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Transgenesis and the Study of Expression, Cellular Targeting and Function of Oxytocin, Vasopressin and Their Receptors

W. Scott Young, 3rd Harold Gainer

Section on Neural Gene Expression, National Institute of Mental Health and Laboratory of Neurochemistry, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Department of Health and Human Services, Bethesda, Md., USA

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Abstract

The neuropeptides oxytocin and vasopressin and the neurons in the hypothalamus that synthesize them have been a rich source for the exploration and understanding of both the brain and the endocrine system. Because of their large size and compact nuclear organization the magnocellular neurons of the hypothalamoneurohypophysial system have traditionally attracted scientists using state-of-the-art techniques, including the subject of this review, transgenesis. We discuss the role of transgenics in deciphering gene elements necessary for the appropriate expression of oxytocin and vasopressin and to deliver exogenous genes, such as green fluorescent protein, selectively to secretory granules in the neurons in the hypothalamoneurohypophysial system. Finally, we review the studies of mice whose genes for oxytocin and, most recently, for the oxytocin and vasopressin receptors have been knocked out through homologous recombination.

Introduction

Large scale sequencing of the human, mouse, and various other eukaryotic organisms' genomes, and the availability of these sequences from public databases, have focused increased attention on the need for model systems to determine the physiological significance of these sequences. The diverse cellular phenotypes found in the mammalian central nervous system (CNS) and their complex connections and interactions make this effort a challenging task often requiring the development of novel mammalian models in which mutations of specific genes are selectively expressed in the CNS in space and time [1–4].

Transgenic mice have been used to model human disease and to understand the regulation and physiological roles of genes [5–8]. Given cellular- and subcellular-specific targeting of genes, one can also use reporters (e.g., green fluorescent protein, GFP) to identify specific neurons in the living state for real-time measurements of neural activity, either in vivo or in vitro [9–14]. Transgenic mice may also be used to perturb a particular system by overexpressing a gene or by reducing the gene's expression and/or effectiveness through antisense or dominant negative expression [15]. Transgenic expression of certain products, such as tumor promoters or fluorescent substances, may allow for the isolation of immortalized and

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W. Scott Young, 3rd
Section on Neural Gene Expression, National Institute of Mental Health, NIH, DHHS
9000 Rockville Pike, MSC 4068, Building 36, Room 2A11
Bethesda, MD 20892-4068 (USA)
Tel. +1 301 496 8767, Fax +1 301 402 6473, E-Mail wsy@mail.nih.gov

homogeneous cells for further study [16–21]. While this can be an effective and straightforward paradigm for the generation of immortalized neurons, it has not been successful for most neuroendocrine neurons in the hypothalamus, in general, and for oxytocin (OT) and vasopressin (VP) neurons, in particular [22, 23]. Finally, transgenic mice may also be used in the attempt to correct defects in mutant mice, either those found accidentally or those produced through homologous recombination or random mutagenesis [24, 25].

Transgenesis in mice has also been accomplished by homologous recombination, usually to produce a mouse whose targeted gene is rendered nonfunctional [25]. As most brain genes seem to be expressed in more than one cell type at one time or another during the life span of a mouse or are widespread in their expression, many 'knocked-out' mice may alter gene expression within the magnocellular neurons (MCNs) through indirect influences. Targeting genes for knockout specifically in the VP and OT MCNs has not been accomplished yet. However, knockouts of a few genes have produced results that are likely to reflect the direct importance of those genes within the MCNs. These studies will be presented below. Finally, since most endocrinological and physiological data have been obtained using rats, it would be highly desirable to perform such experiments on transgenic rats. In fact, significant efforts towards this goal have been made in studies [26–28] on the rat hypothalamoneurohypophysial system (HNS). However, at this time, studies using transgenic rats still lag behind those with transgenic mice, and we are not aware of any studies using homologous recombination to study the rat nervous system.

OT and VP Gene Expression in the Hypothalamus

OT and VP were first identified as neurohormones in the HNS [29, 30]. These peptides are now known to act in other regions of the nervous system, and to control complex behaviors [31–33]. Whereas OT and VP fibers are found throughout the CNS, expression of the OT and VP genes occurs almost exclusively in the hypothalamus, the principal sources being the MCNs of the paraventricular (PVN) and supraoptic (SON) nuclei. In addition, there are parvocellular neurons in the PVN that synthesize corticotropin-releasing factor (CRF) and coexpress VP that is secreted into the portal circulation to stimulate release of ACTH from anterior pituitary corticotropes [34–36]. Parvocellular neurons in the suprachiasmatic nucleus, the central circadian clock in mammals [37], also synthesize VP and may be involved in regulating some circadian rhythms [38]. There are other parvocellular neurons in the PVN that regulate autonomic functions via projections to the brainstem and spinal cord [39, 40]. Finally, some species have parvocellular neurons elsewhere, such as in the amygdala and bed nucleus of the stria terminalis, that express VP or OT [41].

The MCNs in the HNS have been among the most intensively studied peptidergic neurons in the CNS [42–52]. The MCNs have served as excellent model systems for the study of peptide neurosecretion mechanisms in vivo in large part due to their compact nuclear organization in the CNS: most of the approximately 12,000 cells in the rodent hypothalamus are located in the two bilateral nuclei, the PVN and SON. The MCNs project via well-defined axonal tracts to the posterior pituitary (i.e., neural lobe), where each axon is estimated to branch into hundreds of nerve terminals [44, 49], and where these axonal branches and terminals represent about 50% of the total tissue mass of the neural lobe. The relatively easy access to the HNS cellular components, the cell 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 objects of many biochemical and physiological studies. Consequently, a substantial database on these peptidergic neurons exists in the literature.

Based on biochemical, morphological and physiological criteria, the HNS neuronal population has been historically divided into two distinct phenotypes, the OT and VP neurons [53, 54]. The two neuronal phenotypes in rats have also been discriminated by their distinct electrical properties [44–47]. In addition to the principal peptides, OT and VP, these neurons and their large dense core vesicles (LDCVs) also contain smaller amounts of other coexisting peptides (e.g., galanin, cholecystokinin, CRF, dynorphin, enkephalin, thyrotropin-releasing hormone) which can vary between cells depending on functional conditions [52, 55], thereby revealing additional heterogeneity within the OT and VP neuronal phenotypes.

There is also plasticity in the expression of OT and VP in the HNS. Several laboratories have reported coexistence of OT and VP in some HNS neurons (i.e., about 1–3% of the total population under normal conditions) [56, 57], increasing to a maximum of 17% after 2 days of lactation [57, 58]. In addition, while the segregation of OT and VP gene expression in separate cells in the HNS is the rule, recent studies using a sensitive RT-PCR analysis of

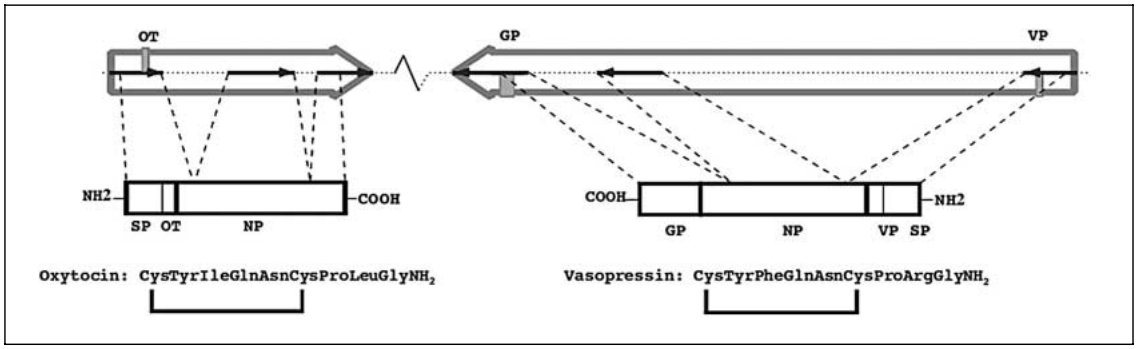

Fig. 1. Organization of the VP and OT genes. Both genes are composed of three exons shown as small solid arrows, separated by two introns (shown as dotted lines between the exons). The genes are present on the same chromosome but are transcribed in opposite directions (shown by large, hollow arrows). Similar gene structures but with variable lengths of intergenic sequences have been found in various mammalian species. The three exons code for preprohormones that contain a signal peptide (SP), VP or OT, neurophysin (NP), and, in the case of VP, a glycopeptide (GP) that are liberated from the preprohormone during processing in the transported dense-core vesicles [92].

single MCNs 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 [58–60]. 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 more than 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 two [60]. Therefore, 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 unclear and will require further study.

The OT and VP Gene Structures

The OT and VP genes each contain three exons and two introns, and both genes are located on the same chromosomal locus (chromosome 2 in mice and chromosome 20 in humans) but are transcribed in opposite directions (see fig. 1). The domain separating the OT and VP genes has been called the 'intergenic region' (IGR) and is relatively short. The IGR in the rat and human is about 10–11 kbp in length [61, 62], whereas in the mouse it is only 3.6 kbp [63, 64]. The rat and mouse IGRs have been sequenced and more than half of the rat IGR is known to be represented by a long interspersed repeated DNA element [65] that is completely missing in the mouse and human IGRs [62, 64]. The principal value of this information has been that the high sequence conservation found both upstream and downstream of the genes (in the IGR) suggested that both of these domains contain regulatory DNA sequences and that the LINE element found only in the rat IGR is not likely to be critical for gene function. This conclusion was confirmed in a subsequent experimental study [66]. Searches for conserved DNA sequences in the human, mouse, and rat IGRs revealed that there were too many candidate conserved sequences to be evaluated by transgenic methods [62, 64].

Transgenic Analysis of OT and VP Gene Expression in the HNS

Studies of the mechanisms that are responsible for cell-specific gene expression of OT and VP in the MCNs of the HNS have been limited by the absence of relevant experimental models in which to examine these issues. The lack of homologous cell lines that express these genes has led most investigators to study their regulation either in heterologous culture systems or in transgenic mice [26, 59, 66–69]. The experimental work using heterologous systems has identified a number of putative regulatory elements in the OT and VP genes. These include those for various nuclear hormone receptors, class III POU proteins, and fos/jun/ATF family members as candidate transcriptional activating factors [69–71]. However, 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 upon

Table 1. VP transgenes

DNA source	Host species	Transgene name	5′ flank kbp	Structural gene	Reporter gene	3′ flank kbp	Selective expression in HNS	Ectopic expression	Ref.
Bovine	mouse	AVP.SV.ER.1.25	1.25	no	5V40T-Ag	–	no	anterior pituitary	22
Bovine	mouse	VP-A	1.25	no	CAT	–	no	ubiquitous	90
Human	mouse	AVP-GH	2	no	hGH	–	yes[a]	ubiquitous in CNS	74
Bovine	mouse	–	1.5–3	yes	Lac-Z	0.2	no	testis, germ cells	26
Mouse	mouse	VP-BGL-pA	1.4	no	Lac-Z	–	no	ubiquitous	unpubl.[b]
Bovine	mouse	VP-B	1.25	yes	–	0.2	yes[a]	ubiquitous in CNS	90
Rat	mouse	V1-minilocus	1.4	yes[c]	no	0.17	no	n.d.	79
Rat	rat	3.VP-GLO.2	3	yes	Lac-Z	0.2	no	no	83
Rat	rat	3-VP-Gal-0.55	3	yes	Lac-Z	0.55	no	no	26
Bovine	mouse	VP-C	9	yes	no	3	yes[a]	pituitary, ovary	90
Rat	mouse	8.2.rVP	3	yes	–	3	yes[a]	lung, pancreas	82
Rat	rat	5-VCAT-3	5	yes	CAT[d]	3	yes[a]	low	27, 83
Rat	rat	11-VCAT-3	5	yes	CAT[d]	3	yes[a]	low	86
Rat	rat	3VCAT-3	3	yes	CAT[d]	3	yes[a]	low	86
Rat	rat	3VCAT-0.2	3	yes	CAT[b]	0.2	yes[a]	low	86
Mouse	mouse	VP-3-CAT-2.1	3	yes	CAT[e]	2.1	yes	little in CNS	66

n.d. = Not determined.
[a] Physiological regulation of transgene expression reported.
[b] Unpublished data, HG lab.
[c] Fused to OT structural gene in 'minilocus' configuration.
[d] CAT inserted in exon 3.
[e] CAT inserted after exon 3.

which cell line is used [69–71]. Consequently, the most relevant data to date has come from studies of transgenic mice with the assays being performed in vivo in the MCNs themselves [28, 59, 67, 69].

The earliest studies of HNS gene expression in transgenic mice made use of the 5′ flanking regions of heterologous VP genes attached to exogenous reporters [22, 71–73]. As noted above, an early attempt to produce an immortalized VP cell line by targeted oncogenesis was made by using a 1.25-kbp bovine promoter region attached to the early region of the tumor virus, SV 40, encoding the large T antigen [22]. This transgene construct (termed AVP.SVER 1.25, see table 1) was not expressed in the HNS, but was in the anterior pituitary, where it produced tumors [22, 72]. The same 1.25-kbp bovine promoter when attached to a chloramphenicol acetyl transferase (CAT) reporter (VP-A) produced an ubiquitous expression of the construct in two lines of transgenic mouse lines, but still no significant expression in the HNS [72]. Russo et al. [74] studied 14 different fusion genes in transgenic mice. One of these contained 2 kbp of the human VP promoter linked to the human growth hormone gene (AVP-GH, table 1). They found an ubiquitous (ectopic) expression of this transgene throughout the mouse CNS, particularly in the cerebral cortex, and also in the hypothalamus and HNS neurons. However, this construct was no more specific or robust in its expression in the HNS than a metallothionein promoter that had been connected to the growth hormone reporter as a transgene. Interestingly, the CNS expression of the latter construct could be eliminated by removing all the introns from the growth hormone reporter gene [74], and only expression in tissues normally characteristic of metallothionein promoter expression (e.g., liver, pancreas, intestine, and kidney) was found with the latter construct.

While the above observations focus on inappropriate (ectopic) expression of these transgenes, they also alert one to the general caveats in the interpretation of transgenic data. Expression in a given tissue can reflect so-called 'position effects' of the transgenes' integration [75], enhancing influences of heterologous sequences [76], or silencing effects [77] that may derive from exogenous sequences present in the constructs. In some cases, the same reporter gene (e.g., Lac-Z) can act as an enhancer (e.g., see VP-BGL-pA construct in table 1) or as a repressor (e.g., see the 3.VP-Gal-0.55 construct in table 1, and OT-BGL-pA in table 2), depending upon the specific configurations of the DNA sequences in the construct. Habener et al. [78] produced a transgenic mouse with a complex construct containing 800 bp of the mouse metallothionein I 5′ flanking region (promoter) and 35 bp of its

Table 2. OT transgenes

DNA source	Host species	Transgene name	5′ flank kbp	Structural gene	Reporter gene	3′ flank kbp	Expression in HNS	Selective ectopic expression	Ref.
Rat	mouse	ROT-1.63	0.36	yes	no	0.5	–[a]	–[a]	unpubl.[b]
Bovine	mouse	b.BOT 6.4	3.0	yes	no	2.6	–[a]	–[a]	89
Bovine	mouse	b.OT	0.6	yes	no	2.5	no	testes, lung	73, 89, 90
Mouse	mouse	OT-BGL-pA	1.3	no	Lac-Z	–	no	no	unpubl.[c]
Bovine	mouse	b.OT.3.5	0.5	yes	no	1.8[d]	yes[b]	testes, lung	89
Mouse	mouse	AI-02	1.05	yes	EGFP[f]	>3.5[g]	no	no	91
Mouse	mouse	AI-01	1.05	yes	EGFP[h]	>3.5[g]	very low	no	91
Rat	mouse	V1-minilocus	0.36	yes	no	0.43	yes[e]	no	79
Mouse	mouse	OT-3-CAT-3.5	0.5	yes	CAT[i]	3.5	yes	no	66
Mouse	mouse	AI-03	1.05	yes	EGFP[j]	>3.5[g]	yes	no	91
Mouse	mouse	JL-01	1.05	yes	IRES-EGFP[k]	0.43[l]	yes	no	91

[a] No transgenic mice were produced.
[b] Unpublished data, WSY lab.
[c] Unpublished data, HG lab.
[d] Fused to bovine VP structural gene with 1.25 kbp VP-5′ flanking and 0.2 kbp VP-3′ flanking regions.
[e] Physiological regulation of expression reported.
[f] EGFP reporter in place of OT-exon 1.
[g] Includes entire IGR plus VP structural gene minus VP-exon 1.
[h] EGFP reporter is immediately after OT-exon-1, plus VP structural gene minus VP-exon 1.
[i] CAT inserted after exon 3, and 3.5 kbp represents entire IGR in mouse.
[j] EGFP inserted in middle of OT-exon 3, fused to entire VP structural gene minus VP-exon 1.
[k] IRES-EGFP inserted into middle of OT-exon 3.
[l] Fused to entire VP-structural gene containing 0.17 kbp of VP-3′ flanking region (IGR).

exon 1 connected through a 14-bp artificial DNA linker to the entire rat VP gene. The authors reported the expression of the fusion gene in appropriate tissues (corresponding to the metallothionein promoter's endogenous expression), but also in the HNS which normally does not express this gene. The authors referred to the HNS expression as 'eutropic expression', and attributed this to a 'synergy' between the metallothionein I promoter sequence and the VP gene sequence. It is clear from the above studies that the heterologous DNA and the reporter's DNA sequences in the transgene can interact with the transgene's 'endogenous' sequences to produce ectopic expression, and sometimes even reproducible expression in relevant tissue, thereby confounding the analysis regarding cell specificity.

The 'Minilocus' Construct and the IGR Hypothesis

In view of the aforementioned complexity produced by exogenous sequences in the constructs, and since it was unclear what part(s) of the genes (including introns and exons) contained the elements that were critical for cell-specific expression, many of the subsequent studies used intact bovine or rat OT and VP genes in transgenic mice. This strategy was based on the fact that while there was great conservation in the gene sequences between the species, there was enough divergence to allow for the production of specific probes to differentiate the expression of the transgene from the endogenous mouse gene. The first report of robust cell-specific gene expression in the HNS of transgenic mice was made using a combined rat VP and OT ('minilocus') construct [79–81]. The construct used was called V1 (see table 2). The results were surprising in that although this construct contained about 4 times more VP 5′-upstream sequence than OT 5′-upstream sequence (the latter being only 0.36 kbp), the rat OT transgene was robustly expressed (at between 10 and 30% of endogenous OT gene expression levels) in mouse OT cells only whereas the VP gene was not expressed at all in the VP cells. The expression of the OT gene in the OT MCNs was subsequently shown to occur with only the presence of VP exon III and a short IGR segment (<600 bp) proximal to the VP gene's 3′-flanking region [Zimmer and Young, unpubl. data, cited in 59].

Later studies on the VP gene [27, 82–84] showed that by extending the 3′-downstream region of the VP gene to 3 kbp, it was possible to get cell-specific expression of these transgenes in VP neurons only. Some of the success-

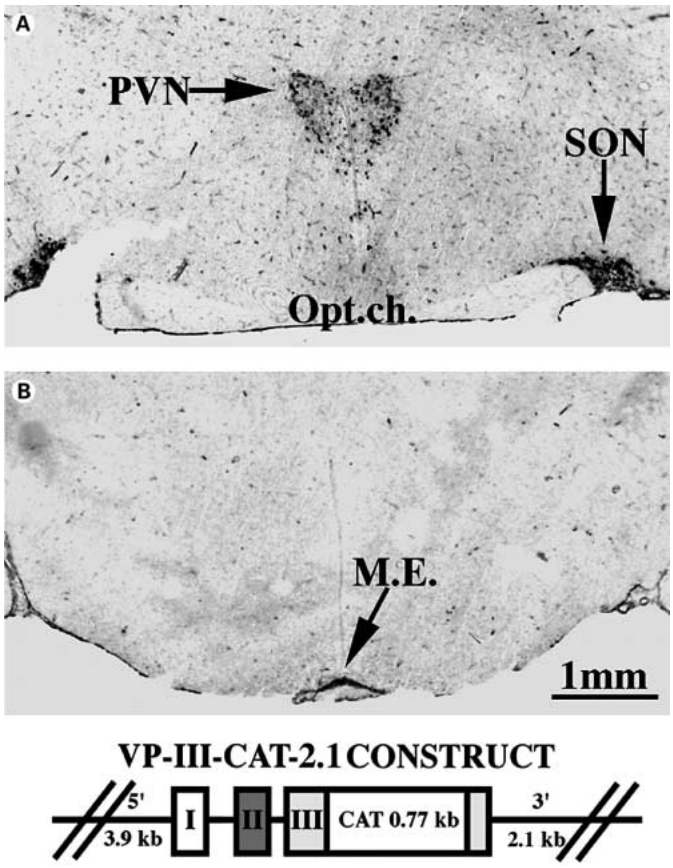

Fig. 2. Expression of 3.5VP-III-CAT-IGR2.1 transgene in mouse. Arrows show areas of CAT expression (immunoreactivity) in HNS neurons in a transgenic mouse. Note that in addition to the expected CAT immunoreactivity in the PVN and SON in the hypothalamus (arrows in **A**), there is expression in axons of passage in internal zone of median eminence (ME; arrows in **B**). Schematic of the transgene is shown below the photomicrographs [66]. Opt.ch. = Optic chiasm.

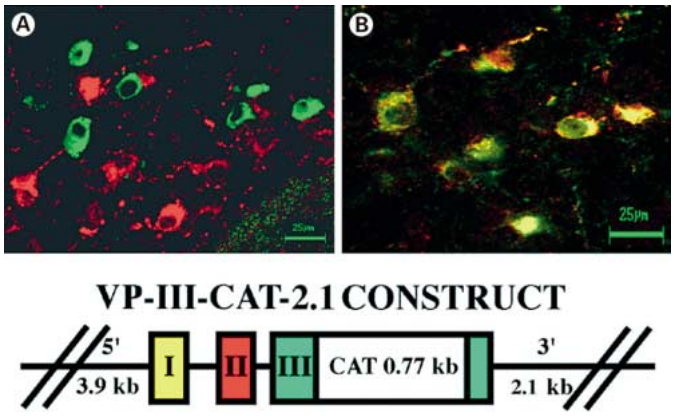

Fig. 3. Merged views of double-label immunofluorescence of CAT immunoreactivity (in red) and anti-OT-neurophysin immunoreactivity (using antibody PS36; **A** in green) or anti-VP-neurophysin immunoreactivity (using antibody PS41; **B** in green) in the SON of a 3.5VP-III-CAT-IGR2.1 transgenic mouse. Note that only the VP cells contain CAT immunoreactivity, and are visualized as yellow in the merged view (**B**). Schematic representation of the transgene is shown below the photomicrographs [from 66].

ful constructs used in these studies (see table 1) contained an exogenous reporter, CAT, inserted into exon III. This indicated that increasing the distance of the putative element in the IGR from the promoter region in the VP by an additional 660 bp was not deleterious for the cell-specific expression of this gene and also suggested that the cell-specific elements for both the OT and VP genes were in the IGR within 3 kbp of the 3′-downstream region of the rat VP gene [59, 83]. These studies of various OT and VP constructs in transgenic mice indicated that constructs containing genomic DNA from 0.5 to 9 kbp 5′-upstream of the OT and VP genes but with no endogenous 3′-downstream sequences do not produce significant expression in the hypothalamic MCNs, and that the IGR contained *cis*-elements that are essential for their cell-specific gene expression in the MCNs. This view point has been called the IGR hypothesis [59, 85].

Given the fact that a CAT reporter could be inserted into exon III of the rat transgene [27, 83], we set out to further examine the IGR hypothesis using CAT-bearing mouse OT and VP gene constructs extended downstream by 2.1 or 3.6 kbp of IGR sequence [66]. The results of these studies were that of five separate founder lines containing the VP-III-CAT-2.1 construct (see table 1) all showed equivalent cell-specific expression of CAT in VP MCNs but no expression at all in OT neurons (fig. 2, 3). All five lines also had some ectopic expression of CAT in the brains, consistently in the large neurons of the reticular thalamic nucleus. Similar data were obtained using a VP-III-CAT-3.6 construct. We also found using a mouse OT gene-CAT construct and the 3.6-kbp IGR domain (table 2) that this OT construct also produced cell-specific expression of CAT, but in this case in OT neurons only and not in VP cells nor in ectopic sites in the brain. Validation of the cell-specific expression of the CAT protein was done by double-label immunofluorescence studies using polyclonal CAT and monoclonal OT- and VP-associated neurophysin antibodies (fig. 3). The above data lend further support to the IGR hypothesis, and indicate that for VP gene expression only 2.1 kbp downstream of the 3′-flanking region was necessary for cell specificity.

In a paper that describes further dissection of the 5′- and 3′-flanking regions of the VP gene, Davies et al. [86] describe the results of experiments that use three modifications of their 5-VCAT-3 transgene (see table 1) to further define the DNA sequences involved in the expression and regulation of this construct in transgenic rats. Their data show that increasing the 5′-flanking region of the transgene to 11 kb, or reducing it to 3 kb, and reducing the 3′-flanking region of the transgene to 0.2 kb did not significantly alter the expression pattern from that which was observed with 5-VCAT-3. With all four constructs, basal expression was typically low, often not measurable, but became detectable and sometimes robust after osmotic stimulation (dehydration for 3 days). Given these data, the authors then present three conclusions: (1) the IGR >200 bases downstream of the structural VP gene does not appear to influence VP expression in the MCNs in the hypothalamus; (2) the putative enhancer for SCN and other parvocellular neuronal expression lies outside the boundaries of their largest construct, 11-VCAT-3, and (3) the 3-VCAT-3 construct was the preferred construct for the cell-specific targeting of VP neurons in the HNS, since it showed the least ectopic expression.

In a recent paper that, in part, addresses the above view that the putative enhancer for SCN and other parvocellular neuronal expression lie outside the boundaries of the 11-VCAT-3 construct, Wells et al. [87] use large amounts of flanking DNA sequences surrounding the rat OT and VP genes in a 44-kbp cosmid construct to generate lines of transgenic rats, with cell-specific expression of a reporter, human growth hormone (hGH), which was inserted into the VP gene. Two transgenic lines were studied, JP17 with 4–8 copies and JP59 with 1–2 copies of the transgene integrated into the rat genome. Both lines showed cell-specific expression in MCNs in the PVN and SON, but parvocellular expression was found only in the JP17 line, presumably because it exhibited much greater hGH expression in the hypothalamus. This was probably due to its greater copy number. The question is whether the 'additional regulatory sequences' used in the 'larger cosmid construct' was responsible for the parvocellular expression observed in the JP17 line. In this regard, their results with line JP59, which contained the same transgene construct but which gave a lower level of VP MCN expression, but no detectable expression in the SCN and other parvocellular neurons, is instructive. An equally reasonable interpretation of these data is that the ability to observe the hGH expression in the nonmagnocellular areas in line JP17 is related to its intense basal expression (perhaps due to fortuitous integration), and that the failure to observe parvocellular expression in JP59 and in previous transgenic studies [28, 71] could simply be due to the low basal expression levels found in those transgenic animals. In this regard, it is important to note that much shorter VP constructs but used at high copy numbers can produce expression in SCN VP neurons in vitro [see 62, fig. 5D].

Some of the transgenes were studied for their ability to be regulated by physiological stimuli in vivo. These studies are denoted in tables 1 and 2 by asterisks. To our knowledge, few of the putative regulatory elements that were identified in the in vitro experiments [69, 88] have been reevaluated in vivo by transgenic studies. In one case, the removal of the putative ERE in the rat OT promoter (–169/–157) in the V1 minilocus construct (V18 construct), which reduced its responsiveness to estrogen by 80–90%, thyroid hormone and retinoic acid in vitro (WSY and J.P.H. Burbach Labs, unpubl. data), also eliminated the transgene's basal expression in the HNS (WSY Lab, unpubl. data). Mutation of bases –168 and –167 from GG to TT (V17 construct) reduced responsiveness to the three hormones by approximately 50% in vitro [88], but had no significant impact on basal transgenic expression (WSY Lab, unpubl. data).

Targeting Reporter Genes to Secretory Granules in the HNS

OT and VP are nine amino acid neuropeptide hormones that are synthesized as part of a larger precursor protein (fig. 1). The VP precursor protein is packaged into LDCVs, or secretory granules, in which the prohormone is processed and transported to the posterior pituitary for secretion of the peptide products [reviewed in 49, 69, 92–94]. The VP prohormone contains the VP peptide followed by the N-terminal signal sequence, and an approximately 10-kD protein called neurophysin, as well as a 39-amino acid glycopeptide at its C-terminus (absent in the OT prohormone). The neurophysins are associated noncovalently with their respective neurohypophysial peptides in the secretory granules [95–97]. Based on the biophysical properties of the nonapeptide-neurophysin complex [97] and its crystal structure [98], it has been proposed that the self-association properties of this peptide-neurophysin complex, as well as the precursor protein, might play a critical role in the sorting of the VP precursor to the regulated secretory pathway and the stabilization of the high concentrations of peptides in the LDCVs against

disulfide exchange and degradation [97–100]. Several studies supporting the hypothesis that the interaction between the peptide and neurophysin in the protein precursor is necessary for its efficient sorting and packaging to the regulated secretory pathway have been reported [reviewed in 101, 102].

The significance of the above information is that it suggests that a relatively intact OT or VP precursor protein will be required for its effective targeting to LDCVs, and this has been the experience in several transgenic studies [13, 27, 66]. Studies of OT and VP peptide hormone secretion from MCNs in the hypothalamus have focused primarily on nerve terminals in the neurohypophysis [93, 94, 103] and, to a lesser extent, on release from magnocellular neuronal dendrites [104–107]. In all of these studies, measurements of secretion of the peptides were made by radioimmunoassay (RIA) of samples collected either by superfusion of the neural lobe or by microdialysis in vivo in the hypothalamus. While the RIA method is very sensitive and has provided much valuable physiological and pharmacological data about the secretion of these neuropeptides, this assay cannot provide information about secretion from individual peptidergic nerve terminals or dendritic processes in these cells. One possible approach would be to develop transgenic mouse or rat models in which constructs such as VP-III-EGFP are targeted to MCNs thereby causing their LDCVs to be fluorescent so that regulated secretion from the processes of these cells could be directly visualized by imaging methods, as has been done in PC 12 cells [108–110]. Recent reports of the successful targeting of an epitope-tagged VP precursor protein to the LDCVs of VP MCNs in transgenic rats [27] and of EGFP-tagged OT precursors to OT MCNs [91] and CAT-tagged OT and VP precursors to OT and VP MCNs, respectively [66], in transgenic mice are encouraging. Given such transgenic neuronal systems containing homologous EGFP-tagged precursor proteins that are efficiently targeted to their LDCVs, the study of peptide neurosecretion from individual nerve endings and dendrites is feasible.

Using the OT-VP minilocus construct (see table 2) as a starting point, four types of transgenic mice, in which the OT genes contained GFP as the reporter gene [111, 112], were generated [91]. Placement of the GFP within the first OT exon, either before or after the signal peptide, yielded little to no expression. However, as with the CAT constructs, placement of the GFP in the third exon (as an in-frame fusion with the carboxyl terminus of the OT preprohormone) resulted in cell-specific expression of GFP in OT neurons. This expression was seen only within the OT neurons as well as their axons and nerve endings in the posterior pituitary [13, 91]. Furthermore, placement of the GFP sequence downstream of a picornavirus internal ribosomal entry site, also in the third exon, allowed expression of the GFP as a separate protein. As expected, the GFP in this construct, synthesized separately from the signal sequence in the pro-OT, was not packaged into vesicles for transport to the pituitary, but was most prominent in the cytoplasm and proximal processes [13, 91]. In summary, constructs with inserts in the third exon could target expression specifically to OT neurons, whereas inserts in the first exon did not. One curious observation was that even in the most robustly expressing line (AI03), the transgene was only expressed in a minority (10–30%) of the OT cells [13, 91]. In contrast, the original V1 construct, which did not contain foreign coding sequence as a reporter, was expressed in over 90% of the OT neurons. Is the reduced expression that was observed with the GFP constructs due to the influence of the reporter? The fact that a similar cell-specific gene expression pattern with a low penetrance was also observed when CAT was used as the reporter in the OT-III-CAT-3.6 construct [66] suggests that another factor was responsible for the low penetrance.

The OT-EGFP (AI03) transgenic mouse in which the fluorescent reporter is targeted to secretory granules in the OT MCNs offered us a unique opportunity to study neurosecretion from the nerve terminals of individual neurons [13]. Electron-microscopic immunochemistry showed that the expressed EGFP fusion protein in the HNS of these mice is restricted to LDCVs containing OT peptides [13]. The assay of secretion employed in these studies was the quantitative imaging of decreased fluorescence (due to exocytosis of EGFP from the LDCVs) in either individual dendritic or nerve terminal processes. We utilized a Zeiss Atto digital fluorescence imaging system with a photometry capacity for quantitative measurements and fast kinetics and demonstrated secretion from individual OT nerve endings (i.e., neurosecretosomes isolated from the neural lobes of AI03 mice) [13]. Recently, single fluorescent LDCVs in the neurosecretosomes from these mice have been visualized by widefield imaging with a very high speed, very sensitive CCD camera (fig. 4).

Si-Hoe et al. [113] and Davies and Murphy [114] have used rats expressing the VP transgene 3-VCAT-3 [86] modified with a Cys67→stop (TGA) codon modification to model the mutation found in a Japanese case of familial neurohypophysial diabetes insipidus [115]. This is an autosomal dominant disorder that presents in early childhood with polydipsia and polyuria as a consequence of

progressive loss of VP secretion. They found that these animals, when exposed to chronic intermittent dehydration to enhance transgene expression, developed swollen and distorted endoplasmic reticulum (ER) in VP neurons [113]. Closer inspection with antibodies to markers of autolysosomes revealed that this ER is undergoing autophagy [114]. Early endosomes and the *trans*-Golgi network appeared unaffected. These investigators hypothesize that the mutant protein is captured by the ER (normal VP passes through the *trans*-Golgi network to the dense core vesicles) and as more and more ER is lost, less VP gets processed leading to the diabetes insipidus [114]. This research is an exciting example of the use of transgenesis to study a human disease.

Complementary Approaches for the Study of Cell-Specific Gene Expression of OT and VP

Comparative Genomics

In a dramatic experiment, Venkatesh and colleagues [116–118] made transgenic rats containing a 40-kbp cosmid sequence derived from the isotocin (IT)-vasotocin (VT) gene locus in the pufferfish, Fugu. Remarkably, they found cell-specific expression of the fish IT gene only in the OT MCNs in the rat HNS, and that this transgene could be physiologically regulated by dehydration in a manner similar to the endogenous gene. This experiment showed that there was sufficient conservation of the cell-specific expression and physiological regulation mechanisms despite an evolutionary distance of 450 million years between these species. They further suggest that the pufferfish genome, because of its high information density (390-Mbp genome with very little 'junk' DNA, and small (about 80-bp) introns [119]), when used in this rodent transgenic assay might provide a unique opportunity to efficiently dissect the key *cis*-elements in the IT-VT locus that are involved in the cell-specific expression and physiological regulation [116–118].

Organotypic Culture-Biolistics Strategies

Over the past 16 years, experiments in transgenic mice and rats have led to the consistent observation that critical enhancers for cell-specific OT and VP gene expression in the hypothalamus are present in the IGR within the first 200–600 bp downstream of the VP gene [reviewed in 28, 69, 84–86]. In order to further dissect this 600-bp IGR into the specific minimal DNA sequences that are responsible for the hypothalamic-specific expression of the OT and VP genes, a simpler and less lengthy method than transgenic analysis would be desirable. Since there are no homologous cell lines available that adequately exhibit the properties of the OT and VP neurons that are present in the mature hypothalamus, we turned to a novel strategy that uses in vitro models in which the distinct neuronal

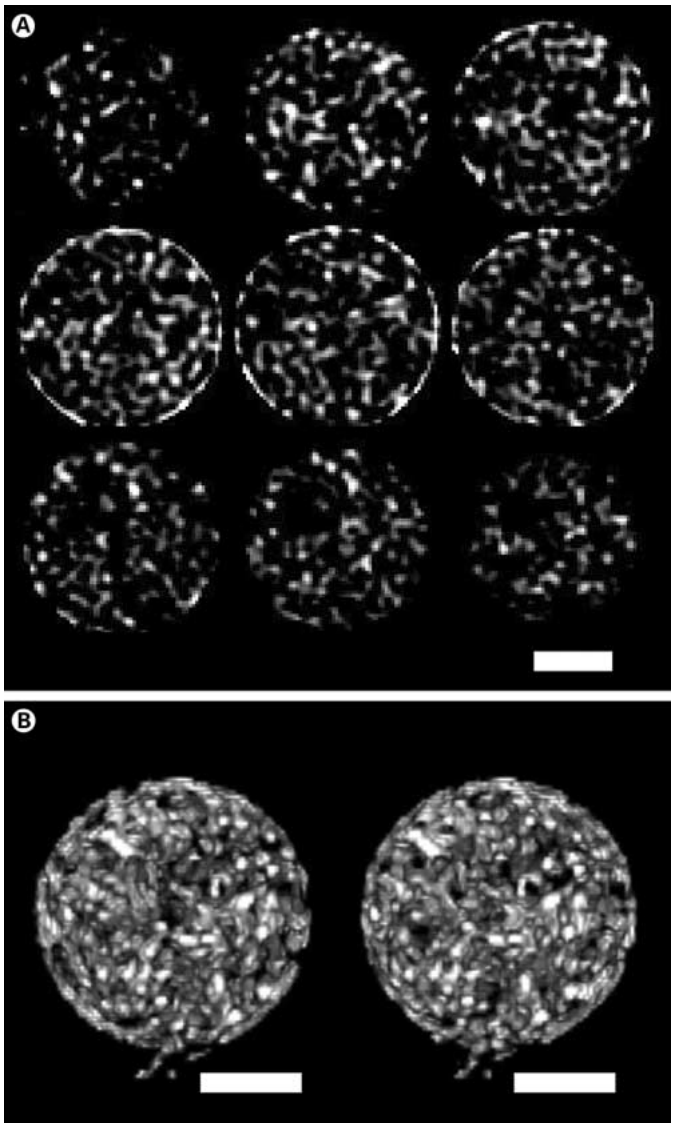

Fig. 4. Fluorescent images of a single living posterior mouse pituitary nerve terminal with vesicles containing the OT-neurophysin-GFP fusion protein expressed from the AI03 transgene [91]. **A** Nine of the twenty-seven optical sections through the terminal along the z-axis spaced 500 nm apart and deconvolved according to the algorithm of Carrington et al. [187]. Individual vesicles are readily discerned. **B** Stereo pair constructed from the entire image set shown in **A**. Calibration bars are 2 μm in each case. Exposures 400 ms (courtesy of Jeffrey Carmichael, Valerie DeCrescenzo, Kevin Fogarty and John V. Walsh of the Department of Physiology and the Biomedical Imaging Group of the University of Massachusetts Medical School).

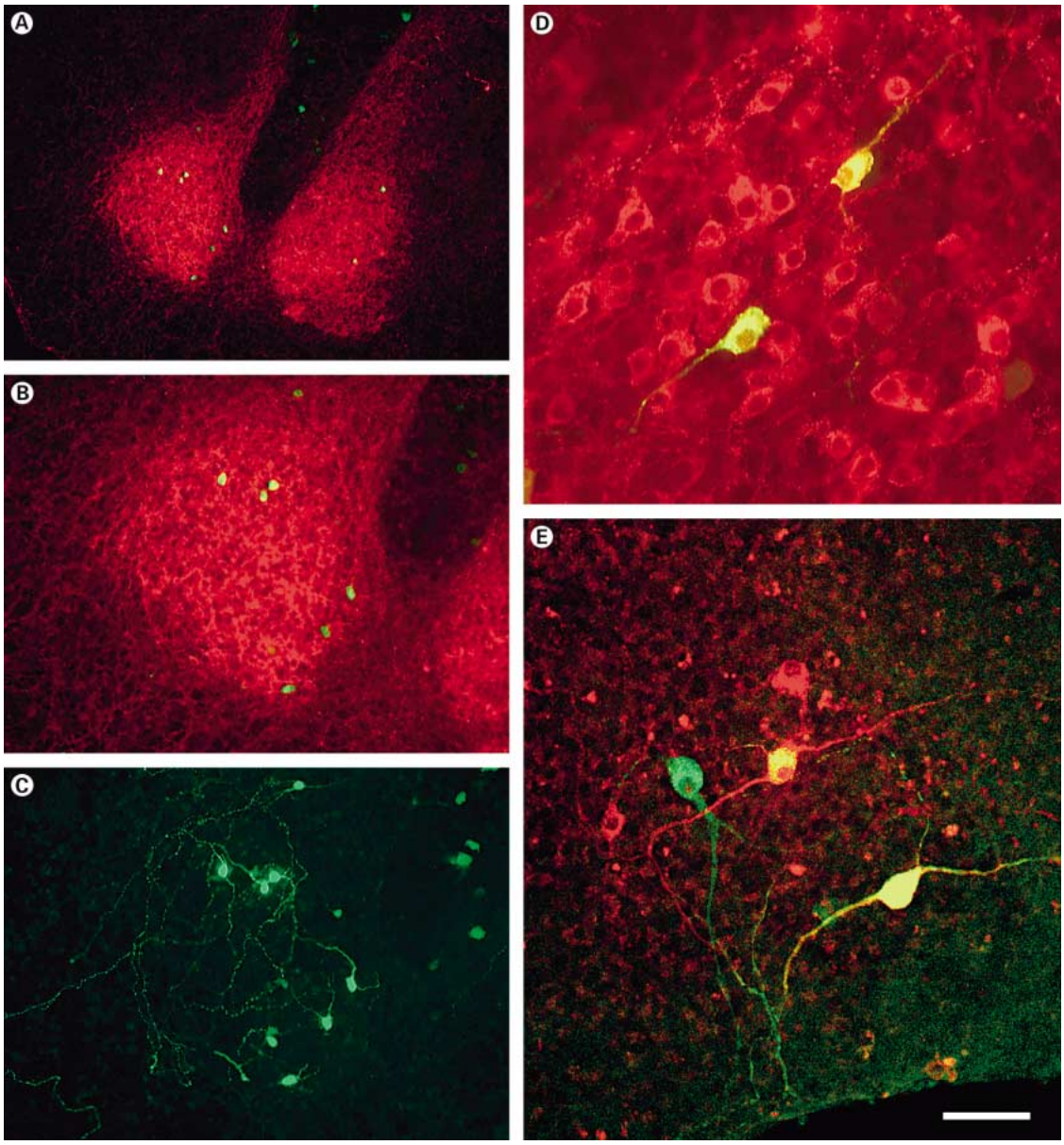

Fig. 5. Biolistic transfections of hypothalamic organotypic cultures using plasmids containing constructs with various cell-specific promoters linked to EGFP reporters. **A–C** Transfection of SCN in culture with neuron-specific enolase promoter driving an EGFP reporter. **D**, **E** A mouse VP gene promoter construct containing a 2.1-kbp IGR sequence (3.5VP-III-CAT-IGR2.1; see text) was used to transfect SCN (**D**) and PVN (**E**) neurons. The two transfected cells in **D** that expressed EGFP appear as yellow cells in the merged view of the green EGFP fluorescence and the red fluorescence representing VP-neurophysin (using antibody PS41) immunoreactivity, thereby showing that the SCN cells which expressed the EGFP also were expressing the endogenous VP gene. Of the three transfected cells in the PVN that expressed EGFP only two contained endogenous VP-neurophysin immunoreactivity. Scale line shown in **E** represents 200 μm in **A**, 100 μm in **B** and **C**, 20 μm in **D**, and 50 μm in **E**.

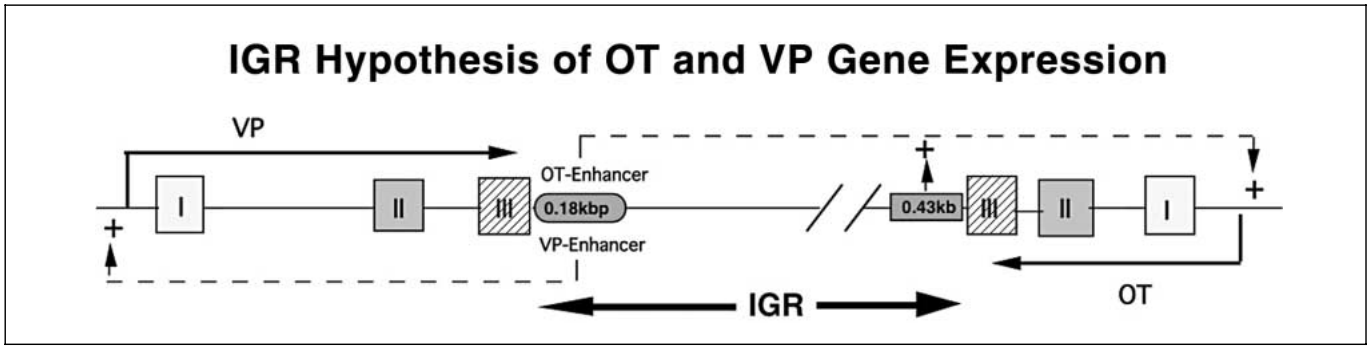

Fig. 6. Regulatory domains in the mouse IGR. Diagram illustrating that the putative enhancer domains in the mouse IGR that are involved in hypothalamic-specific gene expression of the OT and VP genes reside in a 178-bp segment immediately downstream of exon III of the VP gene. A second putative OT enhancer domain also appears to be located within the 430-bp segment immediately downstream of exon III of the OT gene [adapted from 131].

phenotypes found in vivo can be easily identified in the cultured system. In the case of the hypothalamus, this means that the distinct neuronal phenotypes expressing the OT and VP genes should be found in identifiable nuclei (i.e., PVN, SCN, and SON) in the in vitro model. Organotypic slice-explant cultures are known to maintain the cytoarchitectonic organization and topographic relationships of the original postnatal brain tissues [120] and this is also true for organotypic cultures of hypothalamus derived from neonatal mice and rats [121]. Applications of this technique to the rat hypothalamus have been very successful for the maintenance of OT MCNs in the PVN and SON [121], and parvocellular VP neurons in the PVN [122] and in the SCN [121–124]. Interestingly, the maintenance of healthy VP MCNs in vitro requires the addition of specific trophic factors to the media [125, 126]. In order to transfect the neurons in these slice cultures with exogenous gene constructs, we used particle-mediated gene transfer (also called biolistics, see fig. 5). Such an approach has been successfully used with cerebellar slices to uncover a calcium-responsive element in the promoters of the calbindin D28 and calmodulin II genes which are expressed in Purkinje cells [127, 128], in the analysis of *cis*-regulatory elements of genes expressed in the cornea [129], and in Langerhans (dendritic) cells in the lymphoid immune system [130].

We used the above organotypic slice explant cultures of rat hypothalamus as in vitro models, and particle-mediated gene transfer (biolistics) transfection methods to identify critical DNA sequences in the IGR between the OT and VP genes responsible for hypothalamic-specific gene expression. We found that reducing the 5′-flanking region in the mouse VP gene from 3.5 kbp to 288 bp did not alter the efficacy of its expression in hypothalamic slices. Following this, all subsequent VP constructs were based on this 288-bp VP gene construct with changes made only to the IGR. These studies, which used various constructs with OT and VP promoters driving EGFP reporter gene expression, demonstrated that the IGR is specific for OT and VP gene expression in hypothalamic slices (including VP cells in the SCN, see fig. 5D) since these constructs were not expressed in transfected slices derived from other regions of the CNS [131]. In addition, the DNA sequences in the IGR responsible for both OT and VP gene expression were found to be located in a 178-bp domain immediately downstream of exon III of the VP gene [131], consistent with the recent findings of Davies et al. [86] in transgenic mice. Interestingly, another domain in the IGR 430 bp immediately downstream of exon III of the OT gene was found to contain a positive regulatory element for OT gene expression in the hypothalamus [131] (fig. 6).

Gene Transfer Using Viral Vectors

An alternative to transgenesis for the transfer of foreign DNA into differentiated neurons in vivo is the use of viral vectors. Several types of modified virus vectors have been used as efficient vehicles to transfer genes into differentiated neurons in the CNS in vitro and in vivo [132–134]. These include herpes-related viruses [135, 136], attenuated adenoviruses [137, 138], adeno-associated viruses [139, 140], and lentiviruses [141]. To date, only adenovirus has been used in the HNS in vivo [137, 138, 142, 143] and the adeno-associated virus has been used to transfect OT cells in vitro [144]. A particularly elegant example of the use of viral vectors in vivo to alter behav-

ior is illustrated by the work of Pitkow et al. [145], who used an adeno-associated viral vector to deliver the VP 1a receptor (V1aR) gene to the ventral pallidal region of the forebrain in male voles in order to facilitate their affiliative behavior. More remarkable still is that transgenic mice incorporating the monogamous vole's gene for the V1aR displayed an increased affiliative response [146]. These mice have increased V1aR expression within areas that also have high levels in the prairie vole (cingulate cortex and laterodorsal and ventroposterior thalamus). These exciting data are consistent with affiliative behavior being defined, at least in part, by the distribution of the V1aR.

The Study of OT and VP Gene Function through Homologous Recombination

As noted above, we are not aware of homologous recombination targeted specifically to the OT or VP MCNs. However, some relatively specific knockouts have been produced that are relevant. Two transcription factors that have been knocked out have profound effects on the OT and VP MCNs. Null mutations of the basic helix-loop-helix-PAS Sim1 [147] or the POU protein Brn-2 [148, 149] genes lead to failure of development of those MCNs. In addition, the mice die shortly after birth and also lack parvocellular neurons of the PVN encoding thyrotropin-releasing hormone, CRF and somatostatin. Absence of Sim1 also leads to a lack of Brn-2 suggesting that Sim1 functions upstream of Brn-2 [147]. Interestingly, adenoviral-mediated overexpression of Brn-2 in the rat PVN does not affect VP expression [150].

Knockouts of the OT and VP Genes

To our knowledge, no report of a knockout of the mouse VP gene has been published, although Majzoub et al. [151] mention on their website producing one. However, there is an extensive literature about the Brattleboro rat that contains a single nucleotide mutation in the second exon of the VP gene leading to neurohypophysial ('central') diabetes insipidus in homozygous mutants. A recent review of how the CNS is affected in the Brattleboro rat is available [152].

Knockout mice have been produced that lack OT. Two lines were initially reported, one created at Baylor and studied at Emory [153] and another made and studied at NIMH [154]. The mice are fertile and able to deliver their litters, but the pups die because they are not able to obtain milk from the dams. Milk is present in the mammary glands and can be released by injecting them with OT, at which point the pups can successfully suckle [153, 155]. Administration of 22.4 U of OT intraperitoneally, which does not change noticeably the mother's nursing behavior, rescues the pups who then have productive suckling within 30 min to 1 h. The failure of the newborn pups to obtain milk is not just due to ineffective suckling, as 6-day-old wild-type (WT) pups placed with the HO (homozygous) dams (that are within 24 h of parturition) are also unable to obtain milk, despite presumably more forceful suckling. In addition, whole-mount examination of the mammary glands from HO dams shows milk accumulation prior to OT administration. Further studies revealed that alveolar density and mammary epithelial-cell differentiation at parturition are similar in WT and OT-deficient dams. However, within 12 h after parturition, about 2% of the alveolar cells in the WT dams incorporate DNA and proliferate, but virtually no proliferation is detected in the HO dams. Continuous suckling of pups leads to the expansion of the lobuloalveolar units in WT but not HO dams. Despite suckling and the presence of systemic lactogenic hormones, mammary tissue in the HO dams partially involutes. These results demonstrate that postpartum alveolar proliferation requires not only systemic lactogenic hormones, such as prolactin, but also the presence of OT in conjunction with continued milk removal [155, 156].

The OT KO mouse also manifests other physiological changes. OT KO mice have delayed spermiation and sperm transfer [157] and reduced stress-induced antinociception [158]. Michelini et al. [159] in a series of papers have investigated salt intake and cardiovascular function in OT KO mice. These mice are modestly hypotensive at rest suggesting a role for OT in maintaining normal blood pressure. They also presented evidence that 'OT extends the functional range of arterial baroreceptor reflex ... and reduces the sympathetic reserve'. The KO mice also consumed much greater quantities of salt, but not water, under both normal conditions and after overnight fluid deprivation, suggesting an inhibitory role for OT in regulating salt appetite [160, 161]. The OT KO also seemed to begin consuming fluid 3 h earlier in the day than the WT mice [161]. Female OT KO mice were found to have enhanced salt intake as well [162].

The distributions of OT and VP receptors in the brains of various species have been correlated with various behaviors [163–165]. Interestingly, the absence of OT has no affect on the distribution of OT receptors in those mice [153]. Furthermore, a number of behaviors, including sex-

ual and maternal, are minimally, if at all, affected [153, 166]. In fact, a centrally administered OT receptor antagonist also does not affect maternal behavior indicating that VP is not compensating for OT [167]. In the 'NIMH' strain (mixed 129SV-C57BL/6J), HO mice exhibit reduced aggression as compared to their WT or HE (heterozygous) littermates, particularly in agonistic bouts within a neutral arena [166]. WT and HE mice do not differ in aggressive behavior. Although the frequency of aggressive encounters between the WT and HO mice are similar, the OT–/– mice spend significantly less of the test time in aggressive encounters than the WT mice, and the average duration of each aggressive encounter is reduced. In other words, the HO mice attack as frequently as the WT mice, but the aggressive encounters end quickly. HO mice do not display reduced defensive aggression [166]. A battery of sensorimotor skills failed to reveal any detectable sensorimotor deficits. The Baylor/Emory strain had reduced olfactory investigation and increased aggression [167]. These different findings on aggression likely reflect strain differences. The Baylor/Emory KO pups made decreased ultrasonic calls upon separation from the mothers, perhaps due to deficient social attachments [167]. These authors cautioned that significant species and, even, strain differences exist with respect to behaviors making interpretations of mouse behaviors in OT KOs problematic.

Perhaps the most striking behavioral deficit in OT KO mice is their greatly reduced ability to recognize a previously encountered mouse. This deficit in social recognition is found in both males [168] and females [169]. Furthermore, infusion of OT into the medial amygdala prior to the first encounter, but not after, helps restore social recognition in OT KOs whereas infusion of an OT antagonist there inhibits this behavior in WT mice [170]. As KOs of the estrogen-α and estrogen-β receptor genes also lead to similar deficits in social recognition and as these genes are found in the medial amygdala and PVN, respectively, it has been proposed that a 4-gene 'micronet' (2 estrogen receptors, OT and its receptor) exists to regulate this behavior [169]. The deficit in social recognition may have a parallel in the human disease, autism [171].

Behavior that appears to be related to human anxiety was also measured using the elevated plus maze. Interestingly, male knockout mice appeared to be less and females more 'anxious' than WT littermates [172, 173].

The OT-deficient mice were also crossed with 2 transgenic lines, demonstrating that an experimentally created null background may be used to examine the effectiveness of particular transgenic constructs related to the knockout gene. The first line, V7A, was similar to the rat transgene with which we first obtained successful cell-specific and physiological expression of OT [79], except that the OTand VP transcriptional units were in the normal 3′-to-3′ orientation. These double homozygous lines had successful lactation restored and reproduced successfully [174]. The other line, V20, used the rat transgene with replacement of the sequence coding for amino acids 41–107 of the rat neurophysin with the human counterpart (seven differences from mouse). This enabled us to determine if this highly conserved neurophysin sequence between OT and VP within the same species is important for proper protein expression and processing. OT knockout animals, into whose genome this transgene was crossed, were rescued (e.g., they lactate successfully) indicating that the conservation of both nucleotide and amino acid sequences in neurophysin is not necessary for proper expression [175].

In an interesting investigation on the interaction between OT and prostaglandins in regulating mouse parturition, Gross et al. [176] crossed a cyclooxygenase-1 (COX-1)-deficient mouse with an OT-deficient line. The cross corrects the delayed parturition found in the COX-1 knockout mice. The COX-1-deficient mice show impaired luteolysis (and, consequently, elevated serum progesterone levels) and delayed induction of uterine OT receptors. The absence of the luteotropic OT in the double knockouts allows luteolysis to occur and therefore promotes parturition.

Knockouts of Genes for OT and VP Receptors

The functions of OT and VP may also be studied through examination of their receptors, or in the discussion to follow, their absence. The single OT (OTR) and three VP receptors (V1aR, V1bR and V2R), all G protein-coupled, have been knocked out. The first one knocked out, the X-linked V2R, is also the best understood, having a prominent role in the maintenance of normal water balance through reducing water loss in the kidneys. This receptor is not found in the CNS and, not surprisingly, the prominent effect was the inability of hemizygous male mice to concentrate their urine [177]. These mice died within the first week after birth secondary to hypernatremic dehydration and had enlarged renal pelvic spaces. These investigators also examined a separate line of mice in which the neomycin resistance gene was removed using flanking loxP sites (see below about this technique) leaving one small loxP site plus the stop codon in the V2R

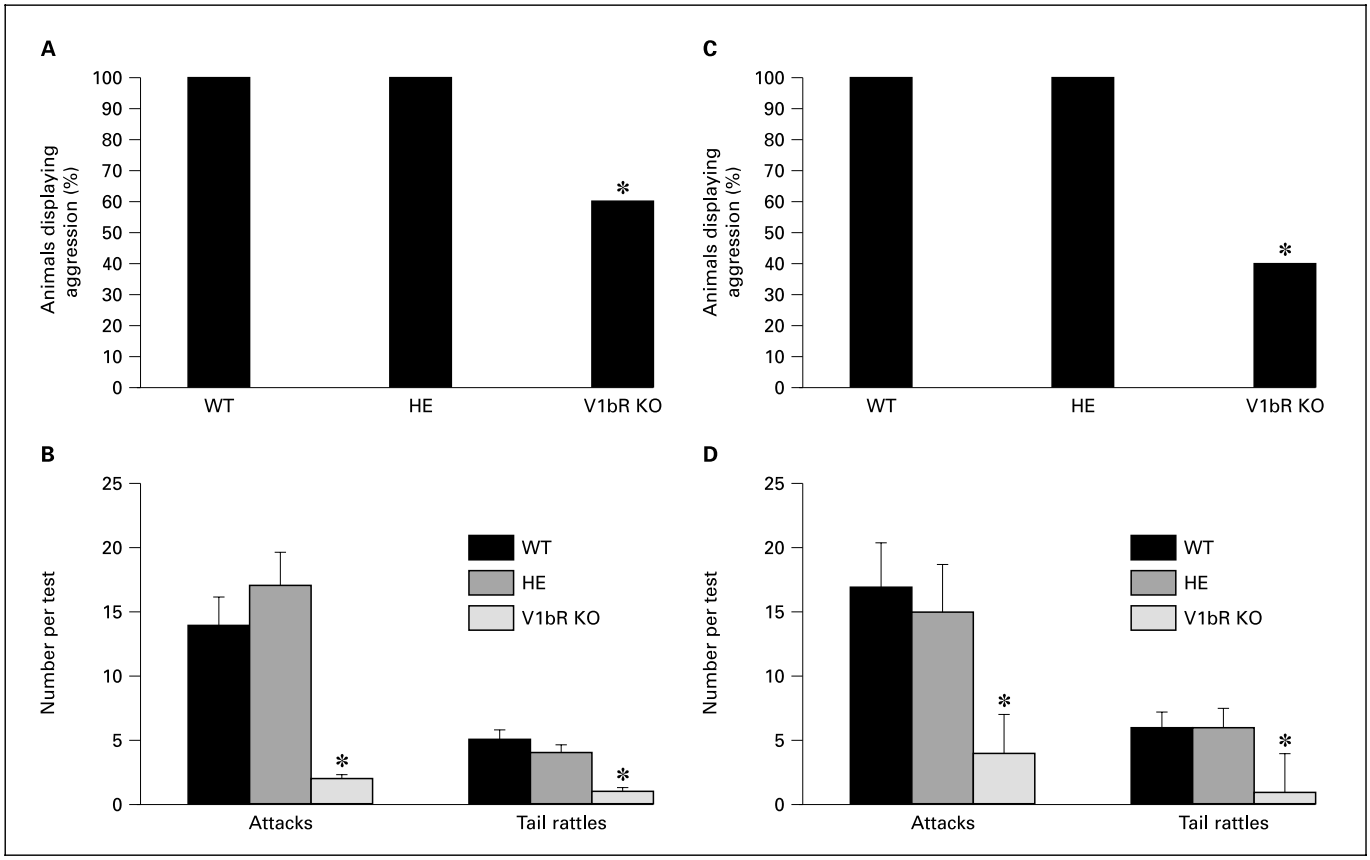

Fig. 7. Male V1bR KO mice are less aggressive than their WT or heterozygous (HE) littermates in a resident intruder paradigm (**A**, **B**) and a neutral-cage paradigm (**C**, **D**) [181]. **A** A significantly lower percentage of V1bR KO males display aggressive behavior than of WT or HE males in the resident-intruder paradigm after three test sessions. **B** Of those mice displaying aggressive behavior, V1bR KO mice display significantly fewer events per test than WT or HE males in a resident intruder paradigm. The means include data only from tests in which aggression was observed. **C** A significantly lower percentage of V1bR KO males display aggressive behavior than of WT or HE males in a neutral-cage paradigm after three test sessions. **D** Of those mice displaying aggressive behavior, V1bR KO mice display significantly fewer events per test than WT or HE males in a neutral-cage paradigm. The means include data only from tests in which aggression was observed. Data are expressed as mean ± SEM. * $p < 0.01$ vs. other groups. A video illustrating the V1bR KO behavior is at http://intramural.nimh.nih.gov/lcmr/snge/Abstracts/V1bRKO.html.

gene. These mice, which had the same phenotype as the ones with the neomycin gene intact, were important to examine in order to rule out any contribution from simultaneous alteration of the RhoGAP ARHGAP4 gene which has an overlapping transcription from the opposite strand [178]. Interestingly, female HE V2R KO mice had a reduced ability to concentrate urine and exhibited polyuria and polydipsia as seen in the human disease of X-linked nephrogenic diabetes insipidus [see references cited in 177].

The V1aR gene has also been knocked out in mice [179]. This initial paper focused on the immune system. While T cell function was intact, the V1aR KO caused a shift from IgM(high)/IgD(high) to the more mature IgM(low)/IgD(high) B cells, greater proliferation of splenic B cells in response to anti-IgM stimulation, and enhanced IgG1 and IgG2b production in response to immune challenge with T-dependent antigen [179]. Interestingly, preliminary evidence suggests that this receptor is not principally involved in mediating VP's well-known role in aggression [180]. Instead, it appears as if the V1bR has this role [181] (see also below).

The vasopressin 1b receptor (also known as V3R) had been sought ever since its pharmacological detection in the pituitary [182]. The cloning of the rat V1bR permitted the study of its localization and it was found widely distributed throughout the brain [183]. However, no function was subsequently associated with the receptor there

due to the lack of specific pharmacological agents. We made a KO of this gene in mice and found two principal defects: a marked reduction in social aggression (fig. 7) and a more modest deficit in social recognition [181]. Major sensorimotor systems are intact in these KO mice, as are a variety of behaviors, including sexual, maternal, feeding and anxiety-like. A function of the V1bR in the brain may be to couple those neurons that receive chemosensory information with those neural circuits that underlie the behavioral response to those cues. Hence, we predict that the coupling mechanism will be highly specific and under strict regulation because only limited behaviors are affected by the V1bR KO [181]. Additional studies are also underway to understand this receptor's role in acute and chronic stress, in view of its prominence in the pituitary corticotropes [184].

Finally, the OT receptor has recently been knocked out [185]. These investigators placed the neomycin resistance gene in the intron after exon 3 with loxP sites 5′ to exon 3 and 3′ to the neomycin resistance gene. This construct resulted in hypomorphic mice with 10% of the normal levels of OTR in the uterus and mammary gland. Pregnancy and delivery were normal but pups died within 24 h due to the lack of milk letdown, similar to what is seen with the OT KOs [153, 154]. Upon excision of exon 3 of OTR along with the neomycin resistance gene, pregnancy and delivery were still normal and the pups still died as before. Reproductive behavior seemed normal in males, but the fertilization rate was lower. Also, KO males attempted to mate with normal females without regard to the female's ovulatory state. A more in-depth description and investigation is eagerly awaited.

Future Directions

There are a number of issues regarding VP and OT gene expression that could benefit from the further use of transgenic animals. For example, little has been done so far to validate in vivo the in vitro work on specific elements within the VP or OT promoters that regulate their gene expression. Targeting of specific sequences that express dominant negatives or other proteins to affect magnocellular physiology should be possible, at least in the OT neurons. In addition to targeting proteins to directly affect neuronal functions, it should also be possible to express cre (cyclization recombination) site-specific DNA recombinase gene of bacteriophage P1 in MCNs. It is likely that a large number of lines will be generated in which genes are flanked by loxP sites ('floxed') so that cre recombinase can be used to excise the intervening sequence, thus inducing a knockout [186]. By crossing with mice expressing the cre recombinase specifically in magnocellular cells with the floxed lines, one should be able to eliminate expression specifically within those cells.

Despite the advances in elimination of the various receptors reported since our last review [85], this avenue of research is still in its infancy. The deficits found so far in the KO mutants need to be examined in more depth with the goal of determining the receptors' precise roles. In many cases, this probably will necessitate the use of cre recombinase or similar systems with both spatial- and temporal-dependent activation. This approach will enable the studies to proceed without worrying about the effect of the complete absence of the gene during various prenatal and postnatal periods.

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