Gene Regulation in the Magnocellular Hypothalamo-Neurohypophysial System

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Burbach, J. Peter H., Simon M. Luckman, David Murphy, and Harold Gainer. Gene Regulation in the Magnocellular Hypothalamo-Neurohypophysial System. Physiol Rev 81: 1197–1267, 2001.—The hypothalamo-neurohypophysial system (HNS) is the major peptidergic neurosecretory system through which the brain controls peripheral physiology. The hormones vasopressin and oxytocin released from the HNS at the neurohypophysis serve homeostatic functions of water balance and reproduction. From a physiological viewpoint, the core question on the HNS has always been, “How is the rate of hormone production controlled?” Despite a clear description of the physiology, anatomy, cell biology, and biochemistry of the HNS gained over the last 100 years, this question has remained largely unanswered. However, recently, significant progress has been made through studies of gene identity and gene expression in the magnocellular neurons (MCNs) that constitute the HNS. These are keys to mechanisms and events that exist in the HNS. This review is an inventory of what we know about genes expressed in the HNS, about the regulation of their expression in response to physiological stimuli, and about their function.
I. INTRODUCTION

The hypothalamic-neurohypophysial system (HNS), this unique collection of diverse peptidergic neurons of the hypothalamus with its major axonal endings in the neurohypophysis, has guided physiological research to novel concepts for over 100 years. This guiding role is evident from our present understanding of neurosecretion, including biosynthesis of neuropeptides and electrophysiology, and of neuroendocrinology, including integration and communication between brain and periphery. The essence of these concepts has been discovered through investigation of the HNS, while proof of principle has been obtained from many other peptidergic systems. In physiological research today, the HNS contributes particularly to our understanding of the way peptidergic systems function at the molecular and cellular level. It tells us about the molecular make-up of a defined neuronal system, the developmental and regulatory functions of specific genes, and the integration of physiological signals to responses at the level of gene regulation. With the total overview of all our genes near, it is to be expected that the HNS will remain to serve as a guiding system to define functions of individual genes it expresses and will allow us to understand further the role and dynamics of gene expression programs during development and physiological functioning of a neuronal system. Here we review the current state of affairs about the HNS at the level of the HNS gene expression and function and their contribution to the physiological functioning of the HNS.

A. Physiology

In the 19th century, endocrinology had become a novel discipline among physiologists. The pituitary gland appeared a rich source of preparations that could elicit a variety of physiological responses when administered to experimental animals. The traditional endocrinologists encountered a conceptual problem when studying extracts with vasopressor (578), antidiuretic (211, 835), oxytocic (166) (from ὠξύς = rapid; τόκος = birth) and milk-ejection activities (589). These were all located in the neural lobe of the pituitary gland, which consisted of neural elements rather than being glandular like the anterior lobe. These biological activities were, in the words of Sir Henry Dale (167) “... as complete a proof of a normal endocrine function for the neurohypophysis as any which has been given, or indeed, has since been given, for any other organ.” Although this notion was accepted, it remained largely unexplained for several decades. Similarly, the vasopressor and antidiuretic activities could be separated from oxytocic and milk-ejecting activities (375), but the nature of the substances, although then already suspected to be peptides, remained elusive for several decades. In the meantime these preparations were essential instruments in the discovery of novel physiological concepts and disease in humans. In particular, in the field of kidney physiology and water homeostasis, antidiuretic preparations appeared to be crucial tools (826, 827; reviewed in Refs. 167, 225, 308, 692). During this period, all elementary physiological properties of the antidiuretic activity and responses of the posterior pituitary gland were discovered that we employ today in current research on the HNS.

In the hunt for “releasing factors” in the 1960s, it was noted that extirpation of the posterior pituitary gland not only affected water homeostasis, but also caused behavioral alterations that could be corrected by replacement of vasopressin (183). De Wied’s vision that vasopressin and derivatives and without endocrine activity could act directly on the brain led to the “neuropeptide concept." This concept encompasses the physiological functions of peptide neural communication and modulation of brain activity (184).

B. Biochemistry

Although neurohypophysial extracts in which antidiuretic and oxytocic activities had been separated (375) were available commercially, e.g., Pitressin or Pitruitin (Parke-Davis) in the 1930s, it was not until the early 1950s that the chemical structure of the peptides responsible for the physiological activities could be identified (192, 193). The discovery and structural elucidation of the nonapeptides vasopressin (VP) and oxytocin (OT) was highly acknowledged; Du Vigneaud received the Nobel Prize for his achievements in 1955. With knowledge of the chemical structure, the route to chemical synthesis and design of analogs was now open and was highly exploited in the years to follow up to today (67, 485).
At the same time, other biochemical studies had identified longer peptides that often carried the biological activities in neurohypophysial extracts (797). This work led ultimately to another milestone, i.e., the elucidation of the biosynthetic pathway of VP and the prohormone concept. The complex termed “Van Dyke protein” was identified to contain VP carried by an associated protein, now known as neurophysin (NP) (2). Soon after, the common origin for VP and NP was proposed from studies on the biosynthesis of VP (654). Furthermore, the dynamic and hormone-NP interactions became elucidated. This culminated in understanding how peptide and NP binding promotes condensation of biosynthetic material and in the physical structure of the complex (643). Now we know that all biologically active peptides are biosynthesized from a precursor protein. Most if not all enzymes that process prohormones into the biologically active parts are also known.

C. Cell Biology

The anatomical structure of the neurohypophysis, being typical for a neural element and very different from the glandular appearance of other endocrine organs, remained exceptional and unexplained to endocrinologists until Ernst Scharrer postulated the concept of production and release of hormones by neurons, which he termed “neurosecretion” (676). This concept was received with great skepticism, and although examples from a variety of vertebrate and invertebrate species accumulated (677), it was not fully appreciated until Bargmann was able to apply Gomori’s pancreas staining to the hypothalamus to reveal neurons that had the appearance of the typical hormone-producing cells, which were so well known in pancreas and anterior pituitary gland (56). Moreover, fiber tracts from these neurosecretory cells appeared to innervate the neurohypophysis. Bargmann and Scharrer formulated the anatomical structure that we now know as the HNS and proposed this structure to be responsible for the biosynthesis of the hormonal entities of the neurohypophysis (57). Early biochemical studies showing hypothalamic synthesis and transport of neurosecretory material supported this notion (720).

The Gomori staining employs chrome-alum-hematoxylin (273) or aldehyde fuchsin (274), which involves oxidized disulfide bonds in proteins. These are highly represented in VP, OT, and their NPs. Refinement of staining for oxidized disulfide bonds improved sensitivity and specificity, for instance, by using pseudo-isocyanine as in the work of Sterba (740), but the application of immunohistochemical (IHC) techniques provided the final answers on the anatomy of the HNS.

D. Outline

From a physiological viewpoint, the core question on the HNS has always been, “How is the rate of hormone production controlled?” Despite a clear description on the physiology, anatomy, cell biology, and biochemistry of the HNS gained over the last 100 years, this question has remained largely unanswered. Obviously, current research in this direction profits tremendously from this wealth of basic knowledge on the HNS. With the birth of recombinant DNA in the 1970s and the creation of techniques to modify specific genes in the germline of experimental animals in the 1980s, new horizons have been obtained to provide answers to this central question. This review is an inventory of what we know about genes expressed in the HNS, about the regulation of their expression, and about their function. Genes relevant to the central question include receptors and signal transduction components that receive and process the message that the organism is in demand of a neurohypophysial hormone. The key players in gene regulatory events, the transcription factors, deserve special attention. They control not only the transcription rate of the neurohypophysial hormone genes, but also determine the molecular makeup of the cell essential for appropriate development and physiological functioning. Finally, the HNS neurons are equipped with a machinery to produce and secrete hormones in a regulated manner. This machinery is complex, involving many different proteins, but we are beginning to know the ones involved in the HNS.

II. THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

A. Organization and Connections

1. Magnocellular nuclei of the hypothalamus

Classically, the hypothalmo-neurohypophysial neurosecretory system is defined as consisting of large neurons (20–40 μm cell body diameter) of the supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei that have axons terminating on the blood capillaries of the posterior pituitary neural lobe (677). The use of immunocytochemistry (621, 728) and injection of retrograde tracers into the neural lobe, the MCNs (379, 700) have since defined accessory magnocellular cell groups in the preoptic area, the lateral hypothalamus, regions near the anterior commissure and the third ventricle, the perifornical nucleus, and the nucleus circularis. The SON is wholly magnocellular, while the PVN is divided into a lateral magnocellular subdivision and a more medial parvocellular subdivision. Parvocellular OT and VP neurons have smaller cell bodies (10–15 μm) and project to the median
eminence (796), brain stem and spinal cord (753), as well as limbic and olfactory areas (108, 314, 729). Parvocellular neurons have different functions to MCNs, and it is not known whether their major genes are regulated by the same mechanisms. The phenotype of null mutants for several transcription factors (see sect. IV.A) indicated that parvocellular neurons expressing corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), and somatostatin may have a developmental origin common to MCNs. This review focuses on the magnocellular HNS. Here it is essential to realize that the magnocellular HNS is by no means a single system. It consists of different MCNs that can produce different products and have different anatomical positions.

2. Architecture of the HNS

The architecture of the HNS has been described in greatest detail for the rat and is exemplary summarized here. OT and VP MCNs are found intermingled in the magnocellular nuclei, although there is some topographical segregation (621, 728). Within the SON, OT MCNs are mainly rostral and dorsal, whereas VP MCNs are found mainly caudal and ventral. The unmyelinated, varicose axons of the MCNs leave the SON dorsomedially and then turn caudally to pass through the dorsal portions of the internal layer of the median eminence and pituitary stalk before reaching the neural lobe (16). As they leave the SON, the axons give rise to collaterals, many of which terminate close to the nucleus where they may contact lateral cholinergic neurons (304, 427, 496). In the magnocellular subdivision of the PVN, OT MCNs are predominantly rostral, whereas VP MCNs are more caudal and lateral. Their axons leave the PVN laterally, then course ventrally and medially over the SON, and pass through the ventral portion of the median eminence and pituitary stalk (16). Collaterals of PVN MCNs terminate within the nucleus or in the nearby perifornical region (304, 495). There is electrophysiological evidence to suggest that some magnocellular axons that terminate in the neural lobe may also give off collaterals to the median eminence, medial amygdaloid nucleus, and lateral septum (603). VP (551) and OT terminals (758) have been demonstrated contacting MCNs containing the same peptide, although the origin of these terminals, and their incidence, remains unclear.

MCNs of the SON have oval cell bodies with one to three thick dendrites that extend toward the ventral surface of the brain (44, 195), although some do project dorsally (575). At the surface of the brain, the dendrites turn in a rostrocaudal direction and can extend for over 200 μm within a ventral lamina of glial cell processes (195). The MCNs of the PVN are essentially the same in morphology, although their dendrites extend medially through the parvocellular subdivision of the nucleus toward the ependyma of the third ventricle (45, 788). In contrast to the SON, which is fairly homogeneous in that there are no other neuronal cell bodies in the nucleus, the magnocellular PVN contains a number of interneurons, at least some of which are GABAergic (51).

The soma and dendrites of the MCNs are sometimes directly adjacent to those of others, although normally interposed between them are thin astrocytic glial processes. During times of high hormone demand, alterations occur in the architecture of the magnocellular nuclei, bringing cell bodies and dendrites into direct contact and increasing synaptic inputs to the neurons (306, 760). MCNs make somatic and dendritic contacts via gap junctions, thus producing cytoplasmic coupling. The extent of this coupling varies with the structural plasticity within the magnocellular nuclei (306).

The varicose appearance of magnocellular axons is due to the presence of large swellings, which, in the neural lobe of the pituitary, bud to form palisades of terminals along the pericapillary basalar lamina (541, 777). This, together with the fact that axons and swellings are themselves capable of releasing hormone (543), has left the definition of terminals in the neural lobe dependent only on their relative size and the presence of translucent microvesicles in addition to the hormone-containing electron-dense granules. Immunohistochemical examination at the light microscope level suggests that VP fibers enter the central region of the neural lobe of the pituitary gland, also termed neurohypophysis or posterior pituitary gland, while OT fibers enter the periphery of the gland (802). However, the fibers become dispersed and terminals of the two cell types can be found intermingled throughout the neural lobe (458). The neuronal elements of the pituitary are, as in the hypothalamic nuclei, interposed by the processes of resident astroglia, the pituicytes (869). The interrelationships of the magnocellular nerve endings, pituicytes, and capillaries also change dramatically during times of increased hormone secretion (306, 760).

3. The HNS and the forebrain osmoreponsive circuit

It is now generally accepted that the HNS forms part of an osmoreponsive circuit with three structures within the lamina terminalis, the front wall of the third ventricle that defines the anterior extent of the hypothalamus (89, 330, 429, 501). In addition to direct projections from the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT), the HNS also receives indirect input from these via the median preoptic nucleus (MnPO; Fig. 1) (501, 525, 622, 661, 671, 690, 775, 863). Tract tracing has revealed that single neurons of the lamina terminalis can project to more than one of the magnocellular nuclei, providing the connections for concerted HNS responses (852). Finally, electrophysiological studies have also provided evidence for connections back
to the lamina terminalis from the HNS, perhaps by collateral excitation of interneurons, thus completing the circuit (330).

Although MCNs are themselves osmosensitive (88, 494), they require input from the lamina terminalis to respond fully to osmotic challenges (428, 330). Neurons in the lamina terminalis are also osmosensitive (89), and because the SFO and OVLT lie outside the blood-brain barrier, they can integrate this information with endocrine signals borne by circulating hormones, such as angiotensin II, relaxin, and atrial natriuretic peptide (516, 585). While circulating angiotensin II and relaxin excite both OT and VP MCNs, atrial natriuretic peptide inhibits VP neurons (221, 736, 847). In addition to an angiotensinergic path from the SFO (360), the OVLT and MnPO provide direct glutaminergic and GABAergic projections to the HNS (565, 888) (Fig. 1). Thus, because inputs from the lamina terminalis can be either excitatory or inhibitory, the view is that the osmoresponsive circuit increases the overall sensitivity of the HNS to osmotic stimuli. Furthermore, a role for nitric oxide (NO) acting throughout the forebrain osmoreponsive circuit to modulate neurohormone release has been suggested (456, 463).

4. Hemodynamic inputs to VP neurons

A fall in arterial blood pressure produces a secretion of VP due to an inhibition of baroreceptors in the aortic arch and activation of chemoreceptors in the carotid body (691). Afferents from these receptors terminate in the dorsal medulla oblongata of the brain stem, including the nucleus of the tractus solitarius (NTS; Fig. 1). The resting inhibitory effect of baroreceptor activity on VP neurons is mediated indirectly via the A6 noradrenergic cell group of the locus coeruleus, the diagonal band of Broca in the forebrain and neurons lying just outside the magnocellular nuclei (76, 361, 362). Release from this inhibition after hemorrhage or direct electrical stimulation of the NTS causes the activation of VP neurons via the stimulatory A1 noradrenergic cell group of the ventrolateral medulla (175, 611). The A1 projects directly to VP MCNs to stimulate hormone release via α1-adrenoreceptors (174).

5. Brain stem inputs to OT neurons

The NTS appears to act also as an important relay for pathways ascending to OT MCNs. Whereas electrical stimulation of the NTS leads to indirect activation of VP neurons via the A1 cell group of the ventrolateral medulla, there is a direct excitatory input to OT neurons that is mediated, at least in part, by the A2 noradrenergic cell group lying within the NTS (175, 611) (Fig. 1). One stimulus that has been used to investigate this pathway is systemic administration of the peptide cholecystokinin (CCK) (171). Physiologically, this octapeptide activates CCK-A receptors on endings of the vagus nerve following distension of the gut after a meal leading to the release, at least in the rat, of OT but not VP into the bloodstream from the HNS (462, 618). With the use of c-fos and tyrosine hydroxylase immunocytochemistry combined with tract tracing, it has been possible to show that this information is relayed in A2 neurons of the NTS that project directly to the magnocellular nuclei of the hypothalamus (109, 429, 581, 628).

Activation of catecholaminergic neurons in the NTS has been associated with a number of other stimuli that cause the release of OT, including administration of nausea-producing agents (580), stress, interleukin-1 (206, 417), and nicotine (499). Furthermore, A2 neurons of the NTS are active during parturition (454) and suckling (725) and are, thus, possibly part of the pathway from the uterus and mammary glands to the HNS (584). These observations suggest that catecholaminergic neurons of the NTS may integrate information from a number of sources. However, it is not apparent whether this involves subsets of NTS neurons. Neuropeptide Y- and somatostatin-containing neurons are activated following CCK (817),
while inhibin neurons appear to mediate at least part of the suckling stimulus (670).

6. Other inputs to the HNS

There are a number of other inputs to the HNS, although the relevance of these is largely unknown (Fig. 1). They include scattered inputs from other hypothalamic nuclei, the preoptic area, septum, and limbic structures (431, 575, 662, 671, 757). The SON receives direct and indirect afferent information from both the suprachiasmatic nucleus (SCN) and the retina (159, 160). These inputs, together with the influence of the pineal gland on hormone release (867), provide substrates for the diurnal regulation of HNS function. There are well-documented direct projections from histamine neurons of the mammillary nuclei to the HNS (205, 853). These involve inputs to VP MCNs via histamine H1 receptors that are electrically excitatory and inputs to OT MCNs via H2 receptors that are electrically inhibitory (889). Interestingly, histamine administered intracerebroventricularly induces c-fos in both OT and VP MCNs and increases the expression of both neurohormone genes (387, 388), but this may not be a direct effect. Roles for the histaminergic input have been implied during pregnancy and parturition (464), lactation (674), dehydration (386, 389), and novelty stress (881). Similarly, direct afferents from the main and accessory olfactory bulbs to the SON have been described (727, 726), the electrical stimulation of which can, like stimulation of the histaminergic input, lead to increased dye coupling between MCNs (306).

B. Cell Biology

1. Overview: the HNS as cell biological model

Over the past 20 years, neuropeptides have become increasingly prominent as intercellular messengers in the peripheral and central nervous system, acting as neurotransmitters, neuromodulators, neurotrophic factors, and/or neurotransmitters (327, 394, 742, 911). In contrast to “conventional” neurotransmitters (e.g., acetylcholine, excitatory and inhibitory amino acids, monoamines, etc.), which are synthesized in nerve terminals and can be packaged locally in recycled small vesicular membranes, the biosynthesis and secretion of neuropeptides such as OT and VP in the HNS requires continual de novo transcription and translation of peptide precursor proteins. The MCNs of the HNS have become prototypical of peptidergic neurons (Fig. 2). These precursor proteins must then be sorted via the Golgi apparatus to the “regulated secretory pathway,” packaged into large dense core vesicles (LDCVs), in which they are posttranslationally processed to the biologically active peptides and axonally transported to nerve terminals before peptide secretion can occur. The secretion process itself may differ in detail between the small clear-cored synaptic vesicle- and LDCV-based secretion systems (825). Different isoforms of secretion-associated vesicular and membrane proteins often appear to be used in each (60, 253, 493), and secretion from LDCVs is rarely, if ever, evoked in response to single action potentials, and typically requires trains (bursts) of impulses to evoke peptide secretion (256, 467). Because conventional neurotransmitter and neuropeptide secretion very often coexist in the same neuron, it is clear that the mechanisms for both processes also must coexist in neurons throughout the nervous system (406).

The MCNs of the HNS, which synthesize and secrete the nonapeptides OT and VP, represent a specialized class of peptidergic neurons called neurosecretory cells (APUD neuroendocrine cells or endocrine neurons), first described over 70 years ago in the fish central nervous system by Ernst Scharrer (675). The HNS neurons have served as important model systems for the study of peptide neurosecretion mechanisms in vivo, in large part due to their relatively compact nuclear organization in the CNS and physically distinct terminal field. The HNS neurons project via a well-defined axonal tract to the neural lobe where each axon is estimated to branch into hundreds of nerve terminals (302). These axonal branches and terminals have been estimated to represent ~50% of the total tissue mass of the neural lobe. The relatively easy access to the HNS cellular components, the cell

FIG. 2. Peptidergic neuron. Cellular and molecular properties of a peptidergic neuron (neurosecretory cell) are shown. The structure of the neurosecretory cell is depicted schematically with notations of the various cell biological processes that occur in each topographic domain. Gene expression, protein biosynthesis, and packaging of the protein into large dense core vesicles (LDCVs) in the cell body, where the nucleus, rough endoplasmic reticulum (RER), and Golgi apparatus are located. Enzymatic processing of the precursor proteins into the biologically active peptides occurs primarily in the LDCVS (see inset), often during the process of anterograde axonal transport of the LDCVS to the nerve terminals on microtubule tracks in the axon. Upon reaching the nerve terminal, the LDCVS are usually stored in preparation for secretion. Conduction of a nerve impulse (action potential) down the axon and its arrival in the nerve terminal causes influx of calcium ion through calcium channels. The increased calcium ion concentration causes a cascade of molecular events (see inset and text) that leads to neurosecretion (exocytosis). Recovery of the excess LDCV membrane after exocytosis is performed by endocytosis, but this membrane is not recycled locally, and instead is retrogradely transported to the cell body for reuse or degradation in lysosomes. TGN, trans-Golgi network; SSV, small secretory vesicles; PC1 or PC2, prohormone convertase 1 or 2, respectively; CP-H, carboxypeptidase H; PAM, peptidyl-glycine α-amidating monoxygenase. [Adapted from Gainer and Chin (253).]
bodies in the PVN and SON and axons in the median eminence by both stereotaxic and micropunch assay methods, and the nerve terminals by their presence outside of the blood-brain barrier in the posterior pituitary, have made these MCNs favorite subjects for many biochemical, morphological, and physiological studies (42, 43, 103, 256, 302, 303). Perhaps the most important virtue of the HNS as a cell-biological model is its requirement for very high rates of peptide biosynthesis and secretion. Because the OT and VP peptides are secreted directly into the bloodstream to act on distant target organs, the mammary gland, and kidney, respectively, substantial quantities of these peptides are secreted to compensate for their dilution in the general circulation. Consequently, transcriptional rates and mRNA levels for these peptides are very high in the HNS neurons, and peptide secretion from LDCVs in the neural lobe is exceptionally robust, thereby allowing for the analyses of all the cell biological aspects of neurosecretory mechanisms in an experimentally favorable context.

2. The OT and VP genes and prohormones

The gene organization of VP and OT genes is peculiar. They are located within the same chromosomal locus on a chromosome at a very short distance from each other (3–11 kb) in a head-to-head orientation (351, 534, 627, 745) (Fig. 3). Although the exon organization is simple, the genomic control regions are complex and likely dependent on large parts of the locus (374). Notably, in the VP-OT locus of the rat, long interspersed repeated DNA elements (LINEs) are present and transcribed. Expres-
A role of these LINEs in the expression of the VP and OT genes is not known. The VP gene consists of three exons and comprises ~2 kb. The structural organization of the VP gene is very similar to the OT gene. The first exon of the VP and OT gene, exon A, codes for a signal peptide, the hormone (VP or OT, respectively), a three-amino acid spacer (-Gly-Lys-Arg-), and the first nine NH2-terminal amino acids of NP (NP-I or NP-II, respectively). Exon B codes for the highly conserved mid-portion of NP (amino acids 10–76), and exon C of the VP gene codes for the remaining COOH-terminal amino acids of NP, a monobasic cleavage site (-Arg-) and the COOH-terminal glycopeptide (GP) of 39 amino acids (Fig. 4). In contrast to the VP gene, exon C of the OT gene only codes for the COOH-terminal amino acids of OT-associated NP-I.

VP and OT are closely related to each other in primary structure and differ in only two amino acids at position 3 and 8. It is generally believed that the mammalian VP/OT family developed from a common ancestral molecule by gene duplication about 450 million years ago (781). The strong conservation of the NP domain of the different VP-related prohormones further strengthens this assumption. The NP domain is remarkably well conserved and exists in all vertebrate and invertebrate species. Especially the exon B-encoded NP portion (NP10–76) shows a considerable homology between different species. Two segments in the NP sequence, which fall within the region encoded by exon B, show 58% homology to each other and 60% at the nucleotide level. This indicates that the primordial NP gene itself arose from a partial gene duplication that extended an initially smaller structure (128). The secondary structure of NP also indicates a two-domain organization of the molecule in terms of the disulfide pairing, that clearly divides the molecule in a NH2-terminal and a COOH-terminal domain (122). NP contains 14 cysteine residues, which are conserved with almost equal interdistances. The NH2- and COOH-terminal domains, with each three cysteine, are held together by the extremely well-conserved amino acids C14QQEENLYPSC54 and a disulfide bridge between Cys10 and Cys54.

### 3. Sorting of OT and VP prohormones into the regulatory secretory pathway

All neuropeptides that are secreted from nerve terminals are first synthesized as protein precursors that are sorted to the regulated secretory pathway, and then processed by enzymatic mechanisms into biologically active peptides before secretion. The first step in the biosynthesis of a peptide is the expression of the gene that encodes the protein precursor. In the cases of the classical neurosecretory peptides, OT and VP, these nine-amino acid-long peptides are first made as part of two separate prohormones of 106 and 145 amino acids, respectively. Their mRNAs are translated on ribosomes attached to the rough endoplasmic reticulum (RER) in the cell body of the neurons, and their resultant precursor proteins undergo cotranslation processing steps in the RER which includes removal of the signal sequence amino acids from their NH2 terminus of the precursor, the formation of disulfide bonds, and the initial stages of glycosylation of the precursor (139, 251).

Although the mechanisms of protein and pro-peptide sorting through the RER, Golgi, and secretory vesicle systems in cells are fundamental issues in cell biology, relatively little work has been done using the HNS. Sorting mechanisms have generally been divided into “receptor-mediated” (118, 445) versus “aggregation-mediated” (339, 769) models. These two views of how secretory

### FIG. 4. Schematic representation of the vasopressin prohormone and the established diabetes insipidus mutations.

The three moieties of the prohormone (VP, NP, and GP) as well as the sequences of the cleavage sites (GKR and R) and the position of the glucon (I) are indicated. SP indicates the signal peptide. The disulfide organization and known secondary elements are included (respectively, above and below the bar representing the prohormone) (146, 643); → = β-strand; coiled springlike pattern = α-helix; __ = loop. FNDI mutations identified in humans (126, 247, 278, 310, 616, 619, 620, 630, 778) are positioned in the prohormone. According to conventions in nomenclature of the familial nephrogenic diabetes insipidus mutations (630), amino acids of each moiety of the VP prohormone were numbered separately. Thus the abbreviation GIR underneath the NP moiety indicates the substitution of the G at position 14 of NP by an R. The symbol Δ indicates the deletion of the indicated residue(s). The asterisk marks the only recessive mutation (PTL); all other mutations are dominant.
proteins are sorted via the trans-Golgi network (TGN) into the regulated secretory pathway (usually via LDCVs) are controversial, and a full elucidation of them is beyond the scope of this review. In brief, the receptor-mediated view conceives of a “sorting receptor” in the TGN membrane that binds a “sorting signal” in the secretory protein to package the latter into the LDCV compartment. The aggregation-mediated model focuses on the self-aggregation of the secretory proteins in the TGN as the principle mechanism.

The experimental data underlying both in the above models usually focuses on specific disulfide-bonded loops, typically in the NH$_2$ termini of the secretory proteins and prohormones, which are essential for proper sorting to the regulated secretory pathway (339, 401, 444, 769). The OT and VP prohormones are extremely rich in disulfide-bonded loops (98), i.e., eight disulfide bridges exist in the ~10-kDa precursor proteins (see sect. III-A). Both the biological functions of the peptides and their structural interactions with the NPs are completely dependent on the integrity of these disulfide bonds (98). The three-dimensional structures of NP-peptide complexes have been elucidated by crystallography (643). This structure emphasizes the function of the disulfide bridges for tightly folding of the NP protein and allowing a “peptide-binding” pocket to accommodate VP or OT by multiple intermolecular contacts.

For many years, the role of the NPs in the OT and VP prohormones has been something of a mystery, and its presumed role as a “carrier protein” (256) has been questioned, since no other prohormone is known to contain such a molecule. Nevertheless, both theoretical considerations (98) and experimental evidence (176) give increasing evidence to the view that the NPs serve as “chaperone-like” molecules serving intracellular transport of the MCNs. Experimental evidence for a role of NP-hormone interaction in sorting to the regulated secretory pathway includes the missorting of a mutant VP prohormone encoding a VP peptide, unable to bind to NP (116). When expressed in neuronal cells, this prohormone is partly retained in the endoplasmic reticulum (ER) and mostly secreted unprocessed via the constitutive secretory pathway. These data favor a mechanism in which the VP and NP domains of the prohormone need to interact for efficient passage of the prohormone through the ER, and for sorting to LDCVs. The intracellular fate of natural VP prohormone mutants causing pathology of the HNS (see sect. uD3) supports such a model.

4. Mechanisms of OT and VP prohormone processing

The prohormones are selected from the Golgi and packaged in LDCVs. After sorting, subsequent posttranslational processing steps involve a variety of proteolytic (endo- and exopeptidase) as well nonproteolytic modifications (e.g., amidation, sulfating, etc.). These steps occur within membrane-bound compartments in the cell, starting within the TGN and then primarily in the LDCV (Fig. 2). The first processing step is to proteolytically cleave the intact protein precursor into its correct peptide fragments. There are at least eight species of prohormone (precursor) convertases (PCs) found in mammalian cells, which are in the subtilisin-like (Kex-2) endoprotease family, that can process precursors of secreted proteins (253, 256, 644, 687–689, 739). Because the PCs usually cleave at paired-basic amino acid motifs, an exopeptidase (carboxypeptidase H/E) that trims the remaining COOH-terminal basic amino acid residue is required in most cells (234, 276). Another critical step in fashioning many biologically active peptides is the conversion of their COOH-terminal glycines into amides by an enzyme system referred to as peptidylglycine $\alpha$-amidating monoxygenase (PAM, see Ref. 199).

All of the above enzymatic mechanisms have been shown to operate in the MCNs of the HNS, and the presence of PCs has been demonstrated (253, 256). The PCs that have been reported to be present in the HNS are PC1 (73), PC2, PC5, and PACE4 (73, 172, 173, 189), and carboxypeptidase H/E (74, 234, 235, 470, 473) and PAM (91, 283, 433, 500, 673) have similarly been located in the HNS. In only a few studies has there been an effort to specifically localize the PC subtypes specifically to either the OT or the VP MCNs. In situ hybridization (ISH) studies have indicated that PC1/3 and PC5 are predominantly present in the VP and OT MCNs, respectively (73, 189). Studies on the regulation of these enzymes’ gene expressions and activities have also been modest in number, although evidence for the osmotic regulation of carboxypeptidase H/E (84, 480) and glucocorticoid regulation of PAM (283) in the HNS has been reported.

Studies on the VP prohormone in cell lines differently equipped with PCs indicated that the VP prohormone can be cleaved first by furin at the level of the Golgi apparatus. This cleavage releases the COOH-terminal glycopeptide. The resulting VP-NP intermediate is cleaved by PC1 and/or PC2 (M. Nijenhuis and J. P. Burbach, unpublished data). One of the most intriguing differential regulations of the processing mechanisms occurs during development. There is a precocious expression of biologically active VP in the fetus, whereas in the OT MCNs there appears to be a cleavage of the OT prohormone to the OT COOH-terminally extended peptide intermediates, but processing of the extended OT forms by carboxypeptidase H/E does not occur until postnatal periods (21, 544). There are also reports of the secretion of the extended forms of OT in primates when they are stimulated by estrogen (23, 24). Because the COOH-terminally extended forms of OT and VP do not exhibit any known biological activities, the functional significance of this regulation of
carboxypeptidase H/E activity in the HNS remains unclear.

5. The neurosecretory granule: central player in neurosecretion

LDCVs serve as the key intracellular sites for OT and VP processing, for axonal and dendritic transport of the peptides to storage and secretory sites, for long-term storage, and ultimately the secretion of the biologically active peptides (Fig. 2). As such, the LDCVs play multifunctional roles in the process of neurosecretion in the HNS. LDCVs are ~160–200 nm in diameter with a high peptide and protein content that cause them to exhibit electron-dense cores (139, 542) when viewed by electron microscopy. It is now clear that all the precursor processing enzymes activities can be found in the LDCVs of the HNS (251) and that these enzymes operate optimally at the mildly acidic condition (pH 5–6) that is found in these organelles (651, 652, 859). This acidic pH of the intravesicular environment is also essential for the binding of the OT and VP nonapeptides by the NPs, which is optimal at pH 5.5 (98, 141, 766), and which contributes to the stability of the intravesicular contents during LDCV storage in the cell. This acidic environment is maintained by a proton-translocating ATPase in the LDCV membrane (651, 652, 678). The LDCV membrane also contains cytochrome b_{561} molecules that presumably contribute electrons to the enzymatic reaction catalyzed by PAM (194, 859).

After their formation in the TGN (Fig. 2), the LDCVs undergo anterograde transport (i.e., away from the cell body) on microtubular tracks (139), probably using members of the kinesin gene family as molecular motors. The kinesin gene superfamily has many members (94, 319), and it is not yet clear which of the specific molecules in this family are associated with LDCV axonal transport. The KIF2 and KIF3 kinesin proteins are reputed to transport vesicles in the 90- to 160-nm-diameter range and thus may be candidates for this function (253). Very few studies have been performed related to the axonal transport of LDCVs, although the association of LDCV transport with microtubules tracks has been indicated by its inhibition by colchicine (254, 255, 592). Direct measurements of LDCV transport rates using pulse-chase analyses have placed it in the "fast transport" category with rates approaching 140 nm/day (139, 223, 592).

In the MCNs of the HNS, most of the LDCVs are stored in the nerve terminals in the neurohypophysis where they are mobilized for secretion by electrical activity (139, 542). However, some LDCVs can also be stored in cell bodies and dendrites, and calcium-dependent secretion can also occur in the dendrites (465, 606). An example of this dual mode of secretion occurs in the OT MCNs in the hypothalamus of lactating animals. As part of the suckling reflex, these neurons secrete large boluses of OT from nerve terminals in the pituitary into the bloodstream to act on their distant targets, the mammary glands. They also secrete OT from their dendrites into the hypothalamus itself for a paracrine function, i.e., to modulate the synchrony of the OT neuron population’s electrical response to suckling to generate bursts of OT release (465, 561).

The molecular mechanisms that are responsible for the secretion of peptides from LDCVs in neurosecretory terminals appear to be very similar to those that underlie secretion from small synaptic vesicles at synapses (493, 640). In both cases, the secretory event is preceded by an influx of calcium ions through a voltage-gated calcium channel located near a secretory vesicle (see Fig. 2, bottom inset) in neurotransmitter secretion, this apposition of the secretory vesicle to the calcium channel is critical, since the secretory event requires a relatively high concentration of calcium ions (10–100 μM) that occurs only immediately adjacent to open calcium channels in the plasma membrane. Secretory vesicles in such locations are said to be in “readily releasable” pools, and at active zones of synapses, they are referred to as being “Docked” (640). While the classification of “Docked” versus “cytoplasmic” reserve small vesicles has clear morphological correlates at synapses, this is not as clear for LDCVs in neuroendocrine cells. Nevertheless, ~1–5% of LDCVs in the posterior pituitary appear to be functionally docked, in that their contents are known to be readily releasable during excitation (139, 542). An intensive research effort is presently underway to identify the molecules that are involved in the cascade of events that cause “Docked” secretory vesicles to fuse with plasma membranes and thereby release their intravesicular contents into the extracellular space. The latter event, termed “exocytosis,” is the fusion event in the final step in secretion. After exocytosis, the nerve terminal membrane is increased in surface area, and this additional membrane is subsequently returned to the cell through a budding process known as “endocytosis.” In recent years, extensive cloning studies have uncovered a very large number of protein families that are associated with either the secretory vesicles membranes or the active zones of secretion in the plasma membrane (see sect. WE for current views about molecular events underlying neurosecretion).

C. Physiological Functions

MCNs are classical neuroendocrine cells, specialized in the synthesis and secretion of vast quantities of the hormones VP and OT. These peptides are transported through the general circulation and exert their effects through interaction with receptors located at distal peripheral sites. The well-known functions of VP and OT in the regulation of salt and water homeostasis and repro-
duction have been studied for over a century (see sect. 1). The physiological function of OT and VP can now be correlated with the presence and properties of the receptors for these neurohypophysial hormones, which are briefly summarized here. For detailed reviews, see References 55 and 684.

1. VP and OT receptors

The OT receptor (OTR) and the VP receptors (V₁R, V₂R, and V₃R) form a subfamily within the much larger superfamily of G protein-coupled receptors (55). The receptors can be distinguished from each other by differential binding affinities for structural analogous of OT and VP and on the basis of their activation of different signaling pathways.

A) OTR. The multiple hormonal and neurotransmitter functions of OT are mediated by the specific OTR, which activates phospholipase C and increases in cytosolic calcium (487). OTRs are expressed in the uterus and mammary gland (381, 648). Expression has also been described in the brain (893), with patterns differing in different species, which may be related to different patterns of sexual behavior (346, 347). There is only one OTR gene. Therefore, the same receptor protein in brain and peripheral organs is expressed. However, posttranslational modification and interactions with downstream signal transduction components may modify OTR signaling (600, 658, 732). Notably, OT MCNs express the OTR (7) (see sect. mD). The OTR can be considered as a “nonselective” receptor for neurohypophysial nonappetidites, since it binds both OT and VP with almost similar affinities.

B) V₁R. The V₁R (formerly known as V₁a) is specific for VP. It activates the Gₛ/₁ family of G proteins, the α-subunit of which regulates the activity of the β-isoform of phospholipase C. Receptor activation has been shown to stimulate phospholipases C, D, and A₂ and, through phosphatidylinositol hydrolysis, to an increase in intracellular calcium and an increase in cell acidification through stimulation of Na⁺/H⁺ exchange (101). V₁R is expressed in the liver, blood vessel smooth muscle cells, and most other peripheral tissues that express VP receptors (447). The V₁R is probably the most common receptor for VP in the brain (588).

c) V₂R. The V₂R is preferentially coupled to the α-subunit of Gₛ, through which it stimulates activity of adenylate cyclase and cAMP production (583). V₂R is expressed only in the kidney of adult rats (447), but the mRNA encoding the V₂R is also expressed in the brain of newborns, although this declines to undetectable 2 wk after birth (318).

d) V₃R. The V₃R (formerly known as V₁b) stimulates phospholipases C and induces an increase in intracellular calcium. V₃ is expressed in the majority of anterior pituitary corticotroph cells, in multiple brain regions, and in a number of peripheral tissues, including kidney, thymus, heart, lung, spleen, uterus, and breast (446). It is of interest to note that MCNs also express the V₃ (314, 338; see sect. mD).

2. Physiological functions of magnocellular VP and OT

There is a vast literature on the physiological mechanisms affected by VP and OT. For the context of this review, a brief summary of the main peripheral physiological functions established for VP and OT from the HNS is given here. For reviews on functions of VP and OT on the brain, mostly mediated by neurons other than MCNs, the reader is referred to Reference 782.

A) SALT AND WATER BALANCE. Mammals respond to changes in the osmolality of their extracellular fluid by altering their behavior and physiology. The behavioral response entails regulation of the salt and water intake through changes in sodium appetite and thirst. The physiological response entails modulation of renal excretion of water and sodium, which are achieved through changes in the plasma concentrations of the antidiuretic and natriuretic hormones VP and OT (617, 686). This, in turn, is achieved through an increase in the secretion of these hormones from MCN nerve terminals (103).

VP is classically known as the antidiuretic hormone. Renal V₂ receptors mediate VP-induced tubular reabsorption of water via induction of intracellular cAMP production in collecting duct cells (583), thereby conserving stores of bodily fluid during times of restricted intake. Whereas no mutations of the OTR, V₁, or V₃ receptors have been found in populations of any species, over 60 different genetic mutations of the V₂ receptor have been described that represent the cause for congenital X-linked nephrogenic diabetes insipidus (NDI) (572, 684). The functionally characterized mutants show a loss of function due to defects in their synthesis, processing, intracellular transport, VP binding, or interaction with the G protein/adenylate cyclase system.

Although OT is best known for its role in reproduction (see sect. mC2b), it also stimulates natriuresis at physiological plasma levels (818, 866, 892), an effect mediated by estrogen-regulated OT receptors in macula densa and proximal tubule cells (99, 586, 587).

B) VASOCONSTRICTION. In animals subjected to hemorrhage, plasma VP concentrations increase to levels sufficient to cause vasoconstriction, thus attenuating the hypotensive response (686). In contrast, whereas hypotension also causes marked increases in plasma VP concentration in human subjects, VP does not seem to contribute to the maintenance of blood pressure (321).

c) CORTICOTROPH REGULATION. VP stimulates adrenocorticotropic hormone release from the anterior pituitary by acting on the V₃R, VP, derived from PVN parvo cellular
neurons, is released into the portal blood in the median eminence, and acts as a potent secretagogue of adrenocorticotropic hormone (32). Evidence suggests that VP released by MCNs may also be involved in the control of ACTH secretion, particularly after acute hyperosmotic and hypotensive stimuli (32, 613). The physiological contribution of VP to modulation of activity of the hypothalamo-pituitary-adrenal axis is particular dominant in situations of chronic stress.

D) REPRODUCTION. OT is involved in parturition (649) and lactation (899). The function of OT in lactation is dual. OT directly contributes to milk production through its stimulating activity on prolactin release (657). At term, OT is released from the pituitary in large pulses. This corresponds to a dramatic OTR upregulation in the uterine myometrium (648), which becomes extremely sensitive to OT. Thus is OT thought to induce contraction of uterine smooth muscle and expulsion of the young. During lactation, OT stimulates the contraction of the myoepithelial cells that surround the alveoli of the mammary gland, resulting in milk ejection.

However, the role of OT in parturition has recently been scrutinized and challenged. Knockout mice devoid of the OT gene (564, 898) are viable and fertile. OT-deficient females give birth normally and appear to demonstrate normal maternal behavior; however, all offspring die shortly after birth due to the dam’s inability to nurse. Thus it appears that OT plays an essential role in milk ejection but not parturition, at least in the mouse. These observations are consistent with the normal delivery seen in humans and experimental animals with complete posterior pituitary dysfunction (27). The finding that the OT knockout mouse (564, 898) gives birth normally has reignited the long-term debate on the role of OT in parturition (649). The fact remains that OT is well placed in mammalian physiology to play a key role in all aspects of reproductive function. The peptide produced in the brain, ovary, uterus, or other peripheral tissues (31, 124, 229, 244, 424, 425, 513, 623) has been implicated in sexual and maternal behaviors, ovarian cyclicity, parturition, and lactation. It should be realized that the appearance of a phenotype of the OTR null mutatioin may require interaction with specific environmental and endogenous cues. Furthermore, the OT gene in the HNS is regulated during reproductive cycles, strongly implying functions for the neurohypophysial hormone.

However, there is ample evidence for a role for OT in parturition (649), and disruption of OT physiology can result in serious dysfunction. For example, OT antagonists delay the initiation of parturition and prolong labor (33). Most transgenic mice expressing a bovine OT gene (bOT3.5; see sect. vA1A) fail to deliver normally (323). High levels of transgene RNA were found in the ovary at the end of gestation and at the beginning of lactation in bOT3.5 mice, correlated with a parturition defect that results in considerable maternal mortality. Thus, whereas OT has a central role in parturition, it is clear that the organism can give birth in its absence and, thus, mechanisms must take the place of OT. The OT knockout mice will be an invaluable tool in the understanding of these mechanisms (649).

D. Pathology of the HNS

1. The homozygous diabetes insipidus (di/di) Brattleboro rat

The Brattleboro (di/di) rat was identified as a mutant of the Long Evans strain, suffering from a hereditary form of diabetes insipidus (784). This disease is transmitted as an autosomal recessive trait. This pathology was shown to be due to a lack of VP production by the HNS. Characteristically, water resorption is greatly impaired in the distal kidney tubules (785), leading to a massive loss of body fluid. The rats can excrete almost 70% of their body weight in hypotonic urine per day and compensate the resulting dehydration by drinking an equivalent amount of water. Urine output, urine osmolality, and water consumption are therefore commonly used to check for homozygosity. The disease state of the animal is reflected by a continuous osmotic stress that affects the MCNs of the HNS.

The genetic defect of the Brattleboro rat is the deletion of a single deoxyguanosine within exon B of the VP gene (680). The resulting frameshift alters the amino acid sequence of the NP moiety from residue 64 onward and the entire glycopeptide (GP) region (Fig. 4 and sect. mB). Due to the new amino acid sequence, 5 of the 14 cysteine residues in NP are missing, as well as additional PC cleavage sites and a recognition signal for glycosylation. In addition, the stop codon within the mRNA is missing, causing translation to continue through the Terrel normally noncoding part of the mRNA into the 3′-poly(A) tail creating a poly-lysine tail at the COOH terminus of the precursor. The poly(Lys) tail may continue as much as 70 Lys residues as estimated from the length of the poly(A) tail (353).

Although the mutant VP gene is correctly transcribed and spliced, mRNA is not efficiently translated (679). Therefore, it has been suggested that the expression of the mutant precursor is inhibited by a block at the translational level. In the di/di rat, the mutant di precursor can be detected in MCNs by IHC (286, 399). Immunoelectron microscopic studies have shown that the mutant precursor is restricted to the ER and small lysosomal-like bodies and does not reach the secretory granules (399).

Several other observations confirm that di/di rats are chronically osmotic stimulated animals. ISH of heteronuclear VP RNA revealed a significant increase in primary VP transcripts in the SON of di/di rats compared with the
wild-type and heterozygous rat (754). No differences in levels of nuclear RNA were found in the PVN of these animals. These data imply an increased transcriptional activity in MCNs of the di/di rat.

Very early data by Sokol and Valtin (730) showed an extreme hypertrophy of perikarya, nuclei and nucleoli of MCNs in the SON of the di/di rat. The appearance of MCNs in the PVN of di/di rats is less different from wild-type cells. However, similar to the MCN neurons of the SON of di/di rats, the nucleolar size is significantly larger in di than in normal rats. The enlarged volume of the nucleoli can be normalized by VP substitution, which indeed suggests an activation of transcription in MCNs of the di/di rat due to osmotic stress (785). In addition, VP substitution of di/di rats has been shown to reduce the level of VP mRNA (895). A continuous osmotic stimulation of the di/di rat is further substantiated by a marked increase in mutant VP mRNA levels in di/di rats compared with the levels of mutant VP mRNA in heterozygous rats. In the heterozygous +/di rat, the mutant VP allele is expressed at levels that are only 5–7% of the expression of the wild-type allele. Instead of the expected twofold increase in mutant VP mRNA in di/di rats, a sevenfold increase has been observed (703).

Upon this continuous osmotic stress, VP gene expression in di/di rats can be further activated by chronic intermittent salt-loading. This is reflected by an increase in mRNA levels for both VP and dynorphin (703). An increase in VP mRNA levels in di/di rats could, however, not be observed after dehydration (483). These data indicate that the transcriptional regulation of the VP gene of the di/di rat is intact.

This, however, seems to contradict the fact that the continuous osmotic stress that affects the di/di rat does not lead to an increased level of VP mRNA compared with wild-type and heterozygous +/di rats. For instance, a decrease in the amount of VP mRNA in the di/di rats has been reported using Northern blot analysis (353, 702, 811) and ISH (285, 504, 895). Others, using similar methods, did not observe any differences in VP mRNA levels between these animals (243, 680, 779).

To explain the relatively low levels of mutant VP mRNA in both the di/di and +/di rat, it has been suggested that the expression of the mutant VP gene is affected at the posttranscriptional level (703). This may relate to the poor translatability of the mutant VP mRNAs that could be caused by the extended translation into the poly(A) tail (353, 679). Moreover, mutant VP mRNAs of di/di rats exhibit an up to 150 nucleotide longer poly(A) tail than normal VP mRNAs (215, 353).

The distribution of the VP neurons in the hypothalamus of the di/di rat is identical to that in wild-type rats, as determined by ISH (215, 504, 779) and in ICH (621, 750). However, the expression of the mutant VP precursor in the di/di rat considerably alters the morphology of the VP MCNs. The larger perikaryal cross sections, up to 90% greater than the VP neurons of the +/di rat, and the increased size of the nucleoli are typical of hypertrophy (540, 786, 895). Biochemically this hypertrophy is reflected by a high metabolic activity as demonstrated by an increased level of cytochrome oxidase (402) as well as glucose utilization (756) and increased staining of a Golgi and lysosomal marker (749). In addition, both the ER and Golgi apparatus of di/di cells are fragmented into small complexes, and increased numbers of lysosome-like bodies are present (350, 540). Whereas VP neurons of the wild-type rat contain large neurosecretory granules of 160-nm diameter, only small granules of ~100 nm are present in di/di cells (540, 859), and these contain the coexisting peptide dynorphin (856).

2. Solitary MCNs of the di/di rat

From 1985 onward, a number of startling papers have described the expression of apparently normal VP gene products in solitary MCNs of the di/di rat. Now, these observations can be clarified by a novel form of RNA mutation, discovered through analysis of the HNS. GP immunoreactivity has been observed in a small number of cells in the SON of di/di rats, suggesting a reversion of mutant VP neurons to the wild-type phenotype (520, 626, 800). Some years later, an extensive study on these reverted VP neurons demonstrated the colocalization of all VP gene products (i.e., VP, NP, and GP) in the same solitary cells together with the mutant di precursor (804). These solitary VP neurons of the di/di rat did not only display a heterozygous phenotype, but they also appeared to transport VP and related gene products into the neural lobe. Furthermore, this study showed an age-related increase in the number of these solitary reverted VP neurons. The number of solitary +/di cells in di/di rats increased linearly with age from only a few cell profiles in young rats to over 120 cell profiles in 2-yr-old adults of either sex. It has been shown by molecular cloning, ISH, and IHC that the apparent +/di phenotype is caused by further mutation of the di VP transcript by a dinucleotide deletion. The deletion involves the loss of a GA pair in one of two GAGAG motifs in the VP transcript (208). The deletion causes the −1 frame of the Brattleboro transcript to restore to the normal frame, but a stretch of 13 or 23 amino acids in the −1 frame remains. Thus this precursor is not identical to wild type. It can, however, be sorted partly to the regulated secretory pathway and results in the biosynthesis of normal VP (209).

Notably, the GA deletions do not exist at the genome level and are considered to be introduced in transcripts by a process termed “molecular misreading” (799). Based on the work on the Brattleboro rat, antisera were raised against predicted +1 frames of several proteins and tested for expression. The results show that molecular
misreading is abundant in several neuropathologies, in particular Alzheimer’s disease (801).

Despite the expression of apparently normal VP gene products, the solitary +/di cells of the di/di rat differ in appearance from the VP neurons of heterozygous (+/di) and wild-type rats. Similar to di/di cells, the ER appears disordered and fragmented into smaller complexes in the reverted +/di cells of the di/di rat, which may indicate a still hypertrophic state (288, 350). The VP immunoreactivity in these solitary cells was located, together with the mutant precursor, solely in the cisternae of the ER and not in any other compartment. Although small (80–100 nm) LDCVs indistinguishable from those in di/di cells, are present, none of them was reported to contain detectable levels of VP, NP, or GP immunoreactivity as determined by electron microscopy (607). This contradicts the presence of immunoreactivity for VP and related gene products in fibers in the neural lobe, which suggests they are axonally transported and thus packaged in granules (804).

The ultrastructural appearance of VP cells of the heterozygous +/di rat is similar to those of wild-type strains, which includes the presence of normal sized (160 nm) LDCVs (540). Thus the VP neurons of the di/di rat that have reverted to the +/di phenotype are different from the +/di VP cells in the heterozygous Brattleboro rat. With regard to the +/di rat, this may be explained by a differential expression of the wild-type and mutant allele, which results in a contribution of mutant VP mRNA of only 5–7% for the PVN and SON, respectively (703). Consequently, a very low amount of mutant precursor is present in VP neurons of the +/di rat.

Several peptides coexist within VP MCNs, which are differentially expressed in di/di and wild-type rats (see sect. wC). As in the wild-type rat, dynorphin, galanin, and NPY are synthesized in VP neurons of the di/di rat, but their turnover is enhanced (371, 641, 701). For example, dynorphin levels in di/di rats are reduced to 25% of wild-type estimates. Also, a reduction of galanin immunoreactivity in the neural lobe of di/di rats has been observed (641). In contrast, angiotensin II (ANG II) and the neuroendocrine polypeptide 7B2 immunoreactive cells are almost totally absent from di/di hypothalami (440, 488), except for a few solitary neurons (245, 325, 380). Remarkably, solitary neurons immunoreactive for ANG II or 7B2 were shown to colocalize, with the reverted neurons expressing VP and related gene products (245, 805), although no explanation for these observations is available. However, Van Leeuwen (798) has proposed the involvement of a compartmentalization of the ER, whereby ANG II, 7B2, and VP are synthesized and translocated within the same ER domains. In di/di cells, their expression would be obstructed by the mutant VP precursor, which then is alleviated by the revertance of phenotype. The peptides that are unaffected in expression (e.g., dynorphin, galanin, and NPY) would utilize other compartments of the ER.

In addition to the solitary neurons that express apparently normal VP gene products, a second subset of “reverted” cells has been described in di/di hypothalami. These solitary cells contain immunoreactivity for VP, OT-related NP, and the mutant di precursor, all located as large aggregates in the cisternae of the RER (607), but no OT, VP-related NP, and GP immunoreactivity could be detected. The number of this type of phenotypically changed neurons is about one-tenth of the +/di reverted VP cells described above. Thus this cell type is very rare among MCNs.

On the basis of the immunocytochemical data, it has been suggested that hybrid VP/OT-NP precursors exist in this second subset of solitary VP neurons of the di/di rat that may be derived by a conversion between the VP and OT genes. This is substantiated by the molecular identification of recombinant VP/OT transcripts in di/di as well as wild-type hypothalami (532). These are hybrids between VP and OT transcripts and encode the NH₂ terminus of the VP precursor fused to the COOH-terminal part of the OT precursor. Thus somatic gene conversion of the VP and OT may occur in both di/di and wild-type rats. Whether this underlies a similar age-related mechanism as the potential genetic alterations in the +/di VP cells of the di/di rat remains to be determined.

In summary, three types of VP MCNs have been recognized in di/di rats: 1) the di/di VP neurons that express the mutant di precursor and include ~4,500 MCNs of the di/di hypothalamus; 2) the solitary +/di VP neurons that express apparently normal VP gene products together with the mutant di precursor due to “molecular misreading.” The number of these cells increases from a few in newborn animals to ~2% of the VP MCNs in 2-yr-old di/di rats. 3) The solitary VP/OT hybrid neurons that express VP/OT hybrid transcripts that encode the NH₂ terminal part of the VP precursor fused to the COOH-terminal part of the OT precursor, due to hybrid mRNA, comprise ~0.3% of the VP MCNs in aged di/di rats.

3. Familial neurohypophysial diabetes insipidus in humans

Familial neurohypophysial diabetes insipidus (FNDI) is the best-known inherited endocrine disease caused by prohormone defects (242, 635). In this autosomal dominant disease, mutations in the VP prohormone cause defects in the synthesis of VP and hence result in a large increase in urine production (polyuria) and fluid intake (polydipsia). In human FNDI, mutations have been observed in either the signal peptide, the VP moiety, or the NP moiety of the VP preprohormone (126, 247, 278, 310, 616, 619, 620, 630, 778). Thirty-two different mutations have been identified in more than 40 pedigrees (Fig. 4).
The disease displays two unexpected features for a deficiency caused by a defective prohormone. First, the disease is dominant, demonstrating that one mutant prohormone allele suffices to cause the defect. Second, the onset of disease symptoms is delayed to several months or years of age. These peculiarities suggest that the mutant human VP prohormone somehow interferes with either synthesis, transport, and processing of the wild-type prohormone or with the viability of VP-producing cells.

Studies in which mutant VP precursors were expressed in cell lines with peptidergic properties (348, 562) showed that mutant proteins were largely retained in the ER and that accumulation of precursor caused morphological and likely functional derangement of the ER. Overexpression of mutant genes resulted in "accretions." It has been indicated that these accretions and the consequent disturbance of the ER are deleterious to the cell and will decrease functionality and/or viability of the MCNs, which express high amounts of VP prohormone in vivo. This hypothesis would explain both the dominant inheritance of human FNDI and the delayed onset. This mechanism bears similarities to observations on other neurodegenerative diseases in which protein aggregates are present as a consequence of altered protein structure, e.g., Alzheimer's disease and prion disease. It does not explain reports of diabetes insipidus symptoms in newborns. In addition to degeneration, it has also been shown that mutant and normal precursors can interact and negatively influence VP synthesis (349). Thus the mutation of a single allele in human FNDI may lead to immediate impairment of hormone synthesis due to protein-protein interactions in the ER, Golgi, and to gradual neurodegeneration as a result of permanent ER obstruction. This mechanism is fundamentally different from diabetes insipidus in the Brattleboro rat, which requires two affected alleles.

III. GENE EXPRESSION

The essential physiological function of the HNS is to convert centrally processed information on the physiological state of the organism into hormonal action on peripheral organs. Key for this function is the reception of chemical signals, transduction and appropriate reaction at the level of the gene and the release processes. Therefore, the expression of genes addressed in the section concerns the peptide "output" genes, the intracellular machinery of their secretion, and the receptors that transduce signals toward cellular activity of MCNs.

A. The Major Neurohypophysial Hormones: VP and OT

Neuronal phenotypes are usually characterized by the specific molecules (e.g., neuropeptides, neurotransmitters, and their associated enzymes) that are consistently expressed in the particular cell type. However, it is now fully accepted that there is significant coexpression of these and other "phenotypic markers" in neurons and that subsets of cellular phenotypes with varying coexpression patterns exist in any given neuronal population (59, 85, 327, 328, 406, 467, 510, 911). Assessments of such phenotypic variability in neurons have been relatively unexplored due to the limitations of the IHC and ISH methodologies that have traditionally been used to assay for expression of specific molecules in cells. IHC and ISH, especially when performed in double-label studies, are nonquantitative and can only detect two (or at the most three) distinct molecules in a given cell. Consequently, these experimental approaches rarely provide insight into the full extent of multiple gene coexpression in neuronal populations.

The major gene products secreted by MCNs are encoded by either the VP gene or the OT gene. The original view was that the VP MCN and the OT MCN represent two strictly separate cell types, each exclusive in the expression of the VP or OT gene, respectively, but a more balanced view with plastic and overlapping expression is arising (see below). Still, the majority of MCNs can be characterized on the basis of the expression of the VP or OT as major output gene, as well as the morphological and electrical properties. The level of expression of the VP and OT genes is very high in MCNs. It has been estimated originally based on DNase protection assays that the average transcript number is on the order of 2,000 molecules VP mRNAs/VP-producing MCN, and between 5,000 and 12,000 OT molecules mRNAs/OT-producing MCN (117). Other estimates of hypothalamic OT and VP mRNA levels and copy numbers per neuron vary widely. Young et al. (895) estimated based on quantitative ISH studies that the MCNs in normal rats contain ~30,000 OT or VP mRNA copies per cell. A similar set of values was obtained by Sherman and Watson (703) who used ribonuclease protection assays of hypothalamic tissue punches. The latter study converted the total mRNA measured per punch to per cell values by assuming, based on previous studies, that there were 4,327 VP cells and 3,233 OT cells in the punched tissues. Kim et al. (380a), using RNA protection assays, reported between 32 pg VP mRNA/µg total RNA in normal rat hypothalamus, and LeMouellec et al. (426b) using quantitative RT-PCR reported 30 pg OT mRNA and 10 pg VP mRNA/µg total mRNA in normal rat hypothalamus. With the assumption of 50–100 µg total mRNA/rat hypothalamus (893) and an estimate of ~7,000 OT and VP cells each per total rat hypothalamus, one can calculate values of 1.26–2.52 attomol OT mRNA/neuron (759,000–1,510,000 molecules/neuron) and 0.36–2.29 attomol VP mRNA/neuron (217,000–1,380,000 molecules/neuron) from these data. These calculations are close to the values found by other workers using quantitative RT-PCR
analyses on single MCNs (874). It should be noted, however, that in none of these studies was the possibility of RNA degradation in the initial sample accounted for, and hence, the explanation for the differences in absolute copy numbers among these studies remains uncertain.

The earliest studies of the MCNs, using IHC or ISH techniques, identified only two distinct cellular phenotypes in the HNS, the OT- and the VP-expressing MCNs (48, 107, 117, 186, 795, 335, 528, 530, 621, 750, 803, 795, 895). These observations and others led to the generalization that expression of the OT and VP genes was mutually exclusive (528). More recently, however, reports of a OT and VP coexpressing MCN phenotype (385, 518), which increased in amount under specific physiological conditions (53, 518), argued strongly against this simple proposition. This OT and VP coexpressing phenotype is normally found at a frequency of ~1–3% in the HNS neuronal population, but increases to 17% in the SON after 2 days of lactation (518).

Recent studies using a sensitive RT-PCR analysis of single MCNs have confirmed the existence of a OT- and VP-coexpressing MCN phenotype in the HNS, which increases to 17–18% of the cell population during lactation, and also showed that virtually all of the MCNs of the OT-phenotype contain some VP mRNA, and those of the VP-phenotype also contain OT mRNA at low levels (252, 270, 271). Separate quantitative RT-PCR analyses of MCNs in normal female rats showed that in the OT and VP MCN phenotypes, the major nonapeptide mRNA species was ~100-fold greater than the minor peptide mRNA. In contrast, in the OT- and VP-coexpressing MCN phenotypes, the ratio of the two peptide mRNAs is around 2 (875). It is presently unknown whether the minor OT or VP mRNA in the MCN is translated or if the synthesized peptide is actually secreted by the cell. Nevertheless, it is clear that expression of these peptide genes is not mutually exclusive in the MCNs, although the physiological significance of this and its underlying mechanisms are obscure and will require further study.

B. Coexpressed Neuropeptides

In addition to the major neuropeptide genes, OT and VP, prominently expressed in the MCNs, many other peptide genes are also expressed in these neurons at lower but varying levels depending on specific experimental conditions (summarized in Table 1) (85, 102, 291, 510, 829). These “copeptides” are differentially expressed between the two major phenotypes. IHC and ISH studies have shown that CRH and CCK are coexpressed in OT cells (121, 491, 492, 519, 602, 792, 859), and galanin, neuropeptide Y, enkephalins, vasoactive intestinal polypeptide (VIP), neurotensin, and several other neuropeptides and secreted polypeptides have also been found expressed in MCNs (Table 1) (418, 491, 492, 793, 846). In some cases, IHC has been performed at the electron-microscopic level, showing that opioid peptides are present in the same secretory granules as VP or OT (492, 857).

Secretion of the coexpressed peptides in the HNS can have modulatory effects on the secretion of OT and VP (510). Galanin release from VP cells has a marked stimulatory effect on CCK from OT cells, and CCK can stimulate both VP and OT secretion (83). Dynorphin on the other hand selectively inhibits OT secretion (82). The copeptides can have additional paracrine functions on pituicytes of the neural lobe. Pituicytes are known to express functional receptors for several of the copeptides and display a markedly plastic morphology in association with secretory activity of the peptidergic terminals (79, 759).

C. Receptors and Postreceptor Components

To sense and respond to changes in the physiological state in which HNS gene products participate, MCNs are equipped with the appropriate sets of receptors and signal
transduction systems. Receptors of different structural families have been demonstrated to be expressed by MCNs, and a number of proteins belonging to different signal transduction cascades have been identified (Table 2).

Initial data suggesting the presence of certain receptors come from studies in which the release of peptide or electrical activity of MCNs was affected by pharmacological agents. In this regard, the reader is encouraged to read recent reviews focusing on these studies (41, 89, 157, 430). In many of these studies it could not be decided if the response was elicited directly through receptors of MCNs themselves, or indirectly through responses of neurons projecting to MCNs. More recently, receptors were demonstrated more directly through ligand binding, IHC, or ISH. In this section we summarize only those receptors for which direct proof of gene expression by MCNs could be provided.

1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) comprise a superfamily of proteins that can bind ligands and initiate signal transduction through the activation of large G proteins. To this superfamily belong receptors for transmitters, neuropeptides, odorants, etc. There are numerous reports describing the effects of substances on the HNS and implying the presence of certain GPCRs on MCNs. We here review only those GPCRs of which the expression in MCNs has been proven by probes or antibodies (summarized in Table 2).

The presence of receptors on MCNs for gene products of MCNs suggest that autoregulatory loops exist. The OT receptor has been identified in OT-producing neurons (6, 783). Its localization suggests that it plays a role in the synchronization of the activity burst typical for OT neurons via dendritic release of OT. Furthermore, the V1a (V1) and V1b (V3) subtypes of VP receptors have been demonstrated in the HNS (314a, 783). They are expressed on VP MCNs (338).

Expression of a number of neuropeptide receptors has been demonstrated. CCK (171) may have a transmitter or modulator role in the hypothalamic magnocellular neurosecretory system. In the rat, the SON and PVN display high-affinity binding for radiolabeled CCK, and exogenously applied CCK depolarizes supraoptic neurons, acting at postsynaptic CCK-B type receptors and evoking VP release from the neurohypophysial perfused hypothalamic explants (356, 357). Binding sites for CCK have also been identified in the posterior pituitary. Transcripts of the CCK-B receptor are relatively abundant in MCNs of SON and PVN (511). The CRH type 1 receptor has been localized to MCNs by in situ hybridization (469). The pituitary adenylate cyclase-activating polypeptide (PACAP) receptor is relatively prominent in the SON (298). The implication of opioid regulation of HNS activity has been substantiated by the demonstration of κ-opioid receptor mRNA in SON and PVN (182, 486). The glucagon-like peptide GLP-1 stimulates release of VP and OT from the HNS. In line with this observation, the GLP-1 receptor is expressed by both VP MCNs and OT MCNs (475, 910). The tachykinin NK-3 receptor is expressed by MCNs in SON and PVN and mediates effects of tachykinins on VP release (187, 197). Of the three cloned galanin receptors, galanin-R1 is expressed in MCNs of SON and PVN, but conflicting data for expression of galanin-R2 have been obtained (289, 393).

### Table 2. Expression of genes for signal transduction components in MCNs of the HNS

<table>
<thead>
<tr>
<th>Class</th>
<th>Gene</th>
<th>Reference Nos.</th>
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<tbody>
<tr>
<td>G protein-coupled receptors</td>
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<tr>
<td>V1b-R (=V2-R)</td>
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<td>CCK-B-R</td>
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<tr>
<td>CRH-R</td>
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<tr>
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<tr>
<td>Opioid-KOR</td>
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<tr>
<td>Galanin-R1</td>
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<tr>
<td>Receptors for growth factors</td>
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</tr>
<tr>
<td>and cytokines</td>
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<td>PRL-R</td>
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<tr>
<td>GH-R</td>
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<td>FGF-R</td>
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<td>Activin-R</td>
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<td>IL-1-R</td>
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</tr>
<tr>
<td>and 2</td>
<td>604, 832</td>
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<tr>
<td>GABAβ-R</td>
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</tr>
<tr>
<td>α-II sodium channel</td>
<td>704</td>
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<tr>
<td>α-Na+ sodium channel</td>
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</tr>
<tr>
<td>β2- &amp; β3-sodium channels</td>
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<tr>
<td>α1A,α2C-Calcium channels</td>
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</tr>
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<tr>
<td>β1-Calcium channels</td>
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See text for definitions.
Several GPCRs for classical transmitters have been found on MCNs in agreement with established neurotransmitter inputs to the SON and PVN. An important link to the noradrenergic innervation of the SON and PVN is the expression of the α2-adrenergic receptor and the α1-, α1D-adrenergic receptors in SON and PVN (771) demonstrated by ISH. The presence of the α2c-adrenergic receptor was indicated by an IHC study (109). Expression of these receptors is altered during lactation, suggesting that MCNs adapt to the altered noradrenergic tone (771).

In primates, the dopamine D1 and D5 receptors are expressed in the SON and PVN. Activation of these receptors acutely affects the activity of SON and PVN neurons (632). Based on IHC it has been suggested that also the dopamine D4 receptor is present in the SON (179). It is not certain from this study if the D4 receptor is expressed by MCNs.

In view of the regulatory inputs to the HNS by excitatory amino acids, the identification of the involved receptor types is of importance. Several subtypes of the metabotropic glutamate receptors, which belong to the superfamily of GPCRs, have been characterized in the HNS. The current view is that MCNs receive prominent inhibitory from GABAergic inputs and lesser excitatory inputs from GABAergic and glutamatergic neurons (873). Both group I mGluRs (mGluR1/5) and group III mGluRs (mGluR4/7/8) are expressed in neurons postsynaptically to the SON, while MCNs themselves primarily contain group I mGluRs. Published anatomical studies are not always consistent and detailed with respect to the post- or presynaptic expression of mGluRs. ISH studies showed expression of mGlu3, mGluR1, and mGluR7 in MCNs in this order with regard to abundance (13, 570). The mGluR1b splice form showed intense staining for mGluR1 and mGluR5 in MCNs by IHC (390). Other reports, however, indicated that there was little or no staining for mGluR1α and mGluR5 in the SON (789, 791).

2. Receptors for growth factors and cytokines

In addition to GPCRs, several other classes of membrane-associated receptors exist. These are often involved in signaling of growth factors, cytokines, and cell-adhesion molecules. Of these classes, a limited number have been found to be expressed by MCNs (summarized in Table 2). Expression of receptors for the anterior pituitary hormones prolactin and growth hormone has been detected in the SON and PVN (52, 148, 524, 601). It is not known if they mediate effects of hormones of pituitary origin. The synthesis of a small, 14-kDa form of prolactin has been indicated (512), suggesting that autocrine responses may exist for MCNs. It has been reported that MCNs contain receptors for the growth factors brain-derived neurotrophic factor (BDNF) (490), fibroblast growth factor (275, 622), and epidermal growth factor (EGF) (472). An activin receptor has been demonstrated in the SON (127), which is of interest in view of the innervation by inhibin which is received by the OT MCNs through a specific neuronal pathway from the caudal nucleus of the solitary tract (669). Neurons acutely isolated from the SON responded to activin-A with alteration of electrical properties (577). The insulin-like growth factor (IGF) receptor is expressed in the SON (9). The only receptor for a cytokine is the interleukin (IL)-1 receptor type I expressed on MCNs (185).

3. Ligand-gated channels

-N-methyl-D-aspartate-type (513) glutamate receptors (NMDARs) are transmitter-gated ion channels that interact with l-glutamate released from presynaptic terminal resulting in a Ca2+ influx through the opened receptor. The NMDARs consist of a complex family of heterodimers consisting of one of the eight NMDAR1 subunit splice variants (NR1 a-h) and one of the four NMDAR2 isoforms (NR2 A-D). Both the NR1 and NR2B receptors have been described in the SON and PVN (162, 163). A differential expression of the subunits has been observed in MCNs (12). In VP, MCNs exhibit an equivalent expression of NR2B, NR2C, and NR2D. In OT MCNs, the NR2B subunit is prevalent over NR2D, and the mRNA levels of NR2C and NR2D are very low. These data suggest that VP and OT MCNs may differ with respect to responsiveness to glutamates. One class of receptors for purines, like ATP and UTP, are ligand-gated ion channels (382). Multiple P2X purine receptors have been detected in the supraoptic region of the hypothalamus. ISH has demonstrated transcripts of the P2X3 and P2X4 receptors in SON and PVN (704). These receptors have been implicated in the ATP-triggered cationic currents and Ca2+ influx in MCNs. There is no evidence as yet that P2Y receptors, the G protein-coupled purine receptors, exist on MCNs.

As an essential inhibitory transmitter, GABA plays a prominent role in most if not all physiological responses of the HNS (430). Of the three types of GABA receptors, the GABA_A, and GABA_B receptors, but not the GABA_C receptor have been shown to exist on MCNs. The GABA_A and GABA_B receptors are chloride channels. Most of these data originate from electrophysiological studies (e.g., Refs. 106, 604). ISH and IHC studies of the GABA_A receptor in the HNS have shown that both OT and VP MCNs contain α1-, α2-, β2-, and γ2-subunits, suggesting that they contain benzodiazepine type 1 and type 2 GABA_A receptor properties (217, 219, 832). GABA_A receptors were demonstrated in the SON and PVN by the use of antibodies directed against α1-, α2-, and β2γ2-subunits as well as by ISH (219). This study indicated a similar subunit composition in OT and VP MCNs, but interestingly suggested receptor heterogeneity between SON and PVN.
neurons. Specifically, SON MCNs express $\alpha_1$, $\beta_1$, and $\gamma_2$-subunits. No other $\gamma$ and $\gamma_2$-subunits were found to be expressed (217, 219). The SON expresses a high level of the GABAB$_1$ subtype (452). An important feature of GABA receptor expression in MCNs is the plasticity in subunit composition in response to altering physiological conditions (430). Rapid switches in the types of subunits inserted into the GABAA receptor, particularly $\alpha_1$-$\alpha_2$, occur during the female reproductive cycle and around parturition (104–106).

4. Cation channels

Eight types of Na$^+$ channel are present in the brain, based on combinations of four $\alpha$-subunits (I, II, III, Na6) and two $\beta$-subunits (I and II), all encoded by distinct genes. Almost all VP and OT MCNs in the SON express the $\alpha$-II and $\alpha$-Na6 mRNAs, whereas $\alpha$-I and $\alpha$-III mRNAs could not be detected (755). Both $\beta_1$ and $\beta_2$ are expressed in MCNs (755).

Recent studies on the calcium channel subunits present in the HNS show that many of the high voltage-activated calcium channel subunit genes, $\alpha_1$A-D, $\alpha_2$, and $\beta_1$-$\beta_4$, are expressed in virtually all the MCNs (271). However, the $\alpha_{1E}$-subunit gene was not expressed at detectable levels in these cells. The expression of all the $\beta$-subunit genes in each MCN may account for the variations in physiological and pharmacological properties of the high voltage-activated channels found in these neurons (224). Data are summarized in Table 2.

D. Secretory Pathway-Associated Proteins

Complex cellular events underlie regulated exocytosis. For regulated release of neurotransmitters, many of the cellular and molecular processes have been dissected in recent years, and protein families participating in these processes have been identified (743). The secretory vesicles are part of a cycle of exocytosis, involving vesicle docking, release upon triggering by a stimulus, and endocytosis. Stimuli generally lead to a Ca$^{2+}$ influx and formation of a Ca$^{2+}$ gradient sensed by Ca$^{2+}$-binding proteins. There are fundamental differences between the exocytosis of transmitters from clear-cored vesicles and that of peptides from dense-cored vesicles (or secretory granules). These differences concern the genesis of vesicles, their synaptic localization, and Ca$^{2+}$ dependence for docking and release (556, 825). Current questions regarding the release of peptides from neurosecretory neurons concern the validity of the generalized mechanism of exocytosis of peptidergic release, and more specifically the identity of the proteins of the peptidergic release machinery, including the Ca$^{2+}$ channels. In view of its well-defined properties, the HNS is a neurosecretory system of choice to answer these questions. Recent studies have addressed these for the HNS. The present status is the demonstration of multiple proteins of the secretory machinery known for clear-cored, synaptic transmitter-containing vesicles. However, there is no evidence as yet for proteins that are unique for peptidergic secretion.

Based on localization, proteins can be classified as 1) vesicle-associated proteins, 2) plasma membrane-associated proteins, and 3) soluble proteins.

1. Vesicle-associated proteins

Synaptobrevin VAMP is the first protein found on the secretory vesicle and occurs in multiple isoforms. Synaptobrevin-1 as well as synaptobrevin-2 were detected in the neurohypophysis (369). In addition, cellubrevin, synaptotagmin-1, synaptotagmin-2, rabphilin 3A, DOC 2B, and to a lesser extent DOC 2A were expressed in the HNS as demonstrated by ISH and/or immunodetection (196, 369, 395, 610, 823, 837).

2. Plasma membrane-associated proteins

Syntaxin I is the prototypic protein present at the plasma membrane and serving as docking site of vesicles during the formation of a large, multiprotein core complex. Syntaxin IB was the predominant member of the syntaxin family detected in the neurohypophysis (369, 395). SNAP 25 was also present on membrane fractions of the neurohypophysis (369).

3. Soluble proteins

Munc 18–1 and SNAP 25 are the two best-known bipartite-interacting proteins with both vesicle- and membrane-associated proteins. Munc 18–1 is expressed in MCNs and present in neurointermediate lobe extracts (209). SNAP 25 was also found in the neurohypophysis (369). Many proteins involved in regulated secretion have not been investigated in the HNS. On the basis of the limited data available now, the composition of secretory-pathway-associated proteins in the neurohypophysis mostly resembles the pattern found for neural tissue in general rather than endocrine glands. Moreover, the similarity to neurons that use transmitters is notable. Most of the essential proteins of the core complex have been demonstrated, and proteins unique to peptidergic neurosecretion have not been identified yet.

Expression of the nonsecreted, cytoplasmic calcium binding proteins, calbindin and calretinin, has also been extensively studied in the MCNs and found to predominate in OT cells (37–40, 527, 833). It has been suggested that the predominance of calcium binding proteins in the OT cell is, in particular, responsible for the intrinsic “fast continuous” firing pattern observed in OT cells in contrast to the “phasic” firing in VP cells (305, 436, 437).
IV. PHYSIOLOGICAL RESPONSES OF GENE REGULATION

A. Development

The present ideas about the mechanisms underlying developmental origin and differentiation of the HNS follow the general notion that neurons are committed early on by an interaction between endogenous gene expression programs and extracellular signals provided by growth factors and contact with neighboring cells. These forces also mediate migratory direction and axonal pathfinding. Although anatomical descriptions of the developing HNS are scarce, details on the developmental events enrolling in MCNs have been contributed recently by studies on mice in which specific transcription factors were inactivated. These studies provide insights in molecular mechanisms of MCN development.

MCNs have been traced back to embryonic day (E) 10.5 using \(^{3}\)H-thymidine incorporation and histochemical methods. Magnocellular progenitors arise in the neuroepithelium of the ventricular region around E10.5 in the mouse and E12.5 in the rat (17–20, 164, 165, 377, 552). They migrate laterally. Using an antiserum against the calcium-binding protein calbindin D-28K, Yamakuni et al. (885) found that these neurons can be detected at E11.5 in the anterior hypothalamus of the mouse as a cluster of cells separated from the ventricular surface. Two streams of cells migrate from this cluster toward the hypothalamus, one following a lateral-ventral direction that will form the SON, and the other following the midline that will form the PVN. Migration takes place between E10.5 and E14.5, whereafter axonal extensions are formed. The first expression of NP has been reported on E13 as earliest time point in the rat (711, 21), and an anatomical distribution on E17 has been provided (858). Innervation of the rat neurohypophysis has been reported to start around E16 (257, 258). In the mouse, an antibody against the glycopeptide revealed immunoreactive cell bodies and fibers at E14.5. These fibers start to innervate the neurohypophysis from E14.5 onward in the mouse (395; N. Korteweg and J. P. H. Burbach, unpublished data). As early as E15.5, VP and OT transcripts are present in different groups of neurons. OT expression is seen first in the PVN at E15.5 and in the SON at E18.5 (364).

There are several intriguing questions to the development of the HNS. First, what is the molecular program required for MCN development? All data on this aspect come from studies on mouse mutants in which transcription factors (Table 3) were inactivated by homologous recombination. Four factors and their phenotypic relationships have been studied in detail. These studies can now be fitted in a hierarchical scheme, suggesting a sequence of transcriptional events intrinsic to the development of MCNs. The knock-out of the POU homeobox gene Brn2 (see sect. viC) results in a defect in migration of early MCNs around E12.5 (552, 683). Consequently, no MCNs are present at birth, and no HNS has been established. A very similar phenotype has been described for a mouse mutant in which the basic-helix-loop-helix (bHLH)-PAS (522) gene Sim1 (see sect. vi) results in a defect in migration of early MCNs around E12.5 (552, 683). Consequently, no MCNs are present at birth, and no HNS has been established. A very similar phenotype has been described for a mouse mutant in which the basic-helix-loop-helix (bHLH)-PAS (522) gene Sim1 (see sect. viD) was inactivated (522), again caused by early apoptosis of MCNs which fail to migrate normally. On the basis of analysis of Brn2 expression in Sim1 mutants, it has been proposed that Sim1 may be required for induction or maintenance of expression of Brn2. This would explain the common phenotype and indicates that Sim1 is upstream of Brn2. In relation to these findings, another bHLH-PAS gene, Arnt2, has been studied in a natural mutant mouse strain (521). This strain, C\(^{112K}\), fails to express the Arnt2 gene due to a deletion in the albino (868) locus. Arnt2 maps within the C\(^{112K}\) deletion. Several bHLH-PAS proteins need to heterodimerize for transcriptional action. The phenotype of the C\(^{112K}\) strain appears identical to the Sim1 null mutant regarding the hypothalamic defects. Moreover, Sim1 and Arnt2 are coexpressed in MCNs and physically interact in vitro (521). These data show that Sim1-Arnt2 heterodimerization forms the transcriptionally active complex that is required for development of the MCNs.

Recently, a fourth gene for a transcription factor, Otp, has been inactivated resulting in a phenotype very similar to those obtained by null mutation of Brn2 or Sim1 (1, 710). This gene is expressed in MCNs (see sect. viC). The homeobox Orthopedia (Otp) null mice lack the morphological features of the PVN and SON nuclei and do not express VP and magnocellular OT. The detailed analysis of the mutant mice lacking one of the four transcription factors.
factors has allowed the proposition of a genetic pathway that outlines the hierarchy of these factors (1, 521, 522). Upstream is Otp that is already active during the proliferation phase of MCN progenitor neuroblasts. Otp is required for the maintenance of Brn2 expression. Parallel to this interaction Sim1 and Arnt2 act upstream of Brn2 and also affect its maintenance. The genetic pathway is presented in Figure 5.

One other mouse mutant has been described from which defects in the development of the HNS may be deduced. This concerns a mouse mutant for the winged helix/forkhead transcription factor Mf3, also termed Fkh5 and HFH-e5.1, which is expressed in the hypothalamus (409, 848). This mutant displays severe developmental defects in diencephalon, mammillary body region, and midbrain. Interestingly, Mf3−/− females cannot eject milk but can be stimulated to give milk after intraperitoneal administration of OT (409). This defect may be due to lack or reduction in OT synthesis or release from MCNs. It has not been established if there is a developmental defect in the HNS, or that this phenotype is due to altered circuitry elsewhere in the brain. Another mutant with defective milk ejection and reduced number of OT-producing MCNs is the paternally expressed zinc finger gene Peg3−/− mouse (434). Mutant females display a dramatic reduction in maternal behavior as well.

Second, do VP- and OT-producing neurons have a common origin, and if so, how is their terminal differentiation determined? The many similarities of VP- and OT-producing MCNs argue in favor of a common progenitor cell for both cell types and a fate-determining event in the course of development. Direct evidence for a common progenitor cell is lacking, however. This is partly due to the fact that the discriminating property, namely, expression of the VP gene or OT gene, is only visible relatively late in development, i.e., after E14.5. With the use of double-label ISH, it was demonstrated that at E16.5 VP and OT transcripts exist in separate populations of neurons (364). The observation that a single cluster of calbindin D-28K positive cells is present in the anterior hypothalamus (552) suggests that a common progenitor may exist. The relatedness of the two magnocellular types is also illustrated in adulthood by the plastic overlap in expression of the VP and OT genes in certain physiological conditions (see sect. III A).

Third, how are axons of the MCNs guided to the formation of the neurohypophysis? It is assumed that the area that will form the neurohypophysis secretes growth factors to attract and guide outgrowing axons from MCNs (683). Four mouse mutants have been described in which MCNs are absent at birth. In one of them, the Brn2−/− mouse, it was reported that the neurohypophysis was extremely hypoplastic (552, 683). Nakai et al. (522) reported that pituicytes were present in Brn2−/− newborn animals, but Schoneman et al. (683) described the total absence of pituicytes leaving a vacated neurohypophysis and an infolding of the intermediate lobe. This discrepancy has not been solved as yet. In the Brn2−/− mice described by Schonemann et al. (683), a normal population of pituicytes is present at E16 but starts to disappear concurrently (772). This finding suggests that MCN axons

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**FIG. 5.** Genetic pathways in the development of MCNs of the HNS. Schematic overview on genetic interactions between transcription factors during the development of hypothalamic peptidergic neurons. Parallel genetic pathways of heterodimerizing bHLH-PAS transcription factor genes Sim1-Arnt2 and the homeobox gene Otp are indicated. The Otp pathway affects neuroblast proliferation, whereas the Sim1-Arnt2 pathway does not. Both pathways are required for the expression of Brn-2, on which the differentiation of magnocellular vasopressin (VP) and oxytocin (OT) neurons and parvocellular corticotrophin releasing hormone (CRH)/VP neurons depends. The requirement of Brn-2 for TRH neurons is controversial. Additional peptidergic hypothalamic phenotypes driven by the pathways are indicated. Somatostatin (ST) neurons in the anterior periventricular nucleus and arcuate nucleus require Sim1, Arnt2, and Otp, respectively. Gonadotropin releasing hormone (GnRH) neurons in the latter nucleus are independent of Otp but require the homeobox gene Gsh1. [Adapted from Acampora et al. (1).]
elaborate a trophic effect on the pituicytes of the neurohypophysis.

In the Sim1<sup>−/−</sup> and Arnt2<sup>−/−</sup> mice, there is a similar absence of MCNs at birth (521, 522). For these mice, the neurohypophysis was reported to be ~50% smaller and to contain 50% less pituicytes. It was noted that the pituicytes in Sim1<sup>−/−</sup> mice were more densely packed (522).

Recently, a mouse mutant was created in which regulated secretion in neurons is impaired through null mutation of Munc 18–1, a key protein in the core complex of synaptic vesicle docking and secretion (824). In these mice there is hardly any axonal innervation by MCNs of the neurohypophysis, which appears expanded and disorganized in pituicytes (Korteweg et al., unpublished data). Taken together, available studies indicate that the pituicytes of the neurohypophysis develop independently but are organized by the axonal innervation from the hypothalamus. If this organizing influence includes maintenance and/or proliferation is uncertain. It will be highly relevant to identify the trophic factors involved in the organizing events, which are possibly growth factors or cell surface molecules. In this respect, the expression of "7365," a protein with EGF and follistatin modules, in the HNS is notable (198) and deserves further attention.

Although the expression of VP and OT genes is relatively low during late embryonic development, the expression is strongly enhanced in the first 2 wk postnatally (15, 808). This enhancement follows the recruitment of physiological control mechanisms in the newborn, such as the regulation of normal diuresis by the end of the second week of age (906). Both the VP gene and OT gene display a similar enhancement, and both SON and PVN MCNs are involved. After the first month there is still a mild increase in levels of VP mRNA and OT mRNA, up to ~3 mo. This increase in mRNA levels that ranges between 1.2- to 2-fold in Wistar rats is correlated with a 2-fold increase in pituitary peptide levels, while plasma peptide levels remain unaltered (809). There may be strain differences, with respect to postnatal development, since the spontaneously hypertensive rat (809) displays a more rapid increase in postnatal VP and OT gene expression (809).

A notable aspect of the postnatal development of the HNS is the maturation of prohormone processing. Although the processing of the VP precursor is complete in all investigated stages of development (from E19 onward), the processing of the OT precursor lags behind significantly (21, 858). At E19, no fully processed OT is found, but rather intermediates of processing such as OT-Gly-Lys-Arg, OT-Gly-Lys, and OT-Gly, indicating that initial cleavage of the precursor occurs, but that trimming by carboxypeptidase H/E and amidation are not fully operative yet. By postnatal day 14, native OT is the end product of processing (21). It is not known if the extended forms of OT serve a physiological function in the developing HNS.

B. Osmotic Regulation

The mature MCNs respond to perturbations in water balance by releasing large amounts of stored VP and OT into the general circulation. This is accompanied by functional remodelling of MCN activity, the molecular basis of which is still far from understood. The snapshot of epigenetic plasticity discussed here is mediated by receptors and coupled transduction system as well as transcription factors of which identified components are described in section vi.

1. Hyperosmolar and hyposmolar stimuli

Studies on the osmotic regulation of gene expression in MCNs have benefited from the exploitation of well-established paradigms for the modulation of the activity of VP MCNs. Experimentally, plasma osmolality can be increased acutely (generally by the intraperitoneal injection of 1.5 M NaCl), or chronically [complete fluid deprivation for up to 3 days, usually referred to as "dehydration," or replacement of the the normal drinking diet with 2% (wt/vol) NaCl for up to 12 days, usually referred to as "salt-loading"]. The VP-deficient Brattleboro rats (731) is also exploited as a model of chronic osmotic stimulation. However, it is possible that developmental effects, rather than physiological responses, might confound analysis in these animals.

Experimental animals can be rendered hyponatremic by a combination of the chronic administration of the VP agonist des-aminot-Arg<sup>8</sup>vasopressin (dDAVP) (639) via osmotic minipumps, and the provision of a dilute liquid diet as the only source of nutrients (639).

2. Transcriptional regulation of VP and OT gene expression

VP and OT release as a consequence of an increase in plasma osmolality leads to a functional demand for increased biosynthesis to replace the depleted stores. A hierarchy of transcriptional and posttranscriptional processes achieves this.

Hyperosmotic stimuli such as dehydration or salt-loading result in an increase in transcription of the VP gene (136, 313) and a concomitant increase in VP mRNA abundance (113, 438, 701, 702, 907). The OT gene is similarly regulated by osmotic stimuli (135, 438, 810). The VP and OT genes are normally expressed in distinct MCNs within the SON and PVN (528), but chronic osmotic stimulation increases the proportion of neurons expressing both VP and OT (385). This genomic response to osmotic stress seems to break down with age. Aged rats (28–30
mo old) subjected to 72 h of dehydration are unable to increase serum VP levels and show no increase in VP mRNA abundance and size, despite normal neuronal activation as measured by c-fos induction (719). This is one of the arguments against a role of c-fos in direct regulation of the VP and OT genes.

Under conditions of hyposmolality, there is no demand for VP synthesis, which is effectively turned off. VP secretion and synthesis cease (639), and VP gene transcription is completely inhibited (227, 313). The inhibition of VP gene transcription is thought to be mediated through a novel form of steroid feedback control. The glucocorticoid receptor (GR) is undetectable in VP and OT MCNs of euhydrated rats, but chronic hyposmolality induces GR expression in VP, but not OT, MCNs (68). GR induction coincides with the onset of corticosterone negative feedback on VP transcription. A bovine VP transgene is negatively regulated by glucocorticoids in transgenic mice, possibly through direct binding of the GR to sequences between 150 and 300 bp upstream of the transcription start site (119). These data support the hypothesis that the VP gene can be directly inhibited by glucocorticoids and that the induction of GR in MCNs suppresses VP expression during periods of prolonged hyposmolality.

3. Posttranscriptional regulation of VP and OT gene expression

Gene expression in MCNs is regulated at the posttranscriptional level in two unusual ways. First, the poly(A) tail lengths of the VP and OT mRNAs are modulated by physiological stimuli. Second, selected gene transcripts may be subject to axonal transport.

A) Poly(A) tail length regulation. After the onset of an osmotic stimulus, the poly(A) tail length of the rat VP mRNA increases in length from ~200 to 400 residues (130). This process is specific to the VP mRNA (and the OT mRNA) and does not affect coexpressed messages (137). The increase in VP and OT mRNA poly(A) tail length is an extremely rapid response to an osmotic stimulus, being apparent with a couple of hours of its onset (137), and coinciding with a burst of transcriptional activity (136). These data suggest that old VP and OT mRNAs, bearing short poly(A) tails, are rapidly degraded following the onset of an osmotic stimulus and are replaced, through de novo transcription, by a homogeneous class of mRNAs with elongated poly(A) tails.

The osmotic effect on poly(A) tail lengths is species specific, having being observed in rat (130) and chicken (145), but not mouse (136). Interestingly, a rat VP transgene mRNA (8.2-rVP; see sect. v.A) increases in abundance following the salt-loading of its murine host, but its poly(A) tail length does not lengthen (279), suggesting that the mouse does not have the recognition or enzymatic machinery required for physiologically responsive poly(A) tail length modulation. Similarly, physiological cues do not alter the the poly(A) tail lengths of OT and VP transgene RNAs of bovine origin in transgenic mouse hosts (30, 322). However, it is not known whether physiological stimuli regulate the poly(A) tail length of the bovine OT RNA when expressed in its homologous species environment. RNAs encoded by the rat VP transgene 5-VCAT-3 accumulate in the rat SON and PVN after salt-loading, but the poly(A) tail length is unchanged (838, 904) (see sect. v.A2A), despite the endogenous VP mRNA increasing in both abundance and size. The 5-VCAT-3 transgene was tagged by the replacement of 72 bp of exon III with sequences from the bacterial chloramphenicol acetyltransferase (CAT) gene. It is possible that the deleted exon III sequences contain RNA cis-acting elements that mediate the physiologically controlled poly(A) length increase. Alternatively, the insertion into exon II of the large CAT-derived insert might disrupt the mRNA structure such that it is no longer recognized by the polyadenylation control machinery.

The increases in VP mRNA abundance and poly(A) tail length can occur independently of each other (30, 134, 137) and are probably separately regulated. For example, chronic ingestion of propylthiouracil, whilst affecting neither plasma osmolality nor VP mRNA size, results in a significant increase in the abundance of the hypothalamic VP mRNA (30). In contrast, treatment with the serotonin-depleting agent phenylchlorophenylalanine (PCPA) blocks the accumulation of VP mRNA after salt-loading but has no effect on the lengthening of the poly(A) tail (134).

The mechanics of poly(A) tail regulation in the SON are not understood. The poly(A) tail length of the VP mRNA appears to be regulated in the cell nucleus (839), and thus only newly synthesized transcripts are subject to this mechanism. This is unlike poly(A) tail length regulation of several genes during early development (861), which operates in the cytoplasm on previously existing mRNA stores. Evidence from Xenopus suggests that the length of the poly(A) tail is governed by cis-acting elements within the nuclear pre-mRNA that presumably interact with factors that control the polyadenylation machinery (169, 615).

Little is known of the signaling pathways that mediate the poly(A) tail effect. Treatment of fetal hypothalamic cultures with stimulators of the protein kinase pathway resulted in an increase in the abundance of the VP mRNA abundance and an increase in the VP mRNA poly(A) tail length (203). Stimulation of the protein kinase C (PKC) pathway also increased the length of the VP mRNA poly(A) tail, but had no effect on VP mRNA abundance (203). It is noteworthy that a specific isoform of PKC, PKC-ζ, is predominantly expressed in the SON, and its mRNA is upregulated by osmotic stimuli (93).
There is no information about the physiological role of the VP mRNA poly(A) tail length shift, although, in accord with well-established notions regarding the function of the poly(A) tail of eukaryotic mRNAs, it has been suggested that translational efficiency and/or message stability may be affected (130, 134, 137).

B) AXONAL TRANSPORT OF RNA. The presence of VP mRNA in the posterior pituitary has been first described by E. Lehmann in her PhD thesis in 1988 (426) and has been described independently by several groups shortly after (138, 474, 503, 529, 531, 535, 549, 714, 773, 774). The description of VP and OT mRNAs in the neurohypophysis of the rat pituitary gland has prompted much interest in their origin, regulation, and function. The metabolism of the VP and OT mRNA populations has been compared with that of the hypothalamic pools. Thus the poly(A) tail length of the pituitary VP and OT mRNAs is short compared with their hypothalamic counterparts (549). Whereas the abundance of the VP and OT mRNAs in the neurohypophysis increases dramatically following an osmotic stimulus, by a magnitude that exceeds that seen in the hypothalamus, the poly(A) tail lengths of these transcripts are unchanged (549). Interestingly, 2 wk of hyposmolality rendered the VP mRNA in the neurohypophysis undetectable, in parallel with a concomitant decrease in hypothalamic abundance and poly(A) tail length. In contrast, hyposmolality had little effect on neurohypophysial, or hypothalamic, OT mRNA levels and size (748). In addition to the mRNAs encoding the major MCN neurosecretory products, other transcripts have also been detected in the neurohypophysis, including those encoding preprogalanin (412), c-fos (716), dynorphin (533), neurofilament (NF-L) (533), the RNA polymerase III transcript brain cytoplasmic 1 (BC1) (767), and tyrosine hydroxylase (714).

VP and OT mRNAs have also been detected in the mouse neurohypophysis (549) and in the bovine neurointermediate lobe (550). Interestingly, sequences contained within a 3.5-kb bovine transgene (VP-B) (30; see sect. vA) are functionally conserved between the bovine and murine genes, being sufficient to mediate physiologically regulated localization in the neurohypophysis of transgenic mice (120).

The question of the cellular origin of mRNAs in the neurohypophysis has remained controversial, with two possibilities explaining their presence in this tissue.

The first explanation is axonal transport from the hypothalamus and localization in neural elements of the neurohypophysis. Whereas an early description of OT transcripts in secretory vesicles (269) was not substantiated, subsequent electron microscopic demonstration of VP mRNA in a subset of axonal swellings in the median eminence and neurohypophysis (773, 774) strongly supports the hypothesis that the VP and OT RNAs are transcribed in MCN cell bodies, then transported down axons to the neurohypophysis. However, such a mechanism demands that the hypothalamic VP mRNAs undergo poly(A) tail shortening during transport, or that transport is restricted to VP RNAs with short tails. Nothing is known about the putative axonal transport mechanism, neither is the function of this process understood. Two hypotheses have been proposed. Bloom and colleagues (368, 474) have suggested that the VP mRNA may act as signaling molecule, carrying the information encoded in the message up and down axons, and maybe even postsynaptically. Gainer and Wray (256) have proposed that the axonal transport of specific RNAs may act as a “molecular garbage shoot,” rapidly carrying older (deadenylated) messages away from the neural perikaryon, but leaving the newly synthesized mRNAs, which bear long poly(A) tails. Older, translationally effected mRNAs would therefore be efficiently replaced at the RER by more efficient molecules.

The second explanation is local synthesis (pituicytes, microglia, vascular or connective tissue), with the level of expression possibly being governed by neuroendocrine factors from the hypothalamus (549). Recently, VP transcripts were identified in a subset of pituicytes (609), consistent with the presence of their translation products in these cells (80, 609). However, it is unclear whether the VP transcripts arise from transcription within the pituicyte, or whether they are transferred to pituicytes from axons. However, the synthesis and release of VP and OT have been observed in long-term cultures of dispersed neurohypophysial cells (355). Furthermore, VP synthesis has been described in a ganglioma of the neurohypophysis (214). Although Mohr et al. (533) could find no evidence of VP gene transcription in the neurointermediate pituitary, the technologies employed might not have been sensitive enough. The possibility of VP RNA synthesis in pituicytes raises the intriguing possibility that the VP mRNA found in axonal swellings is synthesized in pituicytes, then transferred to axon terminals, rather than originating from the hypothalamic perikarya. Such a process would not demand the poly(A) tail shortening mechanism required of a hypothalamic origin.

4. Regulation of coexpressed neuropeptides

A number of molecules have been identified in MCNs that appear to be components of the neurosecretory apparatus, but their functions remain unknown. Some of the genes encoding these molecules are regulated as a consequence of osmotic stimuli (Table 1).

The chromogranin/secretogranin family of acidic proteins is widely distributed in secretory vesicles of endocrine and neural tissues. Secretogranin II is expressed only in VP MCNs (29), where salt-loading prompts a large increase in mRNA levels (479, 480, 697). Chromogranin B expression in SON and PVN is also
increased in the SON and PVN after prolonged dehydration (697).

VGF is a nerve growth factor-induced protein that was first identified in PC12 cells. The role of VGF is not known, but it may be processed into a 30-amino acid neuropeptide (peptide V) (46). VGF mRNA levels increase markedly in the SON and PVN after salt-loading (479).

Genes encoding neuropeptides coexpressed with VP and OT are regulated by osmotic stimuli. The functions of these peptides are largely unknown, although roles as autocrine or paracrine modulators of VP and/or OT release have been suggested. Alternatively, coexpressed peptides might have a related role in the regulation of pituitary plasticity and function (see sect. M).

A) OPIOID PEPTIDES. Peptides derived from VP and dynorphin precursors coexist within neurosecretory vesicles of MCNs. After an osmotic challenge, dynorphin mRNA levels increase in the SON and the PVN, but not the SCN (438, 701). Dynorphin-like immunoreactivity in the neurohypophysis is reduced following prolonged salt-loading, suggesting peptide release (829). Dynorphin, coreleased with VP, is probably a local modulator of neuropecretion in the neural lobe; dynorphin (1—8) has been implicated in the local restraint of OT release (82, 672), and dynorphin (81) can delay the VP-induced mobilization of intracellular calcium in pituicytes (79).

The level of enkephalin mRNA is increased in MCNs after prolonged salt-loading (438, 846) or after the stress of intraperitoneal hypertonic saline (438). The role of enkephalins in MCNs and the neurohypophysis is not understood (672).

B) NPY. NPY colocalizes with VP and OT in MCNs, and a high density of NPY-binding sites is found in the neurohypophysis. Salt-loading for 12 days increases posterior pituitary NPY content (359), and this correlates with a marked increase in NPY-like immunoreactivity in hypothalamic MCN cell bodies (359) and a massive upregulation in NPY mRNA in the SON and PVN (7, 600 and 3,200%, respectively) (359, 418). Evidence suggests that NPY selectively and potently enhances evoked VP secretion as part of an autostimulatory feed-forward loop (416).

C) CORTICOTROPHIN RELEASING FACTOR. Corticotrophin releasing factor expression is barely detectable in MCNs of euvhynated rats, but expression is rapidly upregulated after the onset of an osmotic stimulus, being detectable after overnight dehydration or salt-loading (397, 438, 486). Expression is predominantly in recognized clusters of OT neurons (344).

D) OTHER NEUROPEPTIDES. Galanin and VP are coexpressed in SON and PVN MCNs (712). Galanin mRNA increases during a prolonged salt-loading (829, 480), coincident with a depletion in posterior pituitary galanin-like immunoreactivity which is probably indicative of release (829). Expression in MCNs of the mRNAs encoding the neuropeptides CCK (171), VIP peptide histidine isoleucine, and neurotensin/neuromedin N are all upregulated after the onset of an osmotic stimulus (846), as well as secretory granule-contained polypeptides secretogranin II, chromogranin A, 7B2, and VGF (478, 790).

5. Neuropeptide processing enzymes

Not surprisingly, the increase in VP and OT biosynthesis due to an osmotic stimulus demands a concurrent increase in the enzymatic activity of the components of the neurosecretory processing machinery. In some cases, this requirement is met by an increase in gene expression. The expression of most of the enzymes in response to an osmotic stimulus, including the prohormone convertases PC2, PC1/3, carboxypeptidase H/E, and PAM, has been studied.

The genes encoding PC2 and PC1/3 both are upregulated after chronic salt-loading (73). Carboxypeptidase H/E, which removes COOH-terminal basic residues exposed by endoproteases, is abundantly expressed in MCNs of the PVN and SON. Chronic dehydration increases levels of carboxypeptidase H/E mRNA in these neurons, but not in other peptidergic areas of the brain (84, 480). PAM mRNA increases in abundance in the SON and PVN of salt-loaded rats (479).

6. Receptors and channels

The presence and diversity of receptors for transmitters and neuropeptides has been described in section mD as summarized in Table 2. A limited number of these have also been studied under conditions of osmotic challenges. The results of these studies are briefly summarized here.

A) CRH RECEPTOR. The mRNA encoding the CRH receptor mRNA is upregulated in the SON and the magnocellular PVN after 24 and 60 h of dehydration, or 12 days of salt-loading (49).

B) CCK RECEPTORS. Levels of CCK receptor mRNA in the PVN and SON increase markedly in salt-loaded animals (511). These observations suggest that CCK can act as receptors located on the somata of MCNs to induce neuronal discharges that are conducted to the neural lobe where they evoke release of VP from neurohypophysial axon terminals (357).

C) NMDA RECEPTORS. The NMDA-1 receptor is equally expressed in VP and OT MCNs (12). The level of NR1 expression in SON and PVN increases with dehydration (177). Whereas OT MCNs predominantly express the NR2B isoform of NMDA-R2, VP neurons express more of the NR2C isoform, potentially rendering them more susceptible to glutaminergic activation (12).

D) IGF-I RECEPTOR. The mRNA encoding the insulin-like growth factor-I (IGF-I) receptor (IGF-I-R) is expressed in SON and PVN MCNs (9). An osmotic stimulus increased expression levels in the SON, suggesting that IGF-I might
be involved in MCN plasticity following a physiological stimulus.

Na\(^+\) channels are selectively regulated by osmolality. Expression of the α-II, α-Na6, β1γ, and β2mRNAs, but not the α-I and α-III mRNAs, are increased after 7 days of salt-loading (755), corresponding to an increase in Na\(^+\) channel protein and Na\(^+\) current, suggesting that changes in gene expression contribute to remodeling of the electrogenic machinery following salt-loading.

7. Enzymes of neurotransmitter synthesis pathways

A) Tyrosine hydroxylase. The mRNA encoding the catecholamine synthesis enzyme tyrosine hydroxylase increases in abundance in SON and PVN following an overnight osmotic stimulus (846).

B) NO synthase. NO is a noxious, highly reactive, and diffusible free radical gas with a short half-life. NO is produced by citrulline from L-arginine in the presence of the electron donor nicotinamide adenine dinucleotide phosphate (370), a reaction catalyzed by NO synthase (NOS). NOS exists in a number of forms: inducible or macrophage NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). All three forms of NOS are expressed in the hypothalamus, but nNOS is by far the most prevalent form (71). High levels of neuronal NOS (nNOS), which can also be revealed histochemically as NADPH diaphorase activity, are found in the MCNs of the SON and PVN (96, 300, 666, 831). Although NOS-containing MCNs express both OT and VP, the former peptide appears to be more predominant (300). NOS mRNA and NADPH-diaphorase staining increase in MNC cell bodies following osmotic stimulation (370, 584a, 607, 655, 830), and NADPH-diaphorase activity also increases in axon terminals in the neurohypophysis (170, 607, 655). These data suggest a role for NOS and NO in MNC plasticity. It would be reasonable to suggest that NO might stimulate VP and/or OT release, but evidence points to a role in the attenuation of release (370, 747, 890), although the site of action is not known. One possibility is that NO released from MCN cell bodies has retrograde effects on afferent inputs. A number of potential presynaptic NO targets have been identified, including soluble guanylyl cyclase, which is stimulated to generate cGMP, a second messenger present in fibers in the SON and PVN (734) which activates a phosphorylation cascade through a cGMP-dependent protein kinase. Staining for cGMP increases in the SON, PVN, and structures of the lamina terminalis after acute osmotic stimulation (456).

8. Cell adhesion molecules

MCNs undergo reversible cellular remodeling and intercellular plasticity in response to physiological stimulation. This is characterized by alterations in 1) the relationship between MCNs and glia, 2) the extent of terminal contact with the basal lamina in the neurohypophysis, 3) the type of synaptic inputs, and 4) the extent of electrotonic coupling between MCNs (301, 761). Some studies on the expression of cell adhesion molecules in the HNS have been reported (759), but the mechanisms that mediate the remodeling of MCN structure and neuron-glia interactions are not understood. Two molecules thought to be involved in the control of cellular morphology, and the interaction of the cell with the extracellular matrix, have been examined in MCNs. Alterations in the patterns of expression of F3 and dystrophin after an osmotic stimulus implicate them in activity-dependent structural plasticity that may mediate exocytosis at axon terminals, and other neurosecretory processes.

F3 is a glycophaspatidylinositol-anchored glycoprotein of the immunoglobulin superfamily implicated in axonal growth. F3 mRNA is expressed in MCNs, and levels increase threefold after an osmotic challenge (759). In MCN cell bodies, F3 immunoreactivity was shown to be present only in secretory granules. In the neurohypophysis, however, F3 was found in granules, on neuronal and glial cell surfaces, and in the extracellular spaces, suggesting release by exocytosis at axon terminals.

In muscle, dystrophin links the extracellular matrix with the cytoskeleton. Dystrophin is also expressed in brain, but its role there is not understood. Dystrophin immunoreactivity is present in the neurohypophysis, but this dramatically decreases after dehydration, appearing instead in axonal swellings in the hypothalamic tract (190).

9. A global approach?

The pattern of gene expression in MCNs is altered by an osmotic stimulus, and we can speculate as to how these changes might affect the functioning of the neuron in response to the environmental stress. However, as yet we have only examined the expression of a small number of genes. It is estimated that there are 70,000–100,000 genes in mammalian organisms, of which maybe 80% are expressed in the brain. How many of these are utilized by MCNs remains unknown. Furthermore, no attempts have been made to globally assess how that overall pattern of gene expression is changed by an osmotic stimulus. The core question, “How is the rate of hormone production controlled?” needs to be refined at the genomic level for this stimulus to “which genes are switched on and off following dehydration.” The patterns of genes affected by osmolality in MCNs will prove to be informative about the cellular events that are essential for coordinate control of hormone production. The components described so far are all part of the sensing potential of MCNs (receptors) and the production and release machinery. A global approach to regulated gene expression has been applied to the intermediate lobe of the pituitary of Xenopus in re-
response to background adaptation (329). Current technology, e.g., DNA chips and expression arrays, will allow such an approach.

C. Reproduction

1. Sex steroid sensitivity and the role of the HNS in reproduction

Concerning the role of sex steroids in the reproductive functions of OT, of critical debate at the moment is if the effects of sex steroid hormones during reproductive cycles are direct on the MCNs themselves or whether they are indirect via steroid-sensitive interneurons. Although it is generally accepted that MCNs do not possess ERα, evidence has been provided for the presence of ERβ in both OT and VP MCNs (see sect. viC). The OT gene promoter, but not the VP gene promoter, possesses estrogen-responsive elements (see sect. viC). However, in the majority of cases, both genes in the HNS are regulated similarly during reproductive cycles (see below), as is the sensitivity of both OT and VP neurons to other stimuli (see sect. viC6), evidence which may favor an indirect effect of sex steroids on the HNS.

Although areas of the brain that are known to project to the HNS contain estrogen-receptive neurons, only one study has combined retrograde tracing from the SON with immunostaining for ERα (834). In summary, this study shows that the majority of forebrain estrogen-responsive neurons that project to the SON are located in the medial preoptic area and lamina terminalis, although with scattered neurons through the hypothalamus. Interestingly, few of the neurons in the medulla oblongata, which project to the SON, contain ERα (834). It will be important to investigate estrogen-responsive neurons in other parts of the brain that project to the HNS and that may play a role in reproductive function, such as the olfactory lobes or the tuberomammillary nuclei (301, 464), as well as to map possible innervations by neurons that contain ERβ.

Recently, the ERβ has been found expressed in MCNs of SON and PVN (708), indicating that estrogens have the potential to act directly on MCNs by regulating transcription. Alternatively, sex steroids may be having direct, but nongenomic effects on MCNs. Sex steroids can cause rapid increases in magnocellular cell electrical activity (10, 555), the release of OT and VP into the circulation (25, 158, 713, 884), as well as intrahypothalamic dendritic release (843).

2. Sex differences and the ovarian cycle

Gonadectomy largely, but not totally, prevents the increase in OT gene expression that is associated with the onset of puberty in rats (147, 523). In addition, treatment of prepubertal rats with either estrogen or testosterone has no effect on OT mRNA. Therefore, factors other than gonadal steroids appear to be involved in the maturation of the HNS at the time of puberty. There is a sexual dimorphism in the size of, primarily, VP MCNs (larger in males) that is related to body weight and does not seem to be dependent on gonadal steroids (477). As the volume of MCNs increases with age and body weight, it is likely that this is a reflection of increased hormone demand associated with water balance.

Plasma concentrations of OT and VP are reported to vary during the ovarian cycle in both rats (158, 231, 663, 713) and humans (25, 230), with highest levels corresponding to peak estrogen levels. There is less compelling evidence for changes in magnocellular cell synthesis over the 4-day rat ovarian cycle. Van Tol et al. (809) have demonstrated an increase in OT mRNA during estrous as measured in extracts of SON micropunches. Changes in HNS peptide content have also been reported (158, 281). However, short-term (2–7 days) estrogen treatment of ovariectomized rats had no effect on OT mRNA in the SON as determined by ISH or Northern analysis (112, 151), although a significant increase was noted after 2 mo of treatment (151). It has been speculated that gonadal steroids may affect OT MCNs indirectly (110), but recently the ERβ has been detected in MCNs, pointing to the possibility that estrogens act on MCNs directly (708).

3. Control of OT during pregnancy and parturition

There is a large accumulation of OT peptide in the HNS during pregnancy, and this is secreted from the pituitary at the time of parturition (125, 191, 238, 241, 405, 685). The general consensus is that this build-up of OT peptide is due to a combination of increased gene expression (155, 464, 807, 908), transcript polyadenylation (909), and an inhibition of hormone release (191, 296). There is little doubt that OT MCNs are activated at the time of parturition (218, 457, 461, 746), although it is has been postulated that OT produced in the uterus itself may contribute to the hormone’s peripheral effects at term (424, 425). Although the rat uterus contains 70-fold more mRNA than the hypothalamus at the end of pregnancy, and this disappears with parturition, the actual OT peptide content of the uterus is small compared with that in the neurohypophysis. Furthermore, the increase in OT mRNA reported in the rat and human uterus does not occur in all animals, such as the pig, cow, mouse, and sheep (87, 352, 548, 844).

In a series of elegant studies, Crowley and co-workers (155, 156) have manipulated sex steroids in ovariectomized virgin rats to mimic the changes that occur during pregnancy: a rise in both estrogen and progesterone, followed by an abrupt decline in progesterone (normally 48 h preterm), while estrogen levels are maintained (100).
In this way, they demonstrated that this pattern of estrogen priming followed by progesterone withdrawal can enhance OT mRNA abundance in the SON and magnocellular PVN (156). Maintenance of progesterone artificially at the end of pregnancy not only delays birth (33), it also prevents the normal rise in OT mRNA (156).

MCN activity and plasma OT secretion is pulsatile during birth, although there is an increase in circulating levels that occurs before fetal passage through the birth canal (240, 267, 316, 746). Pulsatile delivery of OT to rats the day before expected delivery leads to uterine contractions and a pattern of neuronal activity similar to that seen during normal parturition (35, 454, 457). The significance of OT pulsatility is probably species dependent (239, 267, 317), although it is likely that the HNS plays a critical role in allowing birth to progress normally (457, 614).

4. Lactation

OT gene expression falls immediately postpartum (155) and then increases during early lactation to cope with the demand of the growing young (438, 464, 807, 908). The level of OT mRNA during early lactation is maintained by the suckling stimulus, suggesting an important role for afferent stimulation (735). Removal of pups during early lactation leads to a decline in OT mRNA that can be attenuated by intracerebroventricular OT infusion, implying an action of centrally released hormone. OT may be released from dendrites and the cell bodies in response to sucking to have this effect (537, 561). In contrast, the expression of OT mRNA in the rat during late lactation shows increasing dependence on the steroid milieu (155). Estrogen levels in the rat are suppressed by suckling for the first week postpartum and then rise gradually as lactation continues, whereas progesterone levels increase during early lactation to peak at around day 10 before declining again (724). Thus around days 10–12 of lactation or ~48 h after interruption of nursing by pup removal (422), the steroid status of the rat is similar to that of the late-pregnant animal, that is, rising estrogen and falling progesterone levels. Both of these situations are accompanied by further increases in OT gene expression, which can be abolished by maintaining progesterone artificially (762).

The fully pulsatile activity of OT MCNs induced by suckling is critical for successful lactation (605). A discussion of the mechanisms that might allow this distinctive patterning to be displayed by the whole population of OT neurons in synchrony is beyond the realms of this review and can be found elsewhere (64, 303, 760). However, it is clear that a number of changes in neuron structure and membrane properties will require complex alterations in gene expression by MCNs that have yet to be elucidated.

5. Modulation of VP and VP-OT colocalization in the HNS

VP mRNA abundance rises in parallel with OT mRNA during pregnancy and lactation, although the increase appears to be less pronounced (155, 438, 807, 908). Lightman and Young (438) also demonstrated an increase in dynorphin mRNA, which is colocalized with VP in MCNs, during lactation. Thus it is interesting that the steroidal paradigm used to mimic late pregnancy does not affect the abundance of VP mRNA (156). The latter result suggests that VP MCNs may not be under the same influence by gonadal steroids, a point that is further confused since 4-day estrogen replacement to ovariectomized rats increased the number of VP, but not OT, MCNs that colocalized immunodetectable levels of other peptides (432). These apparent discrepancies may be explained if colocalized peptides can be regulated independently by afferent inputs that may or may not themselves be steroid sensitive. It is true that VP neurons need estrogen to respond fully to an osmotic stimulus (58, 154), which may be dependent on estrogen-sensitive neurons of the lamina terminalis (834).

Finally, as with a chronic osmotic stimulus or adrenalectomy (53, 385), the percent of MCNs that are found to express both VP and OT transcripts increases from 2% in virgin rats to 17% in lactating rats (518). In addition, a transitory colocalization of OT and VP peptides in the pituitary and median eminence at the time of parturition was not recorded during lactation (367).

6. Modulation of inputs and HNS functions

During pregnancy and lactation, the HNS of the female rat adapts to carry out the specific duties associated with birth and lactation. However, the system needs to continue its role in the maintenance of homeostasis that will require the modulation of other functions. For example, during pregnancy, there is an expansion of blood volume and a decrease in plasma osmolality that is maintained by a comparable reduction in thresholds for drinking and HNS hormone release (441). Despite this decrease in the set point about which the animal is osmoregulating, there is no change in the sensitivity of the HNS to osmotic stimulation. During lactation, plasma osmolality and the threshold for hormone release return to normal, even though there is greater demand on fluid turnover due to milk production. However, in contrast to pregnancy, during lactation the sensitivity of the HNS to osmotic stimuli is reduced (210, 220, 297, 315, 392). In addition, during lactation, there is a reduction in the sensitivity of the HNS to other stimuli, such as stress (132, 315, 391, 506). Thus changes that occur in the HNS gene expression during reproductive cycles may reflect various adaptations of the system.

As well as the short-term, presumptive nongenomic
effects of steroidal status on MCN firing (10, 555), there are longer term electrophysiological effects that may reflect changes in inputs, receptor expression, or membrane properties. Among other things, the morphological changes in the HNS, which are in place by the end of pregnancy and which are maintained during lactation, involve synaptic adaptation of the input onto MCNs (761). These changes, which are dependent on ovarian steroids (536), involve glutaminergic and GABAergic terminals (200, 266). Glutamate or GABA measured by dialysis of the SON does not vary during birth or the suckling of pups (312, 832), although an effect may be achieved by these transmitters through regulation of their receptors (106).

Evidence for modulation of HNS function by other inputs includes changes in the transcriptional activity of presumptive afferent neurons (Fig. 1). For example, noradrenergic neurons of the brain stem NTS are activated around parturition (34, 454), and this corresponds to an increase in norepinephrine release in the SON (312). Similarly, histaminergic neurons that project to the HNS show an increase in the abundance of mRNA for the synthetic enzyme, histidine decarboxylase, that parallels the increase in OT mRNA (464). This is interesting because histamine can increase the synthetic activity of OT MCNs (388). NO has an inhibitory effect on hormone release (370, 747). The mRNA for its synthetic enzyme, nNOS, increases in all structures in the forebrain osmoreceptive circuit during lactation and, thus, NO may be involved in the reduced sensitivity of the HNS at this time (464).

V. TISSUE-SPECIFIC EXPRESSION AND PROMOTER PROPERTIES

To find clues to the molecular mechanisms of gene expression and regulation in the HNS, various available approaches have been taken. These approaches are general to current molecular biology. However, the HNS poses specific problems rendering some of the approaches inadequate. To appreciate the state of affairs in gene regulation in the HNS, some of these approaches and their application to the physiology of the HNS are briefly discussed below.

A. In Vivo Models

The term transgenesis is used very broadly to describe the introduction of cloned DNA into any living cell. Over the past 10–15 years, profoundly important techniques have been developed that enable new genes to be introduced into whole mammalian organisms. The resulting animals are called transgenic animals, defined as any animal into which cloned genetic material has been transferred.

Before the current revolution in transgenic research, the only method available to study the regulation and function of mammalian genes within the context of the whole organism was to utilize mutants that arose spontaneously in nature. The study of such mutations has been termed “forward genetics,” from phenotype to gene. However, this process, the molecular identification and characterization of the mutated gene, is technically difficult. Furthermore, mutations arise opportunistically in nature, and those affecting particular systems are difficult to screen for and identify; the only mutation known to affect the magnocellular HNS is the Brattleboro rat (see sect. uD1), which suffers from hereditary hypothalamic diabetes insipidus (787) because of a single base pair deletion in the VP gene (680).

Gene function and regulation can also be studied in cultured mammalian cells, and evaluation of gene expression in such systems is relatively straightforward. However, because there are no appropriate cell lines available that correspond to the differentiated neurons of the adult magnocellular HNS, researchers have resorted to heterologous cell types. Furthermore, the activity of a specific gene at the cellular level does not yield satisfactory information about the regulation of the gene among the complex physiological interactions of the whole animal. Even the best cell culture systems cannot possibly simulate tissues and organ systems and predict responses to sophisticated environmental stimuli. No cell line, nor culture system, can ever model the plasticity evoked by the panoply of developmental and physiological stimuli to which a magnocellular HNS neuron is normally exposed.

Transgenic animal systems combine the virtues of cell culture and congenic breeding strategies while avoiding the negative aspects of each system; a defined genetic lesion can be studied within the physiological and developmental integrity of the whole animal. This has enabled the development of the concept of “reverse genetics,” from gene to phenotype. With the use of transgenic techniques, a characterized genetic sequence can be evaluated within the context of the whole animal without any prior knowledge of its regulation or function.

1. Germline transgenesis

To apply a reverse genetic approach to mammals, we must be able to introduce a defined genetic change into the germ line such that 1) every cell of an organism will carry the change, and 2) the change will be transmitted to subsequent generations. An unlimited number of genetically identical offspring carrying the same genetic modification are thus available for experimental analysis.

Two methods of making germline transgenic animals are relevant to this review: microinjection of fertilized...
one-cell eggs and manipulation of embryonal stem cells to make knockout mice (547).

The process of microinjection results in the integration of the transgene into the host chromosomes. Integration is an additive process; the host genome gains new information. The host genome is unchanged, except at the locus of integration, where deletions may occur, possibly interrupting endogenous genes. Integration takes place through nonhomologous recombination of the transgene into the host chromosomes, and there is no specificity with respect to either the host or the transgene DNA. A transgene is expressed with a spatial and temporal pattern that is a function of the cis-acting elements that it contains. However, the chromosomal position of the transgene can affect expression, resulting in transcriptional repression or ectopic activation.

Embryonal stem (ES) cells are pluripotential cells derived from of the primitive ectoderm of the mouse blastocyst. With the use of appropriately designed targeting vectors, specific mutations (for example, null mutants, known as knockouts) can be introduced into target genes in ES cells via homologous recombination. The mutated ES clone can then be introduced into a blastocyst, where it will colonize the inner cell mass and contribute to the development of a chimera. Breeding of a germline chimera with a wild-type mate results in the generation of a pure line that is heterozygous for the mutation, and brother-sister matings of these will give rise to homozygous mutant animals. The phenotype elicited by targeted mutation can then be studied.

A) MICROINJECTION OF FERTILIZED ONE-CELL EGGS. One-cell fertilized eggs are harvested from donor females that have usually been hormonally stimulated to produce a large number of eggs before mating. Cloned DNA fragments are then directly introduced into one of the two pronuclei using a fine injection needle. A few hundred molecules of DNA are introduced into the nucleus. Surviving eggs are transferred to the natural environment provided by a pseudopregnant recipient female. A pseudopregnant recipient is an estrus female that has been mated with a sterile or vasectomized male. The animal carries only unfertilized eggs but is physiologically prepared to carry implanted eggs through pregnancy. Pups are born 20–21 days later, 10–50% of which are transgenic as identified by genome analysis.

The process of microinjection results in the integration of the transgene into the host chromosomes. Integration is an additive process; the host genome gains new information. The host genome is unchanged, except at the locus of integration, where deletions may occur, possibly interrupting endogenous genes. Integration takes place through nonhomologous recombination of the transgene into the host chromosomes, and there is no specificity with respect to either the host or the transgene DNA. There is usually only a single integration site per nucleus, but that integration site might contain between 1 and 1,000 tandemly arranged copies of the transgene. The integration process is essentially random; the experimenter has no control over the site of integration, nor the copy number of the transgene.

A transgene is expressed with a spatial and temporal pattern that is a function of the cis-acting elements that it contains. However, the chromosomal position of the transgene can affect expression, resulting in transcriptional repression or ectopic activation. The structure of a microinjection transgene depends entirely on the aims of a particular experiment. A transgene should operate like any other gene in the targeted cells of an organism, and hence, structural elements must be appropriately recognized by the transcriptional, posttranscriptional, and translational machinery of the host. Transgenesis has extensively been applied to VP and OT genes in the HNS (see sect. Va2).

B) MANIPULATION OF ES CELLS (INCLUDING KNOCKOUTS). ES cells are pluripotential cells derived from the primitive ectoderm of the mouse blastocyst. As with any mammalian cell culture, DNA can be introduced into ES cells. ES cells can be genetically altered, and these altered cells can be introduced into a blastocyst where they will contribute to the normal development of a chimeric mouse. If the germ line of the mouse has been colonized by the ES cell descendants, then, at the next generation, a pure line of heterozygous genetically altered mice can be derived.

ES cells can be altered in two ways: 1) nonhomologous recombination and 2) homologous recombination. Nonhomologous recombination is by far the most common mode of integration of exogenous DNA into the chromosomes of mammalian cells. Integration is at random, and there is no specificity with respect to either the host or input DNA. The host gains new information, with the rest of the genome remaining essentially unchanged, except for deletions or other rearrangements around the integration site. Homologous recombination involves an exchange of information between the input DNA and homologous host sequences, mediated by Watson-Crick base-pairing and host recombination enzymes. Homologous events are very rare compared with nonhomologous events. Screening systems have been developed that enable clones bearing these rare homologous events to be isolated.

With the use of appropriately designed targeting vectors, specific mutations (for example, null mutants, known as knockouts) can be introduced into target genes in ES cells. The mutated ES clone can then be introduced into a blastocyst, where it will colonize the inner cell mass and contribute to the development of a chimera. Breeding of a germline chimera with a wild-type mate results in the generation of a pure line that is heterozygous for the mutation, and brother-sister matings of these will give rise to homozygous mutant animals. The phenotype elicited
by targeted mutation can then be studied. Homologous recombination has been instrumental in the delineation of genetic pathways of development of the HNS (see sect. ivA) and refining physiological functions of OT (see sect. ivC).

C) TRANSGENIC RATS. Most transgenic studies have been performed on mice, but it is the rat that continues to be the species of choice for studies in neuroscience and physiology. However, physiological techniques are being adapted to this species to exploit the growing collection of mutant animals available. In contrast, the anatomy of the rat brain is well mapped, and the structure, function, and regulation of the rat CNS have been the subject of detailed study for many years, and numerous effective behavioral paradigms have been developed. The large size of the rat makes it easily accessible for a whole range of physiological measurement and intervention, but its reproductive capacity and gestation time are equivalent to the mouse. Rats can be transformed by microinjection of fertilized one-cell eggs (903). Although ES cell-like cultures have been described (341), it has not been possible to derive chimeric rats from these cells (341); thus gene knockouts are not yet possible in these species. Transgenic rats have allowed us to link physiological studies to control regions of the VP-OT link gene locus (see sect. vA3).

2. Cell specific expression of the VP-OT locus in transgenic mice

A number of VP and OT transgenes of both rat and bovine origin have been introduced into mouse and rat hosts (Fig. 3). The rationale behind these experiments has been to define chromosomal regions necessary for appropriate transgene expression in MCNs. It is then possible to further delineate the regulatory sequences responsible by making further deletions until appropriate expression is lost.

A) VP TRANSGENES. The first attempt to define the sequences required for the cell-specific expression of the VP gene involved the expression in a transgenic mouse of a construct (AVP.SVER1.25; Fig. 3) consisting of 1.25 kbp of the bovine promoter directing the expression of the early region of the DNA tumor virus SV40, which encodes the oncogene large-T antigen (546). This transgene elicited tumors in the anterior lobe of the pituitary, apparently of somatotrope origin (737). However, no expression was detected in the hypothalamus, suggesting either that the proximal 1.25 kbp gene expression was not identical to that of the endogenous VP gene. First, the level of expression of the transgene in hypothalamic VP MCNs (903). 5-VCAT-3 consists of the rat VP structural gene, containing reporter sequences in exon III derived from the bacterial CAT gene, flanked by 5 kbp of upstream and 3 kbp of downstream sequences. The CAT sequences provide a unique nucleic acid reporter of transgene RNA expression, which was used in Northern and ISH analyses (903). 5-VCAT-3 is regulated by cell-specific cues in the hypothalami of transgenic rats, being confined to VP, but not OT, MCNs in the PVN and SON. However, the pattern of 5-VCAT-3 transgene expression was not identical to that of the endogenous VP gene. First, the level of expression of the 5-VCAT-3 RNA was lower than the endogenous VP transcript in the hypothalami of physiologically unstimulated animals. Second, expression in the SCN was, at best, negligible. These data suggest that the transgene lacks enhancers that mediate high basal VP expression, particularly in the SCN.

Progress toward the fine-structure delineation of the regulatory sequences required to direct expression to...
MCNs has been slow, but there is some evidence that flanking sequences downstream of the structural gene are important. When these sequences are deleted (transgenes 3-Vβgal-0.2 and 1.5-Vβgal-0.2) (904, 903) (Fig. 3), expression in the hypothalamus cannot be detected.

b) OT Transgenes. The first OT transgene to be introduced into mice (ROT1.63, Fig. 3) consisted of the entire rat OT structural gene flanked by 0.36 kbp of upstream sequences and ~0.4 kbp of downstream sequences (Fig. 3). However, transgenic mice were not represented in the resulting pups, suggesting that the presence of this transgene was somehow incompatible with normal development (W. S. Young, personal communication). Similarly, Murphy and co-workers (322) introduced a bovine OT transgene into mice consisting of the structural gene, flanked by ~3.0 kbp of upstream and 2.5 kbp of downstream sequences (bOT6.4; Fig. 3). Despite injecting over 1,000 fertilized one-cell mouse eggs with this construct, and screening over 150 of the resulting pups, no transgenic animals were identified (322).

Ang et al. (31) then introduced a smaller bovine OT transgene (bOT) into fertilized one-cell mouse eggs and derived three transgenic mouse lines. bOT differed from bOT6.4 only in that the sequences upstream of the start of transcription consisted of 0.6 kbp, rather than the 3.0 kbp found in the latter transgene. Distal upstream sequences, present in bOT6.4, but absent in bOT, must therefore be detrimental to normal embryonic development. The 4.2-kbp bOT transgene was consistently expressed in testicular Sertoli cells and in as yet unidentified cells in the lung. The expression of bOT in mouse Sertoli cells contain abundant OT RNA (31), a feature that is present in bOT6.4, but absent in bOT, must therefore be detrimental to normal embryonic development. The 4.2-kbp bOT transgene was consistently expressed in testicular Sertoli cells and in as yet unidentified cells in the lung. The expression of bOT in mouse Sertoli cells was shown to mimic a bovine expression pattern. Bovine Sertoli cells contain abundant OT RNA (31), a feature that is brought to the mouse by the cattle transgene. Although the endogenous murine OT gene is not expressed in Sertoli cells, these cells contain trans-acting factors that can interact with cis-acting elements residing in the bovine transgene (but not, presumably, the endogenous mouse gene) leading to a high level of expression. In contrast, the expression of bOT in the lung does not correspond to either a murine or a bovine pattern. In neither species has OT RNA been detected in lung (31). The consistent expression of the bOT transgene in lung must therefore be a consequence of the release of this gene fragment from the action of repressor elements, absent from the transgene but present in the normal context of the bovine genome, that act to prevent expression in this organ in the adult.

No expression of bOT could be detected in the transgenic mouse hypothalamus. Initially it was thought that the transgene must be missing elements crucial for expression in OT MCNs. However, subsequent experiments have suggested that bOT contains too much sequence information, rather than too little. Transgene bOT3.5 (Fig. 3) is the same as bOT except that it lacks ~0.7 kbp of distal downstream sequences. Like bOT, bOT3.5 is expressed in testis and lung. Surprisingly, expression of bOT3.5, unlike bOT, is detected in the hypothalamus (322). ISH experiments have shown that transgene expression in the hypothalamus is confined to magnocellular cells in the PVN and SON that also express the endogenous mouse OT gene. Transgene expression, like that of the endogenous OT gene, is excluded from neurons expressing the endogenous VP gene. Thus downstream sequences present in bOT, but absent from bOT3.5, contain one or more repressor elements that prevent the detectable expression of the former transgene in hypothalamic OT MCNs. It follows that in the normal genomic context of the OT gene, the activity of these repressors must be repressed or overridden.

c) Interactions between the VP and OT Genes. Because of the close linkage between the VP and OT genes in all species examined, it has been postulated that regulatory interactions between sequences within the two transcription units may be important in the overall control of the expression of the locus. This concept has received support from studies on transgenic mice (322, 548, 896, 897). As described above, no transgenic mice were derived from the microinjection of fertilized one-cell mouse eggs with a rat transgene consisting of the entire rat OT structural gene flanked by 0.34 kbp of upstream sequences and ~0.4 kbp of downstream sequences (transgene ROT1.63; Fig. 3). Young et al. (897) then generated mice bearing a minilocus transgene (V2; Fig. 3) consisting of construct ROT1.63 linked to sequences from the rat VP gene (transgene RVP3.55) consisting of the structural gene flanked by ~1.4 kbp of upstream sequences and 0.4 kbp of downstream sequences. In the V2 minilocus transgene, the two genes were arranged in the opposite transcriptional orientation (VP 3’ to OT 5’) to the native genes (VP 3’ to OT 3’). On its own, the RVP3.55 was not expressed in transgenic mice (Young, personal communication), suggesting that sequences necessary for appropriate expression are missing. Similarly, the VP component of the V2 minilocus was transcriptionally silent. However, the OT transcription unit present in V2 was expressed in OT MCNs (62, 588). These data are indicative of regulatory communication between elements contained in either of the two transcription units of the OT-VP locus. Elements within the VP component must in some way repress the deleterious effects of the OT portion, allowing transgenic mice to be generated. Similar results have been obtained with a minilocus transgene organized in the native transcriptional orientation (256), suggesting the presence in the VP component of an orientation-independent enhancer of OT promoter activity. The close linkage of the VP and OT genes within their locus may thus be important for cell-type specific expression. The orientation of the genes within the locus, while apparently not important for cell-type specific expression, may contribute to the maintenance of locus structure.
Similarly, it has also been shown that the bovine VP and OT genes can affect the expression of each other. Two transgenes containing segments of both the VP and OT genes, VP-B/bOT3.5 and Vpro/bOT, have been examined (Fig. 3). The VP-B/bOT3.5 transgene consists of the VP-B and bOT3.5 constructs linked 3' to 3' (322) (Fig. 3).

The VP-B construct (Fig. 3), consisting of the bovine VP structural gene flanked by 1.25 kbp of upstream and 0.2 kbp of downstream sequences, is expressed in all neuronal tissues examined (30) (see above). As described above, bOT3.5 is expressed in OT MCNs, testis, and lung (322). In mice containing the VP-B/bOT3.5 transgene, expression of the VP component could not be detected in any central or peripheral tissue. Although expression of the OT component of the transgene was prominent in testis and lung, no expression could be detected in hypothalamus. These data suggest that sequences within the OT component of the transgene prevent expression of the VP portion in neuronal cell, whereas sequences within the VP-B repress the hypothalamic expression of bOT3.5.

The Vpro/bOT transgene consists of the 4.2-kbp bOT construct, which on its own is expressed in testicular Sertoli cells and lung, linked to the 1.25-kbp promoter of the bovine VP gene (548) (Fig. 3). This promoter, as part of transgene VPA (Fig. 3), directs the expression of a CAT reporter to all central and peripheral tissues in transgenic mice (30) (see above). However, when linked to bOT, sequences in the VP promoter are unable to direct ubiquitous expression from the OT promoter; rather, bOT expression is found, as before, in the testes and in a new site, the cortex. Thus the sequences within the VP promoter that, on their own, are able to direct the ubiquitous expression of a reporter are not strong enough to confer an expression pattern on the neighboring OT promoter. However, interactions between sequences in the OT gene and the VP promoter consistently result in expression in a novel ectopic site, the cortex. Because no OT expression has ever been reported in cortex, these interactions must be ineffective in the normal genomic context of the OT gene.

3. Physiological models

A) PHYSIOLOGICAL REGULATION OF TRANSGENE EXPRESSION. VP transgenes of both bovine and rat origin, in both mouse and rat hosts, when expressed in hypothalamic MCNs, are subject to regulation in response to physiological cues. Salt-loading for 7 days significantly increased the level of bovine VP mRNA in the hypothalami of VP-C mice (Fig. 3), and also increased in the number of MCNs expressing the transgene (30). Interestingly, although expression of the VP-B transgene (Fig. 3) is not confined to VP MCNs, it is upregulated in the PVN and SON as a consequence of 7 days of salt-loading, suggesting that the regulatory sequences mediating physiological regulation are contained within the confines of VP-B (30) (Fig. 3).

Grant et al. (279) showed that 3 days of dehydration increased hypothalamic expression of the rat 5-VCAT-3 transgene in mice (Fig. 3). The physiological regulation in rat hosts of the rat VP gene-derived 5-VCAT-3 transgene has been well defined (838, 903). Five days of salt-loading, an osmotic stimulus that increases the level of the endogenous VP mRNA 2-fold, evokes an exaggerated effect on transgene expression, increasing the level of the transgene encoded RNA 20-fold. This is not a consequence of transgene copy number, because different lines, with different numbers of transgene copies, show the same exaggerated physiological response. It has been postulated that this exaggerated effect is due to the release of the transgene from the action of repressor sequences, absent from the transgene, but present in the normal context of the VP gene, that attenuate the physiological response.

Physiological stimuli are known to affect the pattern of OT gene expression in the hypothalamus, and investigators have asked if these same stimuli alter transgene expression. Young and co-workers (896, 897) have shown that the hypothalamic expression of the OT component of the rat OT-VP minilocus transgene (V2; Fig. 3) increases fourfold during lactation. Similarly, the level of bovine OT mRNA encoded by the bOT3.5 transgene (Fig. 3) increases in the hypothalamus during salt-loading, in parallel with the endogenous mouse OT mRNA (322). Thus the sequences mediating the physiological upregulation of bovine OT gene expression reside within the confines of the bOT3.5 transgene.

B) PHYSIOLOGICAL ENGINEERING. As well as aspects of gene regulation, transgenic experiments can be used to address problems in gene function and the role of gene products in the overall physiology of the organism (136). Thus far, little of functional consequence has emerged from transgenic studies on either the VP or the OT gene. For a transgene to participate in the normal (or abnormal) physiology of its host, it must, of course, be translated and processed into a biologically active peptide. Young and co-workers (896, 897) and Belenky et al. (62) have shown that the rat NP encoded by the OT component of their rat VP-OT minilocus transgene was being synthesized, packaged into neurosecretory vesicles, and transported to the posterior pituitary. It was, of course, not possible to distinguish between transgene derived, rat OT, and the endogenous mouse nonapeptide in the hypothalamus and pituitary. Presumably the transgene-derived peptide is contributing to the normal OT physiology of the transgenic mice; there have been no reports that this is disturbed in these animals.

The incorporation of the CAT reporter into exon III of the 5-VCAT-3 transgene (Fig. 3) placed a unique hexapeptide (DRSAGYGLFKDRKEK, abbreviated to DR-12-EK) at the COOH terminus of a modified prepropeptide.
molecules. The facile but dramatic regulation of the can predictably regulate the level of expression of these manipulated drinking diet of the transgenic rats we compared with the endogenous VP gene, means that by simple expression in parvocellular neurons. Second, the exaggeration in support of models of neurohypophysial homeostasis that suggest that pituitary VP peptide levels increase hypothalamic DR-12-EK levels, in parallel with the hydrophylic peptide “tag” is thus well tolerated and does not disrupt VP production or disturb salt and water balance. An osmotic stimulus was shown to increase hypothalamic DR-12-EK levels, in parallel with transgene RNA levels, but changes in posterior pituitary DR-12-EK levels were more complex. After 5 days salt-loading, DR-12-EK levels fell, as would be expected if its release was coordinate with that of VP. However, after 10 days of salt-loading, posterior pituitary DR-12-EK levels increased, despite the lower level of VP. This probably reflects the greater response of the transgene to osmotic challenge at the RNA level, increasing the proportion of DR-12-EK-containing translation products transported to the posterior pituitary relative to those derived from the endogenous gene. These observations are further evidence in support of models of neurohypophysial homeostasis that suggest that pituitary VP peptide levels passively reflect changes in hormone release and synthesis (227) and that the availability of mRNA is the primary determinant of pituitary VP content in the basal state (226).

Attempts have been made to develop expression systems based on the rat VP and OT transgenes that would target expression of any protein or RNA to magnocellular hypothalamic neurons, with a view to analyzing the physiological consequences. A derivative of the 5-VCAT-3 transgene has been used to direct the hypothalamic expression of foreign genes in the rat. Two properties of the 5-VCAT-3 transgene are very useful for functional studies. First, expression in magnocellular cells is separable from expression in parvocellular neurons. Second, the exaggerated response of 5-VCAT-3 to osmotic challenge, compared with the endogenous VP gene, means that by simple manipulation of the drinking diet of the transgenic rats we can predictably regulate the level of expression of these molecules. The facile but dramatic regulation of the 5-VCAT-3 transgene in precisely defined neurons of the rat brain is in marked contrast to previously described neuronal promoters that, when expressed in mice, are active in diverse cell types and cannot be controlled. However, as yet an effective expression system for the HNS has not been reported, despite attempts to achieve this. The first vector built, 5-VCAT-3 (Sal I), was derived from 5-VCAT-3 by the site-directed deletion of the VP prepropeptide translation start sites in exon I, and their replacement by a unique Sal I restriction endonuclease site, into which any gene sequence can be inserted. Two cDNAs were cloned into this site: a cDNA encoding an inhibitor of protein kinase A (PKi(κ); 579) and a cDNA encoding a putative inhibitor of protein kinase C (PKC-α, HINT-1; Ref. 597).

No transgene-derived RNA could be detected in any tissue of numerous independently derived rat lines bearing the cDNA constructs (840). These data suggest that disruption of exon I with cDNA has a deleterious effect on VP gene expression. This is possibly due to recognition of a premature termination codon into exon I, and the subsequent degradation of the message by nonsense-mediated decay (309).

Similar results have been obtained in transgenic mice bearing mouse OT gene-derived expression vectors (901). Four transgenes, all based on the mouse VP-OT locus and bearing an enhanced green fluorescent protein (eGFP) reporter, were introduced into mice. In two of the transgenes, eGFP was introduced into exon I of the OT gene. In one case, eGFP was inserted in-frame with the OT coding sequences to produce a truncated OT-eGFP fusion. In the second case, eGFP was inserted upstream of the OT prepropeptide coding sequences in the 5'-untranslated region. In the two remaining transgenes, eGFP was inserted into exon III. First, eGFP was inserted in-frame with the COOH-terminal region of NP. Second, EGFp was also placed into exon III after a picornovirus internal ribosome entry site (IRES). The IRES acts as a “ribosome landing pad” (598), directing ribosome binding to an appropriately positioned translation initiation codon in an mRNA cap- and Kozak box-independent manner. Expression of this transgene generates a bicistronic RNA directing the translation of the wild-type OT prepropeptide, and, from the downstream cistron, eGFP. Neither of the transgenes with insertions in exon I were expressed efficiently. In contrast, both of the transgenes with insertions in exon III were robustly expressed in the PVN and SON. These results demonstrate that, like the VP gene, the OT gene is inefficiently expressed if exon I is disrupted by sequences without introns. However, like the VP gene, disruption of OT exon III is tolerated. The efficient IRES-mediated translation of eGFP in the SON and PVN demonstrates that this element can be used to direct the expression of unaltered foreign proteins in magnocellular neurons from...
their own cistron, avoiding problems with function and targeting that may be associated with fusions.

c) KNOCKOUT MICE. There have only been a few reports on the effects of specific gene knockouts on MCN function. Some transcription factor knockouts have profound effects on HNS development. These are described in section IV.

Knockout mice have been generated that lack the OT gene (564, 654). These studies show that OT is only required for nursing but is not essential for parturition or reproductive behaviors. OT-naive mice of both sexes are viable and fertile. OT-deficient females give birth normally and appear to demonstrate normal maternal behavior; however, all offspring die shortly after birth due to the dam’s inability to nurse. Thus it appears that OT plays an essential endocrine role in milk ejection but not parturition, at least in the mouse (see sect. uC2v). These mice display a very selective behavioral deficit, social amnesia, indicating that OT is necessary for the normal development of social memory, which is a central function of OT (221a).

4. Comparative genomics of the VP-OT locus: the rat and the pufferfish

Identifying regulatory elements in mammalian genomes is a tedious exercise because of the large amount of “junk” DNA that is interspersed in the intergenic and intronic sequences. Nearly 90% of the 4,000 Mbp of the rodent genome is comprised of repetitive elements, and this has slowed transgenic studies on the regulatory sequences contained within the VP and OT locus. In contrast, the pufferfish, Fugu, has a compact genome of 390 Mbp that contains very few repetitive elements, yet this teleost has a gene repertoire comparable to that of mammals. The majority of introns in the pufferfish are small (modal value 80 bp), and repetitive sequences account for <10% of the genome (97). Thus the pufferfish genome is an attractive model for identifying and characterizing regulatory elements through comparative genomics. Although the fish brain is anatomically not identical to that of mammals, there are distinct MCNs in the preoptic nucleus of the fish that express the isotocin (IT) and vasotocin (VT) genes, the teleost equivalents of OT and VP, respectively. IHC and ISH studies have shown that the fish IT and VT are expressed in separate MCNs (277, 340). Fugu VT and IT genomic sequences have been cloned and found to be closely linked on a single cosmid, along with at least six other genes (815). Comparison of the sequence of the Fugu VT-IT locus with that of the rat VP-OT locus (681) revealed numerous homologies that might correspond to conserved regulatory elements. To test the functions of these homologies empirically, transgenic rats were produced that contain a 40-kbp pufferfish genomic fragment derived from the IT-VT locus. The IT gene was shown to be expressed only in rat OT neurons of the SON and PVN (815). Furthermore, the expression of the IT gene is upregulated in response to the withdrawal of dietary water in parallel with the endogenous OT gene. These results are important for a number of reasons. They demonstrate conservation of regulatory mechanisms between two species that separated 400 million years ago. The regulatory sequences and cognate trans-acting factors responsible for cell type and physiological regulation have been conserved; this is all the more remarkable given that the two species, rat and Fugu, face profoundly different environmental challenges. Putative regulatory sequences can be readily identified by species comparison; nonsignificant sequences have had ample time to randomize by mutation and translocation, and conserved sequences in the noncoding sequences are likely to have a role in gene expression and regulation. High gene density in the Fugu and a dearth of repetitive sequences is an additional advantage in transgenic studies. A combination of comparative genomics using the compact genome of Fugu and transgenesis in rats is thus a useful tool for identifying and characterizing conserved gene regulatory events. Furthermore, this approach will enable the identification of fundamental and conserved components of the limbic and brain stem systems that control body fluid homeostasis (550).

5. Somatic transgenesis and gene therapy

A) THE GERMLINE TRANSGENIC APPROACH: PROBLEMS AND SOLUTIONS. In a transgenic animal, the genetic change represented by the transgene is manifested throughout development, from conception onward. The lack of temporal or spatial specificity can complicate the interpretation of the phenotypes resulting from a transgenic experiment (557) for three reasons: 1) the overall phenotype observed may be a summation of transgene effects in different tissues at different times. The parts of the overall effect might be very difficult to dissect.

2) An early or severe effect of a transgene might preclude the study of subsequent or downstream processes involving the same gene.

3) Transgenic animals are genetic “reactionisms.” Epigenetic responses to a genetic lesion can have physiological effects (645). Thus a phenotype may be due to endogenous genes being switched on or off in response to the transgene or knockout, rather than being a direct effect of the primary genetic lesion. In contrast, many investigators have invested effort and resources into the generation of a knockout mouse only to find that the homozygous null mutant animal has no overt or obvious phenotype. The knocked-out gene may be “redundant” or other genes, possibly of the same family, may take over the function of the knocked-out gene (324, 566, 645, 765).

With the consideration of these problems, consider-
able effort is being directed toward the development of transgenic systems that avoid the germline by introducing genes directly into somatic cells. Thus the germline is not affected, and the genetic change will not be passed on to subsequent generations. Somatic gene transfer has matured into a valuable tool for basic studies in HNS research and has attracted considerable interest in relation to its possible clinical application (so-called gene therapy).

b) ANTISENSE. The application of antisense oligonucleotides to somatic cells has the potential to be an alternative to germline gene-knockout technologies. Attempts have been made to block gene expression in magnocellular HNS neurons by the application of large quantities of antisense single-stranded DNA oligonucleotides corresponding to the sequence of a target gene or its mRNA. Some striking results have been obtained. Injection into the brain of oligonucleotides complementary to the VP mRNA, chemically modified to increase their stability in vivo, resulted in a rapid reduction in VP biosynthesis and induced diabetes insipidus-like symptoms (228, 509, 716). Similarly, chemically modified antisense oligonucleotides corresponding to the OT mRNA had rapid effects on the level of systemic OT (366, 560) and blocked lactation and corresponding to the OT mRNA had rapid effects on the level of systemic OT (366, 560) and blocked lactation and sucking (366, 715). However, these effects were seen without any changes in OT biosynthesis (366, 715). Furthermore, the OT antisense oligonucleotides elicited effects that were clearly unrelated to OT biosynthesis, including reduced electrophysiological excitability, reduced CCK- (171) and electrically stimulated OT release, and inhibition of CCK-induced cfos expression (558, 559).

Antisense technology is highly controversial and remains unproven in most circumstances. Although it is possible that antisense molecules are able to specifically interfere, through Watson and Crick base-pairing, with their intended targets, it has never been proven that an oligonucleotide can knock out just one gene product and that all of the other expressed genes in the target cell remain unaltered. Antisense molecules, particularly oligonucleotides that have been chemically modified to increase their stability and efficacy, can have profound nonsequence specific effects on cells. Modified oligonucleotides can bind avidly to proteins, and breakdown products can inhibit cell proliferation. Central administration of chemically modified oligonucleotides into the brain have been shown to elevate body temperature, suppress food and water intake, and inhibit nighttime activity; pyrogenic effects accompanied by elevated concentrations of circulating corticosterone; and an increase in the synthesis of interleukin 6 mRNA in the brain and spleen (682).

A biological effect seen as a result of applying an antisense molecule might therefore be the result of the following (95): 1) a specific Watson and Crick interaction with its intended target; 2) a specific Watson and Crick interaction with an unintended, but unidentified, target; 3) a non-antisense interaction with another RNA or RNAs; 4) an interaction with protein; and 5) a nonspecific effect on cell proliferation or metabolism.

Although antisense technology may one day mature into a valuable tool in both basic research and gene therapy, most of the results obtained to date must be viewed with caution.

c) VIRAL VECTORS AND GENE TRANSFER. Viruses have evolved to deliver efficiently foreign nucleic acid into a host cell. As such, modified viruses make ideal vectors for the delivery of new genes into cells. Several types of viruses have been used as vehicles to transfer genes into differentiated neurons in the central nervous system in vivo and in vitro (717). These include herpes-related viruses (213, 222), attenuated adenoviruses (202, 264), adeno-associated viruses (376, 872), and lentiviruses (212), just to mention a few. To date, only adenovirus has been used in the HNS in vivo (262–264), and adeno-associated viruses have been used to transfect oxytocin cells in vitro (378). The experience with adenoviruses is discussed in this section and with adeno-associated viral vectors in section vB3a. Derivatives of adenovirus (Ad) type 5 are being increasingly exploited as a means to transfer genetic material into the brain. To make an Ad capable of safely delivering new genes to a host cell, two technical advances were needed: 1) the development of Ad5 derivatives that can accommodate large transgenes and 2) the development of Ad5 derivatives that are replication incompetent, that is, they are able to infect cells and deliver their DNA, but within the host that DNA will not replicate.

Both of these ends were achieved by deletion of regions of the viral genome required for viral DNA replication. The resulting virus will not propagate autonomously and is of a size that a transgene insert of up to 8 kbp can be incorporated. Replication-deficient viruses are propagated in a helper cell line that provides the missing viral gene products in trans. Transgenes transferred into cells are efficiently expressed, and cell-specific promoters can function in the context of the backbone of the viral genome (299, 633). The main problem encountered with Ad gene transfer is a consequence of the expression of viral proteins. These elicit a host immune response that eliminates the infection and limits the duration of transgene expression. However, it turns out that this is not a great problem in the brain, which is protected by the blood-brain barrier. Injection of an Ad vector expressing β-galactosidase resulted in discrete and high-level expression in the parvocellular and magnocellular cells of the PVN (262). Expression of the reporter protein lasted for 3 mo. The inflammatory response, as assessed by T-cell, MHCI, and MHCIi immunocytochemistry, was much smaller than that seen after peripheral administration of Ad, and subsided 14 days after injection. It was subsequently demonstrated that Ad vectors could mediate the
long-term correction of inherited genetic defects affecting the HNS (263). A single injection into the SON of an Ad expressing the VP cDNA under the control of a constitutive and powerful viral promoter-enhancer resulted in the long-term (over 4 mo) correction of the symptoms of diabetes insipidus in the VP-deficient Brattleboro rat (see sect. uD1). This work has also demonstrated the utility of the Brattleboro rat as a model for CNS gene therapy; the efficacy of a treatment can be monitored by the trivial and noninvasive assessment of water balance.

D) RNA-MEDIATED GENE TRANSFER. It has been reported that injection of synthetic VP mRNA into the hypothalamo-neurohypophysial tract of the Brattleboro rat can reverse the symptoms of diabetes insipidus for up to 5 days (368, 660). VP mRNAs with a short or absent poly(A) tail were more effectively expressed (474). Interestingly, the VP RNA transported to the axonal compartment from the hypothalamic cells bodies also has a short poly(A) tail (see sect. ivB3A). It was thus suggested that, as magnocellular HNS neurons could apparently selectively accumulate, retrogradely transport and translate exogenous mRNA, intra-axonal transcripts might participate in a novel mode of neuronal signaling.

B. In Vitro Models

1. Heterologous and homologous systems

Homologous cell lines generated either by spontaneous mutation or by targeted tumorigenesis (514, 612) are the ideal in vitro model systems for the study of the regulation of specific genes. Because homologous cell lines, by definition, are derived from cells which normally express the genes of interest, they should have the transcription factors and signaling mechanisms that are relevant to its regulation in that cell type. Given such lines, it is relatively easy to identify regulatory elements in the gene, and highly feasible to isolate the transcriptional activation factors acting at these domains. However, in only one neurosecretory cell system in the central nervous system, i.e., the GnRH system, has productive, immortalized stable cell lines been successfully generated by targeted tumorigenesis (514, 612). The GT-1 cell line, in particular, has been successfully used for the analysis of GnRH gene regulation (854, 860, 862). With the use of a similar strategy in earlier experiments, a 125-kbp 5'-untranslated region of the bovine VP gene fused to SV40 large-T antigen as a transgene in mice, failed to produce a hypothalamic, VP-synthesizing cell line (546). Instead, tumorigenesis was ectopic, in the endocrine pancreas and the anterior pituitary (546, 737). This failure in targeting was probably due, in part, to the absence of critical VP gene domains necessary for specific expression (see sect. vA1). However, more recent efforts using SV40 large-T antigen-bearing constructs containing these critical VP-gene domains also failed to produce tumors in the HNS of transgenic mice, although ectopic tumors were also induced in this case (H. Gainer, unpublished data).

Given the absence of HNS-relevant cell lines, investigators in the field have turned either to heterologous systems or to transgenic mice to study OT and VP gene regulation (see Ref. 841 and sect. vA1). Various heterologous cell lines have been employed, including small-cell lung carcinoma lines that express the VP gene (820, 822), mouse P19 embryonic carcinoma cells (3), monkey kidney CV1 cells (591), human MFC-7 breast tumor cell lines, mouse neuroblastoma neuro 2a cells, human embryonic kidney HEK-293 cells, and human choriocarcinoma JEG-3 cells (see Refs. 115, 116, 841). The choice of the heterologous cell line to be used is typically governed by which steroid or other receptor is needed in the experiment and its presence in the given line. Alternatively, the receptor gene and target gene constructs can be cotransfected into cell lines that contain neither but that are easily subjected to cotransfection.

Experimental work using heterologous systems has successfully identified a number of putative regulatory elements in the OT and VP genes. These include various nuclear hormone receptors, class III POU proteins, and fos/jun/activating transcription factor (ATF) family members as candidate transcriptional activating factors involved in the regulation of the OT and VP genes (116, 841), (and sects. vB and vC). Interpretation of the data from heterologous cell lines used for this purpose has been complicated by the observations that the behavior of the identified cis-motifs in the 5'-untranslated regions of these genes often differs depending on which cell line is used, and consequently, the most relevant data to date have come from studies done in transgenic mice with the assays being performed in vivo in the MCNs themselves (see sect. vA1). The transgenic mouse approach, although highly effective, is relatively expensive and lengthy, and investigators continue to search for cell lines or primary neurons that express OT or VP endogenously and that can easily be transfected with exogenous constructs. Several neuronal systems other than HNS neurons have been reported in the literature to contain one or both of these peptides. However, reports that the Neuro2A cell line contained VP (54) and the repeated reports that half of the small sensory neurons in the rat dorsal root ganglion contain colocalized OT and VP (358, 372, 373) have not been confirmed (292; Liu and Burbach, unpublished data; Jeong and Gainer, unpublished data for Neuro2A).

2. Primary MCN tissue culture models

The absence of homologous OT and VP expressing cell lines for use in gene expression studies has raised considerable interest in the possibility of using cultures of primary MCNs for the transfection of various gene con-
structs. Not only would this be a valuable complement to transgenic studies, but it would also afford an opportunity to study the regulation of both the endogenous and the transfected genes as well as secretion in these cells under rigorously controlled extracellular conditions. Thus far, the challenge has been to maintain healthy MCNs in tissue culture over long periods. There are two general approaches to the long-term tissue culture of neurons, i.e., dissociated cell and organotypic tissue culture.

A) DISSOCIATED HYPOTHALAMIC TISSUE-CULTURED NEURONS. It is generally known that dissociated cell cultures survive best when the starting material is derived from embryonic central nervous system. This is also true for hypothalamic neurons, and hence, most work has utilized brain tissue from E13–19 mice or rats (65, 365, 498, 718, 816, 855). The neurons that survive in these cultures typically express very little VP (188, 365) and modest, if any, OT under basal conditions. However, VP (but not OT) expression can be induced in these cultures by continuous treatment with forskolin plus IBMX (498, 569), and large NP-immunoreactive neurons can be detected under these stimulated conditions (498, 718). Although there is no doubt that there is VP expression being induced by cAMP in neurons in these cultures, this dependence on continual extrinsic cAMP stimulation is not a normal property of the VP MCN phenotype. It is possible that the CRH neurons, which can express VP under some circumstances in vivo and are also known to be abundant in these cultures (152), might be the sources of the stimulated VP. In any case, the consistent absence of any significant OT-expressing neurons in these dissociated cultures strongly suggests that at least these MCNs are not thriving under these conditions.

An alternative approach is to use the neonatal hypothalamus as the source of the starting tissue (66, 72, 451, 476, 864, 887). Although cellular yields are generally lower than when fetal brain tissue is used for the preparation of dissociated cells, there are nevertheless some advantages. Principal among these is the fact that the neonatal hypothalamus is sufficiently large and the MCNs well-enough differentiated at this time to permit separate microdissections of the PVN and SON regions (72, 887). Because the SON contains only magnocellular OT and VP neurons, culturing neurons from this nucleus would therefore ensure one of the magnocellular identity of the surviving neuronal population in vitro. Recent experiments using astrocyte feeder layers and the application of growth factors (e.g., BDNF) has yielded about 60 each of well-differentiated OT and VP MCNs per neonatal SON pair dissected after 14 days in vitro (408). These MCNs have robust dendritic and distinct axonal processes and can survive for weeks in culture, thereby providing excellent models for electrophysiological and pharmacological studies. However, their small and variable yields make them inadequate for systematic molecular studies.

B) ORGANOTYPIC HYPOTHALAMIC SLICE-EXPLANT CULTURES. A possible alternative to dissociated cell cultures is the use of slice-explant or “organotypic” cultures derived from postnatal brain (Figs. 6 and 7). The earliest attempts to organ culture rodent hypothalamic explants made use of Maximow double coverslip chambers in the lying drop position (216, 770). Although these cultured tissues could survive for long periods in vitro, they were relatively inaccessible to modern electrophysiological and whole-mount IHC and ISH techniques, and hence, were not further developed nor extensively used. More recently,
Gahwiler and colleagues (248, 250) have pioneered and extended the use of so-called “organotypic” slice-explant culture system in which 400-μm slices of neonatal (PN 2–10) rat brain are attached to a coverslip by a plasma clot and then cultured for weeks. This method has several distinct advantages. First, it permits the culturing of brain tissues at times when neurons are already differentiated (i.e., from neonatal to adult stages). Second, the organotypic nature of the slices, even after weeks in culture, allows for the unequivocal identification of many cell types by correlating their topographic locations in the culture with slices from the intact brain as well as with their other phenotypic characteristics (e.g., their endogenous peptides). Third, many cell types (e.g., MCNs) that cannot easily be maintained by dissociated culture methods can be cultured with better yields by this approach. Finally, the cultures are thin, typically to just a few cell layers and therefore are very useful for electrophysiological, immunocytochemical, and immunohistochemical analyses.

The first systematic effort to produce organotypic hypothalamic cultures made use of the roller-culture method (249, 871), and this was successful in the long-term maintenance of OT MCNs in the PVN (870, 871) and parvocellular VP neurons in the SCN (870). CRH neurons in the PVN are also well preserved by this method (70). However, there were still problems with the roller technique for the culture of the MCNs. Although OT MCNs do very well in these cultures, VP MCNs survived poorly, if at all. In addition, the sizes of the initial 400-μm slices needed to be relatively small, so as to remain adhered to the coverslips during rotation. The attrition rate of individual PVN and SON cultured slices, largely due to the slices slipping off the coverslips during rotation, was >50%. This also precluded the routine inclusion of the SON together with the PVN in a single slice. Finally, the slice explants in roller cultures are initially fastened to the coverslip by a plasma clot, which appears to stimulate astrocyte growth over the surface of the thinned slice. Although this did not interfere with the study of endogenous gene expression in neurons in the slice, it made transfection of the neurons with exogenous genes significantly more difficult.

Recently, a variant of the organotypic culture technique in which the slices are incubated on top of Millipore filters at an air-media interface has been described (741). A major advantage of this technique is that as many as five 400-μm slices can be placed on one Millipore filter (this encompasses nearly the entire neonatal hypothalamus), and large slices containing both the PVN and SON in one slice are easily maintained. Because these cultures are “stationary,” the attrition rate is very low (<5%). This “stationary” variant of the organotypic culture technique has proven to be very effective for the culture of the SCN (63) and the HNS (334). The slices in these cultures thin to less than 80–100 μm, maintain the cytoarchitectural features of the tissue slice even after weeks of culture, and also permit the long-term survival of large numbers of MCNs (334). Cultures derived from neonatal rat hypothalamus contain ~400 OT cells and 200 VP cells per hypothalamus after 15 days in vitro. Similar survival values (380 OT cells and 600 VP cells) were found per mouse hypothalamus after 15 days in vitro (334). Hence, the stationary slice-explant culture method appears to pre-
serve large numbers of highly differentiated MCNs in long-term culture, and these cultures should be excellent in vitro models for physiological and pharmacological studies and analysis of gene expression (Figs. 6 and 7).

3. Transfection of primary cultures

Conventional methods such as calcium phosphate and lipid-mediated cell transfection are not generally successful when used with primary neuronal cultures and especially organotypic cultures. This may, in part, be due to the presence of a reactive astrocyte layer that covers the cultured slice explant, thereby preventing access of the DNA-containing vehicles to the neurons in the slice. Two approaches appear to be promising with respect to the transfection of slice-explant cultures: the use of viral vectors and particle-mediated gene transfer. These are described below.

A) VIRAL VECTORS. One possible approach to transfect the OT and VP neurons in the cultures is the use of viral vectors. Various viral vectors, inducing retroviral, Herpes, adenoviral, and adeno-associated viral (AAV) vectors, have been widely discussed and used for gene transfer into the central nervous system both in vivo and in vitro (376, 717). In addition, adenoviral (262) and AAV (872) vectors have been successfully used to infect MCNs in the rat PVN in vivo (see also sect. VA1E).

In preliminary experiments, AAV was used as a vector to transfect hypothalamic neurons in the organotypic cultures, since this vector is known to be relatively non-pathogenic (376, 876). The recombinant adeno-associated virus vector, AAV-LacZ, that was used has been described (505). The vector is replication incompetent with 96% of the wild-type viral genome, including the replication and encapsulation genes, having been removed and replaced with a cytomegalovirus promoter-LacZ cassette. With the use of the above vector construct with either a cytoplasmic or nuclear-targeted LacZ reporter driven by a cytomegalovirus promoter, robust transduction of both glia and neurons in slices was found, with no obvious deleterious effects even after 7 days posttransduction. With the use of the AAV vectors containing nuclear LacZ, OT cells in culture were quantitated by double-label immunocytochemistry. With the use of of 0.5 \times 10^7 transducing units per culture, ~88% of the OT cells present in the cultured slices were transfigured, with no apparent deleterious effects on the cells (378).

The above data indicate that the AAV vector is a highly efficacious vector for gene transfer into neurons in slice-explant cultures. However, this vector has two disadvantages that limit its use. The first is its insert size limitation of <5 kbp, therefore making many desirable but larger OT and VP constructs not compatible with this vector. The second disadvantage is that it is difficult to obtain high titers of AAV constructs. This would preclude its use as a rapid assay in deletion construct analyses, since the virus production itself would be rate limiting. It is likely that the adenovirus vector described in section VA1E will be equally efficacious and will provide a more practical approach in vitro.

B) PARTICLE-MEDIATED GENE TRANSFER. Particle-mediated gene transfer, also known as “biolistics,” uses helium pressure to accelerate DNA-coated microparticles (usually 1- \mu m gold particles) at high velocity into cells (764). This method was originally used to transfect plant cells, but has now been extended to various eukaryotic and mammalian cells in vivo and in vitro. Recently, this particle-mediated transfer method has been used to transfect neurons and glia in slices of cerebellum, visual cortex, and hippocampus (46, 47, 443, 502, 764) that were cultured on microliter inserts similar to those used in stationary slice-explant cultures. This technique has many applications, and its use has ranged from evaluating dendritic growth in developing visual cortex (502) to the identification of cell-specific transcriptional control elements in the calbindin promoter (47). Rat hypothalamic as well as hippocampal slice-explants have been effectively transfected by this method, and although the transfection incidence is low and random (i.e., the plasmid-coated 0.6- to 1- \mu m gold particle has to penetrate the nucleus of the cell without damaging it), the method could be used for quantitative analysis of deletion constructs (764). In these experiments, biolistics was used to transfect neurons in stationary hypothalamic slice-explant cultures with a variety of promoters [e.g., cytomegalovirus, Rous sarcoma virus, glial fibrillary activating peptide, \alpha-tubulin, and various \alpha_{1B}-calcium (N) channel promoter constructs] coupled to various reporters (e.g., LacZ, luciferase, and GFP). This method is especially useful when Golgi-like visualization of only a few cells are needed per slice (502) or when there are abundant cells of a given phenotype present in the slice (47). One major advantage of this method is that any DNA construct, of any size and in any configuration, can be used virtually immediately for transfection. Therefore, in principle, this can provide a very rapid assay, comparable to the use of cell lines for deletion-construct analysis.

Preliminary experiments using the above particle-mediated gene transfer technique were used to transfect OT and VP constructs that contain the 5’-promoter regions, three exons with their introns, a CAT reporter inserted at the end of the third exon, and 3.6 kbp of the 3’-mouse intergenic region that have been previously expressed in transgenic mice in OT or VP neurons, respectively (252), into HNS neurons in stationary organotypic cultures. Because there are only ~300 OT or VP MCNs present per filter, and the biolistic procedure is random and with a low probability “hit rate,” only a small number of HNS cells might be expected to be transfected (i.e., containing the immunoreactive CAT reporter). A modest
but not rare transfection of neurons that had the expected morphologies of OT and VP neurons was found to be successfully transfected, and these were specifically located in regions of the slice (e.g., PVN, SON, and SCN) where such neurons were to be expected. Preliminary double-label IHC indicates that the CAT fusion protein encoded in the OT and VP constructs was expressed in the OT and VP MCNs, respectively (763), showing that organotypic cultures used together with particle-mediated gene transfer will be a valuable in vitro complement to the transgenic mouse approach (see sect. vA1) for the study of OT and VP cell-specific gene expression and regulation.

C. The VP Promoter

The cell-specific expression and physiological regulation of the VP gene has been amply documented, but the cis-acting sequences and cognate trans-acting factors within the promoter that mediate these expression patterns are not yet understood. This is in part due to the difficulty in studying gene expression in a tiny group of neurons within the brain for which no corresponding cell line exists. As a consequence, studies have relied on either transgenic animals (see sect. vA) or heterologous cell lines (see sect. vB) of dubious physiological relevance.

Whole animal studies in rats and mice (see sect. vA) have shown that both the cell-specific and the physiological regulation of VP gene expression can be mediated by sequences within transgenes consisting of the structural gene, flanked by at least 3 kbp of upstream sequences, and 3 kbp of downstream sequences. However, precise mapping of the elements responsible has yet to be attempted. Computer programs can be used to scan these sequences for potential transcription factor binding sites, but such analyses are meaningless unless followed up with direct experiments.

Physiological observations in the whole animal have prompted studies on the effects of particular signaling pathways or transcription factors on the VP promoter in heterologous cell lines.

1. cAMP

CAMP levels in the SON increase after the onset of an osmotic stimulus (133). Interestingly, these in vivo effects can be mimicked by SON explants cultured in hyperosmotic media, suggesting that the cells of the SON are intrinsically osmosensitive and that they respond to an increase in osmolality by increasing intracellular CAMP levels. Young et al. (900) have demonstrated that hyperosmotic stimuli increased the SON levels of the mRNAs encoding both the stimulatory and inhibitory guanine nucleotide binding protein \( \alpha \)-subunit (G\(_{s}\alpha\) and G\(_{i}\alpha\)) and increased cholera toxin-stimulated adenylate cyclase and G\(_{i}\alpha\). Furthermore, application of cAMP agonists to in vitro cultures of fetal hypothalamus (203, 718) and in hypothalamo-neurohypophysial explants (498) results in an increase in VP RNA levels.

VP promoters respond positively to CAMP after transfection into cultured cells. The luciferase activity in P19 embryonal carcinoma cells that were transiently transfected with \(-174\) to \(+44\) of the 5’-flanking region of the human VP gene linked to the firefly luciferase gene was stimulated about twofold by a CAMP analog (820).

Similarly, the rat VP gene promoter responds to stimulation of the protein kinase A pathway following transient transfection into human choriocarcinoma JEG-3 cells (354) and stable integration into mouse corticotrope AtT-20 cells (203). Two CAMP-responsive elements were identified, located between \(-227\) and \(-220\) as well as \(-123\) and \(-116\), relative to the start of transcription (354). Upregulation is blocked by cotransfection with a dominant negative form of the CAMP response element binding protein (CREB), suggesting a role for this transcription factor in the CAMP response.

The bovine VP gene is strongly responsive to CAMP agonists (591), and a CRE, which binds to members of the API transcription factor family, is located between \(-120\) to \(-112\) within the proximal promoter. Footprinting with SON extracts of salt-loaded and edehydrated rats showed binding to this region, indicating that this element may play a role in the response of the VP gene to changes in water balance (591).

2. Glucocorticoids

The increase in VP gene expression in the parvocellular neurons as a result of stress is well known (396, 471, 667, 668). Expression of the VP gene is suppressed by adrenal glucocorticoids as part of a negative-feedback loop. Similarly, VP mRNA expression increases in PVN but not SON MCNs after the glucocorticoid withdrawal of adrenalectomy (744). In a small-cell lung carcinoma expressing low levels of VP mRNA (GLC8) (821), responses to glucocorticoid and CAMP appear to show mutual dependence. Under basal conditions, glucocorticoids decrease the VP mRNA content of GLC8 cells, but under forskolin-stimulated conditions, glucocorticoids increase the VP mRNA content further (822). Similar findings in the small-cell lung carcinoma cell line H69 were done using IHC (237). This finding may be relevant for the in vivo expression of VP in the parvocellular PVN, where the effects of glucocorticoid and stress may be modulated by activity of synaptic input.

In studies involving cotransfection in heterologous cell lines, no effect of the ligand-activated glucocorticoid, mineralocorticoid, or androgen receptors was found (3). However, glucocorticoids were shown to suppress the
increase in VP promoter activity mediated by cAMP analogs (354). This effect was shown to be dependent on the glucocorticoid receptor but has not been shown to require binding of the receptor to DNA. A bovine VP transgene is negatively regulated by glucocorticoids in transgenic mice, possibly through a direct binding of the glucocorticoid receptor to sequences between 150 and 300 bp upstream of the transcription start site (119).

The GR is usually undetectable in VP and OT MCNs of euhydrated rats, but chronic hyposmolality induces GR expression in VP, but not OT, MCNs (68). GR induction coincides with the onset of corticosterone negative feedback on VP transcription (68). These data support the hypothesis that the VP gene can be directly inhibited by glucocorticoids and that the induction of GR in MCNs suppresses VP expression during periods of prolonged hyposmolality.

3. Action of transcription factors

In view of the availability of VP gene promoters of various species and recognition of homologous elements for over a decade, there is a striking lack of progress in identifying transcription factors that act on the VP promoter. As outlined above, factors downstream in the protein kinase A pathway and glucocorticoid receptors have been implicated in regulation of promoter activity. Along these lines, the VP promoter has been studied for responsiveness of other factors related to CREB and the GR, respectively. First, in heterologous transfection experiments, inhibitory forms of the CREM family (178, 664) are able to reduce the cAMP response of the rat VP promoter (Burbach, unpublished data), suggesting that these and other factors of the CREB/CREM/ATF families may modulate expression of the VP gene in vivo. Second, the VP gene also displays responsiveness to other members of the nuclear hormone receptor family. The rat and human VP promoters are suppressed by 50–70% through ligand activation of the androgen and estrogen receptors (3). The human VP promoter, but not the rat VP promoter, displays a mild response to the ligand-activated thyroid hormone receptor (THRα) (3). The promoter regions involved in these responses as well as the in vivo significance have remained unexplained.

A significant, recent finding has been the activation of the VP promoter by the bHLH-PAS transcription factor CLOCK together with its coactivator BMAL1 (through a conserved G box at −150 nt in the VP promoter, albeit the finding has not been reproduced (see sect. viD)).

D. The OT Promoter

Early physiological observations on the stimulation by estrogens of the HNS (636, 638) have prompted initial studies on the OT promoter. The studies aimed to test the potential of the OT promoter to respond to estrogen and have led to the elucidation of a broad regulatory potential of the OT gene to respond to a wide variety of nuclear receptors and related orphan receptors. Less attention has been given to other regulatory mechanisms and to cell-specific expression, but there are candidate factors...
to participate in these aspects of OT gene expression (Fig. 8).

1. Nuclear hormone receptors (NHRs)

Multiple estrogen response element-like sequences composed of hexanucleotide AGGTCA motifs and variations thereof are present in the proximal 5’ flanking region of cloned OT genes. The AGGTCA motif is part of binding sites for all members of the nuclear receptor superfamily, except the steroid hormone receptors GR, MR, PR, and AR (61, 593). Various combinations of this motif ranging from single hexanucleotides, direct or inverted repeats with spacing varying from one to at least six nucleotides provide target elements for nuclear hormone receptors (593). The specificity of binding is determined in part by the spacing and orientation of repeats, but a large overlap in specificity exists among these factors (593, 780). This suggests that many members of the nuclear receptor family may have the potential to interact with the OT gene and regulate its expression. This family consists of true as well as orphan receptors (116) and potentially contains many members able to act and to interact on the OT gene, some of which have been characterized in the HNS (Table 3 and sect. VI).

The human and rat OT promoters can be stimulated by the ligand-activated ERα, ERβ, THRα, RARα, and RARβ in a variety of cell types (4, 115, 545, 624, 625). These studies pinpointed as binding sites and regulatory sequences for ER, THR, and RAR in the proximal region, approximately between nucleotides −195 to −70 (Fig. 8). Overall, this region appears a complex regulatory domain of the OT gene, providing separate as well as synergistic actions of nuclear receptors. Each receptor appears to employ a different combination of AGGTCA-like motifs in this promoter region and displays a different activating potential. In the appropriate cellular context, the strongest activators are ERα and ERβ. The core element for all the receptors investigated so far is the region between nucleotides −172 and −148 of the rat OT gene. This region contains a variant of the palindromic estrogen response element (ERE), but also integrates a direct repeat with no spacing (DR0) (115). Deletion results in complete loss of 3,3’,5-triiodothyronine (T3) responsiveness and most of the responsiveness to estradiol (E2) and retinoic acid (RA). The −172/−148 hormone response element synergizes with the proximal elements for the estrogen responsiveness (110) and are essential for the positive regulation by RA (442, 625).

Investigations of the OT promoter in particular have pointed toward a role of nuclear orphan receptors in the regulation of this gene (116). The bovine OT gene that is unresponsive to E2 (8) binds at the analogous −172/−148 element three orphan receptors of the bovine corpus luteum granule cells: COUP-TF I, ARP-1, and SF-1 (849–851). The levels of these factors may be responsible for the regulation of the endogenous OT gene in this tissue. The orphan receptor SF1 is one particular isoform of the SFI/ELP gene that codes at least for three isoforms, addressed here are ELP1, ELP2, and ELP3 (563). In the hypothalamus, ELP3/SF-1 is the predominant form (342, 468). All forms can activate the rat OT promoter, but the splice variant ELP1 is the most potent activator and acts through multiple elements in the −195 to −70 region. ELP orphan receptors also activate the promoter of the rat galanin gene that is coexpressed with the VP gene in MCNs (153). This orphan and the orphan receptor TR4 (see below) are factors with constitutive activating properties on the OT gene. Other orphan receptors displayed repressing activity.

The orphan COUP-TF I lacks intrinsic activity on the rat OT promoter, but it represses the induction by E2, T3, as well as RA (114). The mechanism of repression of hormone responses of the rat OT gene by COUP-TF I is the competitive binding of COUP-TF I and hormone receptors to the same region of the target gene. The binding of COUP-TF I in vitro was stronger than that of the ER to the −172/−148 element. By mutagenesis of the binding region it was shown that ER and COUP-TF I have different binding sequences, but these are integrated within the −172/−148 region. The ERE is the inverted repeat of the imperfect AGGTCA motif separated by three nucleotides; the COUP-TF I binding site is the direct repeat with zero spacing (DR0). The integration of responsiveness to E2, T3, and RA and the sensitivity to orphan receptors like COUP-TF within a single element is a unique feature of the rat and human OT genes that allows competitive binding of factors with different preferred response elements.

ROR/RZRα activates the OT promoter through the −172/−148 composite HRE (150). Other orphan receptors displayed repressing activity. The related orphan receptors COUP-TFI, COUP-TF II (449), and Ear2 lack intrinsic activity on the rat OT promoter, but it represses the induction by E2, T3, as well as RA (149). The mechanism of repression of hormone responses of the rat OT gene by COUP-TF I is the competitive binding of COUP-TF I and hormone receptors to the −172/−148 element. The TR4 orphan receptor is a member of the nuclear receptor family belonging to a subfamily of orphans including COUP-TF I and II and RXRs which was first identified by PCR in SON fragments (143). Like the majority of nuclear receptors, TR4 binds to AGGTCA motifs and is able to do so as dimer (421, 423) or monomer. The configuration of the motifs seems always a direct repeat, although the spacing can vary (320, 421, 423). Furthermore, TR4 activates as well as represses transcription as reported for a number of potential target genes (Table 1). Therefore, we determined the optimal binding site of the TR4 dimer and studied its effect on the activity of the OT gene.
TR4 activated the OT promoter in Neuro2A cells ~30-fold when using the −360 to +16 fragment of the rat OT gene. Serial 5′-deletions, consecutively taking away AGGTCA-like motifs, did not affect the responsiveness of the OT promoter significantly until −77, indicating that the −112 to −77 region contains TR4-sensitive elements. Indeed, this region contains variants of the AGGTCA motif in single configuration, suggesting that the activation of the OT promoter is through monomer interaction with these elements. Indeed, monomer binding was observed in bandshift analyses using a partial TR4 protein and random oligos, in addition to dimer binding. Selection of the DNA bound by the TR4 dimer demonstrated a preference for a DR1 element interspaced by a G.

Despite the great regulatory potential of nuclear receptors on the OT promoter (Fig. 8), little is known about the action of ER, endogenous factors in the HNS that employ parts of this capacity. ERα is not present in magnocellular OT neurons of the rat (49), but ERβ reportedly is expressed in SON and PVN (708). This finding needs further refinement, since studies on the in vivo uptake of labeled E2 and in vivo effects of E2 have failed to demonstrate a direct effect on MCNs (110). However, there is a parvocellular OT-expressing cell group projecting to hindbrain and brain stem region that is responsive and may contain ERβ (151). Estrogen may have a regulatory role in other tissues where the OT gene is expressed. In particular, in the uterus that displays a marked upregulation of OT gene expression before delivery, the ER may influence the OT gene (424). THRs have been localized in SON and PVN (93, 752). Indeed, a small but significant influence of thyroid hormones on OT gene expression in vivo has been found in euhydrated rats (5). However, a significant interaction between effects of thyroid hormones and estrogens has been described recently (181).

A large number of orphan receptors exist (116). They can be found in almost every tissue. Depending on the cell type, they may participate in the expression of the OT gene as exemplified by the role of SF-1 in bovine granulosa cells (849, 851). In the magnocellular neuron, no orphan receptors were found to be expressed at high level, but several may exist at low abundance (143, 449) or may be induced by physiological stimuli, e.g., Nur77 (455).

2. POU-homeodomain proteins

The 5′-flanking region of the rat OT gene, a region containing POUIII binding sites, has been recognized. Testing fragments of the OT gene in bandshift assays for binding to nuclear extracts of cells expressing the POTIII protein Brn-1, Brn-2, Brn-4, or Oct-6, only the region spanning nucleotides −1535 to −1270 bound POUIII protein (Fig. 8; H. S. A. Van Schaick and J. P. H. Burbach, unpublished data). Cotransfection of expression vectors encoding Brn-1, Brn-2, Brn-4 or Oct-6 and a luciferase reporter construct containing 4 kb of the 5′-flanking region of the OT gene resulted in a marked cell type-dependent effect. In a neuroblastoma cell line Brn-1 and Brn-4 repressed, while Oct-6 stimulated expression of the reporter gene. In the placental cell line JEG3, however, Oct-6 repressed promoter activity, and Brn-1, -2, and -4 were inactive, while in 293 human embryonic kidney cells no activity was exerted by any of the POUIII proteins. Preliminary data suggest that some of these effects of the POUIII proteins may not be due to direct interaction with DNA elements, but rather due to protein-protein interactions. These data underscore the need for homologous cell systems to study complex transcriptional mechanisms related to cell-specific expression of the VP and OT genes.

VI. TRANSCRIPTION FACTORS AND POTENTIAL FUNCTIONS

Transcription factors are the key players in gene regulatory events. They allow and control transcription and are the end point of cascades that are initiated by extracellular signals. Moreover, they determine programs of cell-specific gene expression. Thus knowing about transcription factors in the MCNs is an essential step in understanding how the HNS acquires and maintains its specific cellular properties and converts signals to alterations in hormonal output and cellular plasticity. Here we describe members of distinct transcription factor families identified in the HNS as well as their potential function, summarized in Table 3.

A. Basic Leucine Zipper Transcription Factors

1. c-fos activity and functional mapping

Reports that the immediate-early gene c-fos is expressed in differentiated PC12 neuroblastoma cells following depolarization- or nicotine-induced calcium influx (280, 539) were followed by the first demonstration of its expression in the intact brain after administration of convulsants (538). Soon after, c-fos mRNA and protein were shown to be induced in the MCNs of the HNS by dehydration or injection of hypertonic saline (136, 269, 656), and the usefulness of immediate-early genes for functional mapping was established by the confirmation that the whole osmoregulatory system in the brain showed c-fos activity (75, 268, 287, 294, 397, 417, 429, 455, 505, 573, 574, 693). Similarly, c-fos IHC or ISH has been used to investigate afferent pathways to the HNS that are involved in hypotension and hypoxia (50, 204, 218, 403, 435, 634, 695, 721, 723), salt appetite (77, 129, 333), noxious stimulation and/or stress (140, 142, 161, 206, 284, 343, 526, 580, 594, 599, 693, 722, 812, 842, 877, 886), and parturition (35, 439, 454, 457). C-fos is also induced in MCNs following
hypophysectomy (828) and by electrical stimulation of either the pontine parabrachial nucleus (404) or the ventrolateral medulla oblongata (705).

This method of functional mapping has been used also to investigate the neuronal pathways that cause the release of OT or VP from the HNS after administration of ANG II or renin (77, 311, 420, 506, 507, 576, 646, 647, 878, 880), endothelin (905), CCK (109, 171, 233, 293, 453, 455, 462, 580–582, 628, 629, 819), endotoxin (201, 595, 842), CRH (28, 343, 812, 813), prostaglandin (410), relaxin (507), insulin (282), and cholinergic agonists (337, 460, 481, 499, 696).

2. Stimulus-transcription coupling of the \( c-fos \) gene

The electrical activity produced in a magnocellular neuron is related to the strength of the stimulus, and this seems to hold true for the induction of \( c-fos \). Roberts et al. (634) showed that the induction of \( c-fos \) protein depends on the level of experimental hemorrhage, whereas Luckman et al. (461) showed that \( c-fos \) mRNA shows a positive correlation to the osmotic pressure of plasma collected from animals given infusions of saline of differing tonicities. Interestingly, the latter study revealed differential regulation of members of the Fos/Jun family of transcription factors in MCNs. In each situation examined, the induction of \( c-fos \) is associated with an increase in electrical activity and the release of OT and/or VP. There are two apparent exceptions to this. First, the administration of histamine induces \( c-fos \) in OT neurons (387), even though histamine has been demonstrated to directly inhibit the electrical activity of OT MCNs (889). However, since Kjaer et al. (387) injected the histamine intracerebroventricularly, it may, thus, be acting on afferents to the OT MCNs. Second, during chronic lactation, OT neurons are electrically active but do not appear to express \( c-fos \) (207, 218, 326, 439, 460).

The lactating female provides an interesting model to investigate stimulus-transcription coupling to the \( c-fos \) gene. Recently, it was shown that, in the rabbit which suckles for only a few minutes in each day, the MCNs do express \( c-fos \) protein during suckling (14). Similarly, if rat pups are removed from their mothers for a period of time and then allowed to resuckle, \( c-fos \) can then be induced in MCNs of the nursing female (207, 454, 722). There are various possible reasons for this, the first of which should not be automatically dismissed: it may be that reuniting the mother with her pups may be a nonspecific stress response by the mother and not a response to suckling. Alternatively, \( c-fos \) protein may be inhibiting its own transcription (665) during the chronic stimulus of lactation, and this is relieved by the period of separation. This is unlikely, because unrelated stimuli can still induce \( c-fos \) in the chronically lactating rat (218, 460). The patterning of neuron activity during lactation means that there is no substantial increase in the number of action potentials (605). It has been shown that calcium entry during depolarization can trigger the induction of \( c-fos \) and, thus, its lack of expression during lactation may be due to insufficient spike activity. However, this would not explain why it can be induced by suckling after a period of separation. Finally, the expression of \( c-fos \) in MCNs may not be dependent solely on electrical activity, but on the afferent receptor-mediated mechanisms. Carbachol stimulation induces electrical activity and \( c-fos \) in MCNs in the lactating rat. However, antidromic stimulation of MCNs with the same number of action potentials produced by the carbachol injection does not induce \( c-fos \) (460). Similarly, direct osmotic stimulation of MCNs that is known to depolarize them without the requirement for afferent input (88, 494) either in vivo or in vitro does not produce \( c-fos \) in these cells (334, 466). Depolarization of cell or slice cultures in vitro by high extracellular potassium can induce \( c-fos \) in MCNs, although this may be subsequent to the release of transmitters into the bathing medium (334, 700). Thus it is possible that the activation of a single intracellular pathway, perhaps by depolarization-mediated calcium entry, is insufficient to induce \( c-fos \) in these neurons, and other pathways are required.

3. Possible target genes of Fos/Jun transcription factors

The Fos/Jun family of transcription factors is part of the basic leucine zipper (bZIP) superfamily and bind to the AP-1 cis-acting site on target genes (415, 653). To act as a transcription factor, \( c-fos \) must dimerize with other members of this family, and these have been shown to be regulated in MCNs after osmotic stimulation (136, 461, 693) and after ANG II injection (78). \( c-fos \) protein is associated with actively transcribing chromatin regions in the cell nuclei of SON neurons (411), and protein extracts of the SON or PVN have increased binding to the consensus AP-1 sequence following osmotic stimulation (86, 131, 891). DNase footprinting has suggested that the OT gene promoter may bind Jun/Jun homodimers (591), but there is no direct evidence to suggest that Fos/Jun can modulate the transcription of either the OT or the VP genes. However, there are a number of possible target genes in MCNs that are now being investigated. Dynorphin is coexpressed in VP MCNs (845) and is upregulated with VP by osmotic stimuli (438, 701). There is a noncanonical AP-1 sequence found in the dynorphin promoter that can regulate gene transcription (554). DNA binding to this element increases in SON extracts after an acute osmotic stimulation, suggesting that dynorphin is a target for Fos/Jun transcription factors in MCNs (131). In contrast, there is no increase in Fos/Jun binding to the enkephalin gene promoter in PVN extracts following the same stimulus (86).
4. cAMP response element binding proteins

In addition to the Fos/Jun genes, there are two other major groups of bZIP transcription factors, the CREB/ATF (517) and the CAAT-box/enhancer binding proteins (C/EBP) (865). Subfamily members dimerize within their own group, but also between groups, adding to the complexity of their actions (290). To date, apart from the constitutive expression of ATF-2 in the SON (78), there are no reports of the regulation of ATFs or C/EBPs in the HNS. In contrast, CREB is constitutively expressed in MCNs, and it is activated by phosphorylation after acute osmotic stimulation (86, 454, 707). CREB phosphorylation can be achieved by the activity of either cAMP-dependent protein kinase A or calcium/calmodulin-dependent kinase (699). cAMP does accumulate in magnocellular nuclei after osmotic stimulation (133, 900), and calcium enters MCNs through voltage-dependent membrane channels during electrical activity (90). There is phosphorylated CREB binding to CRE sequences found in the enkephalin promoter after acute osmotic stimulation (86), but this has not yet been demonstrated for the VP gene promoter that does possess a CRE (533). However, the VP promoter is stimulated by cAMP in heterologous expression systems, suggesting that it is a target for phosphorylated CREB (354, 591, 820). It appears that an early translation of some factor, and not just posttranslational phosphorylation, is required to allow osmotically induced increases in VP gene transcription (187).

Another gene in the CREB family produces a number of CRE modulators (CREMs) by cell-specific alternative splicing (232). At least two of the CREM gene products, CREMa and CREMb, appear to be constitutively expressed in the HNS (515). Furthermore, an inducible isoform transcribed from a second promoter in the CREM gene and termed ICER (738) is produced in MCNs by an acute osmotic stimulus (459). This was the first demonstration of the induction of ICER in any neuron, and since it is a dominant-negative regulator of CREs, its effect on cAMP-responsive genes is not clear. In addition to controlling late genes, CREB has been implicated in the regulation of immediate-early genes, such as c-fos and NGFI-B (698). Indeed, there is an increase in binding of phosphorylated CREB to the CRE within the c-fos promoter after acute osmotic stimulation (86). Furthermore, the time courses for the induction and disappearance of c-fos and ICER are consistent with a role for CREB and ICER in producing the transient expression of c-fos and other inducible transcription factors (232, 459).

B. Nuclear Hormone Receptors

Nuclear hormone receptors (NHRs) comprise a large family of transcription factors that are often activated by small lipophilic ligands, such as steroid and thyroid hor-

mones, vitamin A and D derivatives, and compounds from the environment. For most of the NHR members, no ligands are known, which are hence termed “orphan receptors.” Many of these orphan receptors have constitutive activity and may not require a ligand to modulate transcription of target genes (448).

Interest in the presence and functions of NHRs in the HNS stems from the physiological observations in humans that estrogen increases the plasma levels of OT-associated NPs (25, 637). For a long time the presence and role of estrogen in the HNS of the rat has remained controversial. Cellular effects of estrogen are clear-cut (259, 760), but effects on peptide and mRNA levels are marginal, and the existence of ERα could not be demonstrated (111). With the identification of a second ER, ERβ, that is expressed in OT MCNs of the SON and the PVN, this issue seems to be clarified (336, 708). Also, VP MCNs of the SON express ERβ. However, the unresponsiveness of OT and VP peptides and mRNA levels are still not explained. It has been indicated that the ERβ is only activated under certain physiological conditions, e.g., specific steroid status. In particular, the interaction with progesterone is significant. OT gene expression, but not VP gene expression, is increased when rats were implanted initially with estrogen, 1 day later followed by progesterone, and treatment was sustained for 12 days and the progesterone implant removed 2 days before analysis (22, 26). This condition mimics the condition of a female rat that is interrupted in suckling for 2 days after lactation for 12 days. Although multiple NHRs members have been identified to be expressed in the rat hypothalamus by PCR cloning, i.e., THRα, RARα, RARγ, RXRα, RXRγ, COUP-TF I, COUP-TF II (ARPI), TR4, GCNF, and an ERR2-like factor (143, 449). Only a limited set of NHRs is expressed in the HNS. As mentioned above, these include ERα and ERβ, in a species-dependent manner, and THRα (92, 93). The orphan receptor TR4 is expressed at low levels in MCNs (143, 450), and others may be induced by physiological stimuli. It has been shown that Nur 77 is induced in MCNs upon hyperosmolality (455).

Similarly to the above-mentioned approach of PCR cloning of transcripts expressed in the hypothalamus, a survey of nuclear receptors expressed in SON fragments was performed by PCR (116). Primers were designed to recognize motifs in the AAGTCα-binding members of the nuclear receptor family. In a small plasmid library (300 clones) generated by PCR, five true receptors were identified: THRα, RARα, RARγ, RXRα, and RXRγ, as well as four orphan receptors: COUP-TF I, COUP-TF II (ARPI), estrogen-related receptor 2 (ERR2), and testis receptor 4 (TR4). In these studies TR4 was identified as a novel nuclear orphan receptor (143). Of these factors, THRα was the most abundant one in the MCNs (92, 93) and is physiologically functional in the regulation of the OT gene in the SON (5). Thyroxin-treated rats with mild hyperthy-
C. Homeodomain Proteins

Concerning the HNS, the focus on homeodomain proteins originates from the observations that the POU homeodomain proteins Brn-2 and Brn-4 are expressed in neurons of the SON and PVN regions (307, 426a). These proteins, together with Brn-1 and Oct-6 (Tst1/SCIP), constitute the class III subfamily of POU proteins (POU III) (295). More recent studies have detailed the expression of Brn-2 and Brn-4 in MCNs (484). Brn-1 is expressed in the vicinity, but is not present in VP- or OT-expressing neurons.

Recently, direct evidence has been obtained for the involvement of Brn-2 in the molecular regulation of the MCNs (552, 683). In these studies the endogenous Brn-2 gene was inactivated by homologous recombination. The presence of Tr4 in the SON has recently been reported (892), indicating that this orphan receptor may participate in the control of gene expression in MCNs. Consequently, we investigated the general regulatory properties of TR4 and its potential to regulate the OT gene (see sect. v.D). These data show that the interaction of TR4 with the OT promoter differs from all other studied nuclear receptors.

The GR has been detected in MCNs of the HNS at elevated levels relatively low compared with hippocampal regions (384). However, GR expression is strongly induced by hyperosmolality (68). There is no significant regulatory role known for glucocorticoid gene expression in MCNs (11).

D. The bHLH-PAS Transcription Factor

Over the past few years, a new class of transcription factor, the bHLH-PAS family, has been identified as being important in the transcriptional regulation of cell lineage specific development (733). Members of the large bHLH-PAS family are characterized by two domains: a bHLH and a PAS domain. PAS domains were first identified as conserved structures in three genes: per, ARNT, and sim, that resemble Otx1 and Otx2 in the homeodomain. A significant difference, however, is the presence of a Glu at position 9 in the third helix, which results in a different DNA binding selectivity compared with Otx1 and Otx2. Otp is expected to recognize the general homeodomain consensus motif TAATGG, of which several are present in the rat VP and OT genes. Heterologous transfection experiments as yet have not indicated a significant influence of Otp on VP or OT promoter activity (unpublished data).
tain expression of the Brm2 gene, which in turn directs the terminal differentiation of neuroendocrine lineages within the PVN and SON, including the MCNs (522).

Sim1 is expressed in the PVN and SON after their terminal differentiation, suggesting a role in addition to the specification of neuronal identity. For example, Sim1 might be involved in the maintenance of neuropeptide expression, or the modulation of neuronal activity following physiological stimulation, perhaps in cooperation with coactivators such as nuclear hormone receptors (272).

Sim1 does not homodimerize; rather, it has been shown to function as a heterodimer with other bHLH-PAS family members (202, 608, 751). The aryl hydrocarbon receptor nuclear translocator 2 (Arnt2) protein has been identified as a putative partner for Sim1 in the HNS (521). Sim1 forms dimers in vitro with Arnt, Arnt2, and Bmal1. However, only Arnt2 is expressed robustly in the SON and PVN, along with Sim1. Arnt is expressed at a low level throughout the brain, and Bmal1, although expressed in the SCN, is not detected in the SON and PVN. Interestingly, mice in which the Arnt2 gene is deleted were generated by irradiation in the 1950s (650). These mice show the same neuroendocrine defects as the Sim1 knockout mice (521). Together, these results implicate Arnt2 as the in vivo dimerization partner of Sim1, and that together these transcription factors control the development of the SON and PVN.

It is interesting to note that the bHLH-PAS protein CLOCK is also expressed in the SON and PVN (363, 383). CLOCK is one of a number of bHLH-PAS transcription factors (including BMAL-1 and the PER family) with roles in the generation of circadian rhythms in the SCN. CLOCK has been shown to form heterodimers with the bHLH-PAS protein BMAL-1 (265) that, in heterologous cell line transfection studies, is able to transactivate the mouse VP promoter through an E-box enhancer element (CACGTG) located around 150 residues upstream of the start of transcription (363). These data need further evaluation, since cell-specific context appears to affect the activity of bHLH-PAS proteins on the VP promoter, which can result in conflicting results (J. P. H. Burbach and D. Murphy, unpublished data). Whether CLOCK partners, in combination with other bHLH-PAS transcription factors, such as Sim1 or Arnt1 and other cell-specific factors, regulate VP expression in MCNs remains to be determined.

VII. CONCLUSIONS AND PERSPECTIVES

The HNS has inspired scientists for over a century, which led to the discovery of several essential physiological and endocrine principles. The central physiological question, “How is the rate of hormone production controlled?” already occupied the pioneers in the HNS (166, 578, 826, 835). Although today we know many details of the HNS, this question is still giving direction to current research. This research puts emphasis into two directions. On the one hand it focuses on the physiological integration of the HNS in the control of peripheral functions by the brain, and on the other hand it aims to understand how this integration is accomplished mechanistically at the level of the cell and the molecules involved. This review has attempted to highlight the latter direction by reviewing what we know about HNS-expressed genes that play a role in receiving and transducing signals from the brain and periphery, and connecting them to the machineries responsible for transcription of the VP and OT genes and for manufacturing and releasing the hormonal output of the HNS.

In view of its integrative function and the diversity of its afferent inputs, the number of membrane receptors actually demonstrated to be expressed by MCNs is surprisingly few. These are mostly involved in immediate control of electrical activity and release and also include receptors important for electrical coupling and synchronized bursting activity of OT MCNs. Despite rapid response of immediate early genes, like c-fos, there is no evidence that these modulate the expression of the VP and OT genes. It is more likely that prolonged activity of afferent inputs plays a role in alterations in expression of these output genes. Although the HNS hormones are encoded by genes that are among the smallest and simplest in exon structure, the genome control of these genes is among the most complex. They display a unique genomic association to each other in a common locus, suggesting that they share common control mechanisms. We know some of the information this locus carries for tissue-specific expression, but it harbors many uncovered mysteries. Knowing about transcription factors that act on this locus may help to unravel some of them. In recent years a few of them have been identified initially by expression studies. None of them is uniquely expressed in MCNs, e.g., Brn2, Brn4, Otp, Sim1, but mice carrying mutant alleles of these factors, except for Brn4, surprisingly show a phenotype restricted to MCN neurons that is shared by multiple factors. This phenotype is an early defect in the migratory phase of embryonic development and a consequent absence of the HNS in newborns. Apparently, there is no redundancy of these factors as in other systems not affected by their mutation, or they are uniquely positioned in a common molecular cascade in MCN development. In addition to classical transgenesis and homologous recombination, dedicated experimental systems, like organotypic slice cultures and viral gene transfer, will be essential tools to further define the role of transcription factors and to clear up the mysteries hidden in the control regions of the VP-OT gene locus. With the moment near that all mammalian genes will be known, it may be expected that crucial regulatory mechanisms can be pinpointed for MCNs and that we know how the HNS
is able to handle and fine tune the physiological demand for its gene products at the level of its genome and all the components that contribute to the appropriate integration and functioning of the HNS.

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