Neuropeptide Y mRNA and Immunoreactivity in Hypothalamic Neuroendocrine Neurons: Effects of Adrenalectomy and Chronic Osmotic Stimulation

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Neuropeptide Y (NPY) coexists with vasopressin or oxytocin in magnocellular neurons of the hypothalamo-neurohypophyseal tract. Using quantitative *in situ* hybridization histochemistry and immunohistochemistry, we have studied the effects of adrenalectomy and chronic osmotic stimulation, either alone or in combination, on NPY mRNA expression and NPY immunoreactivity in magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, and arcuate nucleus (Arc). Adrenalectomy and chronic osmotic stimulation each increased NPY mRNA levels in magnocellular neurons of the PVN and SON, while the combination of both treatments had an additive effect. In the Arc, only the combination of adrenalectomy and chronic osmotic stimulation increased NPY mRNA levels. Chronic osmotic stimulation also resulted in a marked increase of NPY-immunoreactive magnocellular perikarya in the PVN and SON. In contrast, adrenalectomy had only minor effects on the number of NPY-immunoreactive magnocellular PVN/SON perikarya. Neither chronic osmotic stimulation nor adrenalectomy affected the number of NPY-immunoreactive Arc perikarya. However, adrenalectomy decreased the number of NPY-immunoreactive nerve terminals in the external zone of the median eminence, while chronic osmotic stimulation increased the number of immunoreactive nerve fibers in the internal zone of the median eminence. The present study provides evidence that adrenalectomy and chronic osmotic stimulation can separately influence NPY gene transcription in magnocellular hypothalamo-neurohypophyseal neurons, while only the combined effect of adrenalectomy and chronic osmotic stimulation increases NPY mRNA expression in neurons of the Arc.

[Key words: neuropeptide Y, salt loading, adrenal steroids, dexamethasone, *in situ* hybridization, neuropeptide expression, magnocellular hypothalamo-neurohypophyseal pathway, arcuate nucleus, supraoptic nucleus, paraventricular nucleus, rat]

The magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei are the main constituents of the hypothalamo-neurohypophyseal system (HNS) (Bargmann and Scharrer, 1951). Two distinct populations of magnocellular neurons synthesize either vasopressin (AVP) or oxytocin (OXY), and a wide variety of physiological stimuli have been shown to influence the activity of both types of neurons within the HNS and the consequent hormone release from the neurohypophysis (for review, see Leng and Bicknell, 1986; Robinson, 1986; Bisset and Chowdrey, 1988; Hatton, 1990). Water deprivation and chronic saline ingestion are potent stimuli to the HNS, resulting in the release of AVP and OXY from nerve terminals in the neurohypophysis (Jones and Pickering, 1969; Brownstein et al., 1980). In addition to AVP and OXY, many other neuropeptides have been shown to coexist within magnocellular neurosecretory cells (Vanderhaeghen et al., 1981; Watson et al., 1982; Brownstein and Mezey, 1986; Meister et al., 1990; Mikkelsen et al., 1992), and the synthesis of these can also be influenced by changes in plasma osmolality (Lightman and Young, 1987; Meister et al., 1990).

Neuropeptide Y (NPY), a 36 amino acid member of the pancreatic polypeptide family (Tatemoto et al., 1982), is abundant in the rat hypothalamus, but not normally present in HNS perikarya (Chronwall et al., 1985; DeQuidt and Emson, 1986; Morris, 1989). The major source of this NPY has been assumed to be the arcuate nucleus (Arc) and projections from the catecholaminergic brainstem (Bai et al., 1985; Sawchenko et al., 1985). We have recently demonstrated that chronic saline ingestion induces the expression of NPY mRNA and immunoreactive NPY in AVP- as well as OXY-containing neurons of the HNS (Larsen et al., 1992a,b). Further corroborating these morphological findings are recent reports showing that salt loading and water deprivation increase the content of NPY in the mediobasal hypothalamus and neurohypophysis (Hooi et al., 1989; Jessop et al., in press), while the number of neurohypophyseal NPY binding sites is drastically reduced during chronic saline ingestion (Larsen et al., 1992a). These data suggest that NPY synthesized in the HNS may participate in the regulation of water homeostasis. In addition to these effects of hypertonic saline on tissue content of NPY, hypothalamic NPY has been reported to be responsive to glucocorticoid manipulation (Corder et al., 1988; White et al., 1990). However, no change in PVN contents of NPY was observed following adrenalectomy (ADX) (Rivet et al., 1989).

At present it is unknown whether the expression of NPY mRNA and subsequent peptide synthesis within magnocellular

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neurons is affected by manipulations with the hypothalamo-hypophyseal-adrenal (HPA) axis. Recently, it has been demonstrated that specific type I and type II glucocorticoid receptors are present within magnocellular perikarya of the rat SON (Kiss et al., 1988; Swanson and Simmons, 1989; Ahima and Harlan, 1990; Ahima et al., 1991), suggesting that the HNS is directly influenced by circulating adrenal steroids. Furthermore, recent *in situ* hybridization histochemistry studies have shown that glucocorticoid treatment significantly increases the content of glucocorticoid as well as mineralocorticoid receptor mRNA in magnocellular neurons of the rat HNS (Swanson and Simmons, 1989).

The present studies further examine the effects of adrenal steroids and osmotic stimulation on the regulation of NPY gene expression and peptide content in magnocellular neurons of the PVN and SON as well as in Arc neurons.

Materials and Methods

Antisera and oligonucleotide probes. Synthetic DNA oligonucleotide probes (48mer) were purchased from Advanced Biotechnology Centre (Charing Cross Hospital, London, UK) and were directed against rat NPY bases 171–218 (Larhammer et al., 1987), rat corticotropin-releasing factor-41 (CRF-41) bases 496–543 (Jingami et al., 1985), and rat AVP bases coding for the last 16 amino acids of the glycopeptide sequence (Ivell and Richter, 1984). A previously characterized rabbit antiserum (code #8182) raised against synthetic NPY was used in all immunohistochemical experiments (Mikkelsen and O'Hare, 1991).

Animals. Male Sprague-Dawley rats weighing 200–250 gm and housed under normal laboratory conditions (12 hr lights on, 12 hr lights off) with free access to food were used in the present study. A total of 46 rats were bilaterally adrenalectomized (ADX) via a dorsal approach, and the same number underwent sham operation where the adrenals were visually identified but left intact (sham-ADX). ADX animals were offered either 0.9% saline ($n = 26$) or 2.0% saline to drink ($n = 14$), while sham-ADX animals were offered either tap water ($n = 26$) or 2% saline ($n = 20$) to drink. A group of ADX animals ($n = 8$) had dexamethasone (DEX) (4 mg/liter; Decadron, MSD, Herlev, Denmark) added to the 0.9% saline they were offered to drink (ADX-DEX). All animals were kept for 12 d after surgery. The animals were killed by decapitation, trunk blood was collected in chilled heparinized plastic tubes and separated by centrifugation, and plasma osmolalities were measured by depression of freezing point on an Osmomat (Clandon Scientific Ltd., Aldershot, UK) within the preceding 2 hr. The effect of 2% saline treatment on plasma vasopressin levels has been described in detail (Jessop et al., 1990a).

In situ hybridization histochemistry. Fourteen sham-ADX, 14 ADX, 8 sham-ADX drinking 2% saline, 8 ADX drinking 2% saline, and 8 ADX-DEX animals were used for *in situ* hybridization histochemistry. Animals were decapitated between 9 and 10 A.M., and their brains were rapidly removed, frozen on dry ice, and stored at -80°C . *In situ* hybridization histochemistry was performed as earlier described (Lightman and Young, 1987). Twelve-micrometer sections were cut on a cryostat and thaw-mounted onto gelatin-coated slides, fixed in 4% formaldehyde in 0.12 M sodium phosphate-buffered saline (PBS; pH 7.4), and rinsed twice in PBS. Hybridizations were initiated by placing the sections in 0.25% acetic anhydride in 0.1 M triethanolamine, 0.9% NaCl (pH 8.0) for 10 min at room temperature, followed by delipidation through a series of graded ethanols (70%, 80%, 95%, 100%) and chloroform. The oligonucleotide probe was labeled with ^{35}S - α -thio-ATP (>3000 Ci/mmol; New England Nuclear-DuPont) using terminal deoxynucleotidyl transferase (Boehringer Mannheim, East Sussex, UK) to a specific activity of 3.5×10^{10} dpm/mol. Sections were hybridized overnight with $0.5\text{--}1.0 \times 10^6$ dpm/100 μl at 37°C in a buffer (pH 7.2) containing 25% (v/v) formamide, $4 \times \text{NaCl/citrate}$ (SSC; $1 \times \text{SSC} = 0.15$ M NaCl, 0.015 M sodium citrate, pH 7.2), $1 \times \text{Denhardt's}$ solution, 10% (w/v) dextran sulfate, 10 mM dithiothreitol, 0.5 mg/ml salmon sperm DNA, and 250 $\mu\text{g/ml}$ yeast tRNA. After hybridization, slides were washed in $1 \times \text{SSC}$ at 55°C 4×15 min and at room temperature for an additional 2×30 min. The sections were next dipped into water, blown dry, and finally exposed to Amersham Hyperfilm for 3 weeks. A series of slides were dipped in Amersham LM-1 emulsion and exposed for 10 weeks before being developed. These sections were subsequently

Table 1. Effect of various treatments on plasma osmolalities

Treatment	Osmolality
Sham-ADX, tap water ($n = 8$)	282 ± 2
2% saline ($n = 8$)	$315 \pm 4^*$
ADX, 0.9% saline ($n = 5$)	282 ± 2
ADX, 2% saline ($n = 6$)	$321 \pm 7^*$

Values are mOsm/kg H_2O , means $\pm$ SEM.

* $p < 0.005$ versus sham-ADX as determined by paired Student's t test.

stained with cresyl violet to counterstain the cell bodies. Autoradiograms were quantified using an image analysis system (IMAGE 1.41, Wayne Rasband, NIH, Bethesda, MD) and grain densities converted to dpm/gm wet weight using ^{35}S brain paste standards as reference. Sections from all animals were hybridized concomitantly and exposed to the same film.

Immunohistochemistry. Twelve sham-ADX rats, 12 sham-ADX rats drinking 2% saline, 12 ADX rats, and 6 ADX rats drinking 2% saline were used for immunohistochemical studies. Except for ADX rats drinking 2% saline, half of animals in each group received colchicine injections (100 μg in 10 μl 0.9% NaCl) delivered into the third ventricle 24 hr prior to fixation. Animals were anesthetized with tribromoethanol (25 mg/kg body weight, i.p.) and perfused transcardially with 100 ml potassium chloride (0.02%) containing phosphate-buffered saline (KPBS; pH 7.4) and heparin (15,000 IU/liter), followed by perfusion with 450 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. Both perfusion fluids were equilibrated to room temperature before use. The brain with attached hypophysis was immediately removed after the perfusion and postfixed in the same fixative at 4°C for 4 hr, followed by immersion in a 30% sucrose solution in KPBS (pH 7.4) for at least 3 d. Forty-micrometer-thick serial cryostat sections were cut and transferred to KPBS at room temperature. NPY immunoreactivity (NPY-IR) was localized on free-floating sections processed for immunohistochemistry by using a streptavidin-biotin-coupled peroxidase method (Hsu et al., 1981). Briefly, the sections were rinsed for 2×10 min in KPBS (pH 7.4) containing 0.25% bovine serum albumin (BSA) and 0.1% Triton X-100 (KPBS-BT), followed by a pretreatment in 1% H_2O_2 in KPBS for 10 min, and then incubated for 30 min in a 5% swine serum solution in KPBS containing 0.3% Triton X-100 and 1% BSA. The sections were then incubated for 24 hr in the primary antiserum at 4°C diluted 1:1000 in KPBS-BT. After incubation in the primary antiserum, the sections were washed in KPBS-BT for 3×10 min followed by incubation for 60 min at room temperature in biotinylated anti-rabbit swine IgG diluted 1:400 (Dakopatts, Glostrup, Denmark). The sections were then washed for 3×10 min in KPBS-BT, and finally incubated for 60 min at room temperature in ABC-streptavidin horseradish peroxidase complex diluted 1:125 (Dakopatts). After washing in KPBS-BT for 10 min, in KPBS alone for 10 min, and in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min, the sections were reacted for peroxidase activity in a solution of 0.025% diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6) and 0.003% H_2O_2 for 20 min. Control sections for single antigen immunohistochemistry were routinely processed by either omitting or replacing the primary antiserum with an equivalent concentration of rabbit preimmune serum.

ACTH measurements. Blood from control and saline-treated rats was collected in chilled heparinized tubes, and plasma was separated after centrifugation, frozen on dry ice, and stored at -20°C for hormone measurements. The method for extracting ACTH from rat plasma with Sep-pak C_{18} cartridges and subsequent measurement by radioimmunoassay has been described in detail (Jessop et al., 1989).

Results

Effect of ADX and saline treatment on plasma osmolality

The influence of ADX, chronic 2% saline ingestion, or the combined effect of these manipulations on plasma osmolalities is given in Table 1. Chronic 2% saline ingestion significantly increased plasma osmolalities in sham-ADX and ADX animals, while plasma osmolalities in ADX animals drinking 0.9% saline were comparable to the levels observed in sham-ADX animals.

Figure 1. Effect of ADX with or without DEX substitution on AVP mRNA levels in the pmPVN, the mpPVN, and the SON. ***, $p < 0.001$ versus control; **, $p < 0.01$ versus control; as determined by two-tailed Student's t test.

Effect of ADX on hypothalamic CRF-41 mRNA and plasma ACTH

ADX resulted in a marked increase in circulating ACTH levels from 259 ± 88 pg/ml to 2211 ± 586 (mean $\pm$ SEM), and this increase was totally inhibited by DEX substitution therapy (57 ± 12 pg/ml). To evaluate further the effects of ADX on the HPA axis, quantitative *in situ* hybridization histochemistry was employed to estimate CRF-41 mRNA levels in the medial parvocellular division of the PVN (mpPVN) and AVP mRNA levels in the PVN and SON. ADX significantly increased CRF-41 mRNA levels in the mpPVN (508 ± 23 vs 99 ± 22 dpm/gm in control animals; $n = 8$), and this increase was totally inhibited by DEX substitution therapy (38 ± 8 dpm/gm; $n = 8$). Due to the close proximity of parvocellular and magnocellular AVP-synthesizing neurons in the PVN and the high levels of AVP mRNA in the latter cell types, a clear distinction between these cell types was impossible to establish from cytoarchitec-

Figure 2. Effect of ADX with or without DEX substitution on NPY mRNA levels in the pmPVN. ***, $p < 0.001$ versus control as determined by two-tailed Student t test, $n = 8$.

Figure 3. Effect of ADX and chronic salt loading either alone or in combination on NPY mRNA levels in the magnocellular part of the PVN (top), the SON (middle), and the Arc (bottom). a, $p < 0.001$ versus control (two-tailed Student's t test); b, $p < 0.001$ versus 2% saline (two-tailed Student's t test); c, $p < 0.001$ versus ADX (two-tailed Student's t test); $n = 8$ in all groups.

tonic criteria. Instead, a topographical distinction between magnocellular and parvocellular areas was employed for the image analysis, and the parcellation introduced by Swanson and Kuypers (1980) was followed. AVP mRNA levels in the PVN were measured in an area corresponding to the posterior magnocellular part (pmPVN) and the mpPVN, while the AVP mRNA in the SON was considered to be exclusively of magnocellular origin. ADX significantly increased AVP mRNA levels in the mpPVN area (4003 ± 110 vs 2250 ± 171 dpm/gm, $\pm$ SEM, in control animals; $n = 8$), while DEX substitution significantly lowered AVP mRNA content in the mpPVN (1578 ± 144 dpm/gm; $n = 8$; Fig. 1). Neither ADX nor ADX-DEX had any effect on AVP mRNA levels in the SON (Fig. 1), while a slight but significant increase in AVP mRNA levels in the pmPVN was observed following ADX.

Figure 4. Dark-field photomicrographs showing cells containing NPY mRNA in the PVN of control (*A*), ADX (*B*), 2% saline-treated (*C*), and ADX rats drinking 2% saline (*D*). All treatments increased NPY mRNA levels in cell bodies within the magnocellular part of the PVN, while ADX apparently also induced NPY mRNA synthesis in the parvocellular parts. Asterisks are placed over the third ventricle. Scale bars, 200 μ m.

Effect of ADX and osmotic stimulation on NPY mRNA expression in hypothalamic neuroendocrine neurons

NPY mRNA levels in magnocellular neurons of the PVN and SON were very low in sham-ADX animals (PVN, 21 ± 1 dpm/gm tissue; SON, 11.91 ± 1.66 dpm/gm tissue; see Figs. 4*A*, 5*A*). However, a significant increase in NPY mRNA in the pmPVN was observed following ADX (166 ± 9 dpm/gm, $\pm$ SEM) that was not evident in animals given DEX replacement (Fig. 2).

Subsequently, the effects of ADX, chronic ingestion of 2% saline, or a combination of both treatments on NPY mRNA levels were investigated in selected hypothalamic areas (Fig. 3). Hypertonic saline treatment significantly elevated levels of NPY mRNA in the magnocellular neurons of the PVN and SON (Figs. 3; 4*B,C*; 5*B,C*), while the combination of ADX and 2% saline treatment resulted in even larger increases of NPY mRNA levels in these neurons (Figs. 3, 4*D*, 5*D*).

Neither ADX nor chronic osmotic stimulation had significant effects on NPY mRNA levels in the Arc (Figs. 3, 6*A–C*), but the combination of both treatments significantly increased NPY

mRNA levels in the Arc from 361 ± 11 dpm/gm in controls to 649 ± 16 dpm/gm (Figs. 3, 6*D*).

Effect of ADX and osmotic stimulation on NPY-IR in hypothalamic neuroendocrine neurons

Normally, NPY-IR perikarya are inconsistently visualized in hypothalamic nuclei, and because of this some animals used for immunohistochemical studies were colchicine treated prior to fixation. Colchicine treatment was, however, not given to ADX rats drinking 2% saline or to animals used for immunohistochemical studies of NPY-IR nerve fibers.

NPY-IR perikarya were not present within either the pmPVN or the SON of sham-ADX rats (Figs. 7*A*, 8*A*), even though they had been colchicine treated. In all animals, no matter what treatment they had been given, a plexus of NPY-IR fibers and terminal-like elements was observed throughout the PVN as well as in the ventral glial lamina of the SON. ADX induced an increase of NPY-IR in few magnocellular perikarya within the PVN and SON (Figs. 7*B*, 8*B*), but this effect was only ob-

Figure 5. Dark-field photomicrographs showing cells containing NPY mRNA in the SON of control (A), ADX (B), 2% saline-treated (C), and ADX rats drinking 2% saline (D). All treatments increased NPY mRNA levels in cell bodies within the SON. Scale bars, 200 μ m.

Figure 6. Dark-field photomicrographs showing cells containing NPY mRNA in the Arc of control (A), ADX (B), 2% saline-treated (C), and ADX rats drinking 2% saline (D). Neither ADX nor salt loading alone increased NPY mRNA levels, while the combined effect of ADX and 2% saline treatment markedly increased NPY mRNA levels in the Arc. Scale bars, 200 μ m.

Figure 7. Photomicrographs showing NPY-IR elements in the PVN of control (*A*), ADX (*B*), 2% saline-treated (*C*), and ADX rats drinking 2% saline (*D*). NPY-IR perikarya are not present in colchicine-treated control animals (*A*), while very few faintly labeled magnocellular perikarya are present in colchicine-treated ADX animals (arrows in *B*). In rats drinking 2% saline and ADX rats drinking 2% saline, a high number of magnocellular NPY-IR perikarya is seen (arrows in *C* and *D*). Scale bars, 100 μ m.

served in colchicine-treated animals. Hypertonic saline treatment induced a marked increase in NPY-IR in magnocellular neurons of the PVN and SON (Figs. 7*C*, 8*C*), and this effect was also observed in rats that had not been treated with colchicine. The combined effects of ADX and hypertonic saline induced an even larger expression of NPY-IR in magnocellular perikarya within the PVN and SON (Figs. 7*D*, 8*D*) than in animals given these treatments separately.

In colchicine-treated animals, a large number of small NPY-IR perikarya was consistently observed within the ventromedial part of the Arc. Neither ADX nor chronic ingestion of 2% saline affected the number of NPY-IR perikarya in the Arc (not shown). The distribution of NPY-IR fibers and terminals in the Arc and the adjacent median eminence (ME) was evaluated in sections from non-colchicine-treated animals. In sham-ADX rats, a dense plexus of NPY-IR nerve terminals was observed in the ventromedial part of the Arc, while slightly lower densities were present within the adjacent ventrolateral and dorsal part of the Arc (Fig. 9*A*). Within the ME, NPY-IR fibers and terminals were predominant in the internal zone (ME_i), while fewer fibers were present within the external zone (ME_e) (Fig. 9*A*). ADX induced a selective decrease in the number of NPY-IR terminals in the ME_e, while 2% saline treatment induced an increase in the number of NPY-IR fibers in the ME_i (Fig. 9*C*), and these changes were even greater in ADX animals drinking 2% saline (Fig. 9*D*). In addition, within the Arc of ADX rats drinking 2% saline a number of small NPY-IR perikarya emerged in the ventro-

medial part of the nucleus (Fig. 9*D*), despite the fact that these animals had not been injected with colchicine.

Discussion

The present study confirms and extends earlier reports that NPY gene expression in the magnocellular neurons of the HNS is enhanced during chronic osmotic stimulation (Larsen et al., 1992a,b), and demonstrates for the first time that levels of NPY mRNA and NPY-IR are subject to negative feedback regulation by adrenal steroids. Only rats drinking 2% saline had elevated plasma osmolarities, indicating that adequate sodium supplementation was given by providing 0.9% saline to ADX animals.

No increase in NPY mRNA was observed in ADX animals receiving DEX, which provides strong evidence that glucocorticoids are responsible for this inhibitory feedback. In contrast to the effect upon NPY mRNA, ADX had little effect upon NPY-IR in magnocellular perikarya of the PVN and SON or in fibers and terminals in the ME_i, suggesting little change in intracellularly stored NPY. This apparent mismatch between NPY mRNA levels and NPY-IR elements in the HNS following ADX may be due to lack of translation of the increased NPY mRNA into peptide. Another possibility is that ADX lowers intracellular storage capacity and/or enhances axonal flow resulting in rapid release of newly synthesized peptide. We have recently observed a similar mismatch in the Arc. Here, a very high number of positively hybridized prosomatostatin mRNA-containing cell bodies was observed, while very few prosoma-

Figure 8. Photomicrographs showing NPY-IR elements in the SON of control (*A*), ADX (*B*), 2% saline-treated (*C*), and ADX rats drinking 2% saline (*D*). NPY-IR perikarya are not present in colchicine-treated control animals (*A*), while very few faintly labeled magnocellular perikarya are present in colchicine-treated ADX animals (*B*). In rats drinking 2% saline and ADX rats drinking 2% saline, a high number of magnocellular NPY-IR perikarya is seen (*C* and *D*). Scale bars, 100 μ m.

tostatin- and somatostatin-immunoreactive perikarya were present (Larsen et al., 1992c). Finally, the effectiveness of colchicine treatment in blocking axonal transport of NPY and the subsequent visualization of NPY-IR perikarya is likely to vary dependent on distance of the neurons from the ventricular system. Circulating levels of NPY are not affected by ADX, and levels of radioimmunoassayable NPY in the PVN region are not altered in ADX animals (Rivet et al., 1989), suggesting that the increase in NPY mRNA expression observed after ADX does not lead to increased peptide content in the HNS. In addition, none of the treatments influenced significantly the plexus of NPY-IR fibers in the PVN or SON. The magnocellular neurons of the PVN and SON receive a major contingent of NPY-IR fibers from the catecholaminergic A1 brainstem area, while the parvocellular PVN subnuclei are preferentially innervated from yet other brainstem areas (Sawchenko et al., 1985). This distant origin of NPY-containing fibers in the PVN and SON may also explain why colchicine administered into the third ventricle had relatively minor effect on the intensity of the NPY-IR in those nuclei.

We could not detect any changes in NPY mRNA or NPY-IR in the Arc following ADX alone. This supports previous work where no change in Arc NPY content was observed a few hours after ADX (Rivet et al., 1989), but there are other reports suggesting that NPY gene expression in the Arc is responsive to

glucocorticoid manipulation. Glucocorticoids increased the content of NPY-IR in the mediobasal hypothalamus and in cultured hypothalamic cells (Corder et al., 1988), while removal of adrenal steroids following ADX has been reported to decrease NPY mRNA levels in tissue blocks containing the Arc (White et al., 1990). One reason for this discrepancy between the latter work and our own may be due to different methods employed to detect NPY mRNA (Northern blot with riboprobes vs *in situ* hybridization histochemistry with oligonucleotide probes). The timing of the studies following ADX may also be important.

Of special interest was our observation that the combined effects of osmotic stimulation and ADX increase NPY mRNA expression in neurons of the Arc, and has additive effects on the accumulation of NPY mRNA in magnocellular neurons of the SON and PVN. The latter result suggests that within magnocellular neurons of the HNS, independent mechanisms regulate the responses to osmotic stimuli and adrenal steroids, respectively. The underlying mechanism behind these results may represent either two separate mechanisms for NPY gene regulation in the same neurons or the accumulative effect of the responses of two independently regulated population of neurons.

Anatomical evidence suggests that besides projections to the ME_c, the Arc sends a substantial NPY input to the PVN (Bai et al., 1985). NPY introduced into the PVN exerts a stimulatory action on the HPA axis as revealed by increased plasma ACTH

Figure 9. Photomicrographs showing NPY-IR elements in the Arc and adjacent ME of control (A), ADX (B), 2% saline-treated (C), and ADX rats drinking 2% saline (D). Scale bars, 100 μ m.

and corticosterone levels (Wahlestedt et al., 1987). The anatomical relationship between NPY-containing terminals and parvocellular CRF-41 motor neurons in the PVN suggests that NPY may exert this effect directly. If this is the case, the logical consequence of ADX would be that NPY mRNA expression is stimulated in NPYergic pathways regulating the HPA axis. A careful examination of the distribution of NPY-IR elements in the Arc and adjacent ME revealed clear differences between variously treated animals, supporting the view that two independently regulated populations of NPY-IR neurons reside in the Arc. In this context, one population of neurons projecting to the ME_c responds to ADX with decreased NPY synthesis while another population projecting to the PVN responds to ADX with increased NPY synthesis. The opposite should hold true for osmotic stimulation. This suggestion is consistent with recent observations that chronic osmotic stimulation is associated with decreased activity of the HPA axis, indicating that synthesis of factors stimulating the CRF-41 motor neurons may be downregulated (Jessop et al., 1990b).

Another osmotic stimulus, water deprivation for 4 d, has been shown to increase NPY mRNA expression in the Arc (O'Shea and Gundlach, 1991). Water deprivation represents a stimulus with both osmotic and volemic components, which in addition to effects on water homeostatic regulatory mechanisms has profound effects on cardiovascular regulation. The possibility that water-deprived animals exhibit altered ingestive behavior should also be considered, and when injected into the PVN, NPY has potent stimulatory effects on ingestive behavior (Stanley and Leibowitz, 1984, 1985). Because of this, the effect of water deprivation is not directly comparable with the presently employed osmotic stimulation paradigm, and consequently the effects on NPY mRNA expression within Arc neurons may differ.

The effects of ADX and subsequent corticosterone substitution on AVP mRNA in magnocellular neurons of the PVN and SON have been subject to controversy. Thus, some studies have not been able to demonstrate any effects of ADX on the AVP mRNA content in magnocellular PVN neurons (Young et al., 1986; Swanson and Simmons, 1989; Grino et al., 1990), while other studies have demonstrated increased AVP mRNA content in these neurons (Davis et al., 1986; Suemaru et al., 1990). A slight but yet significant increase of AVP mRNA levels was observed in the pmPVN in the present study, whereas AVP mRNA levels in the SON were unaffected. The distinction of magnocellular neurons from parvocellular neurons was made merely from topographical criteria rather than morphological criteria, and not all cell bodies contained in the pmPVN are magnocellular (Swanson and Sawchenko, 1983). Therefore, it is possible that the slight increase in AVP mRNA induced by ADX within the magnocellular part of the PVN is actually confined to parvocellular CRF-41/AVP neurons projecting to the ME. From the available evidence, it seems obvious that AVP synthesized in magnocellular neurons plays little, if any, role in the ACTH response to ADX.

In conclusion, NPY mRNA and peptide are present in the rat hypothalamo-neurohypophyseal pathway. After osmotic stimulation and ADX, levels of NPY mRNA in magnocellular neurons of the SON and PVN are increased, while levels of NPY-IR appear to increase to the greatest extent following osmotic stimulation. Further studies are in progress to investigate the physiological role of hypothalamo-neurohypophyseal NPY in the regulation of neurohypophyseal hormone release.

References

- Ahima R, Krozowski Z, Harlan R (1991) Type I corticosteroid receptor-like immunoreactivity in the rat CNS: distribution and regulation by corticosteroids. *J Comp Neurol* 313:522–538.
- Ahima RS, Harlan RE (1990) Charting of type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system. *Neuroscience* 39:579–604.
- Bai JM, Yamano M, Shiotani Y, Emson PC, Smith AD, Powell JF, Tohyama M (1985) An arcuate-paraventricular and dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. *Brain Res* 331:172–175.
- Bargmann W, Scharrer E (1951) The site of origin of the hormones of the posterior pituitary. *Am Sci* 39:255–259.
- Bisset GW, Chowdrey HS (1988) Control of release of vasopressin by neuroendocrine reflexes. *Q J Exp Physiol* 73:811–872.
- Brownstein MJ, Mezey E (1986) Multiple chemical messengers in hypothalamic magnocellular neurons. *Prog Brain Res* 68:161–168.
- Brownstein MJ, Russell JT, Gainer H (1980) Synthesis, transport and release of posterior pituitary hormones. *Science* 207:373–378.
- Chronwall BM, DiMaggio DA, Masari VJ, Pickel VM, Ruggiero DA, O'Donohue TL (1985) The anatomy of neuropeptide Y-containing neurons in rat brain. *Neuroscience* 15:1159–1181.
- Corder R, Pralong F, Turnill D, Saudan P, Muller AF, Gaillard RC (1988) Dexamethasone treatment increases neuropeptide Y levels in rat hypothalamic neurones. *Life Sci* 43:1879–1886.
- Davis LG, Arentzen R, Reid JM, Manning RW, Wolfson B, Lawrence KL, Baldino F Jr (1986) Glucocorticoid sensitivity of vasopressin mRNA levels in the paraventricular nucleus of the rat. *Proc Natl Acad Sci USA* 83:1145–1149.
- DeQuidt ME, Emson PC (1986) Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system. I. Radioimmunoassay and chromatographic characterization. *Neuroscience* 18:527–543.
- Grino M, Guillaume V, Bourdoursque F, Conte-Devoix B, Maltese JY, Oliver C (1990) Glucocorticoids regulate peptidyl glycine α -amidating monooxygenase gene expression in the rat hypothalamic paraventricular nucleus. *Mol Endocrinol* 4:1613–1619.
- Hatton GI (1990) Emerging concepts of structure-function dynamics in adult brain: the hypothalamo-neurohypophyseal system. *Prog Neurobiol* 34:437–504.
- Hooi SC, Richardson GS, McDonald JK, Allen JM, Martin JB, Koenig JJ (1989) Neuropeptide Y (NPY) and vasopressin (aVP) in the hypothalamo-neurohypophyseal axis of salt-loaded or Brattleboro rats. *Brain Res* 486:214–220.
- Hsu SM, Raine L, Fanger H (1981) The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29:577–580.
- Ivell R, Richter D (1984) Structure and comparison of the oxytocin and vasopressin genes from the rat. *Proc Natl Acad Sci USA* 81:2006–2010.
- Jessop DS, Eckland DJA, Todd K, Lightman SL (1989) Osmotic regulation of hypothalamo-neurointermediate lobe corticotrophin-releasing factor-41 in the rat. *J Endocrinol* 120:119–124.
- Jessop DS, Sidhu R, Lightman SL (1990a) Osmotic regulation of methionine enkephalin in the posterior pituitary of the rat. *Brain Res* 516:41–45.
- Jessop DS, Chowdrey HS, Lightman SL (1990b) Inhibition of rat corticotrophin-releasing factor and adrenocorticotrophin secretion by an osmotic stimulus. *Brain Res* 523:1–4.
- Jessop DS, Larsen PJ, Mikkelsen JD, Lightman SL, Chowdrey HS (in press) The effects of adrenalectomy and hypertonic saline on neuropeptide Y content in the posterior pituitary of the rat. *Neuroendocrinology*, in press.
- Jingami H, Mizuno NB, Takahashi H, Shibahara S, Furutani Y, Imura H, Numa S (1985) Cloning and sequence analysis of cDNA for rat corticotrophin-releasing factor precursor. *FEBS Lett* 191:63–66.
- Jones CW, Pickering BT (1969) Comparison of the effects of water deprivation and sodium chloride imbibition on the hormone content of the neurohypophysis of the rat. *J Physiol (Lond)* 203:449–458.
- Kiss JZ, van Eckelen JAM, Reul JMHM, Westphal HM, DeKloet ER (1988) Glucocorticoid receptor in magnocellular neurosecretory cells. *Endocrinology* 122:444–449.
- Larhammer D, Ericsson E, Persson H (1987) Structure and expression

- of the rat neuropeptide Y gene. *Proc Natl Acad Sci USA* 84:2068–2072.
- Larsen PJ, Sheikh S, Mikkelsen JD (1992a) Evidence for neuropeptide Y in the magnocellular hypothalamo-neurohypophyseal pathway is involved in the regulation of water homeostasis. *Brain Res* 573:181–189.
- Larsen PJ, Jessop DS, Chowdrey HS, Lightman SL (1992b) Neuropeptide Y messenger ribonucleic acid in the magnocellular system of the rat is increased during osmotic stimulation. *Neurosci Lett* 138:23–26.
- Larsen PJ, Bersani M, Holst JJ, Møller M, Mikkelsen JD (1992c) The distribution of different molecular forms of somatostatin in the hypothalamus and posterior pituitary of the Mongolian gerbil (*Meriones unguiculatus*). *J Neurosci* 12:946–962.
- Leng G, Bicknell RJ (1986) The neurohypophysis. In: *Neuroendocrinology* (Lightman SL, Everitt BJ, eds), pp 177–196. Oxford: Blackwell.
- Lightman SL, Young SW III (1987) Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. *J Physiol (Lond)* 394:23–39.
- Meister B, Cortes R, Villar MJ, Schalling M, Hökfelt T (1990) Peptides and transmitter enzymes in hypothalamic magnocellular neurons after administration of hyperosmotic stimuli: comparison between messenger RNA and peptide/protein levels. *Cell Tissue Res* 260:279–297.
- Mikkelsen JD, O'Hare MMT (1991) An immunohistochemical and chromatographic analysis of the distribution and processing of pro-neuropeptide Y in the rat suprachiasmatic nucleus. *Peptides* 12:177–185.
- Mikkelsen JD, Schmidt P, Sheikh SP, Larsen PJ (1992) Non-vasopressinergic, non-oxytocinergic neuropeptides in the rat hypothalamo-neurohypophyseal tract. *Prog Brain Res* 91:367–371.
- Morris BJ (1989) Neuronal localization of neuropeptide Y gene expression in rat brain. *J Comp Neurol* 290:358–368.
- O'Shea RD, Gundlach AL (1991) Preproneuropeptide Y messenger ribonucleic acid in the hypothalamic arcuate nucleus of the rat is increased by food deprivation or dehydration. *J Neuroendocrinol* 3:11–14.
- Rivet JM, Castagne V, Corder R, Gaillard R, Mormede P (1989) Study of the influence of stress and adrenalectomy on central and peripheral neuropeptide Y levels. *Neuroendocrinology* 50:413–420.
- Robinson ICAF (1986) The magnocellular and parvocellular Ot and AVP systems. In: *Neuroendocrinology* (Lightman SL, Everitt BJ, eds), pp 154–176. Oxford: Blackwell.
- Sawchenko PE, Swanson LW, Grzanna R, Howc PRC, Bloom SR, Polak JM (1985) Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. *J Comp Neurol* 241:138–153.
- Stanley BG, Leibowitz SF (1984) Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sci* 35:2635–2642.
- Stanley BG, Leibowitz SF (1985) Neuropeptide Y injected into the paraventricular hypothalamus: a powerful stimulant of feeding behavior. *Proc Natl Acad Sci USA* 82:3940–3943.
- Suemaru S, Hashimoto K, Ogasa T, Ota Z, Hirakawa M, Kawata M (1990) Effects of hyperosmotic stimulation and adrenalectomy on vasopressin mRNA levels in the paraventricular and supraoptic nuclei of the hypothalamus: *in situ* hybridization histochemical analysis using a synthetic oligonucleotide probe. *Acta Med Okayama* 44:233–241.
- Swanson LW, Kuypers HGJM (1980) The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol* 194:555–570.
- Swanson LW, Sawchenko PE (1983) Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. *Annu Rev Neurosci* 6:269–324.
- Swanson LW, Simmons DM (1989) Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. *J Comp Neurol* 285:413–435.
- Tatemoto K, Carlquist M, Mutt V (1982) Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 256:659–660.
- Vanderhaeghen JJ, Lotstra F, Vandesande F, Dierickx K (1981) Coexistence of cholecystokinin and oxytocin-neurophysin in some magnocellular hypothalamic-hypophyseal neurons. *Cell Tissue Res* 221:227–231.
- Wahlestedt C, Skagerberg G, Ekman R, Heilig M, Sundler F, Håkanson R (1987) Neuropeptide Y (NPY) in the area of the hypothalamic paraventricular nucleus activates the pituitary-adrenocortical axis in the rat. *Brain Res* 417:33–38.
- Watson SJ, Akil H, Fischli W, Goldstein A, Zimmerman EA, Nilaver G, Wimersma Gredanius TB (1982) Dynorphin and vasopressin: common localization in magnocellular neurons. *Science* 216:85–87.
- White BD, Dean RG, Martin RJ (1990) Adrenalectomy decreases neuropeptide Y mRNA levels in the arcuate nucleus. *Brain Res Bull* 25:711–715.
- Young SW III, Mezey E, Siegel RE (1986) Vasopressin and oxytocin mRNAs in adrenalectomized and Brattleboro rats: analysis by quantitative *in situ* hybridization histochemistry. *Mol Brain Res* 1:231–241.