Gene expression in the rat supraoptic nucleus induced by chronic hyperosmolality versus hyposmolality

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Glasgow, Eric, Takashi Murase, Bingjun Zhang, Joseph G. Verbalis, and Harold Gainer. Gene expression in the rat supraoptic nucleus induced by chronic hyperosmolality versus hyposmolality. Am J Physiol Regulatory Integrative Comp Physiol 279: R1239–R1250, 2000.—Magnocellular neurons of the hypothalamo-neurohypophysial system play a fundamental role in the maintenance of body homeostasis by secreting vasopressin and oxytocin in response to systemic osmotic perturbations. During chronic hyperosmolality, vasopressin and oxytocin mRNA levels increase twofold, whereas, during chronic hyposmolality, these mRNA levels decrease to 10–20% of that of normoosmolar control animals. To determine what other genes respond to these osmotic perturbations, we have analyzed gene expression during chronic hyper- versus versus hyponatremia. Thirty-seven cDNA clones were isolated by differentially screening cDNA libraries that were generated from supraoptic nucleus tissue punches from hyper- or hyponatremic rats. Further analysis of 12 of these cDNAs by in situ hybridization histochemistry confirmed that they are osmotically regulated. These cDNAs represent a variety of functional classes and include cytochrome oxidase, tubulin, Na+–K+–ATPase, spectrin, PEP-19, calmodulin, GTPase, DnaJ-like, clathrin-associated, synaptic glycoprotein, regulator of GTPase stimulation, and gene for oligodendrocyte lineage-myelin basic protein. This analysis therefore suggests that adaptation to chronic osmotic stress results in global changes in gene expression in the magnocellular neurons of the supraoptic nucleus.

vasopressin; oxytocin; neurophysin; PEP-19; gene for oligodendrocyte lineage-myelin basic protein; magnocellular neuron

VASOPRESSIN (VP) release from the magnocellular neurosecretory cells (MNCs) of the hypothalamo-neurohypophysial system (HNS) is the primary homeostatic mechanism by which the body regulates systemic osmotic changes (3, 10, 32, 42). The MNCs respond to acute hypertonic conditions by rapidly releasing VP and oxytocin (OT) from the posterior pituitary into the systemic blood circulation (32), followed by an increase in the synthesis, processing, and transport of VP and OT in the MNCs (10, 47). During chronic hyperosmotic stress, the MNCs respond to the increased requirement for VP and OT release by upregulating the synthesis of VP and OT mRNA by twofold (35, 40).

Conversely, in response to hyposmotic stress, the HNS release of VP and OT is reduced to virtually zero (42), and, after 6 days of hyponatremia, the levels of OT and VP mRNA have decreased to <10–20% of normonatremic animals (33). Therefore, MNCs dramatically upregulate and downregulate OT and VP mRNA levels in response to the physiological conditions of hyper- and hyposmotic stress, respectively.

Central to understanding this adaptive regulation of peptide secretion and synthesis is ascertaining whether the dramatic differences in VP and OT mRNA levels under these two conditions result from general changes in gene expression in the magnocellular neurons or if they are specific only for VP and OT and related genes. To evaluate this, we used differential screening of cDNA libraries from supraoptic nuclei (SON) punches from hyper- and hyponatremic rats. We chose these conditions for study, because there is an ~10-fold difference in VP and OT mRNA levels between these conditions. Additionally, we used mRNA isolated from the SON for our screen, because this nucleus is composed primarily of VP and OT expressing magnocellular neurons and thus maximizes the “signal-to-noise” ratio in the libraries.

We report here the results of these screens, and a subsequent analysis by in situ hybridization histochemistry (ISHH) of the differential expression of 12 diverse cDNAs obtained from SON punches from hyper- and hyponatremic rats. Our results support the view that the opposing conditions of chronic hyper- versus hyponatremia cause global changes in magnocellular neuron gene expression in addition to the already known changes in VP and OT gene expression.

MATERIAL AND METHODS

Animals. Adult male Sprague-Dawley rats weighing 250–275 g were housed individually in wire-mesh cages in a
temperature-controlled room (21–23°C) with lights on from 7:00 AM to 7:00 PM. Animals were fed solid food or liquid diet as described in Induction of hyper- and hyponatremia. All procedures were carried out in accordance with the National Institutes of Health guidelines on the care and use of animals and an animal study protocol approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee and the Georgetown University Animal Use and Care Committee.

Induction of hyper- and hyponatremia. To induce hypernatremia, rats were given 2% NaCl solution ad libitum as their only drinking fluid for 7 days. Daily saline intake was monitored. Hyponatremia was induced as described previously (41, 44). Rats were fed with 40 ml/day (70 kcal/day) of a nutritionally balanced liquid diet (AIN-76, Bioserv, Frenchtown, Nj) each morning. After 2 days on a liquid diet, osmotic minipumps (Alzet model 2002, Alza, Palo Alto, CA) containing 1-desamino-8-D-arginine vasopressin (DDAVP; Rorer Pharmaceuticals, Fort Washington, PA) were implanted subcutaneously using methoxyflurane anesthesia to deliver DDAVP at a rate of 5 ng/h. On the day of osmotic minipump implantation, the rats were given a more diluted preparation of the liquid diet (70 kcal in 60 ml) but thereafter resumed the more concentrated formula (70 kcal in 40 ml).

Blood samples were drawn from all rats via jugular puncture for measurement of plasma osmolality and sodium and potassium concentrations using ion-specific electrodes (Beckman Electrolyte 2 Analyzer, Brea, CA). The physiological parameters of the rats that were used in this study are shown in Table 1. The body weights (g), plasma Na⁺ levels (mM), plasma K⁺ levels (mM), and plasma osmolalities (mosmol/kgH₂O) were measured in each animal after 7 days of treatment (average values are shown in Table 1).

Tissue isolation and mRNA extraction. The rats were euthanized by decapitation, and the brains were rapidly removed and frozen in liquid nitrogen. SON tissue was isolated from the hypothalamus using conventional techniques and stored at −70°C. mRNA was isolated directly from the SON tissue using the PolyATtract System 1000 mRNA purification kit (Promega). The volume of mRNA solution was then reduced using the PolyATract System 1000 mRNA purification kit (Promega). The volume of mRNA solution was then reduced to 10 μl in a speed vacuum, and 1 μl in a speed vacuum, and 1 μl was removed for random-primed RT. The resulting random-primed cDNA was used to confirm, by gene specific RT-PCR (12), that VP mRNA was detected by autoradiography after 3 days. Differentially hybridizing colonies were picked and grown for cDNA purification and sequenced. The identity of each clone was determined by comparing its DNA sequence to the nonredundant GenBank database. We used the nonradioactive ISHH protocol as described by W. Scott Young, III and E. Va Mezey (see http://intramural.nimh.nih.gov/lcmr/instr.html). The amplification cycles were as follows: 1) hold at 94°C for 10 min; 2) run 25 cycles at 94°C for 30 s, 42°C for 30 s, and 72°C for 4 min; 3) add 2.5 U AmpliTaq Gold, then run 25 cycles at 94°C for 30 s, 42°C for 30 s, and 72°C for 4 min; and finally 5) hold at 72°C for 20 min, and then hold at 4°C. After amplification, 5 μl were run on a 1.5% agarose gel containing ethidium bromide to confirm amplification. There was an intense smear of cDNA between 0.4 and 1.2 kb. The remaining amplified cDNA was purified by phenol extraction and ethanol precipitation; this procedure resulted in the generation of large amounts of cDNA (~15 ug) from individual SON tissue punches. One microgram of amplified cDNA from each SON tissue punch was pooled according to treatment group.

To generate cDNA libraries, 1-μg aliquots of the pooled cDNA was digested with EcoRI and the fragments between 0.4 and 1.2 kb were purified by agarose electrophoresis and then ligated into the EcoR I site of the pZErO-2 vector (Invitrogen) or the pBluescript SK’ II vector (Stratagene). The plasmids were then used to transform electrocompetent Escherichia coli HB10 ElectroMax cells (LTI) by electroporation and plated at ~300 colonies per 100-mm plate. Duplicate lifts to Hybond N+ (Amersham) filters were made from each plate. One filter was incubated with hypernatremia-specific probe generated by random-primed 32P labeling (Stratagene, Prime It II Kit) of amplified cDNA from the SON of hypernatremic rats. The other filter was incubated with hyponatremia-specific probe. Hybridization was at 42°C overnight in hybridization solution containing 50% formamide. Final washing conditions were 65°C in 0.2× sodium chloride-sodium citrate (SSC) and 1% SDS for 30 min. Hybridization was detected by autoradiography after 3 days. Differentially hybridizing colonies were picked and grown for cDNA purification and sequenced. The identity of each clone was determined by comparing its DNA sequence to the nonredundant GenBank database by Basic Local Alignment Search Tool (BLAST) searching.

Table 1. Physiological parameters of control, hyper-, and hyposmolar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Plasma Na⁺, mM</th>
<th>Plasma K⁺, mM</th>
<th>Plasma Osmolality (mosmol/kgH₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normonatremic (n = 4)</td>
<td>288 ± 4</td>
<td>134.8 ± 1.1</td>
<td>5.4 ± 0.3</td>
<td>302 ± 2</td>
</tr>
<tr>
<td>Hyponatremic (n = 5)</td>
<td>254 ± 17</td>
<td>99.6 ± 1.2</td>
<td>5.2 ± 0.2</td>
<td>322 ± 2</td>
</tr>
<tr>
<td>Hyponatremic (n = 5)</td>
<td>244 ± 30</td>
<td>147.1 ± 2.5</td>
<td>5.1 ± 0.4</td>
<td>317 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats.
4% formaldehyde for 30 min, then 0.25% acetic anhydride and 0.1% triethanolamine-HCl (pH 8.0) for 10 min, transferred through graded ethanol, and air dried. In situ hybridization was in 50 µl hybridization buffer containing 100 ng denatured probe incubated at 55°C for 18–24 h. After hybridization, the slides were incubated with 20 µg/ml RNase A at 37°C for 30 min, then washed in 2×, 1×, and 0.5× SSC, with two final washes in 0.1× SSC at 65°C. The sections were incubated with sheep anti-DIG-AP (1:1,000; Boehringer Mannheim) at 4°C overnight and then developed with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyolphosphate (NBT/BCIP; Boehringer Mannheim) for 30 min to 24 h until the signal was visible.

RESULTS

The strategy underlying this differential analysis of gene expression in SONs from hyper- versus hyponatremic rats is outlined in Fig. 1, and the results of this analysis are shown in Table 2. We isolated 37 cDNA clones that were enhanced in the hyper- versus hyponatremic SONs. In Table 2, the clones are identified as known genes based on very close nucleotide sequence similarities to the indicated sequences in the GenBank database. The 26 differentially expressed genes in Table 2 represent a variety of different func-

![Diagram](http://apregu.physiology.org/Downloaded from 10.220.33.1 on October 6, 2016)
tional classes and are arranged in the Table according to their well-established functions. We also isolated 11 clones that had no significant sequence similarity to any sequences in the database (see Table 2).

The genes identified in Table 2 are presumed to be expressed in the SON at higher levels during hypernatremia compared with hyponatremia, because they were isolated from the hypernatremic cDNA library in this differential screen. To confirm that the genes identified in Table 2 are in fact expressed at higher levels in the SON during hypernatremia, we subsequently used ISHH, using the riboprobes described in Table 3, to examine the mRNA expression levels for 12 of these genes in hyper- and hyponatremic rats. Coronal sections through the SON were used to examine the relative expression of 12 of the cDNAs by ISHH. Several features of these brain sections are shown in Fig. 2. ISHH using a βIII-tubulin probe is shown in Fig. 2, A, C, E, and G, as a typical example of the ISHH experiments. Stained cells of the SON, paraventricular nucleus (PVN), and cortex are labeled in Fig. 2 A. The levels of βIII-tubulin RNA in the SON increase during hypernatremia and decrease during hyponatremia compared with normonatremic rats (Fig. 2, C, E, and G). In Fig. 2B, the OT and VP cells of the SON and PVN are visualized by hybridization of a neurophysin riboprobe that specifically labels these nuclei. Figure 2, D and F, demonstrate the well-known increase in neuronal markers in the SON during hypernatremia.

Table 2. Differentially expressed clones isolated from the hypernatremic cDNA library

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>~ Size, bp</th>
<th>Accession Number</th>
<th>% Identity</th>
<th>Gene Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>3B7*</td>
<td>400</td>
<td>M27315</td>
<td>100</td>
<td>(mitochondrial) cytochrome-c oxidase subunit II</td>
<td>energy production</td>
</tr>
<tr>
<td>3B7*</td>
<td>300</td>
<td>X54087</td>
<td>99</td>
<td>cytochrome-c oxidase subunit VIIa</td>
<td>energy production</td>
</tr>
<tr>
<td>R44</td>
<td>400</td>
<td>U30838</td>
<td>71</td>
<td>voltage-dependent anion channel 2 (mouse)</td>
<td>energy production</td>
</tr>
<tr>
<td>3B42</td>
<td>800</td>
<td>K00750</td>
<td>97</td>
<td>cytochrome-c</td>
<td>energy production</td>
</tr>
<tr>
<td>9B/E14*</td>
<td>700</td>
<td>U16850</td>
<td>91</td>
<td>calmodulin-I, 3'-UTR (human)</td>
<td>signal transduction</td>
</tr>
<tr>
<td>3B/E1</td>
<td>450</td>
<td>X14159</td>
<td>99</td>
<td>protein phosphatase-2A catalytic subunit</td>
<td>signal transduction</td>
</tr>
<tr>
<td>3B/E5</td>
<td>250</td>
<td>D78674</td>
<td>91</td>
<td>phospholipase A2 (mouse)</td>
<td>signal transduction</td>
</tr>
<tr>
<td>O24*</td>
<td>400</td>
<td>X84047</td>
<td>99</td>
<td>guanine nucleotide-binding protein, Goα</td>
<td>signal transduction</td>
</tr>
<tr>
<td>I31*</td>
<td>400</td>
<td>U67188</td>
<td>91</td>
<td>G protein signaling regulator RGS5 (mouse)</td>
<td>signal transduction</td>
</tr>
<tr>
<td>9B/E6*</td>
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<td>M24852</td>
<td>94</td>
<td>neuron-specific protein PEP-19</td>
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<td>3B/E8*</td>
<td>750</td>
<td>M38395</td>
<td>95</td>
<td>phosphoprotein F1-20 (mouse)</td>
<td>exocytosis</td>
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<tr>
<td>I68*</td>
<td>900</td>
<td>S45663</td>
<td>100</td>
<td>synaptic glycoprotein SC2</td>
<td>exocytosis</td>
</tr>
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<td>700</td>
<td>U53092</td>
<td>87</td>
<td>Dnaj-like protein RDJ1</td>
<td>chaperone</td>
</tr>
<tr>
<td>I61*</td>
<td>600</td>
<td>J02701</td>
<td>99</td>
<td>Na+ -K+ -ATPase β-subunit</td>
<td>ion transport</td>
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<tr>
<td>9B/E4</td>
<td>500</td>
<td>AF030253</td>
<td>77</td>
<td>VGAT</td>
<td>GABA transporter</td>
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<tr>
<td>9B21</td>
<td>1000</td>
<td>S66190</td>
<td>92</td>
<td>prostatic acid synthetase</td>
<td>structural</td>
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<tr>
<td>R46</td>
<td>700</td>
<td>V01227</td>
<td>98</td>
<td>a-tubulin</td>
<td>structural</td>
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<tr>
<td>9B/E17*</td>
<td>800</td>
<td>U47634</td>
<td>85</td>
<td>β-tubulin class III, β-3 (human)</td>
<td>structural</td>
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<tr>
<td>3B/E13*</td>
<td>700</td>
<td>J04828</td>
<td>96</td>
<td>αII-spectrin</td>
<td>structural</td>
</tr>
<tr>
<td>R42</td>
<td>500</td>
<td>M63285</td>
<td>96</td>
<td>matrin 3</td>
<td>structural</td>
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<tr>
<td>I89</td>
<td>577</td>
<td>Y00441</td>
<td>100</td>
<td>β-microglobin</td>
<td>structural</td>
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<tr>
<td>3B33</td>
<td>500</td>
<td>X05080</td>
<td>99</td>
<td>β-globin</td>
<td>oxygen carrier</td>
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<tr>
<td>I9</td>
<td>295</td>
<td>M83118</td>
<td>85</td>
<td>factor VIII-associated protein, f8a (mouse)</td>
<td>unknown</td>
</tr>
<tr>
<td>R37</td>
<td>1000</td>
<td>X99993</td>
<td>97</td>
<td>microvascular endothelial differentiation gene</td>
<td>unknown</td>
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<tr>
<td>3B25</td>
<td>600</td>
<td>D84482</td>
<td>64</td>
<td>PMSG-induced ovarian mRNA</td>
<td>unknown</td>
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<tr>
<td>3B/E6*</td>
<td>400</td>
<td>K00512</td>
<td>97</td>
<td>Golli-MBP</td>
<td>unknown</td>
</tr>
</tbody>
</table>

Eleven clones, 3B/E7, 3B/E9, 3B/E11, 3B/E14, 3B/E18, R18, 119, I5, I59, I45, and I57 did not have significant nucleotide sequence similarity to any sequence in the database. * These clones were selected for further analysis by in situ hybridization histochemistry. UTR, untranslated region; VGAT, vesticular GABA transporter; Golli-MBP, gene for oligodendrocyte lineage-myelin basic protein.

Table 3. cDNA clones used to generate ISHH riboprobes

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Vector</th>
<th>Antisense Primer</th>
<th>Gene Name</th>
<th>Insert Size, bp</th>
<th>Region Of Overlap, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>9B/E17</td>
<td>pBluescript</td>
<td>T7</td>
<td>βIII-tubulin</td>
<td>~800</td>
<td>5'-UTR: 345, Coding: 349, 3'-UTR: 349</td>
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<tr>
<td>3B7</td>
<td>pBluescript</td>
<td>T7</td>
<td>mitochondrial COII</td>
<td>485</td>
<td>5'-UTR: 345, Coding: 349, 3'-UTR: 349</td>
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<tr>
<td>I61</td>
<td>pZErO-2</td>
<td>T7</td>
<td>Na+ -K+ -ATPase β1</td>
<td>639</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
</tr>
<tr>
<td>3B/E13</td>
<td>pBluescript</td>
<td>T7</td>
<td>αII-spectrin</td>
<td>763</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
</tr>
<tr>
<td>9B/E8</td>
<td>pBluescript</td>
<td>T7</td>
<td>PEP-19</td>
<td>593</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
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<tr>
<td>9B/E14</td>
<td>pBluescript</td>
<td>T3</td>
<td>Cal 4.0-kb mRNA</td>
<td>692</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
</tr>
<tr>
<td>O24</td>
<td>pZErO-2</td>
<td>SP6</td>
<td>Goα</td>
<td>424</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
</tr>
<tr>
<td>3B/E2</td>
<td>pBluescript</td>
<td>T3</td>
<td>Dnaj-like protein</td>
<td>~700</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
</tr>
<tr>
<td>3B/E8</td>
<td>pBluescript</td>
<td>T3</td>
<td>F1-20AP-3</td>
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<td>I68</td>
<td>pZErO-2</td>
<td>T7</td>
<td>SC2</td>
<td>854</td>
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<tr>
<td>I31</td>
<td>pZErO-2</td>
<td>T7</td>
<td>RGS5</td>
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<tr>
<td>3B/E6</td>
<td>pBluescript</td>
<td>T7</td>
<td>Golli-MBP</td>
<td>408</td>
<td>5'-UTR: 321, Coding: 349, 3'-UTR: 349</td>
</tr>
</tbody>
</table>

ISHH, in situ hybridization histochemistry; COII, cytochrome-c-oxidase II; Cal, calmodulin.
rophysin mRNA in the SON during hypernatremia, visualized by our nonradioactive ISHH technique. In contrast, during hyponatremia, there is a decrease in neurophysin mRNA in the SON (Fig. 2, D and H). These changes in neurophysin mRNA levels visually confirm earlier studies describing an increase in VP, OT, and neurophysin mRNA during hypernatremia (35, 40) and a decrease in these mRNAs during hyponatremia (33).

**ISHH analysis of differentially expressed genes in the hypernatremic SON.** Visual inspection of the intensity of the reaction product in the SONs of hyper-, normo-, and hyponatremic rats provides the basis of a qualitative rating scale for relative RNA expression levels for the 12 cDNAs that were analyzed by ISHH. We arbitrarily assigned a value of 2+ to the βIII-tubulin RNA expression level in normonatremic rats (Fig. 2C). During hypernatremia, this level increases to 3+ (Fig. 2E), whereas during hyponatremia, the βIII-tubulin RNA level decreases to 1+ (Fig. 2G). For the neurophysin probe, the normonatremic value was assigned as 3+ (Fig. 2D) and to 4+ (Fig. 2F) during hypernatremia and falls to 1+ (Fig. 2H) during hyponatremia. This semiquantitative rating scale was used to suggest the relative RNA changes in the SON of the 12 cDNAs from the differential screen that were selected for further analysis (Table 4).

In this study, our primary aim was to study the differential expression of genes between the opposite conditions of hyper- versus hyponatremia. The nor-
Table 4. Relative intensity of DIG-ISHH staining in the SON

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Gene Name</th>
<th>Hypernatremia</th>
<th>Hyponatremia</th>
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<tbody>
<tr>
<td>9B/E17</td>
<td>β\textsubscript{II}-tubulin</td>
<td>++ +</td>
<td>+</td>
</tr>
<tr>
<td>3B7</td>
<td>oxidase</td>
<td>+++++</td>
<td>+++</td>
</tr>
<tr>
<td>I61</td>
<td>Na\textsuperscript{+}-K\textsuperscript{+}-ATPase β1</td>
<td>+++-</td>
<td>-</td>
</tr>
<tr>
<td>3B/E13</td>
<td>αII-spectrin</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>9B/E6</td>
<td>PEP-19</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>9B/E14</td>
<td>Cal 4.0-kb mRNA</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>O24</td>
<td>Go\textsubscript{α}</td>
<td>++++++</td>
<td>++</td>
</tr>
<tr>
<td>3B/E2</td>
<td>DnaJ-like protein</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>3B/E8</td>
<td>F1-20/AP-3</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>IVE8</td>
<td>SC2</td>
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<td>+</td>
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<tr>
<td>I31</td>
<td>RGS5</td>
<td>+/-</td>
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<tr>
<td>3B/E6</td>
<td>Golli-MBP</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

Values represent qualitative evaluations of the DIG-ISHH staining intensity in the supraoptic nuclei (SON). See Figs. 2 and 3 for examples of ISHH staining (β\textsubscript{II}-tubulin in Fig. 2, A, C, E, G, and PEP-19, DnaJ-like protein, and Golli-MBP in Fig. 3). + Symbols indicate semiquantitative rating scale for relative RNA changes in SON of 12 cDNAs from the differential screen that were selected for analysis. See text for discussion.

Highly upregulated in the SON during hypernatremia (Table 4).

We also isolated clones for several differentially expressed genes involved in Ca\textsuperscript{2+}-mediated signal transduction. The expression of three of these clones, 9B/E6 (PEP-19), 9B/E14 [calmodulin (Cal)], and O24 (Go\textsubscript{α}) were examined by ISHH. Note that PEP-19 mRNA levels fall dramatically during hyponatremia (Fig. 3, A and B; Table 4). Clone 9B/E14 has high sequence similarity to the 3′-untranslated region (UTR) of human Cal. Although the sequence is not in the GenBank database, most of the 3′-UTR of the rat large Cal mRNA, the 4.0-kb species, has been sequenced, and it is nearly identical to clone 9B/E14 where they overlap (31). The region of the Cal 3′-UTR that overlaps with clone 9B/E14 is specific for the large 4.0-kb mRNA species that is preferentially expressed in mature neurons of the brain. Our ISHH results show Cal 4.0-kb mRNA 3′-UTR hybridization in the brain, with higher levels in the SON and hippocampus. Clone O24 is identical to the 3′-UTR of rat Go\textsubscript{α}, the stimulatory α-subunit of the trimeric GTPase protein and shows considerably more mRNA in the SON under hypernatremic conditions (Table 4).

We also examined the expression of three genes isolated from our screen whose functions are poorly understood. Interestingly, these genes have all been located in the synapse, which suggests a role in secretory functions. Our ISHH results show that clone 3B/E2 (DnaJ-like protein, RDJ1), clone 3B/E8 (phosphoprotein F1–20/clathrin assembly protein 3 AP3), and clone IVE8 (synaptic glycoprotein, SC2) are each expressed in neurons but also, to a lesser extent, in glia. All of these synapse-associated genes are also differentially regulated by hyper- versus hyponatremia (Fig. 3, C and D; Table 4).

Finally, we isolated two genes that are highly expressed in glial cells, clone I31 [regulator of trimeric GTPase protein stimulation gene 5 (RGS5)] and clone 3B/E6 [gene for oligodendrocyte lineage-myelin basic protein, designated as the gene for oligodendrocyte lineage-myelin basic protein (Golli-MBP), see Ref. 4]. This was not unexpected as the “punched” samples of the SONs also included glial cells from the nucleus itself and the optic chiasm. The expression pattern of RGS5 as revealed by ISHH shows strong labeling in the optic chiasm and the corpus callosum (not shown), indicating expression mostly in glia. In the SON, labeling is restricted to cells interspersed between the MNCs, presumably in glia. This is the only clone in which there did not appear to be a difference in staining intensities in the SON between the hyper- and hyponatremic rats (see Table 4 and DISCUSSION).

Clone 3B/E6 has a 377-bp insert that has 97% identity to the rat MBP 3′-UTR sequence, which starts at the position of the translation stop codon. The transcription unit containing this region is complex, consisting of 11 exons and at least two transcriptional start sites (4). The MBP mRNAs include at least five alternatively spliced isoforms that are transcribed from the promoter site 1. All five of the MBP mRNAs...
contain 3′-UTR sequence that is similar to clone 3B/E6, and they are all specific to differentiated oligodendrocytes. The clone 3B/E6 ISHH reveals strong hybridization to glial-containing structures, such as the optic chiasm and corpus callosum. However, there is also strong hybridization in the MNCs of the SON (Fig. 3, E and F). The MNC staining probably represents hybridization of the clone 3B/E6 riboprobe to Golli-MBP mRNA, which is transcribed from promoter site 2 and produces at least three alternatively spliced mRNAs, two of which contain the 3′-UTR sequence of clone 3B/E6. Although Golli-MBP proteins were first described from the oligodendrocyte lineage, they are also expressed in many neuronal cells (23). The Golli-MPB mRNA(s) recognized by clone 3B/E6 ISHH is highly expressed in MNCs and is also regulated under conditions of hyper- and hyponatremia (Fig. 3, E and F; Table 4).

DISCUSSION

Our goal was to identify genes that were regulated in the SON in response to the osmotic stresses of hyperosmolality and hyposmolality. This approach resulted in the isolation of many potentially differentially expressed genes that were upregulated during hyperosmolality compared with hyposmolality. These include a wide spectrum of genes, including mitochondrial genes, structural genes such as β-tubulin and α-spectrin, as well as genes involved in Ca2+-mediated signal transduction and exocytosis.

One potential caveat with the interpretation of our results is the fact that the hyper- and hyponatremic rats differed in several important experimental characteristics. Specifically, the hyponatremic rats were infused with the VP V2-receptor agonist DDAVP and were fed a liquid diet to achieve water loading, whereas the hypernatremic rats were fed normal chow along with 2% NaCl drinking solution. Obviously, this introduces some potential additional variables to consider in interpreting the subsequent responses. However, these models represent the best-established animal models of hyper- and hyponatremia currently available for study, and it would be impossible to control for some of the variables unique to these models (e.g., one cannot induce hyperosmolality in a rat infused with DDAVP because of the subsequent antidiuresis induced by this agent). Furthermore, both of these models have been well characterized in the literature, and, more specifically, they have already been used to report the upregulation of VP and OT gene expression that occurs with hyperosmolality and the downregulation of these genes that occurs with hyposmolality (33).
The absence of VP V₂ receptors in the SON and PVN, as well as in the whole brain, argues against a direct effect of DDAVP on magnocellular neurons. However, a recent report has demonstrated that V₂ agonists can increase intracellular Ca²⁺ in magnocellular neurons of the SON (16). Nonetheless, any such excitatory effects of DDAVP on magnocellular neurons appear to be insufficient to override the effects of the induced hyposmolality to markedly inhibit AVP and OT secretion (43) and synthesis (33). Consequently, although our results must be qualified with the acknowledgment that factors other than osmolality may be influencing some of the changes in gene expression, nonetheless they represent the best available well-documented models for studying such sustained changes in plasma osmolality.

Several studies have documented the increase in VP and OT mRNA levels in response to osmotic stress (10), and reports have shown coordinate regulation of VP or OT mRNA with other coexpressed peptides, such as dynorphin, galanin, neuropeptide Y, and signal transduction-associated proteins (i.e., Goₐ) (26, 47). Our results suggest that most genes expressed in the MNCs of the SON respond to chronic hyperosmolality by increasing a variety of mRNA levels. In comparison, the changes in gene expression occurring in the SON under conditions of hyposmolality have not been extensively studied. It has been shown that OT and VP mRNA levels decrease to 10–20% of normonatremic rats during chronic hyponatremia (33). We did not isolate any clones that were expressed at higher levels in the hypo- versus hyperosmotic cDNA library. One gene, the glucocorticoid-receptor gene, has been shown to be upregulated during hyponatremia (2). However, this was not detected in our screen, most likely because the PCR parameters used here were not optimal to amplify this mRNA. In this regard, a similar failure to detect changes in the OT and VP mRNAs was due to inefficient amplification of these mRNAs with these PCR parameters. In fact, we used this effect to minimize the otherwise expected predominance of VP and OT clones resulting from our screen. These results emphasize the limitations of detection of this technique and would significantly be improved by the use of a cDNA microarray approach. Nevertheless, our results suggest that most genes are downregulated in MNCs in response to hyponatremia. In areas of the brain other than the HNS, the overall impression was that the expression of these genes was largely unaffected by osmotic stress. There may be a general, though slight, increase in mRNA levels in the brains of hyponatremic rats but not nearly as dramatic as the changes that we observe in the SON.

Overall, these findings suggest that the gene regulation mechanisms (transcription or mRNA turnover) operating in these cells during hypo- and hyperosmolality are global in nature. In addition to highlighting the concept of global regulation of VP and OT mRNA levels in MNCs in response to osmotic stresses, these studies reveal, for the first time, the presence and regulated expression of several specific genes in MNCs. Some of these genes are well known and might be expected to be present in MNCs but had not previously been described in this system. Other genes that were found in the MNCs in this analysis were of unknown function, and others found were novel in that their sequences have not yet been reported in the database. Specific details of some of these genes are discussed below.

“Housekeeping” genes expressed in the SON. Two genes isolated in our screen, tubulin and spectrin, are components of the cell cytoskeleton and are found in most animal cells. Another housekeeping gene, Na⁺-K⁺-ATPase, is the primary molecular machine for maintaining the Na⁺-K⁺ gradient across the plasma membrane of all cells. The isoforms of these genes that we isolated from the rat SON are known to be generally found in neurons, so that the presence of these genes in the MNCs is to be expected (15, 25, 38). Their regulation during hyper- and hyposmolality (Fig. 2, E and G; Table 4) underscores the concept that during osmotic stress, many common genes expressed in the MNCs of the SON are being coordinately regulated in response to the secretory activity of the neurons.

In addition to the Na⁺-K⁺-ATPase, cytochrome-c oxidase is related to energy consumption in cells (45). Cytochrome-c oxidase II histochemistry has been used previously in the HNS to examine the activity of MNCs and has suggested that there is a hyperactivity of MNCs in the chronically dehydrated Brattleboro rat (22). Our results with cytochrome-c oxidase II ISHH confirm that the synthesis of cytochrome-c oxidase II RNA is high in the MNCs and, furthermore, show that cytochrome-c oxidase II RNA levels are much higher in cells during hypernatremia than in the less-active state of hyponatremia (Table 4). This, therefore, suggests that cytochrome-c oxidase II is involved in the metabolic activity of the MNCs in the SON in response to osmotic stresses.

Signal transduction-related genes in the SON. A novel finding in this study is the expression and regulation of PEP-19 in the SON (Fig. 3, A and B; Table 4). PEP-19 is a neuronal peptide in the camstatin class of Cal-binding proteins, which consist of PEP-19, neurogranin/RC3, and neuromodulin/GAP-43/B50 (36). The function of the camstatins seems to be to modulate Ca²⁺-Cal signaling (11). The camstatins may play a neuroprotectant role in their ability to buffer excess Ca²⁺ influx activation of the Ca²⁺-Cal signal transduction pathway. A potential role for PEP-19 in osmotic stress could be to modulate the effect of hyperactivity of osmosensitive efferents on the OT/VP MNCs during chronic hyperosmolality. The camstatins show region-specific expression and subcellular localization. RC3 is localized to dendrites and soma, GAP-43 is localized to axons, and PEP-19 is more evenly distributed (11). PEP-19 is abundant in the cerebellum and olfactory bulbs but is found at lower levels in other brain regions (51). We now show that PEP-19 mRNA is abundant in the HNS and that PEP-19 mRNA expression is regulated in MNCs by chronic osmotic stress.
There are two forms of Cal mRNA produced by alternative polyadenylation. In the rat brain, the larger 4.0-kb form is predominately expressed in neurons (30), and this appears to be the isoform that we have isolated from the rat SON. Extensive sequence identity between the rat and human Cal 4.0-kb mRNA 3'-UTR sequence suggests that this region is important for the regulation of Cal in neurons, possibly by targeting this message for rapid turnover (34). The Cal mRNA was dramatically upregulated during hyper- versus hyponatremia (Table 4).

Previous studies have shown an increase in Goα mRNA in the SON of hypernatremic rats compared with normonatremic rats (48). Our results are consistent with and extend these studies in that there appears to be an even larger increase in Goα mRNA levels in the SON of hypernatremic rats compared with the hyponatremic condition (Table 4).

Secretion-related genes. Synaptic glycoprotein SC2 was originally isolated by screening a rat brain cDNA expression library with antibodies directed against concanavalin A-binding synaptic junctional glycoproteins (20). Subsequent ISHH analysis indicates that SC2 is also expressed in nonneuronal tissues. Furthermore, a human SC2 homolog has recently been isolated from a human erythroid stem-cell cDNA library (24). Although SC2 expression is not restricted to neurons, SC2 may have a function in the synapse in neuronal cells. Several molecules, such as spectrin and Cal, which are also widely distributed, play important roles at the synapse. It is interesting that an open reading frame from the yeast Saccharomyces cerevisiae genome potentially codes for a protein with substantial similarity to SC2 (19). This suggests that SC2 may have a very basic and highly conserved cellular function. Our ISHH results indicate that SC2 is highly expressed in MNCs of the HNS and that SC2 is regulated in response to hyper- and hyponatremia (Table 4). However, further study is needed to identify a function for SC2 in the SON cells.

The clathrin-associated protein 3, F1–20/AP-3, also known as AP180, NP185, and pp155, is a neuron-specific clathrin-associated protein that is primarily localized to the synapse (46). ISHH and immunocytochemistry shows a complex, highly localized, neuronal expression pattern (37). Our ISHH experiments with clone IV68 extend these observations by showing strong expression in the SON as well as regulated expression in response to hyper- and hyponatremia (Table 4).

DnaJ-like proteins are partners of the heat shock protein (HSP) chaperone HSP 70-kDa protein. The DnaJ-HSP70 chaperone system functions in a wide variety of cellular activities, such as nuclear transport, transport to the golgi, synaptic function, correction of protein folding and/or targeting of incorrectly folded proteins to the ubiquitin-protein degradation system (7, 21, 39). In neurons of spinocerebellar ataxia type 1 mutant mice, it has been shown that DnaJ-like proteins (HDJ-2/HSDJ) promote the recognition of misfolded polyglutamate repeat ataxial proteins (6). Our ISHH results indicate that DnaJ-like protein mRNA is present in both glia and neurons and that, in the SON, it is regulated by osmotic stress (Fig. 3, C and D; Table 4).

Glia-associated genes. When isolating SON tissue by the punch procedure, there is a small amount of contamination by the nearby optic chiasm. Additionally, there are glial cells within the SON itself. Thus, as noted earlier, the presence of glial-associated genes in our SON cDNA libraries was not unexpected. The glial-associated genes that we isolated from our hypernatremic SON library are RGS5 and Golli-MBP. The RGS proteins represent an important class of regulator proteins for signaling from trimeric G proteins (1, 49). RGS5 from mouse is expressed at the highest levels in kidney and skeletal muscle, with only low expression in brain (5). Recently, the expression of RGS5 in the rat brain was described using a 220-bp PCR fragment in ISHH experiments (14). Although not shown, they report strong expression of rat RGS5 in the SON and PVN, as well as in glia. However, our ISHH results with clone I31 revealed a glial expression pattern without a strong SON or PVN staining (not illustrated). Because there is a relatively short overlap between clone I31 and the rat RGS5 probe, the discrepancies between these results may be due to alternatively spliced forms of the RGS5 mRNA. If an RGS5 splice variant is highly expressed in glia, in contrast to the lower expression levels of a different variant in the SON, this could lead to a dominant glial hybridization pattern. Further research will be required to resolve this issue. This complexity could obscure any regulation of expression in the SON.

The Golli-MBP derives from the MBP gene (which is a major component of the oligodendrocytic myelin sheath). There are five major isoforms of MBP, ranging from 14 to 21.5 kDa, that are generated by differential splicing from seven exons transcribed from a single promoter site. However, the transcription unit is more complex because there is at least one more promoter region upstream of the MBP promoter. A novel group of proteins that contain both upstream exons and MBP exons is produced by transcription from this promoter and termed genes for Golli-MBP (4). The function(s) of these genes are unknown, but their early expression in oligodendrocytes, their expression in specific neurons, and their dynamic subcellular localization (e.g., sometimes nuclear, other times axonal) indicate that their function is different from MBP itself (23).

On the basis of the strong hybridization of clone 3B/E6 in MNCs and in the optic chiasm (Fig. 3, E and F), this suggests that this probe is recognizing both MBP mRNA in glia and the Golli-MBP mRNA in the MNCs. The major form of Golli-MBP is BG21, which lacks the MBP 3'-UTR sequence that is recognized by clone 3B/E6 (4). Therefore, clone 3B/E6 appears to be recognizing the less-abundant J37 or TB 28 splice variants of Golli-MBP. The normal expression patterns of these two proteins has not been reported, so this is the first evidence that these genes are strongly expressed and regulated in the MNCs in the HNS.
In summary, we have isolated over 37 genes from the hyperosmolar rat SON that are upregulated compared with SONs from hypossmolar rats. Of these, the nucleotide sequences of 26 genes were found in the GenBank database (see Table 2), and 11 were not. We further characterized the expression of 12 specific genes by a nonradioactive ISHH protocol. Most of these genes, which appear to be of relatively high abundance, are involved in a wide variety of basic functions, such as energy metabolism, signal transduction, and secretion. Hence, we conclude that the mechanisms regulating the synthesis of VP and OT mRNA in response to chronic osmotic stress are accompanied by a more global regulation of genes that subservive many functions in the MNCs. This view is consistent with reports that large volume changes are selectively found in the MNCs (27–29, 50) during conditions of increased (e.g., hyperosmolality and lactation) and decreased (e.g., hyponatremia) OT and VP gene expression and secretion.

The differential gene-screening approach that we have used here has, for the first time, revealed the wide variety of genes other than the secreted peptides that are regulated in the MNCs under these conditions (see Table 2). These include genes involved in energy metabolism, signal transduction, chaperone functions, ion transport, etc., all of which might be expected to be involved in these cells’ dramatic biochemical and physiological adaptations to osmotic stress. Particularly intriguing for future study is the possible function of the Golli-MBP, which generally is unknown, but whose mRNA is so abundant in the MNCs (Fig. 3, E and F).

To our knowledge, the only similar differential gene-screening study in a neuroendocrine tissue that has been done used melanocytes from Xenopus laevis frogs that had been adapted to white versus black backgrounds (18). In black-adapted frogs, these cells synthesize and secrete massive quantities of melanocyte-stimulating hormone and contain 20- to 30-fold greater quantities of the precursor (proopiomelanocortin) mRNA than the white-adapted melanocytes. In that study, which focused on probing secretory protein mRNAs in melanocytes, cDNA libraries from the intermediate lobe of dark-adapted frogs were probed using cDNA probes from melanocyte mRNAs in white- and dark-adapted frogs. These authors found and characterized 12 genes that were differentially expressed in dark-adapted animals, most of which were associated with the peptide secretory pathway in melanocytes. The issue as to whether there was a more global upregulation in genes expressed in these cells was not addressed because the authors included a subtraction step using liver cDNA to exclude “nonspecific” cDNAs from their analysis. That there probably was a global upregulation is suggested by the large hypertrophy of melanocytes in dark- versus white-adapted frogs (8), comparable to that described above for the MNCs.

As a result of their favorable anatomic topography and dramatic and sustained changes in gene expression in response to chronic physiological conditions (such as osmotic stress), the MNCs offer insights into neuronal gene regulation not easily obtained in other neuronal systems. It is very likely that the global changes we observed during physiological adaptation in MNCs will also be present in other neuronal systems but will be within the noise level of measurements. Newer methods of differential analysis, such as the use of cDNA microarrays (13), may make comparable studies of other neuronal systems more feasible in the future.

**Perspectives**

Endocrine and neuroendocrine cells are specialized to perform a relatively specific function: to synthesize and secrete a single-peptide hormone and, in some cases, several biologically active peptides in response to physiological needs. One measure of the degree to which they can accomplish this very focused function is their ability to markedly upregulate hormone synthesis in response to chronic stimulation. The HNS is an excellent example of this phenomenon, because synthesis and secretion of VP and OT is increased many-fold in response to conditions of sustained hyperosmolality and dehydration (35, 40). To increase production and secretion of VP and OT efficiently requires not only upregulated transcription and translation of the VP and OT prohormones but also of a wide range of other proteins necessary to support the intense cellular activity needed to maintain high levels of hormone synthesis and secretion. The finding that chronic hyperosmolality is accompanied by increases in the volume of magnocellular neurons (17) has generally been felt to be an indication of such a “global” upregulation of many different gene products during chronic osmotic stress, and our present studies now confirm this presumption by showing upregulated expression of a wide variety of genes involved with energy metabolism, signal transduction, exocytosis, and cell structure. Conversely, endocrine and neuroendoendocrine cells are also known to be adept at shutting off hormonal secretion under inhibitory conditions (43). This is accompanied by markedly reduced levels of mRNA for VP and OT in animal models of chronic hyposmolality (33). In contrast to hyperosmolality, magnocellular neurons have recently been found to have a significantly decreased cell volume in response to sustained hyposmolality (50), suggestive of a global downregulation of many genes during periods of low synthesis and secretion of VP and OT. The present gene expression results are also consistent with this view.

Our present studies enabled us to see these global changes in gene regulation more easily by virtue of the anatomic localization and robust secretory capacity of the magnocellular neurons and also because our differential screening strategy magnified these changes by comparing a maximally stimulated secretory state with a maximally inhibited secretory state, in contrast with normal conditions that are characterized by intermediate baseline levels of cell synthetic and secretory activity. Nonetheless, it is likely that this represents a universal cellular mechanism and that other endocrine and neuronal systems will show similar aspects of coordinated global gene regulation in relation to exter-
nal stimuli. Perhaps the most intriguing question raised by these studies is what factors coordinate these global changes in gene regulation, not only the upregulation during chronic stimulation, but also the profound downregulation during states of chronic inhibition? Further studies should be directed at elucidating these mechanisms and determining their specificity for different genes and different patterns of cell activity.

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