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Regulation of Hypothalamic Magnocellular Neuropeptides and their mRNAs in the Brattleboro Rat: Coordinate Responses to Further Osmotic Challenge

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A paradigm was developed for the chronic osmotic stimulation of homozygous diabetes insipidus rats of the Brattleboro strain, a strain that fails to synthesize vasopressin. This study examines the adaptation of 2 sets of coexisting peptide hormone magnocellular neurons in the hypothalamo-neurohypophyseal system (HNS) of Long Evans (LE), Brattleboro heterozygote (HZ), and Brattleboro homozygote (DI) rats: (1) the arginine^8-vasopressin (AVP)/dynorphin (DYN) neurons, and (2) the oxytocin (OT)/cholecystokinin (CCK8) neurons of the paraventricular and supraoptic nuclei, which project to the posterior pituitary. The regimen of chronic intermittent salt-loading (CISL) involved the replacement of 2% saline for normal drinking water for 18 hr/d. This protocol effectively increased plasma levels of AVP and OT in LE and HZ rats, oxytocin in DI rats, and maintained the posterior pituitary in a state depleted of AVP, OT, CCK, and peptides derived from pro-dynorphin: DYN A 1–17, DYN A 1–8, and DYN B 1–13. The ratio of pituitary DYN A 1–17 to DYN A 1–8 content in DI rats or in LE, HZ, and DI rats following 6 d of CISL suggests a preferential release of DYN A 1–17 during periods of chronic secretory activity. In response to chronic secretory activity, mRNAs for AVP, OT, DYN, and CCK increased 1.5–2-fold in all 3 AVP rat strains, with mRNAs for coexisting peptide hormones displaying parallel increases. Mutant AVP mRNA in the DI rat was expressed at very low levels and DYN mRNA in very high levels, with each of these mRNAs continuing to be regulated by CISL in a normal manner. These results suggest a regulatory relationship between AVP and OT neurons, in which vasopressin neurons are feedback-regulated by AVP, most likely via plasma osmolarity, and that oxytocin neurons are modulated by peptides derived from pro-dynorphin.

The hypothalamo-neurohypophyseal system (HNS) is a neuroendocrine pathway comprised of magnocellular neurons within the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, which project to the posterior pituitary, where their secretory products have access to the circulatory system. Magnocellular neurons of the HNS are classically known for the synthesis of the blood-borne nonapeptide hormones arginine^8-vasopressin (AVP) and oxytocin (OT), 2 of the original peptide hormones thought to be synthesized as part of larger (Sachs and Takabatake, 1964), and perhaps common (Fawcett et al., 1968), precursors, along with their respective binding proteins, neurophysins I and II. Although the PVN contains nearly every peptide hormone found in the CNS, many of which have been suggested or shown to coexist to some degree with AVP or OT, the coexisting peptide pairs of AVP and pro-dynorphin (DYN; Watson et al., 1982), and OT and cholecystokinin (CCK; Vanderhaeghen et al., 1981) represent the 2 predominant neuronal types described thus far in the magnocellular hypothalamus. Electron-microscopic immunocytochemical evidence revealed that these peptide pairs coexist within neurosecretory granules in terminals of the posterior pituitary (Martin et al., 1983; Whitnall et al., 1983), results that convey, in these 2 instances at least, that coexistence connotes cosecretion. Only recently, however, have studies begun to describe the coordinate or parallel regulation of expression exhibited by the synthesis and secretion of coexisting peptide hormones (Höllt et al., 1981; Deschepper et al., 1983) and their mRNAs (Sherman et al., 1986a).

One hallmark of secretory studies on peptidergic cells, be they purely neuronal as in the nigral-striatal system, purely endocrine as in the anterior pituitary or adrenal, or a combination of both as in the HNS, is the apparent coupling between secretory activity and the expression of the mRNA coding for the peptide being secreted. The neuron or cell adapts to periods of persistent secretion by increasing its capacity to synthesize secretory products. In neurons containing multiple transmitters, such as the AVP/DYN neurons of the Sprague-Dawley rat, chronic secretory activity induced by hyperosmotic challenge increased the cytoplasmic pools of pro-vasopressin/neurophysin I and pro-dynorphin mRNAs in a parallel manner (Sherman et al., 1986a). It remains unclear, however, whether the coordinate regulation

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of AVP and DYN mRNAs implies a similarly coordinated function for AVP and DYN peptides. The very low concentration of pro-dynorphin-derived peptides relative to AVP effectively limits their likely target sites to regions proximal to AVP/DYN terminals in the posterior pituitary. The identification of kappa opioid receptors in this region (Bunn et al., 1985; Gerstberger and Barden, 1986; Mansour et al., 1986) has raised speculation that 2 kappa-binding peptides derived from pro-dynorphin, DYN A 1–17 and DYN A 1–8, may be acting as neuroendrocine secretory modulators (Tweedle and Hatton, 1980; Bicknell and Leng, 1982; Gerstberger and Barden, 1986; Bondy et al., 1987; Brady and Herkenham, 1987). Studies examining the function of DYN peptides have been hampered by the lack of specific kappa receptor antagonists. A possible alternative would be to examine the regulation of DYN peptides and mRNA in a system devoid of AVP. Extension of the classic chronic salt-loading paradigm to the Brattleboro rat, therefore, afforded the unique opportunity to study the regulation of DYN peptides and mRNA, and their regulatory relationship with OT/CCK neurons, in the absence of AVP.

Diabetes insipidus rats of the Brattleboro strain fail to synthesize biologically active AVP in the HNS (Valtin et al., 1965), and, as a consequence, suffer almost constant hyperosmolarity caused by an inability to regulate water balance. Although the genetic basis of this mutation in AVP synthesis has been elucidated (Schmale and Richter, 1984), until recently, the Brattleboro rat's most active services have not been in expression studies, but in studies examining the *role* of AVP peptides: in development and learning, in water and sodium balance, in stress or in the maintenance of cardiovascular tone, or in its relative contributions as an anterior pituitary-releasing hormone. Beyond these physiological experiments, however, the Brattleboro rat is now beginning to yield valuable information that is concerned less with vasopressin itself than with general regulatory mechanisms involved in the transcription, translation, and cotranslational processing of an mRNA that contains a frame-shift point deletion (Schmale et al., 1984; Ivell et al., 1986). Because of our interests in the physiological regulation of coexisting peptide hormones and their mRNAs in the HNS, and in the cellular responses to, and the regulation of, mutant mRNA expression, we have developed a paradigm that permits the additional chronic osmotic challenge of the HNS in the Brattleboro rat. This paradigm not only allows for the examination of changes in mRNA expression, pro-hormone processing, and pituitary peptide content in a secretory pathway that is already greatly stimulated, but allows for this analysis in an environment where one key regulatory feedback component (AVP) is missing.

Materials and Methods

Materials

Nytran (0.45 μm) membrane was obtained from Schleicher & Schuell (Keene, NH). Oligo(dT)-cellulose, type 3, was obtained from Collaborative Research (Lexington, MA). Cesium chloride, electrophoresis-grade agarose, proteinase K, and most enzymes necessary for DNA digests and nick-translation were obtained from Bethesda Research Labs (Gaithersburg, MD). All deoxy- and ribonucleotide triphosphates were purchased from Pharmacia (Piscataway, NJ). Ribonuclease-free glycogen (901 393) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Cytidine 5′-triphosphate, tetra-triethylammonium salt, and alpha-^{32}P at >600 Ci/mmol were obtained from ICN Biomedicals (Costa Mesa, CA). Guanidine thiocyanate and morpholinopropane sulfonic acid (MOPS) were obtained from Fluka Chemical (Hauppauge, NY). The lithium salt of dodecyl sulfate (LiDS) was obtained from Aldrich Chemical (Milwaukee, WI). The 37% formaldehyde solution (Mallinckrodt, Paris, KY) was stored at 4°C and used within 14 d of opening. In all of the following methods, the use of phenol implies redistilled phenol buffered with Tris-HCl, pH 8.0; the use of chloroform implies 24:1 chloroform : isoamyl alcohol; and the use of phenol/chloroform implies a 1:1 (vol/vol) mixture of phenol and chloroform.

Methods

Chronic intermittent salt-loading and dissections. Nine-week-old male Long Evans (LE) rats and male Brattleboro heterozygotes (HZ) and homozygotes (DI) were purchased from Blue Spruce Farms (Altamont, NY). Brattleboro DIs were bred in DI mothers mated with DI males, while HZs were bred from LE mothers mated with DI males. The rats were individually caged on a 12 hr light–dark cycle (0600 on, 1800 off) and maintained with free access to water and Purina Lab Rodent Chow for 7 d prior to salt-loading. The chronic intermittent salt-loading (CISL) regimen involved the substitution of 2% (wt/vol) sodium chloride (0.34 M NaCl) for drinking water from 1600 hr until 1000 hr, with fresh, normal water present for the remaining 6 hr. The amount of water or saline consumed and the animal's weight were recorded at the end of each time period (1000 and 1600 hr). On day 6, the animals were killed by decapitation at 1000 hr. The hypothalamic magnocellular nuclei (PVN and SON) were fresh-dissected from ~1.0 mm sagittal sections, using a modification of the Palkovits punch procedure, as previously described (Sherman et al., 1986c). Posterior pituitaries (neural lobe plus adhering intermediate lobe) were dissected and frozen on dry ice. Anterior pituitary tissues were obtained by dissecting the anterior lobes lateral to the remaining intermediate lobe after the neurointermediate lobe had been removed. All tissues were frozen on dry ice and stored at −80°C until use.

Radioimmunoassays (RIAs). Individual posterior pituitary lobes were homogenized in 2.0 ml of an ice-cold mixture of methanol:0.1 *N* hydrochloric acid (MeOH:HCl; 1:1 vol/vol) and centrifuged at 25,000 × *g* for 20 min. The supernatants were lyophilized and redissolved in 1.0 ml of MeOH:HCl. All samples and peptide standards were diluted in MeOH:HCl. The efficiency of peptide recovery for this extraction procedure was determined by the addition of known amounts of ^{3}H-DYN A 1–17, ^{3}H-DYN A 1–8, or ^{3}H-DYN B 1–13 peptides (the gift of Richard Houghten, Scripps Institute) to posterior pituitaries prior to homogenization. The recoveries were greater than 95% for all 3 pro-dynorphin peptides.

DYN A 1–17, DYN A 1–8, and DYN B 1–13 (Bachem, Torrance, CA) were labeled by chloramine-T-mediated iodination. Iodination reaction mixtures were applied to Sep-Pak cartridges (Waters Associates, Milford, MA), washed with 0.1% trifluoroacetic acid (TFA), and eluted with 60% acetonitrile in 0.1% TFA. The iodinated peptides were subsequently purified by high-performance liquid chromatography (HPLC), using a Waters μ-Bondapak C_{18} reverse-phase column (3.9 × 300 mm). The column was eluted with a linear gradient of 15–40% acetonitrile in 60 min. The purified iodinated peptides were diluted with MeOH:HCl and stored at −20°C.

DYN RIA procedure. To polypropylene tubes were successively added (1) 0.1 ml of peptide standard or sample diluted in MeOH:HCl, (2) 10,000 cpm of the iodinated peptide in 0.1 ml of RIA buffer (150 mM sodium phosphate buffer, pH 7.2, containing 0.1% bovine serum albumin, 0.9% sodium chloride, 0.1% Triton X-100, and 0.01% sodium azide), and (3) the appropriate dilution of antiserum in 0.1 ml of RIA buffer. The mixture was vortexed and incubated at 4°C for 24 hr. The bound, labeled antigen was separated from the free, labeled antigen by the addition of 0.6 ml of a charcoal slurry [5 gm of carbon, Norit A (J. T. Baker, Phillipsburg, NJ), 0.3 gm of Dextran, M_r 77,800, 15 ml of swine serum (Gibco, Grand Island, NY) in 100 ml of 150 mM sodium phosphate buffer, pH 7.2]. After centrifugation at 7000 × *g* for 10 min, the supernatants were decanted and counted on a TM Analytic 1290 gamma counter (Elk Grove Village, IL) for 1 min each. Samples were assayed in duplicate and each assay was performed at least twice.

Antisera against DYN A 1–17, DYN A 1–8, and DYN B 1–13 were raised in our laboratory by immunizing rabbits with thyroglobulin-conjugated peptides, as previously described (Khachaturian et al., 1982). The properties of each antiserum were tested under the above RIA conditions for cross-reactivities to similar and dissimilar peptides. The antiserum against DYN A 1–17 (serum 54, bleed 7, dilution 1:100,000) had an IC_{50} of 10 fmol/tube. The molar cross-reactivity to DYN A 1–8, leucine^{5}-enkephalin, α-neo-endorphin, and DYN B 1–13 was less than 0.001%. The cross-reactivity to DYN A 1–13 was 30%, but only

Table I. Body weights (grams) during a 6-day time course of Chronic Intermittent Salt-Loading.

Body Weight	Day 0 4:00p	Day 1 10:00a	Day 1 4:00p	Day 2 10:00a	Day 2 4:00p	Day 3 10:00a	Day 3 4:00p	Day 4 10:00a	Day 4 4:00p	Day 5 10:00a	Day 5 4:00p	Day 6 10:00a
LE Control	275 ± 10 (21)	288 ± 11 (13)	283 ± 12 (13)	296 ± 11 (13)	289 ± 11 (13)	304 ± 11 (13)	296 ± 11 (13)	304 ± 11 (13)	299 ± 11 (13)	312 ± 10 (13)	305 ± 11 (13)	317 ± 9 (21)
LE Salt-Loaded	290 ± 10 (21)	287 ± 10 (13)	294 ± 10 (13)	289 ± 9 (13)	295 ± 10 (13)	294 ± 10 (13)	302 ± 10 (13)	297 ± 10 (13)	307 ± 10 (13)	303 ± 9 (13)	310 ± 9 (13)	307 ± 8 (21)
HZ Control	296 ± 10 (21)	294 ± 15 (13)	305 ± 12 (13)	313 ± 13 (13)	307 ± 13 (13)	322 ± 13 (13)	315 ± 12 (13)	325 ± 12 (13)	320 ± 12 (13)	334 ± 12 (13)	328 ± 12 (13)	337 ± 11 (21)
HZ Salt-Loaded	319 ± 8 (21)	312 ± 8 (13)	319 ± 8 (13)	310 ± 9 (13)	321 ± 9 (13)	312 ± 9 (13)	324 ± 9 (13)	314 ± 8 (13)	328 ± 8 (13)	318 ± 8 (13)	334 ± 7 (13)	323 ± 6 (21)
DI Control	227 ± 7 (21)	231 ± 10 (13)	224 ± 11 (13)	238 ± 10 (13)	225 ± 10 (13)	244 ± 10 (13)	232 ± 10 (13)	247 ± 10 (13)	236 ± 10 (13)	251 ± 10 (13)	241 ± 11 (13)	258 ± 7 (18)
DI Salt-Loaded	247 ± 5 (21)	210 ± 4 (13)	241 ± 7 (13)	205 ± 6 (13)	240 ± 7 (13)	204 ± 6 (13)	242 ± 8 (13)	205 ± 6 (13)	240 ± 7 (13)	205 ± 5 (13)	242 ± 7 (13)	209 ± 4 (21)

All values are Mean ± S.E.M. LE = Long Evans HZ = Brattleboro heterozygote DI = Brattleboro homozygote

Parenthesis indicate (n).

7% to DYN AB 1–32. Thus, this antibody has poor recognition of the N-terminus of DYN A 1–17, but clearly recognizes the midportion and C-terminus of the peptide. The antiserum to DYN A 1–8 (serum 73, bleed 10, dilution 1:300,000) had an IC_{50} of 6 fmol/tube. Its cross-reactivity to α-neo-endorphin was 0.01%, while DYN A 1–17, DYN B 1–13, and leucine^5-enkephalin had cross-reactivities of less than 0.001%. This antibody is mainly C-terminus-directed. The antiserum to DYN B 1–13 (serum 94, bleed 7, dilution 1:50,000) had an IC_{50} of 15 fmol/tube. The cross-reactivity with DYN A 1–8 was 0.002%, and with DYN A 1–17, α-neo-endorphin, and leucine^5-enkephalin was <0.001%. This antibody is also mainly C-terminus-directed.

Assays for AVP and OT were performed as previously described (Seif et al., 1978; Amico et al., 1981). Neurointermediate lobes (NILs) were extracted using the same procedure described above. For plasma assays, blood was collected from the severed neck into Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing K_3EDTA on ice. The plasma supernatants, recovered after centrifugation at 4°C, were made 0.1 *N* in HCl, frozen on dry ice, and stored at −80°C. Prior to assay, acidified plasma samples were fractionated on C_{18} Sep-Pak cartridges, as previously described (Cahill et al., 1983). Samples were assayed in duplicate. The OT plasma RIA was linear from 0.2 to 50 microunits. The smallest amount of AVP that could be distinguished from zero with 99% confidence was calculated to be 0.46 fmol/ml.

Assays for CCK8 were performed essentially as previously described (Geola et al., 1981), using NILs extracted as described above. The antisera (5135) were raised in rabbits against synthetic, unsulfated CCK8 (Squibb and Sons, New York) conjugated to bovine serum albumin. This antiserum did not distinguish between CCK8 and gastrin17, but the absence of detectable gastrin17 in rat brain and NIL (Beinfeld et al., 1980) obviated the need for a more specific CCK8 assay. The assay used synthetic, unsulfated CCK8 as the iodinated trace, and synthetic sulfated CCK8 as the standard. Samples were assayed in triplicate. The assay was linear from 0.21 to 46 fmol CCK8.

Isolation of total RNA, total nucleic acids, and poly(A)RNA. For the quantitation of pro-dynorphin mRNA, total cytoplasmic RNA was isolated from the pooled tissues of 3 animals, using a modification of the CsCl/guanidinium isothiocyanate method, as previously described (Sherman et al., 1986a). The polyadenylated RNAs were isolated with oligo(dT)-cellulose in a microbatch procedure (Sherman et al., 1986a, c). The finaly poly(A)RNA fraction was ethanol-precipitated from 0.25 M sodium acetate with 20 μg glycogen. Data from our laboratory and others (Bird, 1986) showed that the recovery of small mRNAs was inefficient using the CsCl/guanidinium isothiocyanate method. For AVP, OT, and CCK mRNAs, therefore, none of which are longer than 750 nucleotides, isolation of total nucleic acids was performed by LET extraction. Single tissue punches or anterior pituitary lobes were homogenized in 0.3 ml LET buffer: 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 1% (wt/vol) of 36.7 mM LiDS containing 0.1 mg/ml proteinase K. The homogenate was incubated at 42°C for 1 hr, extracted once with buffered phenol, once with phenol : chloroform, and once with chloroform (each at 4°C). Total nucleic acids were recovered by ethanol precipitation. Specific mRNA recoveries improved with LET extraction (by approximately 15–20% for AVP mRNA). Relative recoveries from control and experimental groups were not significantly affected (assayed by Northern analysis; data not shown). All RNAs and nucleic acids were stored under 70% ethanol at −20°C until use.

Northern gel analysis. Poly(A)RNAs were fractionated on a vertical 1.5% agarose gel containing 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 5.0 mM sodium acetate, and 1.0 mM Na_2EDTA. Details of sample preparation, electrophoresis, passive transfer to Nytran membranes, hybridization with nick-translated cDNAs, and washing have been described elsewhere (Sherman et al., 1986a, c). Northern membranes from the single-punch total nuclei acids were probed for AVP, OT, and CCK (not necessarily in that order). After a good exposure was obtained for a particular probe, the membranes were washed in 0.1% SDS at 90–100°C for 15 min. For subsequent probes, membranes were prehybridized and hybridized as usual. Figure 2 schematically illustrates the portions of pro-vasopressin and pro-oxytocin cDNA that were isolated and labeled by nick-translation for peptide-specific hybridization probes. Pro-dynorphin mRNA was detected with a nick-translated 1516 bp SacI fragment of the rat dynorphin gene, corresponding to a midportion of the main exon (Civelli et al., 1985; Sherman et al., 1986a). Anterior pituitary proopiomelanocortin (POMC) mRNA was detected with a 939 bp mouse cDNA (Roberts et al., 1979). Procholecystokinin mRNA was detected using a 527 bp rat cDNA (Deschênes et al., 1984). Northern autoradiograms were quantitated on a Loats image analysis system as previously described (Sherman et al., 1986a, c), with the final total integrated optical density (IOD) values expressed relative to the IOD value for the LE control of that mRNA.

Results

Chronic intermittent salt-loading

For diabetes insipidus animals of the Brattleboro strain, CISL was the most potent osmotic challenge that still ensured adequate body weight maintenance. Brattleboro DI rats could not survive 48 hr on 2% saline, yet, when given access to fresh water for a short period during each day, could maintain a relatively constant overall body weight for a 6 d regimen. As shown in Table I, the daily body weight measurements for salt-loaded DI rats were comprised of diurnal weight fluctuations, while on 2% saline, of approximately 36 gm: −36.45 ± 0.98 gm from 1600 to 1000 ($n = 84$; SEM); while on fresh water, these measurements were +35.70 ± 1.08 gm from 1000 to 1600 ($n = 70$). This is

Table II. The amount of water and 2% saline (ml) ingested during a 6-day time course of Chronic Intermittent Salt-Loading.

Drinking	Day 0 4:00p	Day 1 10:00a	Day 1 4:00p	Day 2 10:00a	Day 2 4:00p	Day 3 10:00a	Day 3 4:00p	Day 4 10:00a	Day 4 4:00p	Day 5 10:00a	Day 5 4:00p	Day 6 10:00a
LE Control	- - -	28 ± 2 (21)	2 ± 0 (21)	27 ± 2 (21)	1 ± 0 (18)	35 ± 2 (18)	2 ± 0 (18)	33 ± 2 (18)	2 ± 0 (18)	37 ± 2 (18)	2 ± 0 (18)	44 ± 2 (18)
LE Salt-Loaded	- - -	**37 ± 5** (18)	12 ± 1 (18)	**35 ± 3** (18)	12 ± 1 (18)	**45 ± 3** (18)	17 ± 1 (18)	**57 ± 4** (18)	16 ± 1 (18)	**58 ± 4** (18)	12 ± 1 (18)	**67 ± 4** (18)
HZ Control	- - -	22 ± 2 (21)	1 ± 0 (21)	23 ± 2 (21)	2 ± 1 (18)	27 ± 2 (18)	2 ± 0 (18)	32 ± 3 (18)	2 ± 0 (18)	37 ± 1 (18)	2 ± 0 (18)	44 ± 1 (18)
HZ Salt-Loaded	- - -	**42 ± 4** (18)	12 ± 1 (18)	**44 ± 5** (18)	15 ± 1 (18)	**46 ± 5** (18)	17 ± 1 (18)	**56 ± 4** (18)	19 ± 1 (18)	**55 ± 3** (18)	20 ± 0 (18)	**67 ± 4** (18)
DI Control	- - -	130 ± 4 (21)	16 ± 2 (21)	130 ± 7 (21)	15 ± 1 (18)	141 ± 7 (18)	22 ± 2 (18)	148 ± 6 (18)	19 ± 2 (18)	156 ± 6 (18)	25 ± 1 (18)	157 ± 5 (18)
DI Salt-Loaded	- - -	**21 ± 3** (18)	59 ± 2 (18)	**35 ± 3** (18)	57 ± 1 (18)	**28 ± 2** (18)	57 ± 2 (18)	**42 ± 3** (18)	57 ± 1 (18)	**36 ± 1** (18)	59 ± 1 (18)	**37 ± 2** (18)

All values are Mean ± S.E.M. **LE** = Long Evans **HZ** = Brattleboro heterozygote **DI** = Brattleboro homozygote

Bold type numbers indicate 2% saline values. Parenthesis indicate (n).

contrasted with control DI animals on continual fresh water: +14.36 ± 0.86 gm (n = 78) during the 18 hr period, versus −10.35 ± 0.70 gm (n = 65) during the 6 hr daytime period. The fluctuations were much more muted for LE and Brattleboro HZ: on average, control LE and HZ rats gained 10.58 ± 1.34 gm (n = 156) during the 18 hr period and lost 4.36 ± 1.28 gm (n = 130) during the daytime 6 hr. CISL LE and HZ animals lost 7.08 ± 0.72 gm (n = 156) and gained 10.12 ± 0.59 gm (n = 130) during the same 2 periods. These weight changes were directly attributable to water loss and gain as a reflection of drinking behavior, shown in Table II.

Although CISL was intended to provide a chronic osmotic stimulus to LE, HZ, and DI rats during this study, the degree to which it could also be serving as a stressor, affecting the hypothalamic–pituitary–adrenal axis, was investigated. Anterior pituitary concentrations of POMC mRNA (relative content per microgram of total nucleic acids) was not significantly increased after 6 d of CISL for LE, HZ, or DI rats (data not shown; n = 4, Student t test: $p > 0.1$). DI animals, however, contained 25% more POMC mRNA than did control LE and HZ rats ($p < 0.01$).

Plasma levels of AVP and OT

The ingestion of 2% saline in LE and HZ animals during CISL stimulated the secretion of AVP and OT. Plasma levels of AVP after the second night of CISL were elevated 3-fold over controls (Fig. 1, left). After 6 d, however, plasma concentrations of AVP were no longer significantly different from that of controls. Plasma levels of OT responded similarly. Control levels of OT for all 3 groups were essentially identical, at 10 μU/ml plasma (Fig. 1, right). Two-day CISL plasma levels of OT were nearly 8-fold greater than controls for both LE and HZ animals, although only 2-fold higher in DI rats. As with AVP, plasma levels of OT decreased to control values after 6 d of CISL for all 3 groups.

Neurointermediate lobe depletions

In parallel with the plasma peptide determinations, the peptide contents of the NIL reflected the depletion that has been shown to result from chronic secretory stimuli. As shown in Table III, LE and HZ NILs were increasingly depleted of AVP after 6 d of CISL, with final AVP contents nearly 30% of control. The depletion of OT from the NIL of LE, HZ, and DI rats paralleled that shown for AVP. With control DI rat NILs containing approximately one-half the initial levels of OT measured in LE and HZ animals, the content was further depleted after 6 d of CISL.

In addition to the nonapeptide hormones AVP and OT, 3 peptides derived from the pro-dynorphin precursor were assayed in the NIL. Although existing within identical neurons, the steady-state peptide levels for the DYNs exhibited less than 0.1% of those measured for AVP. The depletion patterns for the 3 peptides, however, were very similar to those observed for AVP and OT (Table III). The steady-state levels of DYN A 1–17, DYN A 1–8, and DYN B 1–13 were significantly decreased in control DI rats compared to LE and HZ, and were further depleted by continued CISL. DYN A 1–8 is derived from DYN A 1–17 (Seizinger et al., 1981). DYN B 1–13 is a dominant product from its domain of the pro-dynorphin precursor, with its measure more closely approximating the amount of total pro-dynorphin synthesized (Cone et al., 1983). The relative molar ratios of DYN A 1–17 and DYN A 1–8 are shown in Table III. Control DI rat NILs contain a significantly lower ratio of DYN A 1–17 to DYN A 1–8 than do either LE or HZ controls, indicating that control DI have a higher resting proportion of DYN A 1–17 than do LE or HZ controls. With CISL depletion, the pro-dynorphin peptides remaining in the NILs for each strain exhibited a significantly increased ratio of DYN A 1–17 to DYN A 1–8 (such that 6 d LE and DI CISL groups were no longer significantly different). NIL pools of DYN A 1–17 were depleted to a greater extent than were DYN A 1–8 pools for all 3 groups following CISL, leaving NILs with a lower resting proportion of DYN A 1–17.

At even lower abundance than the pro-dynorphin peptides was CCK octapeptide (CCK8; CCK 26–33), derived from preprocholecystokinin. CCK8 displayed a parallel depletion pattern to its more abundant "cell-mate," OT (Table III).

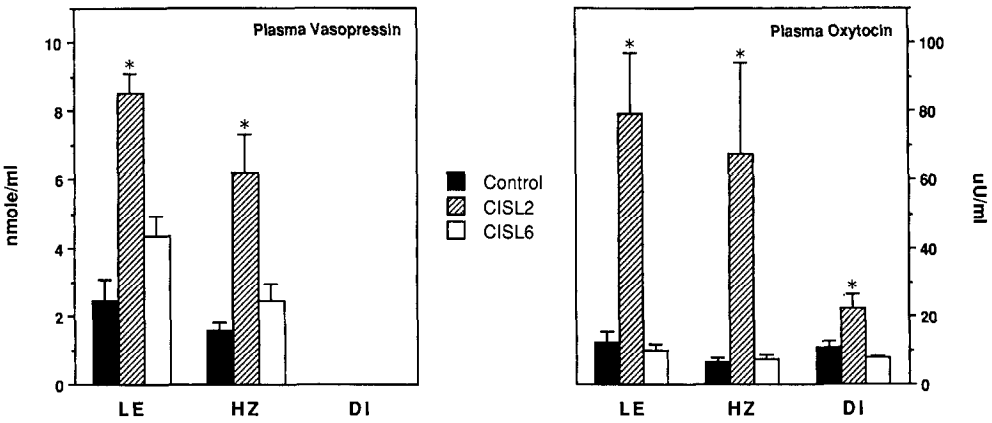

Figure 1. Concentrations of AVP and OT peptides in plasma during chronic intermittent salt-loading (CISL). AVP and OT levels in plasma collected at 1000 hours from Long Evans (*LE*), Brattleboro heterozygotes (*HZ*) and Brattleboro homozygotes (*DI*) after 2 (*CISL2*) and 6 (*CISL6*) d of CISL are compared to controls. Control and CISL6, $n = 12$; CISL2, $n = 4$. CISL2 significantly differed from both control and CISL6; $p < 0.05$ (*). Controls did not differ from CISL6; $p > 0.1$. For OT, in this case 1.0 μU $\approx$ 0.1 nmol.

mRNA quantitation

Figure 2 schematically illustrates the portions of AVP and OT cDNA used as probes for the specific detection and quantitation of AVP and OT mRNA by Northern analysis. These clones were isolated from a Sprague-Dawley hypothalamic cDNA lambda gt10 library. Sequencing of several isolated cDNAs for AVP and OT gave complete agreement with the proposed mRNA sequences for AVP and OT deduced from the Sprague-Dawley rat AVP (Schmale et al., 1983) and OT (Ivell and Richter, 1984) genes. A single base substitution in the 3′ untranslated region of the OT cDNA was the only detected difference: a G to A substitution at position 981 (Ivell and Richter, 1984). Completed sequences and comparative analyses of these cDNAs are presented elsewhere (Sherman and Watson, 1988). The AVP and OT cDNAs share a 176 bp midregion of 98.3% homology, at the extreme 3′ end of which is a convenient SacII restriction site that was used to isolate the nonhomologous 3′ ends of the AVP and OT cDNAs for use as specific hybridization probes. Details of this procedure can be found in the legend to Figure 2.

Figure 3 shows representative Northern autoradiograms from the SON for AVP and DYN mRNAs (*A*), and from the PVN for OT and CCK mRNAs (*B*). Quantitation results for each of these mRNAs are summarized in Figures 4 and 5. Each of the 4 mRNAs displayed a unique distribution between the 2 magnocellular nuclei. AVP and DYN mRNAs were 2–2.5-fold more abundant in the SON than in the PVN for the 3 rat strains, each belonging to or derived from the LE strain [as opposed to the 1.5× factor observed in control Sprague-Dawley rats (Sherman et al., 1986a, c)]. In contrast, OT mRNA was more equally distributed between the 2 nuclei, and CCK mRNA was much more abundant in the PVN.

The control Brattleboro DI expressed detectable levels of the deletion-containing AVP mRNA in both the PVN and SON, but at 45% the concentration was observed in control LE and HZ rats. The amount of AVP mRNA in the control HZ animals was not significantly different from control LE rats. Cytoplasmic levels for each of these messages was induced by the CISL paradigm (Fig. 4, right). The quantity of DYN mRNA was very high in the control DI rats, greater than the induced, salt-loaded values attained by either LE or HZ animals. This high level

Table III. The depletion of neuropeptides from the neurointermediate lobe during 6-days of Chronic Intermittent Salt-Loading

	Vasopressin	Oxytocin	CCK8	Dyn B 1-13	Dyn A 1-17	Dyn A 1-8	Ratio[a]
	nmole/pit	U/pit	fmoles/pit	pmole/pit	pmole/pit	pmole/pit	Dyn A 1-17:1-8
LE Control	9.78 ± 0.91	6.63 ± 0.50	148.50 ± 12.02	5.48 ± 0.46	1.58 ± 0.11	2.78 ± 0.26	1 : 1.75 ± 0.07[c]
LE Salt-Loaded	3.73 ± 0.44*	2.88 ± 0.61*	71.29 ± 8.70*	3.12 ± 0.36*	0.84 ± 0.12*	1.70 ± 0.22*	1 : 2.12 ± 0.13[b]
HZ Control	6.18 ± 1.03	7.51 ± 0.90	122.14 ± 15.65	5.56 ± 0.57	1.36 ± 0.18	2.51 ± 0.32	1 : 1.95 ± 0.15[c]
HZ Salt-Loaded	2.16 ± 0.44*	2.60 ± 0.27*	60.26 ± 6.50*	1.77 ± 0.29*	0.44 ± 0.08*	1.03 ± 0.18*	1 : 2.42 ± 0.16[b]
DI Control	---	3.59 ± 0.30	58.14 ± 8.15	2.49 ± 0.32	0.98 ± 0.13	0.97 ± 0.09	1 : 1.19 ± 0.19
DI Salt-Loaded	---	1.28 ± 0.28*	25.31 ± 4.31*	0.82 ± 0.21*	0.36 ± 0.08*	0.57 ± 0.11*	1 : 1.79 ± 0.14[b]

All values are Mean ± S.E.M. n = 12 **LE** = Long Evans **HZ** = Brattleboro heterozygote **DI** = Brattleboro homozygote

[a]Values for Dyn A 1-17 and Dyn A 1-8 were divided by the Dyn A 1-17 value for each animal [b]significantly different from control, $p < .05$

[c]significantly different from DI Control, $p < .05$ *significantly different from control, $p < .005$

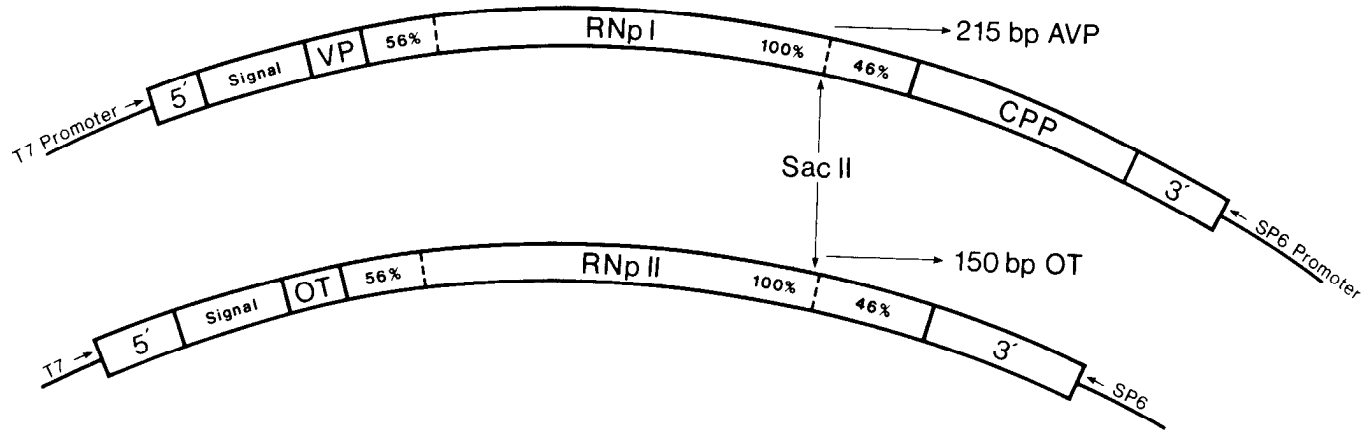

Figure 2. Schematic of cDNAs utilized for the generation of AVP and OT cDNA probes. Nucleotide sequences of both cDNAs can be found in Figure 1 of Sherman and Watson (1988; the following paper). AVP probes: pGEM3–AVP4c has rat AVP cDNA subcloned into EcoRI site of pGEM3 plasmid, oriented as shown. The long poly(A) tail was removed by using the AhaIII (DraI) site just 5′ to the poly(A) tail, with blunt-end ligation to the HincII site in the polylinker of pGEM3. A 215 bp AVP-specific cDNA could be excised with restriction cuts at the indicated SacII site within the cDNA, and the HindIII site in the polylinker. OT probes: pSP64–OXY3c has rat OT cDNA subcloned into EcoRI of pSP64 plasmid, oriented as shown. This cDNA has a 16 bp poly(A) tail, which was not removed. A 150 bp OT-specific cDNA was excised by restriction cuts at the indicated SacII site in the cDNA, and the SacI site in the polylinker. These same plasmids, linearized with SacII, could be used for the generation of AVP- or OT-specific cRNA probes with SP6 RNA polymerase for use in *in situ* hybridization studies (Sherman et al., 1986b) or, linearized with HindIII, to generate full-length cRNA probes for solution–hybridization RNase protection studies (Sherman and Watson, 1988).

could be induced still further with CISL. Consistent with previous data in the Sprague-Dawley rat, the SON response to salt-loading for AVP and DYN mRNAs was greater than that observed in the PVN.

OT mRNA levels in the control DI were significantly higher than the values measured in control LE and HZ rats (Figs. 4, 5). In contrast to the values for DYN mRNA, the control levels of OT mRNA were not as high as the CISL-induced mRNA levels in both the LE and HZ rats; 6 d of CISL induced levels of OT mRNA in the DI rat significantly higher than those induced in LE and HZ, however. Beyond the proportional difference between the 2 magnocellular nuclei, CCK mRNA concentrations were up-regulated in a like manner to OT only in the PVN (Figs. 4, 5). The detectable levels of CCK mRNA in the SON were very low, with the changes following CISL not reaching significance ($p > 0.1$; Fig. 5).

Discussion

In many aspects, diabetes insipidus rats of the Brattleboro strain can be regarded as a model for the very-long-term, chronically osmotically challenged rat. These animals exhibit significantly higher than normal plasma [Na^+] and osmolarity (Opava-Stitzer et al., 1982; Valtin, 1982) from as early as 14 d postnatally (Dlouhá et al., 1982). As opposed to that of the salt-loaded rat, however, this high plasma osmolarity is not due to the ingestion of highly osmolar fluids, but to the renal inability to concentrate sodium in the absence of AVP, with the subsequent secretion of too dilute urine (Valtin and Schroeder, 1964; Valtin et al., 1965). This inability to adequately manage fluid balance made studies identical to chronic salt-loading studies in Sprague-Dawley rats impossible. DI rats could not survive for more than 48 hr when given 2% saline instead of water. In the development of a suitable chronic osmotic challenge for the DI Brattleboro rat, we elected to exaggerate their already existing osmotic periodicity: a continuous moderate dehydration interrupted by periods of hydration or even overhydration (Valtin and Schroeder, 1964; Valtin et al., 1965). In the paradigm, "chronic intermittent salt-loading" (CISL), experimental animals were dehydrated for 18 hr/d with 2% saline as drinking water, and then allowed to rehydrate on normal fresh water for the remaining 6 hr. As shown in Table I, a CISL regimen over 6 d resulted in the maintenance of body weight for DI rats, and a relatively healthy rate of growth for LE and HZ animals. Even though DI animals preferred not to drink the 2% saline, consuming little more than half as much as LE and HZ rats (Table II), they were not able to survive a similar paradigm in which they were deprived of anything to drink for the 18 hr period.

Not surprisingly, CISL in LE and HZ rats proved to be a more moderate osmotic stimulus than continuous salt-loading. Concern, however, that salt-loading or the behavioral changes induced by the increased daytime drinking could be serving as effective stressors, especially in the Brattleboro DI, prompted us to investigate some parameters of the stress response. Control DI rats contained significantly higher anterior pituitary POMC mRNA content. The 25% increase was small, however, when compared to the 75% increase induced by chronic, intermittent foot-shock stress (Höllt et al., 1986). Neither this value nor the control levels of POMC mRNA in LE or HZ anterior lobes significantly increased after 6 d of CISL. Because DI rats have been shown to have low resting levels of plasma corticosterone

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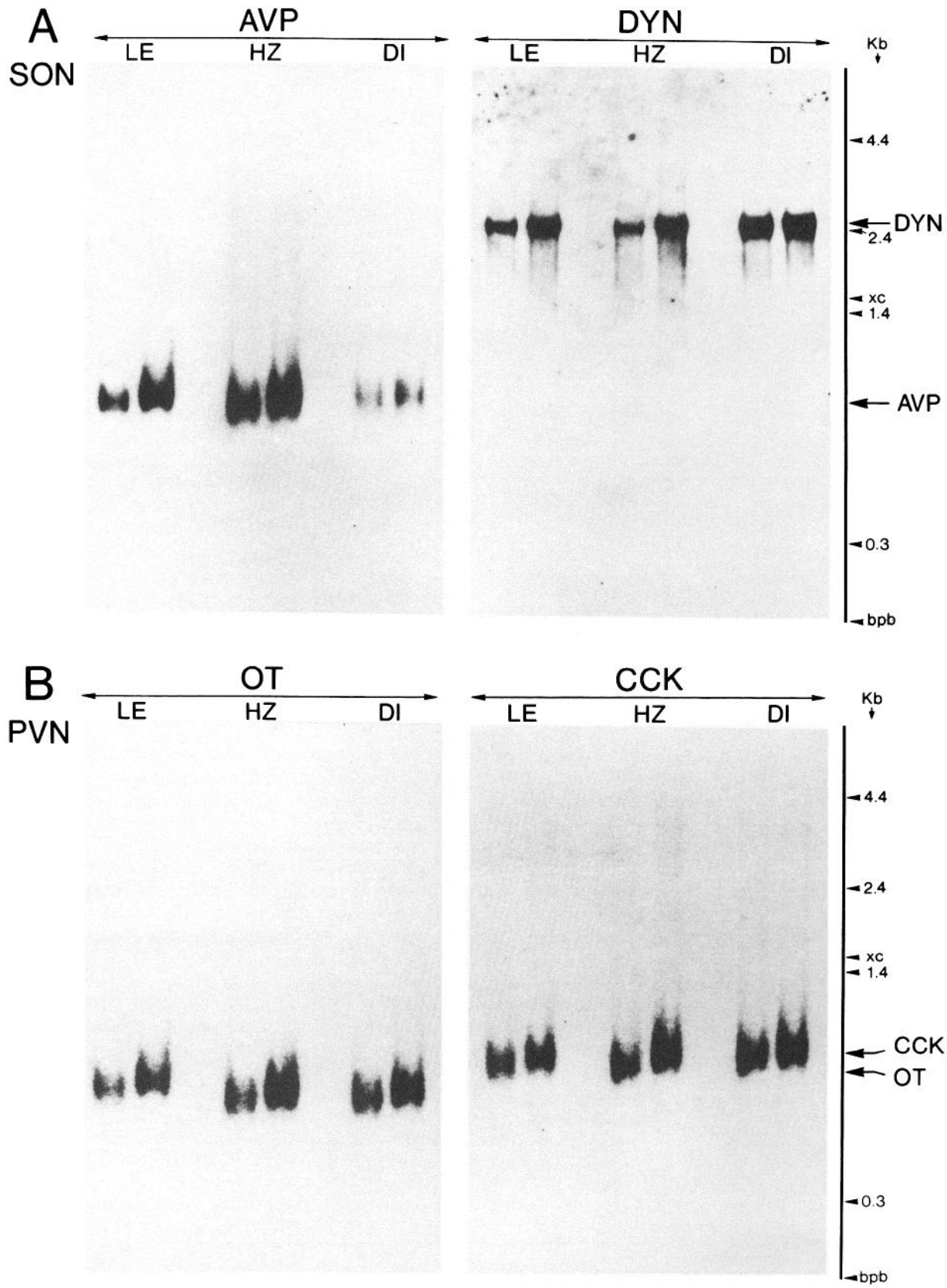
Figure 3. Identification of hypothalamic preprohormone mRNAs by Northern analysis. Representative Northern autoradiograms from coexisting hormone mRNAs are shown. *A*, Vasopressin (AVP) and dynorphin (DYN) mRNAs in the supraoptic nucleus (SON). *B*, Oxytocin (OT) and cholecystokinin (CCK) mRNAs in the paraventricular nucleus (PVN). For each group—Long Evans (*LE*), Brattleboro heterozygotes (*HZ*), or Brattleboro homozygotes (*DI*)—control mRNAs are compared to mRNAs after 6 d of chronic intermittent salt-loading (left versus right, respectively). For AVP, OT, and CCK, each lane represents the total nucleic acids from one nuclei punch (either one PVN or SON), whereas the DYN Northern



uses poly(A)RNA from the pooled nuclei of 3 rats (6 nuclei). The OT and CCK Northerns are the same, probed first with nick-translated OT cDNA and second with CCK cDNA, as described in Materials and Methods. RNAs were run with single-stranded RNA molecular-weight markers (56205SA; Bethesda Research Labs, Gaithersburg, MD). After passive transfer to Nytran, the markers were detected with a ^{32}P nick-translated 1 kbase DNA ladder (5615SA; Bethesda Research Labs). *xc,* Xylene cyanol; *bpb,* bromophenol blue. Exposures: AVP, 9 hr with intensifying screen at −80°C; OT, 18 hr with screen; CCK, 67 hr with screen; DYN, 18 hr without screen at 22°C.

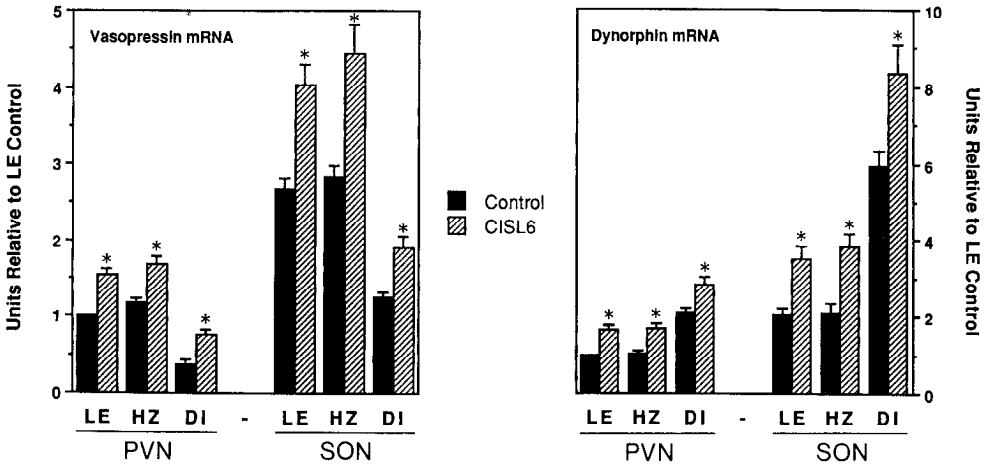


Figure 4. Relative expression of vasopressin (AVP) and dynorphin (DYN) mRNAs following 6 d of chronic intermittent salt-loading (*CISL6*). Autoradiographic Northern bands were quantitated as described in Materials and Methods, with the total integrated optical density expressed relative to the values for LE control. Each determination for vasopressin represents the total nucleic acids from one magnocellular nuclei punch, whereas each dynorphin is the pooled nuclei from 3 animals. Values are expressed as mean ± SEM, with $n = 4$ for vasopressin, and $n = 3$ for dynorphin. Note: There can be no comparison between AVP and DYN relative values in this figure.

(Milne et al., 1982), which is partially attributable to the lack of AVP (McCann et al., 1966), it is difficult to assess whether the small increase in anterior pituitary POMC mRNA indicated a stress component of diabetes insipidus or a response to elevated corticotropin releasing hormone (CRH) secretion brought on by adrenal insufficiency (McCann et al., 1966; Sawchenko et al., 1984; Loeffler et al., 1985). Nevertheless, it did not appear that CISL served sufficiently as a chronic stressor to explain the results observed.

The consumption of 2% saline during CISL induced the secretion of AVP and OT from the posterior pituitary in a manner similar to that of other chronic osmotic paradigms, such as regular salt-loading and water deprivation (Negro-Vilar and Samson, 1979, 1982; Zerbe and Palkovits, 1984). In each case, for AVP in the LE and HZ, and for OT in LE, HZ, and DI animals, the plasma level for the nonapeptide hormones increased after 2 days of CISL (Fig. 1). This high rate of secretion could not be maintained for 6 d, however, by which time plasma concentrations of AVP and OT had decreased to near normal levels. The inability to maintain high plasma concentrations was due to the depletion of the posterior pituitary stores necessary for the initial high rates of secretion. NILs were not maximally depleted by 2 d of CISL, displaying a high degree of content variability (data not shown). With 6 d of CISL, however, neural lobe depletions were clearly demonstrated (Table III) for both AVP and OT, for the 3 peptides derived from pro-dynorphin, and for CCK8, consistent with depletion patterns observed many times previously with physiological stimuli known to affect hypothalamic magnocellular neurons (Negro-Vilar and Samson, 1979; Höllt et al., 1981; Deschepper et al., 1983; Sawchenko et al., 1984). The Brattleboro HZ contained a slightly, but significantly, lower control level of AVP in the NIL than did LE rats, although concentrations of OT in both plasma and pituitary were not significantly different. Control DI Brattleboros had significantly less OT NIL content than did LE and HZ, a partially depleted state consistent with the high plasma osmolarity of DI animals (Opava-Stitzer et al., 1982; Valtin, 1982). The low resting NIL OT content for DI rats could possibly account for the weak plasma OT response to 2 d of CISL, shown in Figure 1.

The depletion patterns of the 3 peptides derived from prodynorphin, DYN A 1–17, DYN A 1–8, and DYN B 1–13, paralleled those of AVP and OT (Table III). The depleted state of DYN peptides from the DI rat NIL, as compared to that from control LE or HZ, suggested an active secretion analogous to that observed for OT. One major difference, however, is that DYN A 1–8 is derived from DYN A 1–17, a precursor-processing variable not present with the AVP and OT precursors. As shown in Table III, the ratios of DYN A 1–17 to DYN A 1–8 in the NIL for each rat strain after 6 d of CISL were significantly different from those of controls; additionally, the ratio for control DI animals was significantly different from that for control LE or HZ. The data indicated that the proportion of DYN A 1–17 depleted from the NIL increased relative to DYN A 1–8 under conditions of chronic secretion induced by either diabetes insipidus or salt-loading. Although these results are tenuous, since they are derived from content rather than release data, they suggest a regulatory dimension not observed previously in the neural lobe.

Opioid peptides derived from pro-dynorphin display a marked preference for the κ opioid receptor subtype (Goldstein et al., 1979), the only opioid receptor subtype found in the rat posterior pituitary (Herkenham et al., 1986; Mansour et al., 1986). Although exogenous opioid agonists have long been known to influence the secretion of AVP and OT (for review, see Forsling, 1985), recent evidence that salt-loading induces the down-regulation of κ-receptors in the rat neurohypophysis (Brady and Herkenham, 1987) demonstrated that endogenous opioids must act at these κ sites as well. *In vitro,* DYN A 1–8 has been shown to bind to κ-receptors in the rat neurohypophysis (Gerstberger and Barden, 1986). Pharmacological doses of κ and/or opiate agonists have been reported to induce a renal diuresis in rats, either by the inhibition of AVP secretion, a direct effect on the kidney, a central action, or a combination of each (Iversen et al., 1980; Lutz-Bucher and Koch, 1980; Huidobro-Toro and Huidobro, 1981; Leander, 1983; Forsling, 1985). Similar effects induced via endogenous opioids in a physiological paradigm have not been convincingly demonstrated. Recently, DYN A 1–17 and 1–8 were shown to specifically inhibit the electrically evoked release of OT from the posterior pituitary *in vitro* (Bondy et al., 1987), confirming an earlier, less specific observation (Bicknell and Leng, 1982). Since DYN A 1–17 has a much greater affinity than DYN A 1–8 for the κ opioid receptor (Young et al., 1986), the increase in the proportion of DYN A 1–17 depleted from salt-loaded posterior pituitaries may be a processing or release mechanism designed to modulate the amount of OT peptide secreted during periods of osmotically induced AVP/DYN neuronal activity. If this hypothesis were true, then

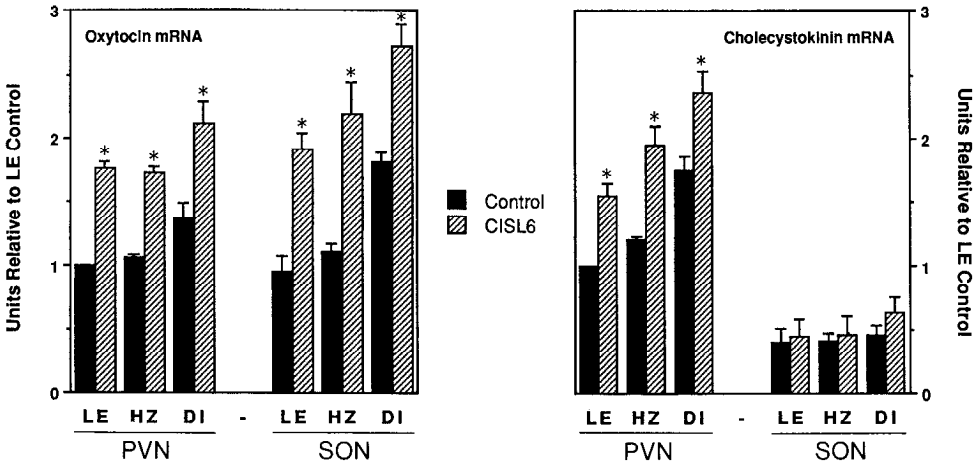


Figure 5. Relative expression of oxytocin (OT) and cholecystokinin (CCK) mRNAs following 6 d of chronic intermittent salt-loading (*CISL6*). Autoradiographic Northern bands were quantitated as described in Materials and Methods, with the total integrated optical density expressed relative to the values for LE control. Each determination for oxytocin and cholecystokinin mRNAs represents the total nucleic acids from one magnocellular nuclei punch. Values are expressed as mean ± SEM, with $n = 4$ for OT and CCK. Note: There can be no comparison between OT and CCK relative values in this figure.

a contributing factor to the low induction of plasma OT at 2 d of CISL, shown in Figure 1, would be the increased presence of DYN A 1–17 at κ-receptors in the DI neural lobe. In the absence of secretion data or specific κ antagonists, however, the existence of such mechanisms remain conjectural. Pro-dynorphin processing changes favoring DYN A 1–17 could also be a mechanism to increase the nonopiate effects of DYN A 1–17 encoded in positions 7–17 (Walker et al., 1982).

The plasma and tissue content data give a partial picture of how AVP and OT magnocellular neurons adapt to 2 types of chronic secretory stimuli, a 6 d regimen of CISL and the prolonged developmental stimulus of diabetes insipidus. Each of these stimuli relies on posterior pituitary secretory product storage pools for the maintenance of high plasma levels of AVP and OT during the first few days of chronic secretory activity. Once these stores are depleted, the neurons must secrete only newly synthesized peptides in recently transported neurosecretory granules. Thus, with continuing demands for secretion, the amount of peptide secreted closely approximates the amount of precursor peptide synthesized. In order to increase, or even maintain, adequate plasma levels of hormone, cells must increase their rates of precursor synthesis, either by increasing the efficiency or capacity (or both) of their translational machinery. The increased cytoplasmic pools of pro-vasopressin, pro-oxytocin, pro-dynorphin and pro-cholecystokinin mRNA in response to CISL, shown in Figures 3–5, are surely part of this mechanism.

Several studies have shown that chronic stimuli for the secretion of AVP, OT, or DYN peptides induce the cytoplasmic levels of their corresponding mRNA (Majzoub et al., 1983; Burbach et al., 1984, 1986; Sherman et al., 1986a, c; Zingg et al., 1986; Van Tol et al., 1987). Although the overall mRNA increases observed in Figures 4 and 5 are more modest than those observed with regular salt-loading, a 6 d course of CISL significantly increased the cytoplasmic pools of pro-hormone mRNAs in both the PVN and SON of LE, HZ, and DI rats. The regulation of AVP mRNA between the LE and the Brattleboro HZ was very similar, despite the fact that the HZ animal contains only one functional allele for the vasopressin gene. Expression of 2 mutant alleles of the AVP gene, as in the DI rat, resulted in less than 50% of control LE levels; yet the ability to up-regulate AVP mRNA cytoplasmic pools following CISL was preserved. The translational, transcriptional, and packaging machinery in DI AVP neurons can be shown to be intact, given the healthy expression of DYN A 1–17, DYN A 1–8, and DYN B 1–13 peptides in the NIL, the marked induction of pro-dynorphin mRNA (Figs. 3, 4), and the previous observation that pro-dynorphin peptides are packaged in neurosecretory granules (Whitnall et al., 1985). The extremely high levels of pro-dynorphin mRNA in the control Brattleboro DI indicate that these neurons are, indeed, chronically driven, as the depleted levels of DYN peptides in the NIL originally suggested (Table III). This can also be seen in the elevated levels of OT and CCK mRNA seen in the control DI. The Brattleboro DI, in terms of both pituitary peptide and cytoplasmic OT, CCK, and DYN mRNA levels, behaves as a chronically salt-loaded animal. One principal difference between the elevated mRNA levels detected in diabetes insipidus and those in chronic salt-loaded rats, however, was that the increased steady-state levels of cytoplasmic mRNAs in the DI acted as a new baseline, from which they could respond to further osmotic challenges by presumed transcriptional increases. At what point during development this new set point is established in the DI rat is an interesting question, and is under investigation.

The high content of CCK mRNA in the PVN versus the SON is relatively consistent with published immunocytochemical and RIA data. Although approximately 80% of the NIL CCK8 originates in the PVN (Palkovits et al., 1984), only about 40% of CCK cells in the PVN are magnocellular neurons projecting to the posterior pituitary (Kiss et al., 1984). The majority of the remaining parvocellular neurons of the PVN project to the median eminence, where CCK8 levels 3 times higher than in the NIL can be found (Palkovits et al., 1984). These data suggest a relatively small CCK8 mRNA content in the SON, as observed (Fig. 5). Earlier studies have shown that median eminence CCK8 content did not display a significant depletion with osmotic stimulation as it did with adrenalectomy (Anhut et al., 1983), behavior similar to that of AVP, OT, and CRF neurons of the parvocellular PVN projecting to the median eminence, which have been shown by *in situ* hybridization in our laboratory to be relatively insensitive to osmotic stimuli (unpublished observations). Given the observed comparable increases of OT and CCK mRNAs in the PVN over a presumed large and stable baseline of parvocellular CCK mRNA, a large disparity between parvo- and magnocellular CCK mRNA contents probably does not exist. This suggests that the PVN magnocellular CCK mRNA

content is higher than the PVN lesion data suggests (Palkovits et al., 1984). This in an issue best addressed by *in situ* hybridization, however.

The unusually high levels of pro-dynorphin mRNA in the control DI were very interesting, and contrasted sharply with the low levels of mutant AVP mRNA. Equivalent levels of DYN mRNA could not be attained by 6 d of CISL, or even by 6 d of continuous salt-loading in the Sprague-Dawley rat (Sherman et al., 1986a). The fact that pro-dynorphin mRNA was increased to a much higher extent than either OT or CCK mRNA in the control DI compared to the salt-loaded LE or HZ suggested that vasopressin neurons are driven to a far greater extent than are OT neurons in the DI rat. This is the only instance in which a clear osmotic dissociation between AVP and OT neurons has been observed with mRNA quantitation methods. These results are consistent with a mechanism whereby AVP serves as an autofeedback regulator of AVP/DYN neurons (perhaps only indirectly, via plasma osmolarity), and DYN serves as a feedback modulator of OT neurons. Although the physiological role of DYN peptides in the regulation of AVP and OT secretion remains unresolved, there is no question that the principal regulator of AVP neurons is plasma osmolarity. In the Brattleboro rat, therefore, the absence of AVP leads to a continual hypersecretion of DYN peptides due to high plasma osmolarity, which, in turn, induces very high levels of DYN mRNA; the continual presence of pro-dynorphin peptides would attenuate the secretion of OT, resulting in only the modest induction of OT and CCK mRNAs observed. It remains unclear why a κ-receptor mechanism that only partially modulates AVP or OT secretion would be physiologically necessary, or how such modulation is affected by the observed down-regulation of κ-receptors following salt-loading. It is possible that such a mechanism would only come into play during periods of chronic AVP secretion, when high plasma levels of OT would be either unnecessary or detrimental. It is also important to realize that other putative secretory modulators, such as CRH, the enkephalins, and dopamine, known to be found in some magnocellular neurons and/or the NIL, undoubtedly have important regulatory roles not yet defined.

Nearly opposite in behavior to that of DYN mRNA in the DI Brattleboro was the low control expression of mutant AVP RNA. Studies using Northern analysis or *in situ* hybridization have shown previously that the DI Brattleboro rat contained AVP mRNA at levels less than 50% of genotypic controls (Majzoub et al., 1984; Fuller et al., 1985a, b; Van Tol et al., 1986). Although other studies have shown that this mRNA is efficiently transcribed, but inefficiently translated *in vitro* (Schmale et al., 1984; Ivell et al., 1986), Figure 4 presents the first evidence that the osmotic regulatability of the mutant AVP gene remains intact. If *in vivo* transcriptional mechanisms remained normal, which has not been shown, a plausible explanation for the low levels of cytoplasmic mutant AVP mRNA would be a shortened mRNA half-life, brought about either by the point frame-shift mutation itself, or by virtue of aberrant translational mechanisms present in the absence of a stop codon. Indirect evidence for such a mechanism has been presented elsewhere in an investigation of independent AVP allele expression in the Brattleboro HZ (Sherman and Watson, 1988).

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