counterstained with thionin. E–F: triple immunofluorescence experiment illustrating a close apposition between a BDA-labeled (confocal microscopy, Alexa 488; panel F) VGLUT2-immunoreactive cluster of terminals (confocal microscopy Cy3; panel G), and a tyrosine-hydroxylase immunoreactive dendrite located within the RVLM (confocal microscopy Cy5, panel E). The merged image

is shown in H. Scale bar in A = 10 μm for A–C and E–H. Scale bar in D = 100 μm ; 200 μm for inset.

Figure 5. Water deprivation increases c-Fos immunoreactivity in VGLUT2 mRNA-containing PVH neurons that innervate the ipsilateral RVLm

A: c-Fos immunoreactivity in the MP region of the PVH in a water-deprived rat. B: c-Fos immunoreactivity in the magnocellular region of the PVH in a water-deprived rat. C: lack of c-Fos immunoreactivity in the magnocellular region of the PVH in a normally hydrated rat. D–E: cluster of CTB-positive PVH neurons (D; Alexa 488 fluorescence) that contain VGLUT2 mRNA reaction product (E; brightfield) and c-Fos immunoreactive nuclei (Cy3 fluorescence, F). CTB was injected into the RVLm to retrogradely-label PVH neurons. Filled arrows indicate triple-labeled cells (CTB+VGLUTmRNA+c-Fos), whereas open arrows indicate VGLUT2mRNA-expressing PVH neurons that are c-Fos immunoreactive. Scale bar in A = 100 μ m for A–C. Scale bar in D = 20 μ m for D–F.

Figure 6. Location of c-Fos immunoreactive and VGLUT2 mRNA-positive PVH neurons with projections to the ipsilateral RVLM

Computer-assisted plots of three classes of CTB-immunoreactive neurons at two representative levels of the PVH in one water-deprived rat. CTB was injected into the RVLM to label PVH neurons that innervate this structure. Note that most of the CTB-labeled neurons activated by water-deprivation (i.e. c-Fos positive) contained VGLUT2 mRNA (stars). For abbreviations see Fig. 3. Scale bar = 500 μm.

Table 1

PVH neurons with projections to RVLM contain VGLUT2 mRNA

n = 7	Level 2	Level 3 (LP)	Level 3 (MP)	All levels
CTB only	4 ± 1	12 ± 3	6 ± 3	23 ± 6
CTB+VGLUT2	72 ± 14	12 ± 2	37 ± 5	121 ± 18
% VGLUT2	94 ± 3 [*]	50 ± 10 [†]	88 ± 5	85 ± 3

CTB-immunoreactive PVH neurons containing VGLUT mRNA *in situ* hybridization reaction product (CTB + VGLUT2) or not (CTB only) were counted in one section per level per rat ipsilateral to the RVLM injection site. These sections were selected to correspond to levels 2 and 3 depicted in Figure 3. All cells present at level 2 were counted regardless of their subnuclear location. At level 3, counts were made separately within LP and MP. The first three columns of the results describe the average number of neurons per section at the levels indicated (mean ± SE for 7 rats). The last column represents the total number of PVH neurons counted within the 2 sections per rat examined.

Kruskal Wallis one way analysis of variance on ranks revealed significant differences overall between regions ($p < 0.01$).

^{*} Bonferroni t-test showed %VGLUT2 in Level 2 different than Level 3 (LP) ($p < 0.01$) but not different than Level 3 (MP).

[†] Bonferroni t-test revealed %VGLUT2 in Level 3 (LP) different than Level 3 (MP) ($p < 0.01$).

Table 2

Water deprivation increases c-Fos immunoreactivity in VGLUT2 mRNA-positive PVH neurons with projections to the ipsilateral RVLM

Water-deprivation n = 4	Level 2	Level 3 (LP)	Level 3 (MP)	All levels
CTB+VGLUT2 / no c-Fos	98 ± 14	12 ± 3	41 ± 4.7	150 ± 21
CTB+VGLUT2 + c-Fos (triple)	29 ± 6	7 ± 2	9 ± 3	45 ± 9**
% Triple	22 ± 2*	40 ± 4 [†]	16 ± 6	23 ± 2**
Control n = 3	Level 2	Level 3 (LP)	Level 3 (MP)	All levels
CTB+VGLUT2 / no c-Fos	39 ± 2	12 ± 4	32 ± 11	83 ± 10
CTB+VGLUT2 +c-Fos (triple)	1 ± 0	0 ± 0.3	0 ± 0	1 ± 0
% Triple	3 ± 0	2 ± 2	0 ± 0	1 ± 0

CTB-immunoreactive PVH neurons containing VGLUT2 mRNA *in situ* hybridization reaction product (CTB + VGLUT2; presumed glutamatergic neurons with RVLM projection) were counted separately depending on whether the nuclei were c-Fos-positive or not. Cell counts were obtained from one section per level per rat (4 water-deprived and 3 control rats). The sections were selected to correspond to levels 2 and 3 depicted in Figure 3. All cells present at level 2 were counted regardless of the subnuclear location. At level 3, counts were made separately within LP and MP. The first three columns of the results describe the average number of neuron per section at the levels indicated (mean ± SE for either 4 or 3 rats). The last column represents the total number of PVH neurons counted in the 2 sections per rat examined.

One way analysis of variance revealed significant interregional differences between regions ($p < 0.01$).

* Bonferroni t-test showed %Triple in Level 2 different than Level 3 (LP) ($p < 0.01$) but not different than Level 3 (MP).

[†] Bonferroni t-test revealed %Triples in Level 3 (LP) different than Level 3 (MP) ($p < 0.01$).

** t-test revealed that the number and percentage of c-Fos neurons with CTB+VGLUT2 was greater in the water-deprived animals than in the control animals ($p < 0.01$).

Table 3

Most RVLM-projecting PVH neurons activated by water deprivation contain VGLUT2 mRNA

Water deprivation n = 4	Level 2	Level 3 (LP)	Level 3 (MP)	All levels
CTB + c-Fos / no VGLUT2	0	3 ± 1	0	3 ± 1
CTB + c-Fos + VGLUT2	29 ± 6	7 ± 2	9 ± 3	45 ± 9
% Triple	100	71 ± 17	100	94 ± 4

CTB-immunoreactive PVH neurons containing c-Fos immunoreactivity (CTB + c-Fos; RVLM-projecting PVH neurons activated by water deprivation) were counted separately depending on whether they contained VGLUT2 mRNA *in situ* hybridization reaction product. Cell counts were made in one section per level per rat in 4 water-deprived rats. Control rats had virtually no c-Fos and are not shown. The sections were selected to correspond to levels 2 and 3 depicted in Figure 3. All cells present at level 2 were counted regardless of their subnuclear location. At level 3, counts were made separately within LP and MP. The first three columns of the results describe the average neuronal counts per section at the levels indicated (mean ± SE for 4 rats). The last column represents the average total number of PVH neurons counted per rat.