Cellular/Molecular

Transcription Factor Expression in the Hypothalamo-Neurohypophyseal System of the Dehydrated Rat: Upregulation of Gonadotrophin Inducible Transcription Factor 1 mRNA Is Mediated by cAMP-Dependent Protein Kinase A

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The supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamo-neurohypophyseal system (HNS) undergo a dramatic function-related plasticity during dehydration. We hypothesize that alterations in steady-state transcript levels might be partially responsible for this remodeling. In turn, regulation of transcript abundance might be mediated by transcription factors. We used microarrays to identify changes in the expression of mRNAs encoding transcription factors in response to water deprivation in the SON. We observed downregulation of 10 and upregulation of 28 transcription factor transcripts. For five of the upregulated mRNAs, namely gonadotropin inducible ovarian transcription factor 1 (Giot1), Giot2, cAMP-responsive element binding protein 3-like 1, CCAAT/enhancer binding protein β, and activating transcription factor 4, *in situ* hybridization was used to confirm the array results, demonstrating a significant increase in expression in SON and PVN magnocellular neurons (MCNs) after 3 d of water deprivation and, in some cases, upregulation in parvocellular PVN neurons. Using a viral vector expressing a potent inhibitor of cAMP-dependent protein kinase A (PKA), we show that the osmotically induced increase in the abundance of transcripts encoding Giot1 is mediated *in vivo* by the PKA pathway. We thus suggest that signaling pathways activated by dehydration in MCNs mediate transcription factor gene activation, which, in turn, regulate target genes that mediate HNS remodeling.

Key words: **supraoptic nucleus; paraventricular nucleus; osmotic stress; plasticity; microarray; *in situ* hybridization; transcription factors; protein kinase A; viral vector**

Introduction

Osmotic stability is aggressively defended in mammals (Antunes-Rodrigues et al., 2004). The hypothalamo-neurohypophyseal system (HNS) mediates neuroendocrine responses to dehydration through the axonal projections from the supraoptic nucleus (SON) and paraventricular (PVN) magnocellular neurons (MCNs) to the posterior pituitary (Antunes-Rodrigues et al., 2004). The antidiuretic hormone vasopressin (VP) is synthesized as part of a prepropeptide precursor in the cell bodies of SON and PVN MCNs (Brownstein et al., 1980; de Bree, 2000). This precursor is processed during anterograde axonal transportation to terminals in the posterior pituitary gland, in which biologically active VP is stored until mobilized for secretion; a rise in plasma osmolality is detected by intrinsic MCN osmoreceptor mechanisms (Bourque et al., 2002; Zhang and Bourque, 2003) and by specialized osmoreceptive neurons in the circumventricular organs that project to the MCNs (Bourque et al., 1994; Bourque, 1998; McKinley et al., 1999; Anderson et al., 2000; McKinley et al., 2004) that provide direct excitatory inputs (van den Pol et al., 1990) to shape the firing activity of MCNs (Hu and Bourque, 1992; Nissen et al., 1994) for hormone secretion (Dyball et al., 1995; Onaka and Yagi, 2001). On release, VP travels through the blood stream to specific receptor targets located in the kidney in which it increases the permeability of the collecting ducts to water, reducing the renal excretion of water, thus promoting water conservation.

Dehydration evokes a remodeling of the SON and PVN, a process known as function-related plasticity (Hatton, 1997; Theodosis et al., 1998). A plethora of activity-dependent changes in the morphology, electrical properties, and biosynthetic and secretory activity of the HNS have been described (Sharman et al., 2004). For example, alterations in the relationship between MCNs and glia, the extent of terminal contact with the basal lamina in the neurohypophysis, the type and weight of synaptic

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inputs, and the extent of electrotonic coupling between MCNs have all been documented (Tanaka et al., 1999; Yang and Hatton, 1999; Miyata et al., 2001a; Miyata and Hatton, 2002; Tasker et al., 2002). The response of the HNS to dehydration represents a unique and tractable model for understanding the processes whereby changes in gene expression mediate neuronal plasticity (Sharman et al., 2004), but the molecular mechanics of these processes remain to be elucidated.

We hypothesize that some of the changes seen in the SON and PVN after dehydration are mediated by changes in the expression of transcription factors. We have thus used microarrays to identify changes in the expression of mRNAs encoding transcription factors in response to water deprivation within the SON. For five of these factors, the microarray data have been confirmed by *in situ* hybridization analysis. We further show *in vivo* that the osmotically induced increase in the abundance of gonadotropin inducible ovarian transcription factor 1 (Giot1) mRNAs is mediated by the cAMP-dependent protein kinase A (PKA) pathway.

Materials and Methods

Animals. All experimental procedures were approved by the University of Bristol Ethical Review Committee and were licensed by the Home Office under the terms of the Animals (Scientific Procedures) Act (1986). Adult male Sprague Dawley rats (280–330 g) were obtained from commercial breeders (Harlan Sera-Lab, Loughborough, UK) and housed in groups of four to five per cage at a constant temperature (22 ± 1°C) and humidity (50–60%) under controlled light/dark cycle (10 h light, 14 h dark, lights on at 7:00 A.M.). They were given access to food (standard laboratory rat chow) and water *ad libitum* for a period of 1 week before experimentation. After the 1 week adaptation period, the rats were randomly assigned to one of three groups. The first group had constant access to drinking water (euhydrated control group), and the second group was deprived of drinking water for exactly 72 h (3 d).

Affymetrix GeneChip analysis. Interrogation of the Affymetrix 230 2.0 Rat Genome array (Affymetrix, High Wycombe, UK) with targets derived from the SON of control ($n = 5$) or dehydrated ($n = 5$) rats has been described (Hindmarch et al., 2006). The Affymetrix Rat 230 2.0 Genome Chips consists of 31,000 oligonucleotide probe sets representing 30,000 transcripts encoded by 28,000 genes. Data were analyzed using GeneSpring software version 7.0 (Agilent Technologies, Stockport, UK). After data normalization (Hindmarch et al., 2006), we asked GeneSpring to compile a list of genes called present in all five independent control (C) or dehydrated (D) SON experiments. All marginal, absent, or unknown calls were excluded. These gene lists were then combined to produce a gene catalog that represent transcripts called present in all five experiments of either the C or the D conditions. Note that some of these genes, although, by definition, called present in all of the samples of one experimental condition, may well be called absent or marginal in some or all of the samples of the other condition. This combined list was then used as the basis for statistical analysis to robustly assess C versus D changes (Welch's *t* test, $p < 0.05$, with Benjamini–Hochberg multiple test correction) to produce a catalog of transcripts that are significantly changed in abundance in the SON as a consequence of dehydration. Note that the false discovery rate of this analysis will be ~5% of identified genes. All raw data are available at the National Center for Biotechnology Information Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) (accession number GSE3110).

Microinjection of adenoviral vectors into the PVN. Rats were anesthetized with a mixture of ketamine (60 mg/kg) and medetomidine (250 mg/kg) via intramuscular injection. The level of anesthesia was checked frequently by assessing limb withdrawal reflexes to noxious pinching. Rats were then placed in a stereotaxic head frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set at −3.3 mm below the interaural point. The skin overlying the skull was reflected back, and a 2 mm burr hole was drilled in the parietal bones overlying the PVN (anteroposterior, 1.8 mm caudal of bregma along the midline) according to coordinates derived from the atlas of Paxinos and Watson (1986). A glass micropipette containing a 1:1 mixture of the replication-deficient adenoviral vectors Ad.CMV.eGFP (titer, 2.7×10^{10} pfu/ml) and Ad.CMV.PKIα (titer, 8.7×10^{11} pfu/ml) was lowered into the right PVN, and the virus mixture (50 nl) was pressure injected over a period of 1 min. The Ad.CMV.PKIα (Wong et al., 2003) virus expresses protein kinase inhibitor α (PKIα), a potent and specific peptide inhibitor of PKA (Dalton and Dewey, 2006), under the control of the human cytomegalovirus (CMV) enhancer promoter. Ad.CMV.eGFP produces enhanced green fluorescent protein (eGFP) under the control of the CMV enhancer promoter. The control virus (Ad.CMV.eGFP only) was injected into the left PVN (50 nl of 2.7×10^{10} pfu/ml). After surgery, anesthesia was reversed with a subcutaneous injection of atipamezole (1 mg/kg), and the rats were returned to their home cages for recovery. After a recovery period of 9 d, the rats were randomly assigned to either the 72 h water deprivation or the control group. After 3 d of water deprivation, rats were stunned and decapitated, and their brains were rapidly removed, frozen over liquid nitrogen, and stored at −80°C until processed for *in situ* hybridization histochemistry. A total of 14 animals were injected, seven of which were dehydrated. To verify the correct placement of injections, representative sets of slides containing the PVN (from each animal) were viewed under a fluorescent microscope (DM IRB; Leica, Milton Keynes, UK) with the appropriate filter to view eGFP. Only animals in which eGFP expression was observed bilaterally within the PVN ($n = 5$ for both control and dehydrated) were subject to analysis. Sections were counterstained with toluidine blue.

Oligonucleotide probes. Oligonucleotide hybridization probes were obtained from GeneDetect (Bradenton, FL). All of the probes were cartridge purified to ensure >95% full length. Antisense probes were either single sequences or were a mixture of three sequences that recognize the same transcript (supplemental Table 1, available at www.jneurosci.org as supplemental material). To confirm the specificity of the antisense probe, control experiments with all corresponding sense RNA probes were performed. No signals were seen with any of the sense probes (data not shown). Oligonucleotides were 3′-end labeled with [^{35}S]dATP (PerkinElmer, Boston, MA) using terminal deoxynucleotidyl transferase (Roche, Lewes, UK). Labeled probes were purified on a probe purification column (Stratagene, La Jolla, CA), and their specific activities were determined by scintillation counting.

Low resolution in situ *hybridization histochemistry.* After 72 h of water deprivation, rats were killed by cervical dislocation (between the hours of 11:00 A.M. and 1:00 P.M.) and decapitated. Brains were rapidly removed and snap frozen over liquid nitrogen. Coronal sections (12 μm) of the hypothalamus were cut on a cryostat (Cryocut CM3050; Leica), thaw mounted on poly-L-lysine-coated microscope slides, and stored at −80°C until processed. On the day of fixation, the slide-mounted brain sections were removed from storage and allowed to equilibrate to room temperature (RT) for 15–20 min. The sections were fixed with 4% (w/v) cold paraformaldehyde in 1× PBS solution (made up within the last 7 d and stored at 4°C) for 5 min at RT and were rinsed twice with 1× PBS. The sections were then placed in 0.25% (w/v) acetic anhydride in 0.1 M triethanolamine HCl/0.9% (w/v) NaCl for 10 min at RT, followed by dehydration for 1 min each in 70% (v/v), 80% (v/v), then in 95% (v/v) for 2 min and 100% (v/v) EtOH for another minute. This was followed by a delipidation wash in chloroform for 5 min, followed by washes in 100% (v/v) and 90% (v/v) EtOH for 1 min each. The sections were then air dried. Labeled oligonucleotide probes (50,000–100,000 cpm) were diluted in 45 μl of a hybridization buffer containing 50% (v/v) deionized formamide, 4× SSC (1× SSC is 0.6 M NaCl and 0.06 M sodium citrate, pH 7.0), 500 mg/ml sheared DNA, 250 mg of yeast tRNA, 1× Denhardt's solution [0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, and 0.02% (w/v) BSA], and 10% (w/v) dextran sulfate. The slides were covered with Parafilm coverslips and then incubated overnight in a humidified atmosphere at 37°C. On the following day, slides were rinsed in 1× SSC, washed in 1× SSC at 55°C for 1 h (four times for 15 min), followed by additional washes in 1× SSC (two times for 30 min) at room temperature, given two quick rinses in distilled water, and dried with a warm stream of air. When dry, slides were apposed to Hyperfilm (Eastman Kodak, Hemel Hempstead, UK) with standard ^{14}C microscales in autoradiographic cassettes for a period of 2 weeks. After development, the

films were placed under the microscope (MZ6; Leica), and the images were captured with the camera and analyzed with NIH ImageJ 1.62 software. The density of the hybridization signal was assessed from x-ray film images by comparing the optical density of the autoradiograms to standard microscales. Each section was corrected for background by subtracting the value obtained from an area outlying the SON and PVN on the same section. Results were normalized and expressed as a ratio of control (euhydrated) levels. Four sections per rat taken at regular intervals through the PVN or SON of each rat from the respective groups were analyzed. Two sections from each rat were used to establish nonspecific binding. All values are given as mean ± SEM. The data were statistically analyzed with Student's *t* test (paired or unpaired as appropriate and when indicated) using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Statistical significance was assumed when $p < 0.05$.

High resolution in situ *hybridization histochemistry.* To obtain high-resolution images, hybridized and washed slides were emulsion dipped rather than being exposed to x-ray film. In the dark, under the safelight, 30 μl of 50% (v/v) glycerol and 6 ml of dH_2O was mixed with K-5 emulsion (Ilford Imaging, Knutsford, UK) to a final volume of 10 ml. The mixture was incubated at 45°C for 20 min until the emulsion had melted. The molten emulsion was then poured into a dipping chamber in the water bath and left for another 20 min to allow any air bubbles to escape. Each slide was dipped into the bottom of the vessel with a steady action, and excess emulsion was drained off. The slides were placed in a rack in the dark, allowed to dry for ~2 h, then placed in black boxes, and exposed at 4°C. After 60 d, in the dark under the safelight, the slides were removed from the black box and placed into metal racks. The racks were then placed in D19 developer for 3.5 min, indicator stop bath for 0.5 min, fixative for 3.5 min, and dH_2O for 5–30 min to wash off the fixative. After development, the slides were dipped in 95% (v/v) EtOH for 1 min, 100% (v/v) EtOH for 1 min, and 0.5% (w/v) toluidine blue for 1–5 min and were rinsed with dH_2O. The slides were air dried and mounted in DPX, covered with coverslips, and observed under the microscope. The processed sections were examined on a Leica DM IRB microscope. Photomicrographs were taken with a Leica DC-300F digital camera using IM50 software (version 1.2; Leica), saved as TIFF images at 300 dpi resolution.

Semiquantitative image analysis was conducted on emulsion-dipped sections. Cell counting was performed at 200× magnification on a Leica DM IRB microscope with C-plan optics. Grain counting was conducted under bright-field conditions at a magnification of 400× using the above microscope and a Leica 300DM digital camera. NIH ImageJ software was used to quantify the number of silver grains expressed in neurons of the SON and PVN. Only cells that expressed five times the background labeling (which was typically 5–15 grains per equivalent cellular area) were assessed. From each treatment group, two sections were selected from the middle region of each nucleus. These selected sections were carefully matched between groups. From each section, 10–15 labeled cells were selected at random from throughout the whole nucleus to determine the number of silver grains expressed in each cell. Data were analyzed with an unpaired Student's *t* test. Significance was assumed when $p < 0.05$.

Results

Microarray analysis reveals differential regulation of transcripts encoding transcription factors in the rat SON after 3 d of dehydration

We generated a gene list (supplemental Table 2, available at www.jneurosci.org as supplemental material) that, with a high degree of confidence, represents a catalog of 2453 transcripts that are significantly changed in abundance in the SON as a consequence of dehydration. We then examined this gene list using GeneSpring gene ontology tables, as well as manual inspection, with a view to identifying mRNAs encoding known transcription factors (supplemental Table 3, available at www.jneurosci.org as supplemental material). Forty-one probe sets gave significant data. Note that the transcripts encoding Crem (cAMP-responsive element modulator), Mlx (Max-like protein X), and Nr1d2 (nuclear receptor subfamily 1, group D, member 2) (also known as Rev-erb-β) were each recognized by two independent probe sets. Thus, in total, 38 transcripts encoding transcription factors were identified, 28 of which are upregulated by dehydration, whereas 10 are significantly downregulated (supplemental Table 3, available at www.jneurosci.org as supplemental material). Note that the false discovery rate of this analysis will be ~5% of identified genes.

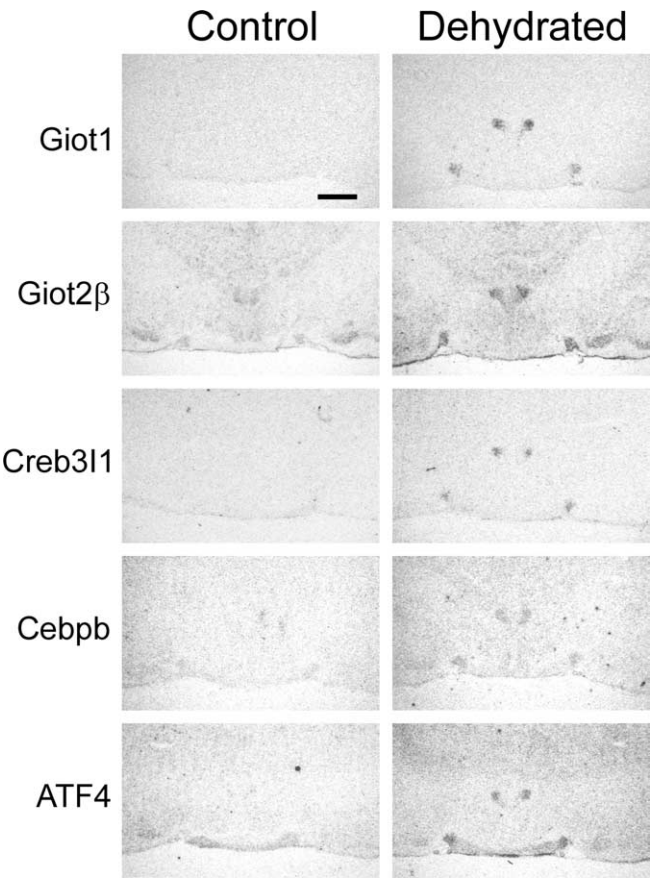

Figure 1. Low-resolution *in situ* hybridization analysis of transcription factor mRNA expression in the control and dehydrated hypothalamus. Scale bar, 1 mm.

In situ hybridization histochemistry confirmation of the microarray results

Five upregulated transcription factor transcripts were selected for additional study on the basis of their novelty and robust osmotic regulation. These are Giot1, cAMP-responsive element binding protein 3-like 1 (Crel3l1) (also known as OASIS), CCAAT/enhancer binding protein (C/EBP) β (Cebpb), activating transcription factor 4 (Atf4) [also known as cAMP-response element binding protein 2 (CREB2)], and Giot2. Note that two alternatively spliced Giot2 transcript variants (α and β) were independently examined. Low-resolution *in situ* hybridization histochemistry was used to validate the microarray data. Representative autoradiograms are shown in Figure 1, and quantification is presented in Table 1. Expression of Giot1 mRNA can barely be detected in the control rat brain, but 3 d of dehydration elicits a robust and significant upregulation specifically in the SON and PVN. No expression can be detected elsewhere in the brain. Even using a triple probe mixture, Giot2α mRNAs could not be detected in the rat brain at the level of the hypothalamus (data not shown). In contrast, in euhydrated rats, the alternatively spliced Giot2β mRNA has a widespread distribution in the

Table 1. Quantification of low-resolution *in situ* hybridization analysis of the changes in transcripts encoding transcription factors in the rat SON and PVN after 3 d of fluid deprivation

	SON		PVN	
	Fold change	*p* value	Fold change	*p* value
Giot1	15.9	0.0002	38.8	6E-06
Giot2β	1.62	0.0008	1.32	0.0745
Creb3l1	3.12	0.0002	2.97	0.007
Cebpb	2.13	0.0033	3.56	0.0663
Atf4	3.15	3E-05	2.69	0.0469

The *p* value was derived from unpaired Student's *t* test; $n = 4–5$.

brain, with expression being particularly evident in the SON and the PVN (Fig. 1) and also in the hippocampus and the cortex (data not shown). Fluid deprivation for 3 d significantly increases expression in the SON, but the increase in the PVN does not quite reach significance (Table 1). There is no significant change in Giot2β transcript levels in the hippocampus and cortex after dehydration ($p = 0.1769$ and 0.4817, respectively). Creb3l1 transcripts are barely detected in the SON and PVN of euhydrated rats (Fig. 1). After 3 d of dehydration, levels of Creb3l1 RNAs increase significantly in the SON and PVN (Fig. 1, Table 1). Creb3l1 transcripts are not seen in any other brain region. Cebpb is weakly expressed in the hippocampus of the euhydrated rat brain (data not shown), but only after dehydration does expression become clearly detectable in the SON and PVN (Fig. 1); fluid deprivation for 3 d significantly increases expression in the SON, but the increase in the PVN does not quite reach significance (Table 1). Atf4 transcripts are barely detected in the SON and PVN of euhydrated rats (Fig. 1). After 3 d of dehydration, levels of Atf4 RNAs increase significantly in the SON and PVN (Fig. 1, Table 1). Atf4 transcripts are not seen in any other brain region. We note that the dehydration-induced increases in transcript expression in the SON described by array analysis (supplemental Table 3, available at www.jneurosci.org as supplemental material) match this confirmatory *in situ* analysis (Table 1) very closely.

Cellular localization of transcripts encoding transcription factors

High-resolution radioactive *in situ* hybridization analysis using radiolabeled probes, followed by emulsion dipping was then used to determine which cell types in the hypothalamus express transcription factor mRNAs in the SON and PVN (Fig. 2) and the different divisions of the PVN (Fig. 3) (supplemental Figs. 1–4, available at www.jneurosci.org as supplemental material) under euhydrated and dehydrated conditions. In the SON, grains were evident over MCN-like cells (Fig. 2), with upregulation after 3 d of dehydration as assessed by grain counting (Table 2). The number of expressing cells significantly increased for Atf4, Creb3l1, Giot1, and Giot2β, whereas expression per cell significantly increased with Atf4, Cebpb, Giot1, and Giot2β (Table 2). In the PVN, Giot1 (Fig. 3), Giot2β (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), Creb3l1 (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), Cebpb (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), and Atf4 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) mRNAs are all widely distributed throughout the nucleus, in both magnocellular and parvocellular divisions. Depending on the transcription factor, dehydration had differential effects on expression in the parvocellular or magnocellular (Fig. 2) divisions of the PVN (Table 2). For example, after dehydration, the number of cells expressing Giot1 transcripts significantly increases in the magnocellular division of the PVN (Fig. 2) but not the parvocellular division. Similarly, expression per cell increases in the MCNs but not the parvocellular neurons. Although the number of parvocellular cells expressing any of the transcription factor RNAs is not changed by dehydration, there is a significant increase in the expression per cell of transcripts encoding Atf4, Creb3l1, and Giot2β (Table 2).

The cAMP-dependent protein kinase A pathway mediates the upregulation of Giot1 in the PVN after an osmotic stimulus

We asked whether a signaling pathway known to be upregulated in the dehydrated HNS impacted on the regulation of the Giot1 mRNA. The activity of the Giot1 proximal promoter has been shown to be induced by cAMP intracellular signaling pathways through a cAMP response element (CRE) located proximal to the start of transcription (Yazawa et al., 2003). Because cAMP pathways are upregulated in the SON and PVN after dehydration (Young et al., 1987; Carter and Murphy, 1989), we predicted that virally mediated *in vivo* inhibition of cAMP-dependent PKA activity (Wong et al., 2003) might attenuate the increase in Giot1 RNA levels in the PVN after dehydration. This is indeed the case; the upregulation of both the Giot1 mRNAs in the PVN after dehydration is significantly inhibited by blockade of PKA activity (Fig. 4). Viral infection per se, as demonstrated by the expression of eGFP from the control virus (Fig. 4*B*), has no effect on the gross anatomy of the PVN (Fig. 4*A*) or on the expression of the Giot1 RNAs (Fig. 4*C*,*D*).

Discussion

That the physiological activation of the HNS by dehydration is accompanied by a dramatic activity-dependent remodeling is well documented (Hatton, 1997; Theodosis et al., 1998). However, the molecular mechanics of these processes are not understood. We hypothesized that alterations in steady-state mRNA levels might be responsible, as least in part, for mediating and regulating SON functional plasticity (Sharman et al., 2004). We have thus used microarrays to compile transcriptome catalogs that, with a high degree of confidence, represent comprehensive descriptions of the RNA populations expressed in the SON (Hindmarch et al., 2006). Furthermore, we identified transcripts that are either upregulated or downregulated as a consequence of chronic dehydration (supplemental Table 2, available at www.jneurosci.org as supplemental material).

Although other processes, such as RNA stability, will be operational, we predict that some of the transcriptome changes seen in the SON after dehydration are transcriptional and are mediated by altered transcription factor activity. There are many ways in which transcription factor activity might be modulated, one of which is a change in the steady-state levels of its mRNA template. We have thus examined our microarray data to identify changes in the expression of mRNAs encoding transcription factors in response to water deprivation. We identified significant changes in the abundance of transcripts encoding 38 transcription factors (supplemental Table 3, available at www.jneurosci.org as supplemental material), 10 of which are downregulated, whereas 28 are upregulated.

A number of the transcription factor mRNAs identified here have been shown previously to be regulated in the SON by an osmotic challenge (supplemental Table 3, available at www.jneurosci.org as supplemental material). For example, the activator protein 1 (AP1) transcription factors Fosl1, c-Fos, and c-Jun have all been

shown to be upregulated in the SON after dehydration (Carter and Murphy, 1990; Sharp et al., 1991; Luckman et al., 1996; Miyata et al., 2001b; Cunningham et al., 2004; Ji et al., 2005; Kawasaki et al., 2005; Penny et al., 2005; Gottlieb et al., 2006). Here we identify JunD as also being upregulated (supplemental Table 3, available at www.jneurosci.org as supplemental material), an observation that we confirmed by *in situ* hybridization and immunocytochemistry (Yao et al., unpublished observation). Interestingly, it has been shown recently *in vitro* that any of the Fos proteins can synergize with any of the Jun family to upregulate the activity of the rat VP promoter via an AP1 *cis*-acting element (Yoshida et al., 2006).

Importantly, we identified transcription factors not previously known to be expressed in the HNS and that are differentially regulated therein by dehydration. Five transcription factors were selected for additional study: Giot1, Crel3l1, Cebpb, Atf4, and Giot2. For all five transcription factor mRNAs, *in situ* hybridization histochemistry confirmed the microarray data, revealing robust osmotic regulation of these transcripts not only in the SON but also, in some cases, in the PVN (Fig. 1, Table 1). Preliminary double-labeling experiments (data not shown) have revealed that VP-expressing MCNs in both the SON and PVN express the transcription factors but that many transcription factor-expressing cells do not contain VP gene product. It is likely that the latter cells express the closely related neuropeptide oxytocin (Xi et al., 1999). Interestingly, in the PVN (Fig. 3) (supplemental Figs. 1–4, available at www.jneurosci.org as supplemental material), Giot1, Giot2β, Creb3l1, Cebpb, and Atf4 mRNAs are widely distributed throughout the nucleus, in both magnocellular and parvocellular divisions. In some cases, dehydration evokes transcript level upregulation in MCNs but not parvocellular neurons (Giot1). In other cases, transcripts are also increased in abundance in parvocellular neurons but only in terms of expression level per cell (Atf4, Creb3l1, and Giot2β); the number of expressing cells does not significantly alter for any factor. The PVN parvocellular division is an important integrative structure that regulates coordinated responses to perturbations in cardiovascular homeostasis, such as dehydration. Through descending projections from parvocellular neurons to the brainstem and spinal cord (Sawchenko and Swanson, 1982), the PVN directly regulates changes in sympathetic nerve activity involved in blood pressure and blood volume regulation (Toney et al., 2003; Antunes et al., 2006). The role of these novel PVN transcription factors in the coordinated cardiovascular response to an osmotic stimulus will be a focus of future studies.

Although we know nothing about the functions of the five transcription factors studied here in HNS physiology, it is pertinent to briefly review what is already known about them in the context of general speculation regarding their putative roles in altering the transcriptional environment of the HNS after dehydration.

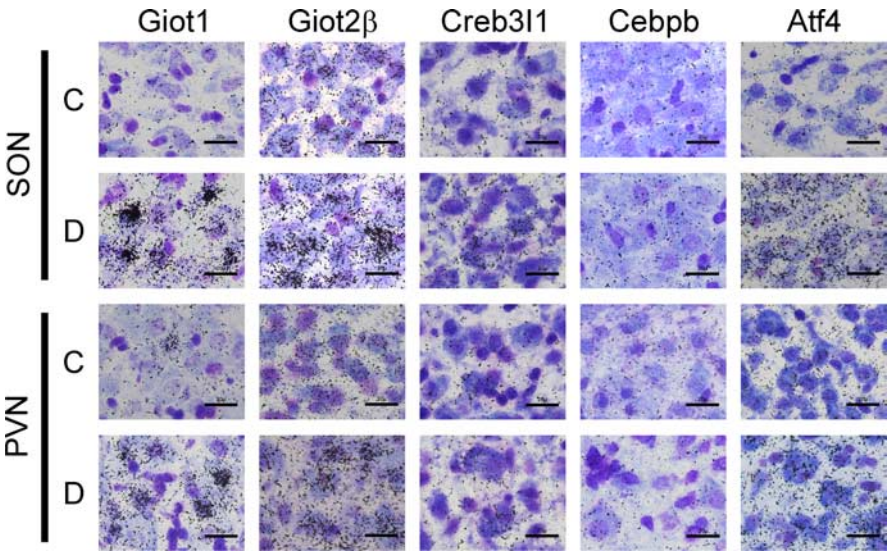

Figure 2. High-resolution *in situ* hybridization analysis of transcription factor mRNA expression in the PVN and SON of control (C) and dehydrated (D) rat brains. Scale bar, 20 μm.

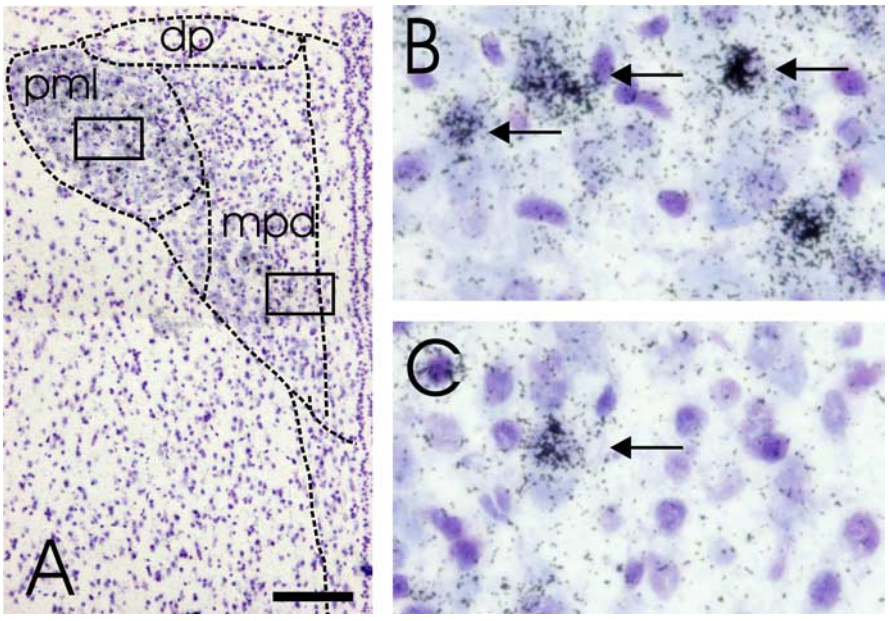

Figure 3. Representative photomicrographs showing the distribution of Giot1 mRNA in the dehydrated rat PVN as demonstrated by *in situ* hybridization histochemistry. ***A***, Neuronal labeling in both the magnocellular and parvocellular compartments of the PVN was observed. A higher-power photomicrograph of the magnocellular subdivision is shown in ***B***, and a higher-power photomicrograph of the parvocellular subdivision is shown in ***C***. Scale bar (in ***A***): ***A***, 200 μm; ***B***, ***C***, 20 μm. dp, Paraventricular nucleus of the hypothalamus, dorsal parvocellular part; mpd, medial parvocellular part; pml, posterior magnocellular part, lateral zone.

Giot1 and Giot2

Both Giot1 and Giot2 are Kruppel-type zinc finger proteins originally identified as being rapidly induced by gonadotropins in immature rat ovary granulosa cells (Mizutani et al., 2001). Two splice variants of Giot2 were identified (Giot2α and Giot12β); only the β variant is expressed in the HNS (this report). Giot1 has

Table 2. Effect of water deprivation on the distribution of cells expressing Atf4, Cebpb, Creb3l1, Giot1, and Giot2β in the SON and PVN of the control (C) and dehydrated (D) rat hypothalamus

	Atf4		Cebpb		Creb3l1		Giot1		Giot2β	
	C	D	C	D	C	D	C	D	C	D
Cells										
PVN (magnocellular)	43 ± 4	59 ± 3*	19 ± 2	25 ± 6	20 ± 2	53 ± 3**	13 ± 2	51 ± 2***	41 ± 2	60 ± 5**
PVN (parvocellular)	21 ± 2	32 ± 7	11 ± 1	12 ± 1	16 ± 2	17 ± 2	11 ± 1	16 ± 2	44 ± 3	50 ± 2
SON	41 ± 2	74 ± 3***	13 ± 3	20 ± 2	28 ± 2	42 ± 3**	13 ± 2	49 ± 3***	37 ± 2	59 ± 7*
Grains/cell										
PVN (magnocellular)	36 ± 3	93 ± 10**	34 ± 3	31 ± 2	31 ± 2	76 ± 3**	83 ± 11	196 ± 13**	83 ± 8	185 ± 10***
PVN (parvocellular)	30 ± 2	60 ± 8*	28 ± 2	31 ± 5	31 ± 5	70 ± 10*	38 ± 3	39 ± 7	47 ± 4	99 ± 6***
SON	44 ± 4	144 ± 10*	34 ± 2	45 ± 3*	28 ± 3	40 ± 5	47 ± 6	219 ± 9***	85 ± 6	204 ± 10***

Cells in the SON, the magnocellular PVN, and the parvocellular PVN were quantified at 200× magnification while the numbers of silver grains were quantified at 400× magnification. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (unpaired Student's t test). Data are expressed as mean ± SEM. $n = 3$–4 per group.

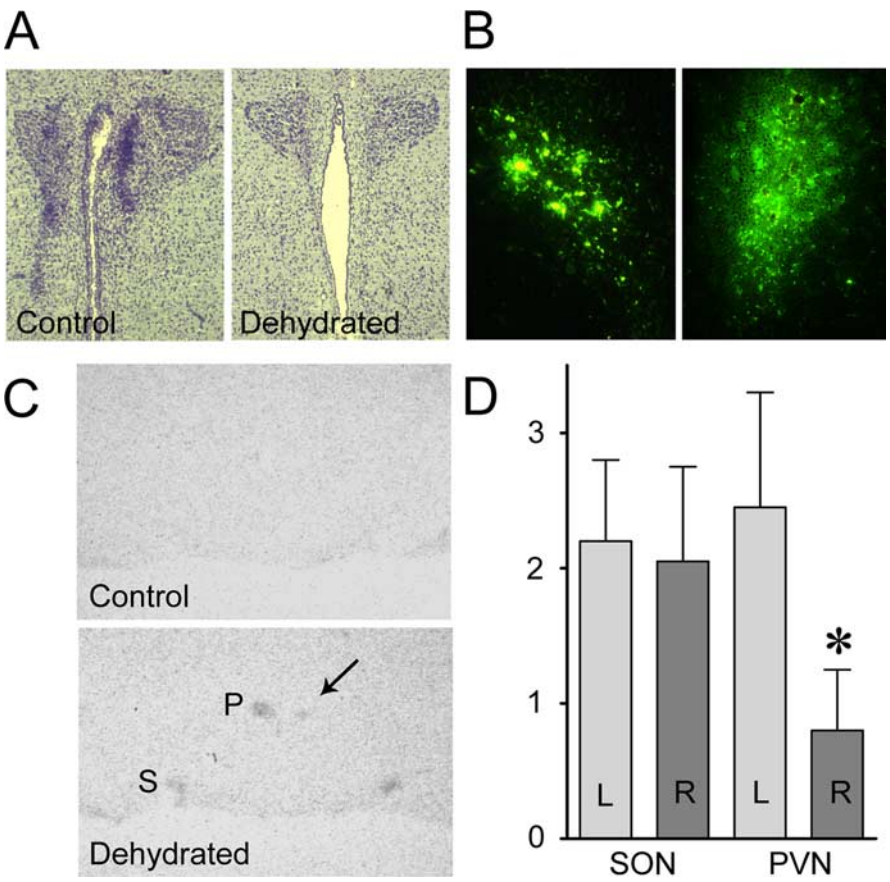

Figure 4. Inhibition of PKA attenuates Giot1 mRNA upregulation in the PVN of the dehydrated rat. The right side of the PVN was transduced with an adenoviral vector expressing a potent inhibitor of PKA and a virus expressing eGFP. The left side of the PVN was injected only with the virus expressing eGFP. ***A***, Representative photomicrographs of the PVN stained with toluidine blue. ***B***, Representative images of bilateral eGFP fluorescence in the PVN after viral transduction. ***C***, Representative low-resolution *in situ* hybridization autoradiogram showing attenuation of Giot1 mRNA induction in the right side of the PVN of dehydrated rats (arrow). No effect is seen in the left PVN (P) or the SON (S). ***D***, Quantification (arbitrary units) of Giot1 mRNA levels in the right (R) and left (L) SON and PVN. *$p = 0.03$, paired Student's t test; $n = 5$.

14 Kruppel-type (Cys)(2)-(His)(2)-type zinc finger domains, Giot2α has 18, and Giot12β has 6. Although Giot1 and Giot2 are remarkably similar (94% amino acid identity), only the latter has a KRAB (Kruppel-associated box)-A domain at the N terminus. Consequently, Giot1, but not Giot2, appears to function as a transcriptional repressor (Mizutani et al., 2001).

Creb3l1

Creb3l1 is a basic leucine zipper (bZIP) transcription factor of the ATF/CREB protein family with a transmembrane domain that allows it to associate with the endoplasmic reticulum (ER) (Omori et al., 2002). The molecule is cleaved at the membrane in response to ER stress, and its cleaved N-terminal cytoplasmic domain, which contains the bZIP domain, translocates into the nucleus in which it activates the transcription of target genes that are mediated by ER stress-responsive and cAMP-responsive elements (Kondo et al., 2005).

Cebpb

Cebpb is a bZIP transcription factor. C/EBP isoforms interact with each other through their leucine zippers to form homodimers or heterodimers that can directly recognize a consensus DNA sequence (TTNNGNAAT) in target genes (McKnight, 2001). In addition, the C/EBPs can also regulate target gene expression through interaction with other transcription factors or coactivators such as p300/CBP (McKnight, 2001). Extracellular signals can induce C/EBP phosphorylation and other posttranslational modifications, thereby modulating its interaction with DNA or with other proteins (McKnight, 2001). Interestingly, Cebpb has been shown to activate the promoter of the corticotropin-releasing factor gene (Stephanou et al., 1997), which is known to be upregulated in the SON and PVN after dehydration (supplemental Table 2, available at www.jneurosci.org as supplemental material) (Lightman and Young, 1987).

Atf4

Atf4 is a ubiquitous bZIP transcription factor that is a member of the ATF/CREB protein family (Rutkowski and Kaufman, 2003). Evidence has been presented that suggests that Atf4 can both activate and passively repress transcription of different target genes (Schoch et al., 2001).

Finally, we asked about the signaling pathways that mediate the dramatic upregulation of Giot1 transcripts in the HNS after dehydration. The activity of the Giot1 proximal promoter has

been shown to be induced by cAMP intracellular signaling pathways through a CRE (Yazawa et al., 2003). Furthermore, the Giot1 promoter has been shown recently to be a target of the orphan nuclear receptor Nr4a1 (nuclear receptor subfamily 4, group A, member 1) (also known as Nur77 or NGFIB) (Song et al., 2006) transcription factor, which is also upregulated in the SON after dehydration (supplemental Table 3, available at www.jneurosci.org as supplemental material) (Luckman, 1997; Kawasaki et al., 2005). Nr4a1 is also known to be under the control of cAMP pathways (Humphries et al., 2004). Because it is well documented that cAMP pathways are upregulated in the SON and PVN after dehydration (Young et al., 1987; Carter and Murphy, 1989), we asked whether their inhibition would attenuate the concurrent increases in Giot1 transcript levels. To achieve this *in vivo*, we introduced an adenoviral vector that encodes PKIα (Wong et al., 2003), a potent and specific peptide inhibitor of PKA (Dalton and Dewey, 2006), into one side of the PVN. A control virus expressing eGFP alone was introduced into the contralateral side of the PVN. PKIα expression significantly attenuated the upregulation of Giot1 mRNAs in the PVN after dehydration (Fig. 4*C*,*D*), strongly suggesting that cAMP pathways are mediating the osmotic regulation of the Giot1 gene. Because Giot1 is significantly upregulated only in PVN MCNs (Table 2), we can conclude that PKA has a specific role in osmotic regulation of Giot1 transcript levels in these neurons. Indeed, the residual PVN expression may be confined to parvocellular neurons. Viral infection per se, as demonstrated by the expression of eGFP from the control virus (Fig. 4*B*), has no effect on gross anatomy of the PVN (Fig. 4*A*) or on the expression of the Giot1 RNAs (Fig. 4*C*,*D*). However, we cannot rule out the possibility that the higher viral load in the experimental side of the PVN may have had a deleterious effect.

To conclude, we used transcriptome analysis to identify transcription factor RNAs that change in abundance in the HNS after dehydration stress. For one of these transcription factors, Giot1, we have shown that cAMP pathways mediate its upregulation in the HNS. In turn, the transcription factors encoded by these transcripts will act on the genome to alter the composition of the transcriptome. To "close the loop," we will now use chromatin immunoprecipitation to identify the target genes of our candidate transcription factors (Wu et al., 2006).

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