Physiological activation of the hypothalamo-neurohypophyseal system (HNS) by dehydration results in a massive release of vasopressin (VP) from the posterior pituitary. This is accompanied by a functional remodeling of the HNS. In this study we used cDNA arrays in an attempt to identify genes that exhibit differential expression in the hypothalamus following dehydration. Our study revealed nine candidate genes, including interleukin-6 (IL-6) as a putative novel secretory product of HNS worthy of further analysis. In situ hybridization and immunocytochemistry confirmed that IL-6 is robustly expressed in the supraoptic (SON) and the paraventricular (PVN) nuclei of the hypothalamus. By double-staining immunofluorescence we showed that IL-6 is largely co-localized with VP in the SON and PVN. In situ hybridization, immunocytochemistry, and Western blotting all revealed IL-6 up-regulation in the SON and PVN following dehydration, thus validating the array data. The same dehydration stimulus resulted in an increase in IL-6 immunoreactivity in the axons of the internal zone of the median eminence and a marked reduction in IL-6-like material in the posterior pituitary gland. We thus suggest that IL-6 takes the same secretory pathway as VP and is secreted from the posterior pituitary following a physiological stimulus.

The hypothalamo-neurohypophyseal system (HNS) consists of large magnocellular neurons (MCNs) of the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei that have axons terminating on blood capillaries of the posterior pituitary (1), along with associated glia, blood vessels, interneurons, and afferent terminals. The HNS is the source of the neuropeptide hormone vasopressin (VP), which has a crucial role in osmoregulation (2). Following the onset of an osmotic stimulus such as dehydration (fluid deprivation), mammals respond to plasma hyperosmolality by reducing the renal excretion of water. Tubular reabsorption of water is controlled by circulating levels of VP. A rise in plasma osmolality is detected by osmoreceptor mechanisms in the circumventricular organs (3–6). Subsequent angiotensinergic (7, 8) and glutamatergic (9–11) excitation of hypothalamic neurons leads to a massive release of stored VP into the general circulation. Through an interaction with V2-type receptors located in the kidney, VP increases the permeability of the collecting ducts to water, promoting water conservation by decreasing the amount of water lost in urine.

Physiological activation of the MCNs by dehydration results in a massive release of stored hormone from posterior pituitary terminals, and a concomitant functional remodeling of the HNS, characterized by activity-dependent secretory, electrophysiological, biosynthetic, and gene expression plasticity (12–14). At the morphological level, this plasticity is manifested as synaptic remodeling, increased direct neuronal membrane apposition and dendritic bundling in the SON, and by changes in the organization of neurovascular contacts in the neurohypophysis (12). For example, alterations in the relationship between MCNs and glia, the extent of terminal contact with the basal lamina in the neurohypophysis, and the extent of electrotocnic coupling between MCNs have all been described (12, 15, 16). However, the mechanistic basis of these effects has not yet been described in terms of the differential expression of genes.

We have used cDNA microarray gene expression profiling technology (17–19) to identify candidate genes that are differentially expressed in the SON following 3 days of dehydration. One of these genes, interleukin-6 (IL-6), has been reported to be involved in the hypothalamic-pituitary-adrenal axis activation (20–22) and therefore was studied in detail. We demonstrate robust expression of IL-6 mRNA and peptide in the SON and PVN, with significant up-regulation following dehydration. The concomitant decrease in IL-6-like immunoreactive material in the posterior pituitary suggests, for the first time, that this cytokine is a secretory product of the HNS.

**EXPERIMENTAL PROCEDURES**

**Animals**—Adult male Sprague-Dawley rats 10–12 weeks old were maintained in standardized conditions in accord with United Kingdom Home Office regulations. Dehydration involved complete fluid deprivation for 3 days.

**Total RNA Preparation**—Each experimental group was compared as replicates of three. For each replicate SONs from 12 rats were pooled before RNA extraction. Animals were sacrificed by cervical dislocation, and brains were removed. SONs were isolated on a coronal slice of 1–2 mm thick and then micro-dissected under microscope. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol.
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Microarray Target Labeling and Hybridization—Targets for cDNA microarrays were generated using 5 μg of total RNA from control and dehydrated rat SON in a standard reverse transcription reaction. RNA was annealed, in 16 μl of water, with 1 μg of 24-mer poly(dT) primer (Invitrogen), by heating at 65 °C for 10 min and cooling on ice for 2 min. The reverse transcription reaction was performed by adding 8 μl of 5× first strand reverse transcription buffer (Invitrogen), 4 μl of 20 mM dNTPs minus dCTP (Amersham Biosciences), 4 μl of 0.1% diithiothreitol, 40 units of RNase OUT (Invitrogen), 6 μl of 3000 Ci/mmol [32P]dCTP (ICN Biomedicals) to the RNA/primer mixture to a final volume of 20 μl. Two μl (400 units) of Superscript II reverse transcriptase (Invitrogen) were then added, and the sample was incubated for 30 min at 42 °C followed by additional 2 μl of Superscript II reverse transcriptase, 50% salmon sperm DNA, 0.5 μl of 5× glycine buffer, and 0.5 μl of 0.5 M EDTA. The samples were incubated at 65 °C for 30 min after addition of 10 μl of 0.1 M NaOH to hydrolyze and remove RNA. The samples were pH neutralized by the addition of 45 μl of 0.5 M Tris, pH 8.0, and purified using Bio-Rad 6 purification columns (Bio-Rad). The NIA neurarray consists of 1152 cDNAs printed on nylon membrane in duplicate (23). The arrays were hybridized with [α-32P]dCTP-labeled cDNA probes overnight at 50 °C in 4 ml of hybridization solution. Hybridized arrays were rinsed in 50 ml of 2× SSC and 1% SDS twice at 55 °C followed by washing one to two times with 2× SSC and 1% SDS at 55 °C for 15 min each. The microarrays were exposed to PhosphorImager screens for 1–3 days. The screens were then scanned with Amersham Biosciences STORM PhosphorImager (Sunnyvale, CA) at 50-μm resolution.

Microarray Data Analysis: z Normalization—ImageQuant software (Amersham Biosciences) was used to convert the hybridization signals on the image into raw intensity values, and the data thus generated were transferred into MS Excel spreadsheets, predesigned to associate the ImageQuant data format to the correct gene identities. Raw intensity data for each experiment was normalized by z transformation. Intensity data were first, log10-transformed and used for the calculation of z scores. z scores were calculated by subtracting the average gene intensity from the raw intensity data for each gene and dividing that result by the S.D. of all the measured intensities. Gene expression differences were then calculated by subtracting the average gene intensity by the difference observed gene z scores. The significance of calculated z differences can be directly inferred from measurements of the S.D. of the overall mean difference distribution. Assuming a normal distribution profile, z differences are assigned significance according to their relation to the calculated standard deviation of all the z differences in any one comparison. To facilitate comparison of z differences between several different experiments, z differences were divided by the appropriate standard deviation to give the z ratios (23).

Immunocytochemistry—Deeply anesthetized rats were perfused with 60 ml PBS and then with 60 ml of 4% (w/v) paraformaldehyde. Brains were then dissected into 4% (w/v) paraformaldehyde and postfixed overnight in the same fixative. Following dehydration for 0, 1, and 3 days, sections were placed in PBS for 1 day, 30-μm (for brains) and 16-μm (for pituitaries) cryostat sections were prepared on Superfrost plus slides (BDH) and incubated overnight in 1:100,000 dilution of horseradish peroxidase-coupled anti-goat (Sigma) secondary antibody for 1 h at room temperature, and bound antibody was visualized by diaminobenzidine reaction. Finally sections were mounted on glass slides, air-dried, and cover-slipped with Vectashield (Vector Laboratories) mounting medium.

Western Blot Analysis—Brain tissues were homogenized in 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM NaCl, 0.2 mAEDTA, 1 μg/ml pepstatin a, 1 μg/ml leupeptin, 1 mM dithiothreitol, 25% (v/v) glycerol. The homogenate was then frozen on dry ice for 5 min, thawed in ice water for 15 min, and centrifuged at 14,000 × g for 15 min. The clear supernatant defined as total protein extract was removed and stored at −70 °C. Protein concentrations were determined using the Bradford method (Bio-Rad).

Oligonucleotide microarray—NIA neurarray probes were hybridized with a fluorescently labeled oligonucleotide probe (Promega, UK) as described previously (24). Slides were washed four times in 1× SSC at 55 °C for 15 min each time and twice in 1× SSC and air-dried, and cover-slipped with Fluoresca (Vector Laboratories) mounting medium.

RESULTS

Microarray Findings—NIA neurarray probes were hybridized in triplicate with targets derived from SON of control rats and rats dehydrated for 3 days. Data analysis by z normalization of the hybridization signals enabled us to identify 9 candidate regulated genes. Four of these genes were up-regulated by dehydration, while five were down-regulated (Table I). Here, we pre-

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sent detailed data for IL-6, a gene identified as being up-regulated by dehydration. To confirm and extend the array data we investigated IL-6 expression at the mRNA and protein levels throughout the HNS, both before and after an osmotic stimulus.

IL-6 mRNA Is Up-regulated by Dehydration in the SON and PVN—Using in situ hybridization we have shown that following 3 days of fluid deprivation of rats, IL-6 mRNA increased significantly by 3.6- (p < 0.001) and 2.3- (p < 0.01) fold, respectively, in the SON and PVN (Fig. 1). The use of a sense probe did not give a signal (data not shown). We noticed that following osmotic stimulus IL-6 mRNA level increased only in the SON and PVN and not in the control tissues hippocampus and piriform cortex where IL-6 has the same mRNA level in control and dehydrated rats. For example, the normalized optical densities of IL-6 mRNA in control and dehydrated piriform cortex were, respectively, 1.89 ± 0.32 and 1.65 ± 0.18. In the dentate gyrus of the hippocampus, the normalized optical densities of IL-6 mRNA were 2.10 ± 0.13 in control versus 2 ± 0.24 in dehydrated.

IL-6 Protein Level Increases by Dehydration in the SON, PVN, and Median Eminence (ME)—As mRNA levels do not necessarily reflect the final steady-state levels of the functional gene product, we used Western blotting and immunocytochem-

**TABLE I**

| GeneBank™ accession no. | UniGene Cluster ID | Gene name | Up/Down | z ratio | S.E. |
|-------------------------|--------------------|-----------|---------|---------|------|
| W99328                  | Hs.8121            | ESTs, highly similar to notch homolog 2 (Homo sapiens) | Up       | 7.84    | 2.83 |
| W30935                  | Hs.111460          | ESTs, highly similar to multifunctional calcium/calmodulin-dependent protein kinase II β2 isoform (H. sapiens) | Up       | 4.05    | 1.35 |
| N98599                  | Hs.93913           | Interleukin 6 (interferon, β2; IL-6) | Up       | 2.6     | 0.44 |
| H45000                  | Hs.74122           | Caspase 4, apoptosis-related cysteine protease | Up       | 2.37    | 0.47 |
| N78582                  | Hs.50732           | Protein kinase, AMP-activated, β2 non-catalytic subunit | Down     | −1.83   | 0.47 |
| AA669443                | Hs.334810          | Eukaryotic translation initiation factor 5 (eIF-5) | Down     | −1.93   | 0.22 |
| AA464067                | Hs.6453            | Inositol 1,3,4-triphosphate 5/6 kinase | Down     | −2.03   | 1.52 |
| H98694                  | Hs.333282          | PI 3-kinase-related kinase SMG-1 | Down     | −2.39   | 0.76 |
| AA668470                | Hs.24950           | Regulator of G-protein signaling 5 (RGS5) | Down     | −2.90   | 0.75 |

**Fig. 1.** IL-6 in situ hybridization analysis. Control (A) and dehydrated (B) rat brain sections were hybridized with an antisense oligo probe corresponding to rat IL-6 as described under “Experimental Procedures.” Film autoradiographs were developed after 4 weeks then scanned. C represents a histogram of the normalized optical densities of IL-6 mRNA. CT, control; DH, dehydrated. p < 0.05 was considered significant. ***, p < 0.001; **, p < 0.01. D and E represent, respectively, non-radioactive ISH of control and dehydrated rat SON. Magnifications, ×8 (A, B) and ×80 (D, E).

**Fig. 2.** IL-6 immunoreactivity in the SON (A, B), PVN (C, D), ME (E, F), and posterior pituitary (G, H) of control (A, C, E, G) and 3-day dehydrated (B, D, F, H) rats. The IL-6 immunoreactivity was detected as described under “Experimental Procedures.” A FITC fluorescent secondary antibody was used. Green cells are IL-6 immunoreactive. Scale bars are: 60 μm (A–D), 100 μm (E, F), and 200 μm (G, H).
istry to assess protein levels and distribution throughout the HNS. Immunocytochemistry showed that following 3 days dehydration, IL-6-like immunoreactivity increased dramatically in the SON, PVN, and internal zone of ME (Fig. 2). Using Western blotting, we have shown 2- (4.18 \pm 1.06 in control versus 8.53 \pm 1.04 in dehydrated, \( p < 0.05 \)) and 1.6- (4.36 \pm 0.01 in control versus 7.07 \pm 0.53 in dehydrated, \( p < 0.05 \)) fold increases of IL-6 protein level in the SON (Fig. 6A) and PVN (Fig. 6A), respectively, following dehydration.

**IL-6 Is Co-localized with VP in the SON**—We then asked whether IL-6 is expressed by VP neurons of the SON and PVN. Using double staining immunocytochemistry, we showed that IL-6 is present within most VP cells in the SON (Fig. 3) and PVN (Fig. 4) of euhydrated and dehydrated rats. In addition, the same technique showed co-localization of VP and IL-6 in the internal zone of the ME (Fig. 5).

**IL-6 Is Released from the Posterior Pituitary following Dehydration**—IL-6 immunoreactivity in the MCN axons of the internal zone of the ME (Figs. 2 and 5) suggests that this protein can take the same secretory pathway as VP. We therefore asked whether IL-6 was present in the posterior pituitary. Using immunocytochemistry (Fig. 2) we have shown that the robust IL-6 staining seen in the posterior pituitary of euhydrated animals decreases dramatically following dehydration, suggesting a release of IL-6 from the magnocellular neuron terminals following an osmotic stimulus. This reduction was confirmed by Western blotting (2.7-fold decrease; 4.84 \pm 0.61 in control versus 1.75 \pm 0.53 in dehydrated, \( p < 0.05 \); Fig. 6).

**IL-6 Circulating Levels do Not Increase following Dehydration**—In accord with previous observations (25), serum IL-6 levels in control, euhydrated rats did not exceed the lower limit of detection of the enzyme-linked immunosorbent assay (16 pg/ml). Dehydration for either 1 or 3 days had no effect on this (data not shown).

**DISCUSSION**

Microarray analysis is an approach for expression profiling that provides the means to perform parallel analysis of thousands of genes in a single assay (26–28). The results provide a semiquantitative assessment of whether the expression of a gene has been up- or down-regulated or remains unchanged. As such, microarrays provide a powerful tool with which to investigate biological specimens to screen for alteration in mRNA levels that accompany, and may regulate, physiological change.

Our cDNA microarray analysis revealed nine genes as being potentially differentially expressed in the SON as a consequence of dehydration (Table I). As microarray findings must be confirmed using independent methodological criteria, one of
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FIG. 5. IL-6 and VP co-expression in the dehydrated rat ME. A, IL-6 expression. B, VP expression. C, IL-6 and VP co-expression. Immunoreactivities were detected as described under "Experimental Procedures." FITC and Texas Red fluorescent secondary antibodies were used to reveal IL-6 (green) and VP (red) immunoreactivities. Yellow cells are both IL-6 and VP immunoreactivities. Scale bars are 80 μm.

FIG. 6. Western blots of IL-6 in the SON (A), PVN (A), and posterior pituitary (B). The Western blots were performed, and IL-6-like immunoreactivity was visualized as described under "Experimental Procedures." Each lane contains 30 μg of total cell protein extracts. Arrows at the right indicate the position of a 26-kDa molecular weight standard. C, control; D, dehydrated.

Genes encoding secreted molecules co-expressed with VP are thought to have roles as autocrine or paracrine modulators of hormone release from the HNS, and the expression of some of these is regulated by osmotic stimuli. For example, peptides derived from VP and dynorphin precursors co-exist within neurosecretory vesicles of MCNs. Following an osmotic challenge, dynorphin mRNA levels increase in the SON and the PVN (31–33), but dynorphin-like immunoreactivity in the posterior pituitary is reduced following prolonged salt-loading, suggesting peptide release (34). Dynorphin, co-released with VP, is probably a local modulator of neurosecretion in the neural lobe (35, 36).

Enzyme-linked immunosorbent assay assay of serum did not reveal a measurable increase in IL-6 levels following dehydration, suggesting that the amount of IL-6 released from the posterior pituitary is insufficient to contribute significantly to the circulating pool. Instead, we suggest that IL-6, like dynorphin, might be involved in VP secretion at the level of the posterior pituitary gland. It is most likely that IL-6 exerts its effect in a paracrine fashion. A recent transgenic study showed that increased level of IL-6 following restraint stress were associated with an increase in plasma VP of GFAP-IL6 mice (37). Using a static rat hypothalamic explant incubation system, it has been demonstrated that IL-6 increases VP release (38) and that the stimulatory effect of IL-6 was blocked by cyclo-oxygenase inhibitors, suggesting the involvement of prostaglandins (38). Similarly, IL-6 increased GABA release from posterior pituitary explants, but only under depolarizing conditions (39). Again, this effect was abolished by incubation of the tissue with indomethacin, an inhibitor of cyclo-oxygenase activity, indicating that prostaglandins could mediate the stimulation of GABA release induced by IL-6 (39). Interestingly, plasma VP levels were also elevated during the 2 h after IL-6 injection in cancer patients, suggesting that IL-6 is a secretagogue of magnocellular VP secretion in humans (20). In addition, IL-6 release might also be involved in the oxytocinergic system, since it has been shown that IL-6 increases oxytocin release (38).

We have used array analysis to identify candidate genes differentially expressed in the rat SON following a 3-day dehydration stimulus. We have thus revealed IL-6 as a novel secretory product of the hypothalamo-neurohypophyseal system, the expression of which is up-regulated following dehydration. Our study has thus demonstrated the utility of array technology for the identification of new candidate genes that might be involved in HNS physiological plasticity. In this study we interrogated only 1152 gene sequences, a fraction of the total number of genes in the rat genome. Clearly our array findings
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represent only a partial picture of the gene expression changes that follow dehydration. Encouraged by our results, we are currently using Affymetrix GeneChips (40) to obtain a more complete picture of the global expression patterns in control and physiologically stimulated SON.

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