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REVIEW

Expression of Neurohypophysial Hormone Precursor Genes in the Mammalian Hypothalamus

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INTRODUCTION

Arginine vasopressin (AVP) and oxytocin (OXT) are mammalian neurohypophysial hormones. AVP has important roles in water and salt metabolism, control of blood pressure, response to stress, and consolidation of memory [1, 2], while OXT is important in reproduction [2] and sodium excretion [3, 4]. They are synthesized in magnocellular and parvocellular neurosecretory neurons in the hypothalamus (Fig. 1). Magnocellular supraoptic and paraventricular AVP and OXT neurons send their axons mainly to the neurohypophysis and release hormones into the circulation. Projections from parvocellular neurons, which are localized in discrete neuronal groups such as the paraventricular and suprachiasmatic nuclei, are widely distributed among various brain loci, e.g., the median eminence, medullar oblongata, spinal cord, and pineal organ [5, 6]. Particular neuronal groups, either magnocellular or parvocellular, are considered to be involved in characteristic physiological roles. It is thus important to clarify the regulatory mechanisms for the secretory activity, i.e., synthesis and release, of neurosecretory neurons in particular neuronal groups, in order to understand their physiological roles.

Immunohistochemistry and radioimmunoassay have been utilized for studying regulation of the secretory activity of neurosecretory neurons. In addition, recent progress in molecular biological

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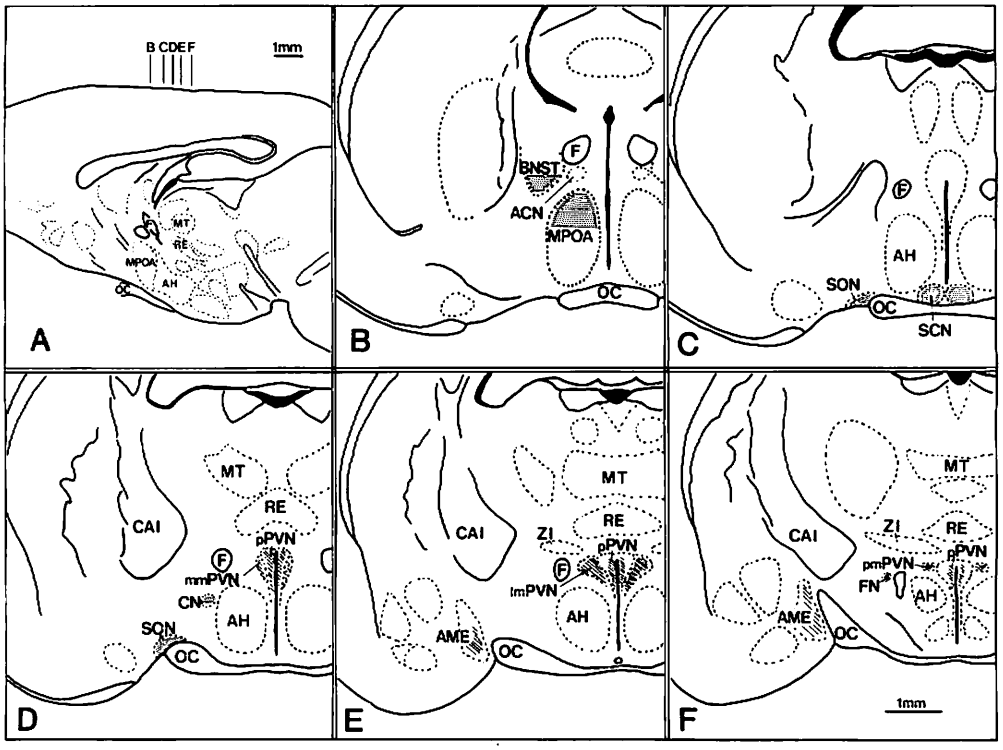

FIG. 1. Schematic diagram showing the rat diencephalon. (Based on Konig and Klippel [186]). *A*, Sagittal view of the diencephalon showing the levels of cross sections in Figs. 1B-1F. *B-F*, Cross sections illustrating the nuclei which produce neurohypophysial hormones (hatched area). ACN, anterior commissural nucleus; AH, anterior hypothalamic nucleus; AME, medial amygdala; BNST, bed nucleus of the stria terminalis; CAI, internal capsule; CN, circular nucleus; F, fornix; FN, fornical nucleus; MPOA, medial preoptic area; MT, medial nucleus of thalamus; OC, optic chiasma; mPVN, magnocellular part of paraventricular nucleus; lmPVN, lateral mPVN; mmPVN, medial mPVN; pmPVN, posterior mPVN; pPVN, parvocellular part of PVN; RE, reunient nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; ZI, zona inserta.

methods has enabled us to study expression of hormonal genes using hybridization techniques, e.g., Northern and dot blot analyses and *in situ* hybridization (ISH). Since the magnitude of gene expression is considered to reflect the level of synthetic activity, these techniques have been applied by many investigators to clarify neuroendocrine secretion, mostly in mammals. Accordingly, this review will focus on studies on the expression of neurohypophysial hormone genes in the mammalian hypothalamus. Where data is available, comment is also made on nonmammalian neurohypophysial systems. The role of extrahypothalamic expression of AVP and OXT genes is also considered together with changes in synthetic activity of neurohypophysial peptides during ontogeny.

STRUCTURES OF GENES ENCODING NEUROHYPOPHYSIAL HORMONE PRECURSORS

The amino acid sequences of AVP and OXT were determined by du Vigneaud's group around 1950 [7]. Then, neurophysins, components of vasopressin and oxytocin precursors, were isolated in many mammalian species [8]. For the vasopressin precursor, a glycopeptide named copeptin was further isolated first as an amino (N)-terminal fragment [9, 10], although it is actually a carboxyl (C)-terminal moiety (see below).

Subsequently, the nucleotide sequences of cDNAs and genes for AVP and OXT precursors were clarified in several mammalian species, including bovine [11–14], rat [13, 15, 16], human

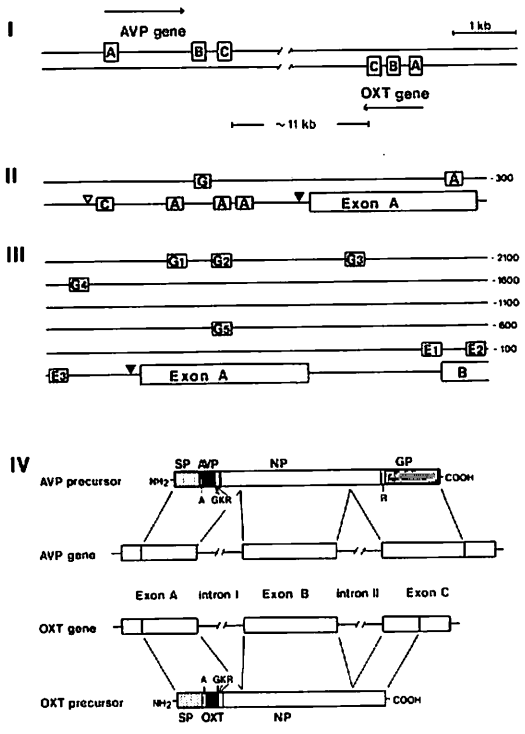

FIG. 2. *I*, Schematic diagram of the organization of the rat AVP and OXT genes. Horizontal arrows indicate the direction of transcription. A, B and C indicate Exon A, B, and C, respectively. *II*, Schematic diagram of the promoter region of rat AVP gene. The number indicates the position relative to the transcription start site (+1). A, AP2 element; C, cAMP responsive element; G, glucocorticoid responsive element; Open triangle, CCAAT motif; closed triangle, modified TATA-box. *III*, Schematic diagram of the promoter region of rat OXT gene. E, estrogen responsive element; G, glucocorticoid responsive element; closed triangle, modified TATA-box. *IV*, Schematic diagram showing the structural organization of the AVP and OXT precursors and their genes. SP (dotted bar), signal peptide; NP (Open bar), neurophysin; GP (hatched bar), copeptin; A, alanine; G, glycine; K, lysine; R, arginine.

[17, 18], porcine [19; only lysine vasopressin], and ovine [20; only OXT]. These studies clarified the organization of AVP and OXT precursors. The AVP precursor is composed of AVP, AVP unique neurophysin and C-terminal glycopeptide, while the OXT precursor contains OXT and neurophysin (Fig. 2D).

Neurohypophysial hormone precursor genes (see Fig. 2) consist of three exons (A, B and C) and two introns [13–17]. Exon A consists of a nucleotide sequence encoding the signal peptide, hormone and N-terminal portion of neurophysin. Exon B encodes the central portion of neurophysin. Exon C encodes the C-terminal portion of neurophysin for the OXT precursor, and C-terminal portion of neurophysin and copeptin for the AVP precursor. The nucleotide sequences of exon B are highly conserved not only among species, but also between AVP and OXT genes. This implies the occurrence of a gene conversion event which has recently affected the AVP and OXT genes.

Human AVP and OXT genes are linked together with 12 kilobases intervening, and are transcribed from the opposite DNA strand [17]. This is also true for rat AVP and OXT genes [21] (Fig. 2A). In the case of the human genom, the AVP and OXT genes are further linked to prodynorphin gene on chromosome 20 [18]. Dynorphin is co-expressed and co-released by rat AVP neurons [22, 23].

The promoter regions have been well investigated in the rat and human genes (Fig. 2). The promoter region of rat AVP gene has one glucocorticoid responsive element (GRE), one cAMP responsive element, four AP2 binding sites, and a CCAAT motif [24]. The CCAAT box binding factor may have a functional cooperativity with a steroid-receptor complex [25]. The AVP mRNA level was increased by elevated intracellular cAMP in the primary dissociated cultures derived from diencephalic tissues of fetal rats [26] and in the human AVP-expressing small cell lung carcinoma cell line GLC-8 [27]. Further, sodium loading increased mRNA levels for guanine-nucleotide binding protein α-subunits in the SON and the PVN [28]. In the SON, these changes were accompanied by increases in the basal cAMP level and adenylate cyclase activity, suggesting that hyperosmotic reception activates the cAMP second messenger system.

The promoter region of rat OXT gene contains three estrogen responsive elements (ERE) and five GREs [29] (Fig. 2C). Following stimulation with 17β estradiol of heterologous fusion gene which consists of a part of the OXT gene promoter and

chloramphenicol acetyl transferase (CAT) gene, two ERE, ERE 1 and ERE 2, induced a 13.6-fold and 8.6-fold transcriptional activation of the CAT activity, respectively. Moreover, the ERE 1 and ERE 2 can act synergistically (34-fold transcriptional activation). These facts suggest that two EREs are functional at least in heterologous gene constructs [29]. Among GREs, GRE 1, which is located more than 2 kb upstream of the transcriptional starting site, represents the functional GRE [29]. Since the single GRE, which is located more than 300 bp upstream of the transcriptional initiation site, alone is almost inactive [25, 30], this element would require additional nearby cis-acting elements for its putative activity. The human OXT gene has one ERE which is identical to ERE 1 in the rat OXT gene, and has two motifs corresponding to the right half of ERE palindrome [31]. The human OXT gene further contains a CCAAT box and a "elegance box" (CTGCTAA) heptamer. The latter is closely associated with functional EREs and is thought to play a supportive role in the control of gene expression by estrogen [32]. Among the EREs of human OXT gene, only ERE 1 induced transcriptional activation in Neuro-2a cells co-transfected with an expression vector for the human estrogen receptor [31].

A corresponding sequence element to the rat and human ERE 1 has also been detected in the promoter region of bovine OXT gene, however, the left half of the element has three variations from the ERE consensus sequence [14]. This bovine element does not function as an ERE [33]. A mouse corresponding element, which differs in two nucleotides from the consensus sequence, is a functional, but a weaker ERE than the rat/human element [31].

Recently, cDNAs and genes encoding neurohypophysial hormone precursors have been cloned in several lower vertebrates such as toad [34], and several species of teleost fishes including white sucker [35–37], chum salmon [38, 39] and masu salmon [40]. The toad vasotocin (VT) precursor contains signal peptide, hormone, neurophysin and glycopeptide, while toad mesotocin precursor consists of signal peptide, hormone and neurophysin, as in mammalian neurohypophysial hormone precursors [34]. Nevertheless, processing between neurophysin and copeptin does not occur in toad VT precursor, despite the presence of the arginine residue between the two moieties, and VT and "big" neurophysin are released from the neurohypophysis [41, 42]. Furthermore, incompletely processed VTs, Hydrin 1 and 2, have been found only in amphibian [43, 44]. These intermediates are roughly as active as VT on water permeability of frog cutaneous and urinary bladder epithelia.

Teleost VT precursors consist of signal peptide, VT and neurophysin [36–40]. The C-termini of neurophysins are about 30 amino acids longer than neurophysins of toad vasotocin and mammalian vasopressin precursors, and are strikingly similar to the copeptins of AVP and toad VT precursors, although these extended regions do not contain a glycosylation site. Teleost isotocin (IT) precursors contain signal peptide, IT and neurophysin [35, 36, 39, 40]. The C-termini of IT neurophysins also extend about 30 amino acids beyond OXT and MT neurophysins. The elongated region includes a leucine-rich core segment at their central loci, and show similarities with the copeptins of AVP and toad VT precursors.

MAGNOCELLULAR NEUROSECRETORY SYSTEMS

(*a*) *Association with water and salt metabolism*

Magnocellular supraoptic and paraventricular neurons whose axons project predominantly to the neurohypophysis have important roles in water and salt metabolism. Sodium loading by oral intake of hypertonic saline and water deprivation are physiological stimuli that can affect magnocellular AVP and OXT neuronal activity. These stimuli increase electrical activity of magnocellular AVP and OXT neurons in the supraoptic (SON) and the paraventricular (PVN) nuclei, and then rapidly elevate the plasma AVP and OXT levels [45, 46]. Changes in expression levels of AVP and OXT precursor genes have been studied by use of semi-quantitative *in situ* hybridization, Northern blot, dot blot, and solution hybridization analyses. For hybridization probes, most investigators have used restriction fragments or synthetic oligonucleotides complementary to the AVP mRNA en-

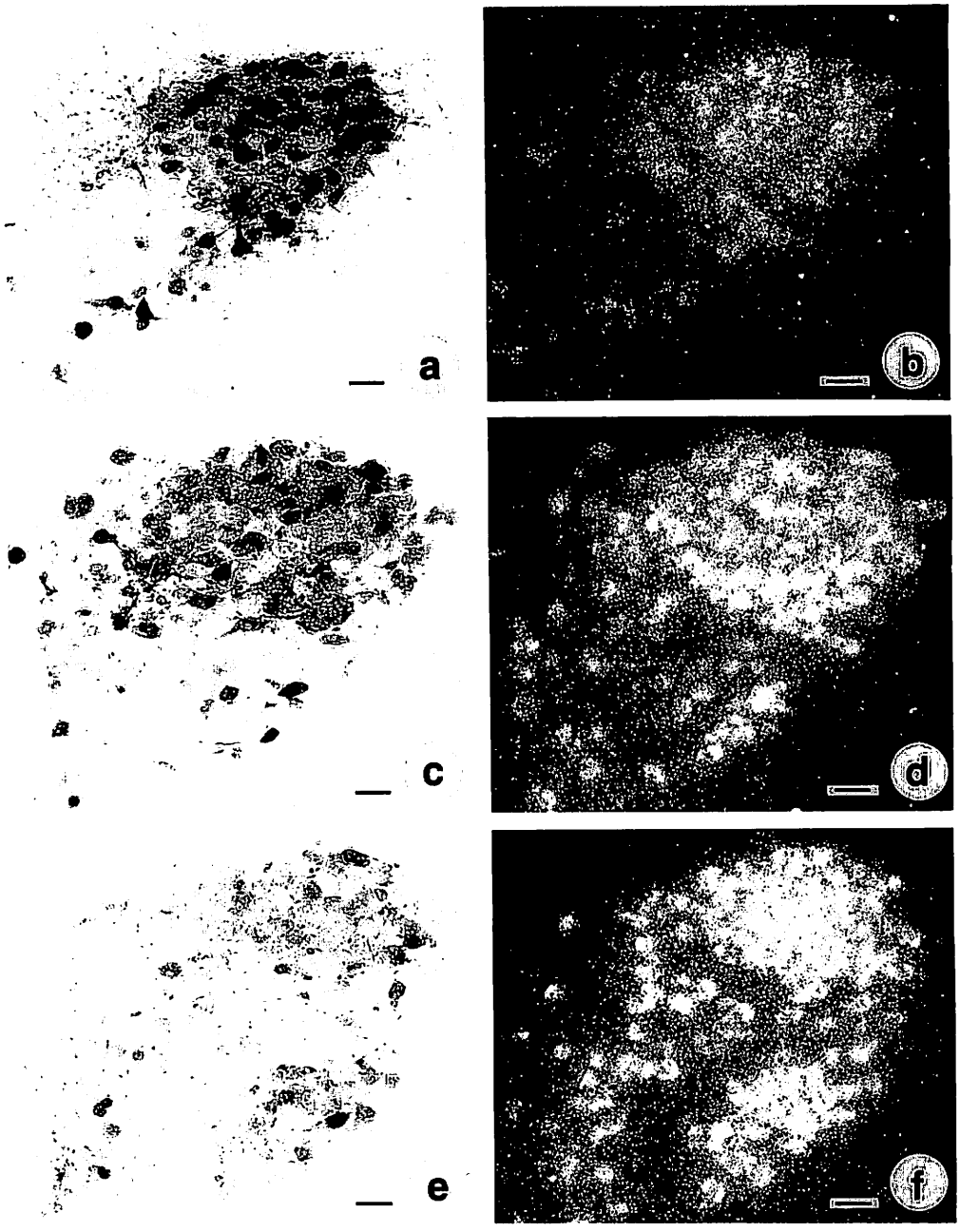

FIG. 3. AVP immunoreactive neurons (a, c, e) and hybridization signals (b, d, f) for the AVP mRNA in the PVN of normal (a, b), 4-day (c, d) and 7-day (e, f) water deprived rats in mirror image sections. Scale bar, 50 μm. Dark-field photomicrographs for hybridization. From reference [57].

coding copeptin and to the 3′-noncoding region of the OXT mRNA, while we have used synthetic oligonucleotide probes complementary to the mRNA loci encoding hormones and N-terminal region of neurophysins [47–49].

The AVP mRNA levels were increased in the magnocellular neurons of the SON and the PVN in the rat following sodium loading [2, 48, 50–56] and

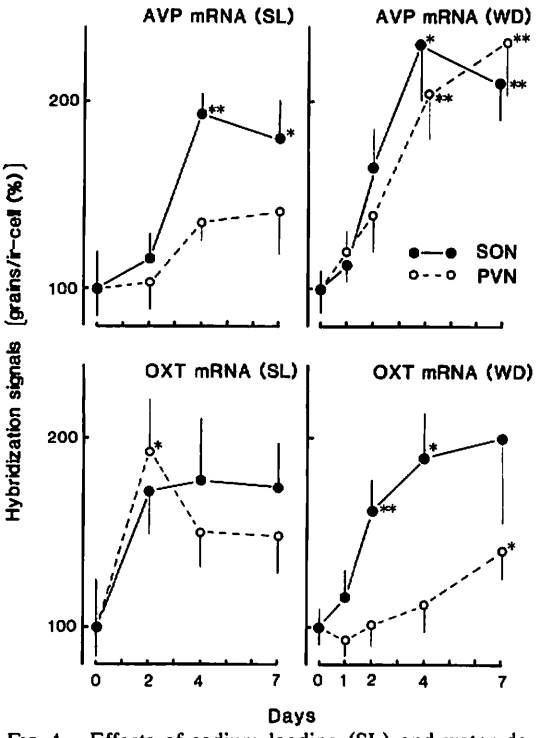

FIG. 4. Effects of sodium loading (SL) and water deprivation (WD) on the AVP and OXT mRNA levels in the SON and the PVN. The mRNA levels are expressed as percent changes compared to the level of day 0. Each point represents the mean ± S.E. (n =7). *, $p<0.05$; **$p<0.01$; by t-test compared to day 0. From references [51, 57].

water deprivation [57–61] (Figs. 3 and 4). The rise in AVP mRNA levels was restricted to magnocellular neurons, and was not found in parvocellular neurons. A significant rise in the AVP mRNA level was observed, in most cases, 48 hr after the onset of stimuli [22, 51, 52, 55–57]. Thereafter, the AVP mRNA level increased further, and attained a peak level by day 7 of the treatment (Fig. 4). The increased level induced by dehydration was reduced following rehydration of the rat, but remained significantly elevated throughout 30 days, although the plasma sodium concentration and osmolality returned to the control levels by 2 days after rehydration [56].

Although the AVP mRNA levels were increased both in the SON and the PVN following sodium loading in the rat, the increase in the PVN was gradual and much less than that in the SON [22, 50, 51, 55]. In contrast, a marked increase in the AVP mRNA level was observed in both the SON and the PVN following water deprivation [57] (Fig. 4). This trend agrees well with the data concerning changes in amino acid incorporation following sodium loading and water deprivation [62, 63], suggesting that AVP neurons in both the SON and the PVN are sensitive to hypovolemic stimulation, and that supraoptic AVP neurons are more responsible for osmotic or sodium regulation than paraventricular neurons.

OXT mRNA levels were increased in the SON and the PVN following sodium loading and water deprivation in the rat [51, 52, 57, 64] (Fig. 4). After osmotic stimulation, marked release of OXT and hypertrophy of OXT neurons have been observed [51, 57, 65]. These results may imply that OXT neurons have some physiological role in water and salt metabolism, in addition to reproduction. Indeed, OXT administration produced dose-related increases in urine flow and Na^+ and Cl^- excretion, and considerably potentiated the natriuretic action of AVP [3, 4].

Dynorphin A is co-stored with AVP in the same secretory granules in magnocellular neurons [23] and binds to κ-receptors on nerve terminals in the neural lobe [66]. Expression of dynorphin gene was increased after sodium loading in the rat [22, 52]. This result indicates coordinated regulation of gene expression for the coexisting peptides, AVP and dynorphin. The fact that loci for the structural genes for human AVP/OXT have extremely close linkage with the dynorphin locus on chromosome 20 [18] supports the idea of coordinated regulation. Dynorphin-related peptides may preferentially inhibit OXT release by a κ-receptor mechanism in the neurohypophysis, and control differential secretion of AVP and OXT [67].

In addition to the increase in the amounts of mRNAs, the size of the AVP and OXT mRNAs was increased following sodium loading and water deprivation in the rat [58, 68–72]. As is mentioned above, the significant increases in the mRNA levels were observed, in most cases, after 48 hr of osmotic stimulation. Whereas, the increases in AVP mRNA size were detected within 2 hr [58, 69–71]. The increases in the size of the mature mRNAs were due to an increase in the poly (A)

tail size, since poly (A) tail removal reduced the length of AVP mRNA of control and experimental groups to exactly the same size [68, 72]. The length of AVP mRNA poly (A) tail began to decline progressively within 8 hr of rehydration, and was returned almost to the normal level by 7 days after water replenishment [68]. Although the function of the poly (A) tail of eukaryotic mRNA is poorly understood, it may be involved in the enhancement of mRNA stability [73, 74] and translational efficiency [75, 76]. Mechanisms involved in an increase in poly (A) tail length include a polyadenylation in the nucleus, a cytoplasmic poly (A) polymerase and protection from nucleolytic attack [77]. A rapid increase in AVP gene transcription after osmotic stimulation did not correlate with the increase in the AVP mRNA level, but coincided with the increase in the poly (A) tail length [58], suggesting that there is a rapid destruction of preexisting AVP mRNAs and their replacement with new transcripts bearing longer poly (A) tails which could be added in the nucleus or the cytoplasm.

Depletion of serotonin with p-chlorophenylalanine (PCPA) blocked the increases in AVP and OXT mRNA levels following sodium loading or water deprivation in rats, whereas the increase in the poly (A) tail length was similar in PCPA-treated and sham-operated animals [69]. After osmotic stimulation, the poly (A) tail length of AVP mRNA in the parvocellular suprachiasmatic neurons increased in parallel with that in the magnocellular supraoptic and paraventricular neurons, whereas there was no change in the amount of AVP mRNA in the suprachiasmatic nucleus (SCN) [70]. In the mouse, an increase in the poly (A) tail length of AVP mRNA was not observed after osmotic stimulation, although there was an accumulation of AVP mRNA [70]. These results suggest that osmotically induced accumulation of AVP mRNA is a consequence of increased transcription and/or the activation of mRNA-stabilizing systems not involving the poly (A) tail. The increases in the amount of mRNA and the length of poly (A) tail may be regulated by different control systems/mechanisms. However, signals which regulate gene expression of neurohypophysial hormone precursors remain to be clarified.

Among lower vertebrates, gene expression of VT precursor was increased following hypo-osmotic stimulation in rainbow trout [78]. The control mechanism responsible for this increase in gene expression after hypo-osmotic stimulation, which contrasts with the increase in AVP and OXT gene expression after hyper-osmotic stimulation in mammals, is not clear at present. Evolution of regulatory mechanisms of gene expression and the physiological roles of neurohypophysial hormones are attractive subjects for study in the future.

(*b*) *Brattleboro rat*

The Brattleboro rat with hereditary hypothalamic diabetes insipidus lacks AVP [79]. An explanation for this syndrome was found within the structure of the AVP gene which has a single base deletion in exon B [16]. This mutation results in a new open reading frame beyond position 66 of the neurophysin. The new reading frame of the mutant gene has no glycosylation site and lacks a stop codon. The mutant AVP gene was expressed at a reduced level in the Brattleboro hypothalamus [80–86], although there were claims that the mutant AVP gene is expressed in the Brattleboro rat at the same level as in the Long-Evans rat strain [61, 87, 88].

Immunohistochemical analysis using antibodies raised against a synthetic tetradecapeptide (CP-14) corresponding to the frame-shifted C-terminal, indicated that a mutant AVP precursor is present in the potential AVP neurons *in vivo*, though at a reduced level [82, 89]. These antibodies identified a product of molecular weight about 26,000 in a reticulocyte lysate system programmed with Brattleboro hypothalamic mRNA [82]. Hybridization signals of the mutant AVP mRNA and immunoreactivities of this large precursor and AVP were restrictively localized in cell bodies [81, 82, 85]. Furthermore, the signals were restricted mostly to the periphery of the cytoplasm. The mutant AVP precursor may be too foreign to be handled by the normal post-translational processing system of the magnocellular neurons. An electron microscopic study showed that immunoreactivity is localized in the endoplasmic reticulum and *trans*-Golgi, but not in neurosecre-

tory granules [90]. Instead, there is heavy staining of lysosomal bodies. Since the mutated precursor appears to contain approximately 70 lysine residues corresponding to the poly (A) tail in normal rats, this highly charged alien moiety may bind onto membrane surfaces and/or may trigger the scavenging mechanisms of the lysosomal system.

Following water deprivation for 24 hr or partial water deprivation (given 75% of their daily *ad libitum* water intake) for 96 hr, the mutant AVP mRNA level was not significantly increased, whereas plasma osmolality increase [84]. This result suggests that the Brattleboro rat has a marked defect in the regulation of AVP gene expression. The defect in AVP gene regulation, however, could be a phenomenon that occurs secondarily to the absence of circulating AVP and the accompanying water diuresis. In contrast to the untreated Brattleboro rat, the Brattleboro rat treated with AVP for 2 weeks exhibited a significant rise in the AVP mRNA level in response to 24 hr of water deprivation [88]. The extent of AVP mRNA regulation following water deprivation in the AVP treated Brattleboro rat was comparable to that observed in the Long-Evans rat, suggesting that the defect in AVP gene regulation is a secondary phenomenon rather than a primary genetic defect.

Northern blot analysis of the Brattleboro and normal rats demonstrated a longer poly (A) tail in the Brattleboro rat than that in the wild-type strain [80, 82]. The elongated poly (A) tail in the Brattleboro rat may be involved in translational efficiency and/or mRNA stability, and could be related to the continuous osmotic stimulation experienced in this strain. It will be necessary to examine whether the length of poly (A) tail of mutant AVP mRNA in the Brattleboro rat reduces to that of AVP mRNA in the wild-type rats after AVP treatment.

In contrast to the mutant AVP mRNA level, the Brattleboro rat contained greater levels of OXT mRNA in the magnocellular regions compared with wild-type rats [85, 86]. The OXT mRNA of the Brattleboro rat also possessed a longer poly (A) tail as compared with that of the wild-type rat [80]. The plasma OXT level rose even more in the Brattleboro than in the wild-type rats in response to an increase in plasma osmolality [65]. This elevated activity of the oxytocinergic system in the Brattleboro rat may be involved in an attempt to maintain plasma osmolality, since OXT seems to have an important role in the control of renal function [3, 4].

(*c*) *Association with reproduction*

Significant changes in hypothalamic OXT mRNA levels have been detected during postantal development, the estrous cycle, pregnancy and lactation in the rat. These changes in OXT mRNA levels were noted throughout the hypothalamus, including both magnocellular and parvocellular neurons [91]. The OXT mRNA level increased 5- to 10-fold from postnatal day 20 (PN20) to PN60 in both sexes coincidently with puberty in the rat [91, 92]. Pregnancy induced a gradual rise in the OXT mRNA level during the late gestational period (after day 18) [93, 94]. This increase at the end of gestation was in accord with increases in hypothalamic and pituitary OXT levels [95]. Throughout the lactational period, the OXT mRNA level remained elevated approximately 3-fold higher than that in the control [91, 93, 94]. The OXT mRNA level in the SON was significantly increased at estrus relative to the other periods of the estrous cycle [93], though cyclic variation in hypothalamic OXT mRNA levels has not been found by other investigators [92]. During lactation and estrus of the sexual cycle, pituitary OXT contents were decreased, whereas plasma OXT levels were increased, suggesting an increased release of OXT [93].

In addition to the rise in the OXT mRNA level during pregnancy and the elevated level throughout the lactational period, hypothalamic OXT mRNA underwent structural changes during these periods. The poly (A) tail length increased during pregnancy and this was maintained during lactation in the rat [96]. This change in size was observed as early as day 14 of pregnancy. The increase in the poly (A) tail length may be involved in translational efficiency and/or mRNA stability, as in the case for osmotic stimulation described previously.

The changes in the OXT mRNA levels correlated well with the peak of estrogen levels at

estrous [97], the gradual increases in plasma estrogen levels during puberty [98], and pregnancy of the rat [99]. Prepubescent gonadectomy on PN20 inhibited, though not completely, pubertal upregulation of the OXT mRNA level [92]. Pubertal treatment of genadectomized animals with estrogen or testosterone abolished the effectes of genadectomy. These results suggest that the increases in OXT mRNA levels are largely dependent upon circulating gonadal steroids. Estrogen treatment also stimulated the release of OXT from the pituitary [100], and altered the distributional pattern of immunoreactive OXT in oxytocinergic cells and fibers in the rat brain [101].

As described previously, the consensus sequence proposed for the estrogen responsive element was found in the 5′-flanking region of the rat and human OXT genes [29, 31, 102] (Fig. 2C). These estrogen responsive elements are capable of conferring estrogen sensitivity on a heterologous reporter gene in cells expressing the estrogen receptor. These facts suggest that estrogen can directly regulate OXT gene expression. However, in the rat, only a subset of oxytocinergic neurons at sexual maturity bound radiolabeled estradiol [102, 103] and expressed estrogen receptor-like immunoreactivity [104]. Estrogen target cells were confined to the posterior magnocellular PVN (mPVN) and to a region ventromedial to the lateral mRVN, which belong to a group of magnocellular neurons projecting to the medulla and spinal cord rather than to the neurohypophysis. Thus, in some of the effects mentioned above, estrogen may affect OXT gene expression in an indirect fashion.

Prepubescent gonadectomy of the rat inhibited pubertal upregulation of the OXT mRNA level, but the effect was not complete [92]. During the lactational period, OXT mRNA levels were increased while estrogen levels rapidly dropped after parturition [99]. These results indicate that a significant component of the increases in the OXT mRNA levels is independent of gonadal steroids. The adrenal gland is a source of steroid hormones, and adrenally derived steroids have been suggested to play a role in the onset of puberty in the rat [105]. Insulin-like growth factor-I was increased during puberty and directly upregulates the OXT mRNA level in the rat granulosa cells [106].

A projection from the nucleus tractus solitaris to hypothalamic oxytocinergic neurons is immunoreactive for the activin βA-chain in the rat, and may be important in the regulation of OXT biosynthesis and mediation of the milk ejection reflex [107]. Although, expression of activin βA-chain gene did not vary as a function of gonadectomy or steroid treatment, it is still possible that significant changes occur in activin mRNA in a small subset of cells that influence OXT gene expression [92].

The changes in AVP mRNA levels closely parallel the profile for OXT mRNA levels during gestation and lactation. The AVP mRNA level increased 2-fold after day 18 of gestation compared to early gestational period in rats [93, 94]. Throughout the lactational period, the amount of AVP mRNA remained elevated at levels approximately 3-fold higher than the control [93, 94]. These observations are very surprising since the increase in neuronal activity with relation to milk ejection reflex is strictly specific to OXT-producing neurons [108].

The stimulation of both OXT and AVP gene expressions in the last phase of pregnancy and during lactation suggests that the two genes are sensitivie to the same regulatory factors. However, it is unlikely that estrogen has direct effects on the AVP gene, since no estrogen responsive elements have been found in the 5′-flanking region of AVP gene (Fig. 2B). It is not clear what functions AVP has in labor or lactation, but there are indications that AVP could stimulate uterine contraction [109].

PARVOCELLULAR NEUROSECRETORY SYSTEMS

(*a*) *Hypothalamo-hypophysio-adrenal axis*

Besides the magnocellular SON and PVN, AVP is synthesized in other magnocellular nuclei, the circular nucleus and the fornical nucleus, and also in many parvocellular nuclei, e.g., the SCN and the parvocellular part of PVN (pPVN) [5] (Fig. 1). In the medial pPVN, AVP and corticotropin re-

leasing factor (CRF) are colocalized [110, 111] and are coreleased from nerve terminals into the capillaries of the hypophysial portal vessel system in the external layer of the median eminence [112]. This AVP promotes ACTH release from the adenohypophysis [113], and synergizes with CRF in this respect [114].

AVP mRNA was colocalized with CRF and CRF mRNA in the medial pRVN neurons, and expression of both AVP and CRF genes was concomitantly stimulated by adrenalectomy (ADX) in the rat [86, 115–117]. Treatment with dexamethasone prevented the increases in the mRNA levels induced by ADX [115]. Glucocorticoids could act directly to affect negative-feedback at the level of the pPVN via the glucocorticoid receptors in these cells [118–120].

Several brain regions which contain high levels of glucocorticoid receptors, including the hippocampus and the lateral septum, have been implicated in the regulation of pPVN neurons [118, 121, 122]. Hippocampal ablation resulted in profound upregulation of the expression of CRF and AVP genes in the medial pPVN of the rat [123], suggesting that the hippocampus provides a prominent inhibitory influence on medial pPVN neurons via fiber systems projecting through the forebrain. Unilateral lesions in the lateral septal nucleus enhanced the ADX-induced expression of AVP gene in the medial pPVN on the side ipsilateral to the lesion [124]. Further, anterior and total deafferentations of the PVN resulted in increases in expression of AVP and CRF genes in the medial pPVN neurons [125]. However, the increase in the AVP mRNA level after deafferentation was limited to a few clearly definable neurons, comprising a less than 2-fold increase when factored across the entire medial pPVN neurons, whereas the AVP mRNA level increased about 8-fold in the medial pPVN after ADX. These results suggest that medial pPVN neurons may be regulated by circulating glucocorticoid levels both directly and indirectly through extrahypothalamic glucocorticoid-receptive neurons. This idea is supported by the fact that elimination of synaptic inputs from the lateral septum to the PVN enhanced the ADX-induced expression of AVP gene [124].

The magnitude of increase in CRF mRNA after the deafferentations of the PVN was 2-fold in the medial pPVN neurons. This change was similar to that seen after ADX. It is thus probably that expression of AVP and CRF genes within medial pPVN neurons is differentially regulated by neuronal and humoral inputs.

(*b*) *Suprachiasmatic neurons*

The SCN is functionally specialized to form a circadian oscillator or a biological clock [126]. SCN neurons exhibit a circadian rhythm in metabolic activity as measured by uptake of 2-deoxyglucose [127]. The dorsomedial SCN (dmSCN) is perhaps principally involved in rhythm generation, whereas the ventrolateral SCN which receives visual afferents from the retina [128] is considered as the locus of entrainment.

AVP mRNA is localized in the dmSCN of the rat that is coextensive with AVP immunoreactive perikarya [129]. The AVP mRNA levels in the dmSCN show diurnal variation. The values were higher in the morning than in the night [130–133], which correlated well with the morning increase in immunoreactive AVP in the cerebrospinal fluid [134]. AVP mRNA in the dmSCN exhibited diurnal variation also in the poly (A) tail length. A single species of mRNA identical to that in the SON and the PVN was expressed in the light phase, whereas a smaller species was expressed in the dark phase [131, 135]. The increase in the poly (A) tail length was consistent with the increase in the mRNA level, suggesting that an elongated poly (A) tail may be involved in translational regulation and/or mRNA stability, as described in the previous section.

Regulatory mechanisms for the changes in the amount and the size of mRNA are not clear. Several neuromodulators including serotonin and melatonin, adrenalectomy and gonadectomy had no effect on the diurnal rhythm of mRNA size [131]. The rhythm observed in the dmSCN AVP neurons seems to be independent from neural connection, suggesting an intimate association between suprachiasmatic AVP neurons and the oscillatory machinery within the dmSCN. This hypothesis is supported by the observation that the AVP mRNA level in the SCN was under circadian control when the nucleus was morphologically

immature and lacking many of the connections found in adult animals. The circadian rhythm of AVP mRNA level in the SCN was observed on embryonic day 21 in the rat [133]. Although immature synapses were first seen within the SCN from late fetal life, the vast majority of SCN afferent and efferent connections were formed postnatally [136].

The AVP mRNA level in the SCN was not affected by osmotic stimulation, however, the size of AVP mRNA was increased within 2 hr after water deprivation in the rat as those in the SON and the PVN [70]. A second smaller band of AVP mRNA was further observed after 2 and 4 hrs of water deprivation. Since dehydration was started during the dark phase of the daily cycle when the animals drink more, the smaller band may represent a de-adenylated SCN species observed during the dark phase [70, 131, 135], suggesting independent regulation of the AVP mRNA size by circadian rhythm and dehydration. The physiological significance of an increase in the poly (A) tail length in the SCN after osmotic stimulation is not clear, but suprachiasmatic AVP neurons may be involved in dehydration-associated behavioral modifications.

EXTRAHYPOTHALAMIC EXPRESSION OF AVP AND OXT GENES

(*a*) *Neural systems*

Expression of AVP and OXT genes has also been detected in several extrahypothalamic neural systems. AVP mRNA was found in the bed nucleus of the stria terminalis (BNST) [137] and the medial amygdala (AME) [138] in the rat (Fig. 1). AVP neurons in the AME project to the lateral septum and the ventral hippocampus [139], and are suggested to be involved in sexual differences in memory and learning.

AVP neurons in the BNST and their projections exhibit sexual dimorphism in the rat. The number of neurons and the density of staining of the cells and their fibers are greater in males than in females [140]. BNST AVP neurons project mainly to the lateral septum and also to the lateral habenular nucleus and the olfactory tubercle [141]. Gonadal steroids appear to influence the development and maintenance of these pathways [142, 143]. AVP neurons in the BNST and fibers in the lateral septum have been considered to be associated with several sexually dimorphic functions, including the regulation of gonadotropin secretion [144], male copulatory behavior, learning and memory [145], and so on.

Castration dramatically decreased the number of AVP mRNA-containing cells and the intensity of hybridization signals in the BNST on the rat [137]. Testosterone treatment of castrated animals reversed the effect of castration. Treatments of castrated rats with supraphysiological levels of testosterone increased the AVP mRNA level over that in castrates treated with physiological levels of testosterone and also that in intact rats. These results confirm that gonadal steroids are critical in the maintenance of the AVP system and support the physiological roles of BNST AVP neurons for sexual functions. Steroid-concentrating cells are localized in the BNST of the rat, and the promoter region of the rat AVP gene contains GRE which serves as a target binding site for a variety of receptors including the androgen receptor [146]. These facts suggest a direct genomic effect of steroids on AVP neurons.

Expression of OXT gene was detected in the medial preoptic area (MPOA) [147] (Fig. 1). The MPOA is considered as the brain area that controls maternal behavior. The most critical region of the MPOA for the expression of maternal behavior lies in the lateral commissural nucleus (LCN) [148] from which OXT neurons project to the lateral septum [149]. In the LCN, the OXT mRNA level was increased in lactating rats relative to pregnant and castrated rats [147]. The factors responsible for the observed increase in the OXT mRNA level are not known. Short-term estrogen treatment elevated the OXT mRNA level in LCN neurons [150]. However, neuronal activity arising from nipple stimulation during suckling may be a major stimulatory factor for the OXT mRNA level, since during lactation both estradiol and progesterone levels are decreased relative to late pregnancy.

(*b*) *Gonadal expression*

AVP and OXT have been immunologically detected in several peripheral organs including the ovary (corpus luteum), testis, adrenal and thymus. AVP and OXT mRNAs have also been demonstrated in the ovary and testis of the rat, bovine, human, ovine and baboon [19, 82, 106, 151–157], suggesting extraneuronal syntheses of these peptides. Sequence analyses of both hypothalamic and gonadal neurohypophysial hormone precursor mRNAs showed that both sequences are identical except that the gonadal mRNAs have a substantially shorter poly (A) tail [20, 82, 158]. Expression of neurohypophysial hormone precursor genes is probably under independent control in the hypothalamus and gonads. In the large cells of the ruminant corpus luteum, there was a conspicuous elevation in OXT gene expression accompanying luteinization on the day of ovulation, following which the OXT mRNA level was markedly reduced [155, 157]. There was no change in the level of OXT mRNA in the ruminant hypothalamus through the estrous cycle.

Except for OXT mRNA in the bovine ovary, the levels of gonadal OXT and AVP gene expression were too low to influence the plasma levels of the hormones, indicating paracrine roles of the peptides [159]. However, little is known about the physiological functions of gonadal OXT and AVP. It has been suggested that luteal OXT may play an endocrine role, stimulating the release of prostaglandins from th uterus [160]. In the rat, AVP affected androgen synthesis *in vitro* [161]. However, the physiological significance of this effect has been questioned, since no effect on testosterone production could be demonstrated *in vivo* [162]. OXT and AVP may also be involved in modulation of seminiferous tubule contractility [163].

In the Brattleboro rat, AVP immunoreactivity (ir) was detected in the gonads, in which the amount of ir-AVP of both the Brattleboro and the Long-Evans rats was approximately equal [164], whereas AVP-ir was almost undetectable in the Brattleboro hypothalamus. The Brattleboro rat expressed the mutant AVP gene which has a longer poly (A) tail than its non-mutant counterpart, but at reduced levels, in the hypothalamus [82]. In the ovary, the mutant AVP gene was expressed at the similar level to the Long-Evans and the Sprague-Dawley rats. Irrespective of rat strain, the poly (A) tail of ovarian AVP mRNA was shorter than that in the hypothalamus [82]. The AVP mRNA isolated from gonads possessed a very short poly (A) tail which would yield up to 10 lysines at the C-terminal of the mutant precursor when translated. This precursor may be sufficiently close to normal to avoid being diverted from the usual processing pathway.

Recently, three novel AVP gene-related transcripts of 0.67, 0.83, and 2 Kb were identified in the rat testis [153, 165]. The 0.67 and 0.83 Kb transcripts contained exon B and C and a novel exon derived from sequences about 7–10 Kb upstream of the hypothalamic transcriptional start site, but not exon A. These two transcripts should be derived by differential splicing of a common pre-mRNA. They were not translationally active, since they do not encode an open reading frame that is capable of being translated, and were not associated with translationally active polysomes [153]. In contrast, the 2 Kb transcript was the only testicular transcript that contained an exon A-related sequence [165]. This transcript may explain the mechanism that enables the Brattleboro rat to produce ir-AVP in the testis but almost none in the brain. The testicular mRNA that produces AVP-like immunoreactivity would be free from any mutational aberrations and, therefore, would be capable of being efficiently translated [165], although sequence determination of the transcript would be required to confirm this hypothesis.

ONTOGENY

Fetal AVP is though to be involved in regulation of the amniotic fluid balance, restriction of blood loss during labor, stimulation of mitosis, and brain development [166–168]. In the rat and the mouse, supraoptic and paraventricular neurons differentiate between embryonic age (E) 12 and E14, and settle between E15 and E18 [169, 170]. The presence of AVP in magnocellular neurons in the SON and the PVN of rat and mouse embryos has been demonstrated by immunological techniques. AVP immunoreactivity was first detected between

E14 and E16 [171–174]. Expression of AVP gene was detected in the SON of the rat and the mouse beginning on E15 [80, 172] or E16 [133, 175, 176] using *in situ* hybridization, while signal was detected from late gestational period using Northern blot analysis [177, 178]. In the mouse, AVP and AVP-NP immunoreactivities were first detected on E14 in the internal layer of the median eminence, while hybridization signals of AVP mRNA and immunoreactivity of AVP and AVP-NP were detected from E15 in the SON, suggesting that AVP precursor is already translated from a small amount of AVP mRNA and that newly synthesized AVP or AVP precursor may be immediately transported to the neurohypophysis [172]. Increased sensitivity of the ISH method may be needed to clarify the onset of synthesis of AVP precursor in the hypothalamo-hypophysial neurosecretory system.

The AVP mRNA levels were markedly increased in both the SON and the PVN just after the birth in the mouse, although the expression level of AVP gene was rather low in the fetus compared to the adult [172] (Fig. 5). Immunoreactivity of AVP neurons was drastically decreased in both nuclei on PN 1 and PN 2, suggesting marked AVP release in the neonates (Fig. 5). A hybridization study demonstrated that AVP mRNA levels in the SON were increased on E21, but not on E19, by osmotic stimulation [179]. This increase in the AVP mRNA level was positively correlated with plasma osmolality. AVP may have an important osmoregulatory role in neonatal animals undergoing drastic changes in water metabolism immediately after the birth, although the maturation of AVP hormonal action appears to be a late developmental event [180, 181].

OXT has also been detected in the rat embryo, but, unlike AVP, noticeable quantities of OXT were first detected at E19 to E20 in the hypothalamus [171]. In contrast, the presence of OXT mRNA was first detected in the SON at E17 and in the PVN at E18 in the rat [176]. The detection of both AVP- and OXT-neurophysins at E16 in the rat [182] is consistent with the mRNA data, suggesting that this difference between first detection of OXT mRNA and OXT immunoreactivity is accounted for by a delay in prohormone maturation. Gainer and his colleagues showed that OXT was cleaved from its precursor on E18 but remained in a C-terminal extended form until E21, when amidated OXT was first detected in the pituitary, whereas processing of AVP precursor in the fetus was immediate and complete [183].

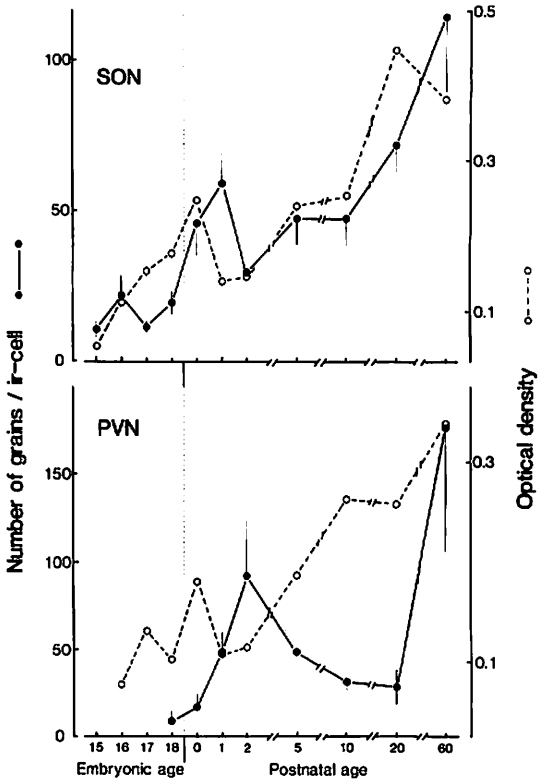

FIG. 5. Changes in the number of silver grains per immunoreactive cell (solid line) and immunohistochemical stainability of AVP neurons (broken line) in the SON and the PVN of developing mice. Each point represents mean±S.E.. Higher optical density corresponds to higher level of immunoreactivity. Note that stainability of AVP neurons is drastically decreased on PN1 (significantly different from the value of PN0, $p<0.001$), whereas the number of silver grains is still high. From reference [172] (Neuroscience, with permission).

CONCLUSION

Neurohypophysial hormone producing neurons are located in many neuronal groups in the brain, including the SON, the magnocellular and parvocellular PVN, the SCN, the BNST, and the

MPOA. Each of them has particular physiological roles. AVP and OXT gene expression is altered by a variety of physiological stimuli. The changes in gene expression coincide well with the predicted roles that the nuclei have. These facts suggest that each neuronal group producing AVP and/or OXT is regulated differentially and specifically by a particular regulatory system. The intriguing question to be clarified is the nature of differences in the regulatory systems/mechanisms of neurohypophysial hormone producing neurons in different neuronal groups.

Studies on promoter regions of AVP and OXT genes and intracellular regulatory mechanisms for transcription of AVP and OXT genes are indispensable to clarify the regulation of AVP and OXT biosyntheses. Up to the present, these studies have been performed using heterologous cell lines and chimeric plasmids containing portions of the promoter regions linked to the reporter gene. This system, however, cannot study relationships between promoter sequences and additional cell-specific transcription factors. The availability of a cell line expressing the endogenous AVP or OXT gene is required. Recently, transgenic mice expressing rat AVP gene [184], rat OXT gene [185], and bovine OXT gene [151] have been produced to study tissue-specific expression of these genes and their regulation. Such molecular techniques will be appropriate tools to realize the regulatory mechanisms of neuroendocrine secretion.

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