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REVIEW ARTICLE

The Hypothalamic–Neurohypophyseal System: From Genome to Physiology

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The elucidation of the genomes of a large number of mammalian species has produced a huge amount of data on which to base physiological studies. These endeavours have also produced surprises, not least of which has been the revelation that the number of protein coding genes needed to make a mammal is only 22 333 (give or take). However, this small number belies an unanticipated complexity that has only recently been revealed as a result of genomic studies. This complexity is evident at a number of levels: (i) *cis*-regulatory sequences; (ii) noncoding and antisense mRNAs, most of which have no known function; (iii) alternative splicing that results in the generation of multiple, subtly different mature mRNAs from the precursor transcript encoded by a single gene; and (iv) post-translational processing and modification. In this review, we examine the steps being taken to decipher genome complexity in the context of gene expression, regulation and function in the hypothalamic-neurohypophyseal system (HNS). Five unique stories explain: (i) the use of transcriptomics to identify genes involved in the response to physiological (dehydration) and pathological (hypertension) cues; (ii) the use of mass spectrometry for single-cell level identification of biological active peptides in the HNS, and to measure *in vitro* release; (iii) the use of transgenic lines that express fusion transgenes enabling (by cross-breeding) the generation of double transgenic lines that can be used to study vasopressin (AVP) and oxytocin (OXT) neurones in the HNS, as well as their neuroanatomy, electrophysiology and activation upon exposure to any given stimulus; (iv) the use of viral vectors to demonstrate that somato-dendritically released AVP plays an important role in cardiovascular homeostasis by binding to V1a receptors on local somata and dendrites; and (v) the use of virally-mediated optogenetics to dissect the role of OXT and AVP in the modulation of a wide variety of behaviours.

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Genome complexity

Life was simpler in the 1950s. Or so we thought. In 1958, Francis Crick (1,2) first enunciated the 'Central Dogma' of molecular biology, which stated that the information contained within the self-replicating DNA sequence of the genes is transcribed into mRNAs, which are in turn translated into proteins. Coupled with the 'one-gene, one polypeptide' hypothesis (3), itself a development of Beadle and Tatum's (4) 'one-gene, one enzyme' concept, the question arose as to how many genes (i.e. protein encoding transcription units) are required to make a human being (5). In other words, how many genes in the genome are transcribed into the sum total of mRNAs (the transcriptome), which are, in turn, translated into the sum total of proteins (the proteome). Based on assumptions

that were perfectly valid at the time, most of which have been subsequently shown to be baseless, Friedrich Vogel, (6) in 1964, came up with a 'preliminary estimate' of 6 700 000 genes. Over the decades, as methods for studying the mammalian genome improved, and as our understanding of genome structure and expression became more refined, this number fell by two orders of magnitude. However, it was not until the release of the first draft of the human sequence in 2001 (7) that the genome could be looked at *in toto*, with a view to accurately calculating the number of genes. The number immediately dropped, and continued falling in subsequent years as the genome sequence was perfected, and as methods to compare it with the transcriptome were developed and improved. Thus, it is now assumed that it takes as few as 22 333 genes to make a mammal, give or take a few thousand, which is a surprisingly small number (5). However, this apparent simplicity belies a remarkable and unanticipated complexity that has itself emerged from genome and transcriptome sequencing and analysis. This complexity has two aspects. First, it is a function of the mechanisms that can result in massive protein diversity from a limited number of genes. Second, it is a function of the complex *cis* and *trans* regulatory functions of what used to be called 'junk DNA', namely that proportion of the genome that does not code for proteins. This complexity will be described, where possible, with reference to the regulation and physiological functions of the hypothalamic-neurohypophyseal system (HNS) and its major neuroendocrine secretory products, arginine vasopessin (AVP) and oxytocin (OXT).

Protein diversity

The 'one-gene, one polypeptide' hypothesis (3,4) has not stood the test of time. It is now evident that, although we may only have 22 333 genes, we have millions of different protein isoforms. A number of mechanisms are responsible, namely alternative exon usage, RNA editing, and post-translational modification of proteins.

Alternative exon usage

Alternative exon usage can be achieved through the mobilisation of different exons at the 5′ end, through the use of different promoters, or through the process of alternative splicing, whereby different exon sequences are included in mature mRNAs, resulting in the translation of related proteins containing different functional motifs. It is estimated that at least 95% of human genes are subject to alternative splicing (8,9). Given that this number is only approximately 25% in nematode worms (10), it is reasonable to assume that an increased level of exon shuffling is contributing to our relative complexity. Nothing is known about the contribution of alternative splicing to HNS function and regulation. However, in the rat testis, the AVP gene is transcribed into precursor RNAs that are processed into a number of mature transcripts (11). One of these transcripts has a structure identical to that of the hypothalamic RNA that encodes the vasopressin prepropeptide, although it is present at such low levels that it can only be detected by the polymerase chain reaction. Apparently in germ cells, alternative AVP-like RNAs are derived from differential splicing events that join transcribed sequences between 3 and 9 kb upstream of the hypothalamic transcription start site to exons corresponding to II and III of the hypothalamic-type RNA. These testis-specific AVP-like transcripts do not appear to be templates for protein synthesis, and hence appear to be noncoding RNAs (ncRNAs; see below) of unknown, if any, function.

RNA editing

Single base pair changes can be introduced into the sequence of an mRNA, thus changing its coding capacity by a single codon, resulting in a change in the corresponding amino acid sequence of the translated protein, or the introduction of a stop codon that results in a truncated translation product (12). Such subtle changes can have dramatic functional consequences. The main mode of RNA editing in mammals is an adenosine-to-inosine transition catalysed in the nucleus by enzymes called 'adenosine deaminases acting on RNAs' (ADARs), of which there are 3 (ADAR1-3). All three ADARs are expressed in the brain, with ADAR3 being exclusively neuronal. RNA editing alters transcripts from loci encoding proteins involved in neural cell identity, maturation and function (13). However, nothing is known about the contribution of RNA editing to HNS function and regulation.

Recently, nonrandom discordances between mRNA sequences and the corresponding genomic DNA sequences were identified in over 10 000 human exon sites (14). All 12 possible nucleotide transitions were seen, with only 23% possibly resulting from conventional ADAR-mediated adenosine-to-inosine RNA editing. Importantly, proteins are translated from these discordant mRNAs. Neither the molecular mechanisms, nor the physiological relevance of this phenomenon are understood but, yet again, a novel, unanticipated and as yet unexplored aspect of genome variation and complexity has been revealed by genome and transcriptome analysis.

Post-translational modification

Translated proteins are subject to a whole gamut of post-translational modifications that can profoundly affect function and activity. These include covalent modifications, such as phosphorylation, or cleavage and processing events that generate different biologically active peptides from the same precursor that can be the subject of differential transportation within the cell. Post-translational processes have, of course, been well documented in the HNS. Indeed, pioneering studies on the sorting, processing and secretion of AVP and OXT in magnocellular neurones laid the foundations for much of what we know today about neuroendocrine mechanisms and functions (15).

Junk DNA

Noncoding DNA separates genes (intergenic noncoding DNA) or splits the coding regions of genes into exons separated by introns (intragenic noncoding DNA). Noncoding DNA, which makes up most

(> 95%) of the genome, used to be called 'junk', a term proposed by Ohno in 1972 (16) who doubted that these mostly repetitive sequences could have any functional role. However, at around the same time, other authors, such as Britten and Davidson (17) were suggesting that intergenic regions (introns had not at that time been discovered) may have a role in 'regulatory processes'. These 'regulatory processes' are now the subject of intense study, and it is now recognised that they provide complexity and subtlety to the gene expression pathway at two levels: *cis* and *trans*.

cis regulation

The intergenic and intragenic DNA of the genome contains *cis*-acting sequences that mediate the regulation of the genes through interactions with the transcriptional machinery. It has been found that *cis*-regulatory sequences evolve much faster than the genes that they control, and the cognate transcription factors that recognise them, suggesting that the divergence between species is driven by mutations in these elements, rather than changes in the expression or function of transcription factors (18). This concept is supported by studies carried out two decades ago on the expression of the bovine OXT gene in transgenic mice (19). Although mice do not express OXT transcripts in their testes, cattle express relatively high levels of testicular OXT mRNA in Sertoli cells, which appears to be translated to give rise to OXT peptide. In transgenic mice bearing an bovine OXT transgene, expression of bovine OXT mRNA is observed in seminiferous tubules. These data suggest that the bovine OXT gene has *cis* elements required for testicular expression, and that these reside within the confines of the bovine OXT gene used in this study. Further, *trans*-acting factors present in murine testicular cells are able to recognise these elements, although they do not express the endogenous OXT gene in this tissue, presumably because of the absence of endogenous cognate regulatory sequences.

That differences in the *cis*-acting elements of a single gene can have profound phenotypic effects is illustrated by studies on central AVP V1a receptors in different species of vole with very different social and sexual arrangements. The prairie vole is highly affiliative, forms enduring, monogamous social bonds between mates, and displays biparental behaviour (20). By contrast, montane and meadow voles are asocial and promiscuous. In male prairie voles, AVP plays a critical role in the regulation of partner preferences. For example, blockade of V1a receptors in the ventral pallidum or lateral septum with antagonists prevents partner preference formation after mating in prairie voles (21,22). It is considered that species-specific V1a receptor expression patterns in the brain mediate different male behaviours. Thus, monogamous male prairie voles have high densities of V1a binding in the ventral pallidum, whereas non-monogamous vole species do not (23). Viral vector mediated overexpression of the V1a receptor in the ventral pallidum of the male meadow voles induces this promiscuous species to display partner preference (24). The molecular basis for these different expression patterns appears to be related to changes in the sequences of the proximal regulatory regions upstream of the transcription start site of the V1a gene, which contains a polymorphic microsatellite element (25,26). Variation in the length (26) or the sequence (27) of this microsatellite might explain species differences in social organisation among voles, with montane and meadow voles having considerably shorter microsatellite elements than prairie voles (26).

trans regulation

One of the great surprises to emerge from the various genome sequencing and transcriptome analysis projects is that most of the genome (up to 93%) is transcribed, not into mRNAs that encode proteins, but rather into an amazingly divergent abundance of ncRNAs (28,29). Furthermore, both strands of the double helix are templates for RNA biosynthesis, and many classical protein coding (sense) mRNAs have noncoding antisense counterparts (30). ncRNAs can also be derived from the intron sequences spliced out from the precursor RNAs of protein coding mRNAs (31). The functions of ncRNAs are currently the subject of intense study, and it is becoming clear that they have numerous roles as *trans*-acting regulators of gene expression pathways at all levels (29,32,33):

Transcription

- ncRNAs can act on protein complexes that mediate chromatin remodelling and histone modifications.
- ncRNAs can form triple helices at promoter regions, thus inhibiting gene expression.
- via direct interactions, ncRNAs can act as transcription factor co-repressors or co-activators.

Post-transcription

- antisense transcripts can block the recognition of an exon by the spliceosome, thus modulating alternative splicing patterns.

Translation

- ncRNAs can be processed by Drosha and Dicer to generate microRNAs (miRNAS). These are incorporated into the RNA-induced silencing complex (RISC) that interacts with mRNAs in a sequence dependent manner. Partial complementarity, usually within the 3′ untranslated region (UTR) of the target mRNA, leads to translational repression. Complete complementarity with the target leads to mRNA degradation (RNA interference). The latter can also be mediated by small interfering RNAs, formed by the Dicer cleavage of hybrids between mRNAs and antisense RNAs.

Post-translation

- ncRNAs can associate with proteins to alter structure and/or to modulate activity and/or intracellular localisation.

Despite the growing realisation of their importance, almost nothing is known about the expression of ncRNAs in the HNS. Neither do we have any inkling of their roles in the regulation and physiological functioning of the HNS. The one exception is the study by Lee *et al.* (34), who used an miRNA microarray to describe changes

in the expression of miRNAs in the mouse paraventricular nucleus (PVN) and supraoptic nucleus (SON) after 10 days of salt-loading (obligatory 2% w/v NaCl drinking diet). Bioinformatic tools were then used to identify possible mRNA targets of the miRNAs that changed in abundance (both up- and down-regulated). This analysis revealed that miR-7b, which the array suggested was up-regulated by hyper-osmotic stimulation, could potentially interact with two highly conserved binding sequences located within the 3′ UTR of the *Fos* gene. miR-7b was then shown to inhibit *c-fos* protein accumulation in phorbol ester-treated NIH3T3 cells, without having any effect on *c-fos* mRNA levels. These data suggest that miR-7b is repressing *c-fos* mRNA translation. *c-fos*, in partnership with a member of the Jun family, forms an activator protein 1 (AP-1) transcription factor heterodimer. That osmotic stimuli induce the expression of the *c-fos* immediate early gene in the SON and PVN is well known (35). It is thus possible that the up-regulation of miR-7b in the SON and PVN as a consequence of osmotic stress acts to restrain AP-1 activity through repressing *c-fos* mRNA translation. The physiological relevance of this in terms of HNS function remains to be determined.

From genome to physiology

As described above, work over the past decade has revolutionised our view of the structure, expression and function of the genome. A pyramid of complexity is evident (Fig. 1), with a relatively small number of protein coding genes giving rise to millions of protein isoforms, each with subtly different functions and expression patterns.

Superimposed on this information flow is a myriad of regulatory interactions with a whole range of different ncRNAs. At the base of the pyramid is the physiological functioning of the organism. Thus, the question to be addressed now is what can this new view of the genome tell us about HNS regulation and function? This was the theme of the symposium entitled 'The Hypothalamo-Neurohypophyseal System: from Genome to Physiology' that was held at the 9th World Congress of Neurohypophyseal Hormones, held in Boston, USA, in July 2011. Five speakers examined the tentative steps being taken to decipher genome complexity in the context of the expression and regulation of established and novel neurohypophyseal hormone genes, and the elaboration and physiological functions of their biologically active translated products. This journey takes us from gene discovery, using transcriptomics and proteomics to reveal potential novel players, through to the use of transgenesis, both germline and somatic, to assess function *in vivo*, and to shed new light [in some cases literally (36)], on HNS physiology.

Cataloguing of the transcriptome, proteome and peptidome of the HNS under different physiological conditions

Transcriptome

As a consequence of the exploitation of transcriptomic technologies, we now have a good idea of the identity and relative expression levels of the sum total of protein coding mRNAs expressed in the SON, PVN and neurointermediate lobe (NIL) of the pituitary (made up of the intermediate lobe and the posterior lobe) under various physiological and pathological conditions, in both rat and mouse. These studies are described and critiqued below.

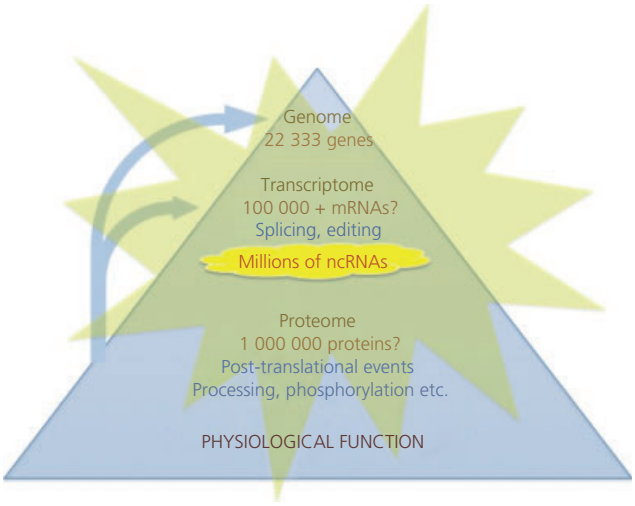

Fig. 1. A pyramid of complexity in genome expression. There are assumed to be approximately 22 333 protein coding genes in the mammalian genome but, through processes such as alternative slicing and RNA editing, this small number can give rise to perhaps millions of protein isoforms, which are then subject to a whole range of post-translational modifications that can alter their activity. Proteins feedback to the genome and transcriptome to control gene expression and transcript maturation. In addition, it is now recognised that the genome hosts the information that give rise to millions on noncoding RNAs (ncRNAs), transcripts that do not code for protein. These ncRNAs impose their regulatory will at all levels of the gene expression pathway (represented by the yellow splodge). It remains to be determined how this pyramid of complexity imposes physiological order.

Transcriptomics of the hypothalamic–neurohypophyseal system: from discovery to function

The HNS is an important integrative brain structure that co-ordinates responses to perturbations in cardiovascular homeostasis. The HNS consists of the large peptidergic magnocellular neurones of the hypothalamic SON and PVN, the axons of which course though the internal zone of the median eminence (ME) and terminate on blood capillaries of the posterior lobe of the pituitary gland (15). In response to a chronic fluid deprivation, which is both a hypovolemic and an osmotic stimulus, the HNS is mobilised to release AVP into the circulation, which acts at the level of the kidney to promote the conservation of water. The SON is a homogenous collection of magnocellular neurones, whereas the PVN is divided into a lateral magnocellular region and more medial sub-division of smaller parvocellular neurones. PVN parvocellular neurones mediate the stress response via projections to the external zone of the ME (37), and, through descending projections to the brainstem, notably the rostral ventrolateral medulla (RVLM), and intermediolateral cell column of the spinal cord (38), the PVN regulates changes in

sympathetic nerve activity involved in the regulation of both arterial pressure and blood volume (39–41). The activity of the HNS is perturbed in rats that have a genetic predisposition to high blood pressure, and this has both a neuroendocrine and an autonomic component. Compared to normotensive Wistar Kyoto (WKY) rats, genetically Spontaneously Hypertensive Rats (SHRs), drink more water, show hypertrophy of the posterior pituitary, have a higher posterior pituitary AVP content, and have elevated plasma AVP levels (42). Evidence suggests that these are primary abnormalities, and are not just a response to the hypertension (42). Furthermore, the PVN excitatory drive to the RVLM appears to be heightened in the SHR compared to the normotensive WKY rat strain (43), and GABA drive in the PVN is decreased in hypertensive models (44).

To obtain understanding of the gene networks that are responsible for HNS regulation and function in both healthy homeostasis and in disease, we have used Affymetrix microarrays (Affymetrix, Santa Clara, CA, USA) to catalogue the genes expressed in the SON, PVN and NIL (which consists of both the intermediate and posterior lobes) of euhydrated male Sprague–Dawley (SD) rats (45), euhydrated female SD rats (46), dehydrated (3 days fluid deprived) male SD rats (45), dehydrated female SD rats (46), normotensive male WKY rats, and SHR rats (C. Hindmarch, J. Paton, D. Murphy, unpublished data). We used the Affymetrix rat 230 2.0 GeneChip probes, which consist of 31 009 oligonucleotide probe sets representing about 30 000 transcripts encoded by approximately 28 000 genes. Separate microarrays (n = 4 or 5) were probed using independently generated targets. For each completely independent replicate, tissue from five rats was pooled for RNA extraction. The raw data (.CEL) from each GeneChip was then loaded into GENESPRING GX11 (Agilent Technologies, Stockport, UK) where it was summarised with MAS5 (which incorporates a scaling normalisation) and transformed to the median of all samples. We first compiled lists of all genes called by the software as being Present on all GeneChips in each experiment; all Absent or Marginal calls were excluded. The stringent filtering results in the generation of lists of RNAs that, with high degree of statistical confidence, are expressed in the SON, PVN or NIL under different physiological conditions (Table 1). The lists are a very valuable resource for physiologists, and can be mined to garner expression information relating to any gene or genes of interest. For example, based on our data sets, we have described changes in the expression of transcription factor mRNAs that occur in the male SD SON as a consequence of dehydration (47), and a comprehensive description of the G-protein coupled receptors expressed in the PVN and SON has recently been published (48).

Table 1. Estimates Derived from Affymetrix GeneChip Analysis of the Number of Genes Expressed in the Supraoptic Nucleus (SON), Paraventricular Nucleus (PVN) and Neurointermediate Lobe (NIL) in Different Strains of Rat and Under Different Physiological Conditions.

Rat	SON	PVN	NIL
SD ♂	16 124	16 144	16 487
SD ♂ dehydrated	15 357	15 678	15 805
SD ♀	13 393		
SD ♀ dehydrated	14 126		
WKY ♂	13 532	15 428	15 516
SHR ♂ hypertensive	15 518	14 853	15 394

SD, Sprague–Dawley; WKY, Wistar Kyoto; SHR, Spontaneously Hypertensive Rat.

We then used GENESPRING to combine Present lists into Experimental lists that were used as the basis for further filtering and robust statistical analysis to identify differentially expressed genes. From the 14 Present lists noted in Table 1, it would be possible to make 182 separate comparisons. However, some are more biologically meaningful than others. Thus, we have described global changes in transcript abundance in the male SD SON, PVN and NIL as a consequence of dehydration (45,47). Similar changes in mRNA expression are seen in the SON of the female SD rats after chronic water deprivation (46). Interestingly, many of the genes regulated in the SON by the hyperosmotic stimulus of dehydration are also regulated in the SON of animals subjected to sustained hypoosmolality (49) but in the opposite direction. We have also examined differences and similarities in gene expression in the SD SON and PVN, both in the euhydrated state and the dehydrated state (45), and we have identified profound differences in transcript expression in the SON when comparing different euhydrated, normotensive strains (WKY versus SD) (50).

Thus far unreported has been our identification of genes whose expression changes in the HNS of normotensive WKY rats compared to hypertensive SHR animals. These genes may contribute to the changes in HNS function seen in hypertensive animals (42–44). One of the genes identified as being up-regulated, at the level of mRNA expression in the PVN, was the α1 catalytic subunit of casein kinase II (*Csnk2a1*). The serine/threonine protein kinase casein kinase II (CK2) is a heterotetrameric holoenzyme composed of two catalytic α1 or α2 subunits, and two regulatory β subunits. Consistent with our array data, Ye *et al.* (51) have shown an increase in membrane bound Csnk2a1 protein in the PVN of SHRs compared to WKY rats. These authors have gone on to use pharmacological tools to test the hypothesis that augmented CK2 activity in SHR PVN contributes to the hypertensive state. Using selective inhibitors, these authors were able to show that CK2 contributes to increased presynaptic and postsynaptic NMDA receptor activity in the PVN of SHR rats, which drives increased vasomotor tone. Furthermore, acute inhibition of CK2 in anaesthetised SHRs reduced blood pressure and lumbar sympathetic nerve discharges. These physiological data confirm the validity of our approach. We are currently validating other genes in terms of their contribution to the phenotypes exhibited by the SHR.

It should be noted that although the gene coverage of the Affymetrix GeneChips is good, it is far from complete, and is dependent upon gene sequences banked in the public databases. Particularly, the vast majority of ncRNAs are not represented, precluding an exploration of their physiological functions. Thus, to maximise the efficiency of data acquisition, and to ensure that all transcription units, sense and antisense, coding as well as noncoding are included in the analysis, we recomend that future transcriptomic studies should employ Illumina deep/short read sequencing

(RNAseq) (Illumina, Inc., San Diego, CA, USA) (52,53), or similar techniques. Advantages are:

- Nonreliance on prior gene identification.
- Highly detailed quantitative and qualitative data in a single experiment, allowing for a complete description of allelic-specific gene expression, novel transcripts and splice variants.
- High performance with more than five log dynamic range with extremely low background and high sensitivity.
- Flexible data re-mining, as new annotations become available, without having to re-run the experiment.

Another caveat relates to the anatomical precision of our analysis. Although we are confining our analysis to finely dissected specific brain regions (SON, PVN and NIL), we acknowledge that our samples will be contaminated with neighbouring tissues. Similarly, even in samples derived by laser capture microdissection (54), the samples are composed of numerous different cell types, including neurones and glia. Still to be addressed is the diversity of gene expression to be found amongst supposedly homogenous neuronal types (e.g. AVP neurones). Single cell sampling techniques coupled with improved target amplification methods and RNAseq will surely answer this question very soon.

To summarise, we and others have obtained large scale data sets that catalogue the genes expressed in the HNS in different physiological and pathological conditions. Tentative steps are now being taken towards a physiological understanding of this information. In many cases, these studies utilise viral vectors targeted to particular brain nuclei with a view to over-expressing a gene of interest, or down-regulating the activity of an endogenous gene using dominant negative mutants or shRNAs. However, the sheer extent of the data derived from a microarray experiment is daunting, and always begs the question of which genes to best study using limited resources. In this regard, unsupervised learning techniques, such as FunNet (http://www.funnet.info) and Weighted Correlation Network Analysis (WGCNA; http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA/) are being developed that construct gene networks from array data. Gene network prediction will allow us to adopt rational criteria for the selection of hub or nodal genes for further study.

Please note that our gene lists are freely available for download (http://www.vasopressin.org); raw data have been deposited in the Gene Expression Omnibus database at the National Center for Bio- technology Information (http://www.ncbi.nlm.nih.gov/geo). Colleagues are encouraged to mine and analyse our data as they see fit. We hope that our data will inform many future physiological studies on HNS function.

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Proteome

The gene catalogues that emerge from transcriptome studies are an incredibly valuable resource for the physiologist as they enable functional targets expressed in a particular tissue, or that change in expression as a consequence of a physiological or pathological transition, to be readily identified. However, it has to be recognised that mRNA levels are not necessarily proportional to the abundance of the encoded protein (55–57). The pathway of gene expression that leads to a steady-state level of the final functional protein is regulated by four fundamental processes, namely transcription, mRNA degradation (or stability), translation and protein degradation (or stability). Recently, this cascade was quantified on a genome scale for the first time (58). Using mouse NIH3T3 cells, simultaneous measurements of absolute (steady-state) mRNA and protein abundance were compared with the half-lives and transcription and translation rates over 5000 genes. In agreement with other authors (59), it was found that transcription rate, rather than trnacript degradation, is the major determinant of mRNA abundance. However, because mRNA abundance accounts for only approximately 40% of the variability in protein levels, and because protein stability appears to have a minor role, at least in the NIH3T3 cell culture system, it would appear that protein abundance is mainly controlled at the translational level. However, although protein stability seems to be less important for rapidly dividing cells, for a long-lived organism, stability varies by four orders of magnitude between proteins, and so becomes really important when determining total protein content; it is the long life of myelin basic protein or collagen, for example, that allows so much of these proteins to accumulate.

These findings highlight the importance of looking directly at the protein, rather than relying on RNA level analyses. This means that hypotheses derived from transcriptomics must be tested at the protein level, and it means an emphasis on proteomic approaches to the study of physiological systems such as the HNS. Indeed, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS) has been used to examine changes in protein abundance in the SON and NIL as a consequence of dehydration (60). Seventy proteins were altered by dehydration (45 in the NIL and 25 in the SON). Six proteins in the NIL (four down, two up) and nine proteins in the SON (four up, five down) were identified using matrix-assisted laser desorption/ionisation MS. Five of these proteins were then characterised using independent methods, and confirmed as being altered in abundance in either the NIL or the SON by dehydration. Interestingly, ProSAAS (proprotein convertase subtilisin/kexin type 1 inhibitor) was shown to be up-regulated in the SON but down-regulated in the NIL. In the SON, ProSAAS was shown to co-localise with both AVP and OXT. The ProSAAS precursor protein is known to be processed into a number of small peptides (61), including Big-LEN (ProSAAS 245–260) and Little-SAAS (ProSAAS 42–59), both of which were shown to decrease in abundance in the NIL as a consequence of dehydration (60). ProSAAS has been shown to coexist with proprotein convertase 1 (PC1), and PC1 mRNA is known to be expressed in both AVP and OXT MCNs of the PVN and SON (62). Pro-AVP is processed by PC1 (63), and ProSAAS is known to inhibit the activity of PC1 (64–66). Thus, ProSAAS is potentially an indirect actor in AVP and OXT processing. PC1 expression is up-regulated in the HNS after dehydration, perhaps to cope with increased AVP and OXT biosynthesis, and a reduction in ProSAAS levels in the NIL would be consistent with this. Furthermore, ProSAAS-derived peptides have also been shown to be secreted (see below), and to have endocrine activities related

to feeding, obesity and circadian functions (61,67,68) (see below). An up-regulation of ProSAAS in the SON, along with a decrease in the NIL, might suggest transport of peptides from the SON to the NIL after dehydration, followed by subsequent secretion from posterior pituitary nerve terminals. The functions of such secreted peptides remain unknown.

Comparison of these proteome data (60) with transcriptome data (45) revealed little overlap, reinforcing the view that there is no necessary correlation between mRNA and protein steady-state levels in the SON and NIL. Furthermore, in contrast to the dramatic and numerous changes seen in microarray analyses of the SON and NIL transcriptomes from comparison of euhydrated and dehydrated rats (45), proteome changes were few, and rather small in magnitude. Part of the problem is that the 2D-DIGE technology is only able to resolve the most abundant (approximately 2000) proteins in the proteome. The dynamic range of 2D-DIGE is also limited, and the technique selects for the most soluble proteins and excludes proteins with extreme pIs or very low or very high molecular weights. Thus, because most proteins are undetectable or hidden by the noise of the abundant minority, interesting molecules known to be modulated by dehydration, such as transcription factors (47) or signalling molecules such as interleukin-6 (69) would not be detected by this method. Furthermore, 2D-DIGE presents but a snapshot of the steady-state levels of the detected proteins. An increase in protein activity, and hence maybe protein turnover, might necessitate an increase in protein synthesis and mRNA biogenesis, although there may not be a change in the steady-state level of that protein.

Peptidome

It is now recognised that the HNS elaborates many peptide hormones in addition to the classical neurohypophyseal hormones OXT and AVP. Given its central role as a neuroendocrine organ, a comprehensive description of all of the secreted peptide products of the HNS, and an exploration of their functions, is vital. Such state-of-the-art neuropeptidomic studies are described below.

Neuropeptidomics of the hypothalamus: from discovery to function

Neuropeptides are critical molecules that modulate the physiological activity of almost every neuronal circuit in the brain. Although much of the research involving neurohypophysial hormones has concentrated on the roles of OXT and AVP and their regulation of systemic physiology and behaviour, the hypothalamus has a myriad of other putative and known neuropeptides whose roles are less well studied. The physiological functions of defined hypothalamic nuclei, such circadian regulation controlled by the suprachiasmatic nucleus (SCN) and water balance, reproduction and affiliative behaviour by the SON, are influenced by a number of neuropeptides.

Although transcriptomics approaches provide exciting detail on gene expression, the biological action of a neuropeptide depends on its precise chemical form and this is not always predictable from genetic information alone. The creation of a neuropeptide is initiated when its RNA is translated into a protein; this process does not stop with protein formation. In the case of a neuropeptide, the protein (the preprohormone) contains a signal sequence. The signal sequence is cleaved from the preprohormone and this targets the remaining protein, a prohormone, to the secretory pathway (70). The prohormone is packaged into dense core secretory vesicles, and a number of processing steps occur to convert the prohormone into one or more bioactive neuropeptides within the single vesicle. These prohormone cleavages occur under control of a suite of intracellular prohormone convertases. Individual prohormones often produce multiple peptides. The specific peptides produced may vary in a cell specific manner, and may change within one cell type under different conditions. Therefore, one often has to make the measurement to determine the precise chemical forms of the expressed neuropeptides from the prohormone. Although recent advances in bioinformatics tools to predict such prohormone processing are improving (71), peptide measurements are still advisable.

Even after decades of dedicated peptide discovery efforts, it appears that many brain peptides remain to be uncovered. The rate of brain peptide discovery is accelerating because of recent technological advances in MS-based peptidomics. A variety of MS methods are utilised for peptidomic and proteomic studies and their description is beyond the scope of this overview (72,73). It is important to realise that the specific instrument used determines the quality of the information obtained and so such details are important when designing experiments.

These MS technologies now allow defined brain regions to be assayed for their endogenous peptides, and enable rare peptides to be discovered. Of importance to studies related to the hypothalamus, several recent studies have created lists of known and unknown peptides from the SCN (74) and SON (75), with many observed peptides being shortened or modified forms of known peptides, although several known peptides have unusual modifications and others are unexpected in these brain regions. We used an MS technology that allows higher confidence peptide assignments and also confirms a number of modifications on the peptides. Within the SCN, for example, more than 100 peptides were identified, with one-third not being previously reported (12 peptides had additional post-translational modifications) (74). Several of the identified peptides were derived from precursors known to be expressed in the SCN, including short forms of peptides from vasoactive intestinal peptide, gastrin-releasing peptide and somatostatin. As mentioned, such neuropeptidomic lists of peptides can reach to hundreds of peptides, even for well-defined brain areas. This makes selecting the most promising peptides for follow-up studies daunting. Therefore, considerable efforts have gone into the developments of protocols to provide additional information. As one example, pre-selecting specific cell types to study simplifies the peptides detected from such targeted samples. Taking this to an extreme, an uncommon but effective approach involves peptidomic investigations of single cells. MS at the single-cell level permits identification of peptides from the same precursor as they should be colocalised within the cell soma, as well as determination of peptides from multiple prohormones colocalised within the same cell, although such studies have rarely used mammalian neurones

(76,77). As one example, magnocellular neurones of the SON have been manually isolated from a hypothalamic slice. Although the small amount of peptide in a single cell allows the detection of only a few peptides, for magnocellular neurones, AVP, neurophysin II and cocaine- and amphetamine-regulated transcript-related peptides have been mass-matched to the SON peptide database (75). By contrast to acute isolation of cells, culturing neurones has the advantage of permitting regeneration of long neurites, which are lost during acute cellular separation and this approach has also been used with SON neurones (78).

Particularly exciting, the measurements of peptide release hint at which peptides are biological active, with the release measurements being from cultured neurones, brain slices or microdialysis sampling. Using the rat SCN, we have measured the peptides released from the SCN in an activity-dependent manner and at specific times of the day (Fig. 2) (79,80). We have created several sampling approaches to collect peptides secreted at discrete locations within the hypothalamus from *ex vivo* brain slices with one approach outlined in Fig. 2. We can distinguish time-of-day or activity-dependent release by comparing releasates after such stimulations. Not unexpectedly, peptides released in response to physiological stimulation include putative SCN peptides, such as AVP, substance P, the somatostatins, neurotensin and galanin, with these assignments confirmed using tandem MS. A surprising result was the detection of multiple proSAAS-related peptides, including the peptide little SAAS (74,79), which had not been associated with the SCN previously. We have subsequently determined a role for this peptide in shifting the phase of the SCN, as well as its detailed co-localisation with other peptides within the SCN (79,80).

Knowing that a novel peptide is only detectable at a specific time of the day or under specific electrical stimulation protocols yields critical information on potential peptide function. For example, the SCN peptide 'little SAAS' exhibits robust retinohypothalamic tract-stimulated release from the SCN, and exogenous application of little SAAS induces a phase delay consistent with light-mediated cues regulating circadian timing (79,81). As the methods of localised sampling and activity-dependent release are further refined, information on dynamics is being combined with information on chemical form and spatial localisation to enable comprehensive understanding of peptidergic signalling.

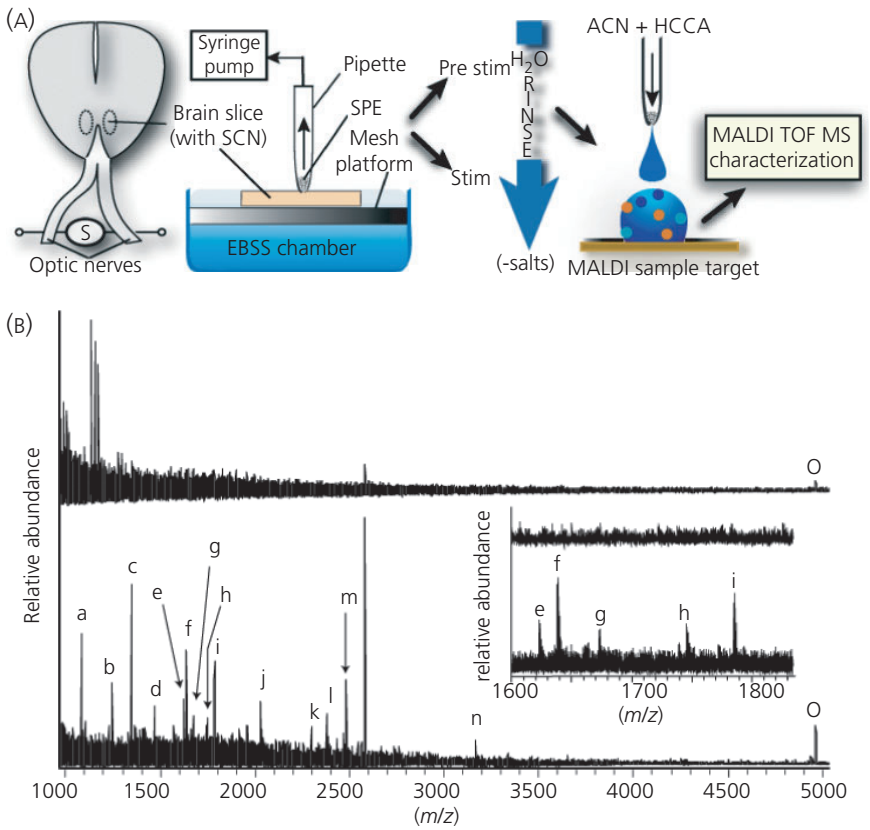

Fig. 2. Approach used for collecting peptide release *in vitro* from a hypothalamic brain slice. These results demonstrate the complexity of peptides released even from a defined brain region such as the suprachiasmatic nucleus (SCN). (A) Sample collection and preparation scheme. (B) Mass spectrometry-based characterisation of SCN releasates. (Inset: a zoomed mass range.) Labelled analytes are: (a) AVP, (b) proSomatostatin 89–100, (c) substance P, (d) PENK 219–229, (e) melanotrophin α, (f) somatostatin 14, (g) pyro-glu neurotensin, (h) big LEN, (i) little SAAS, (j) unknown 2028.02 m/z, (k) PEN, (l) unknown 2380.10 m/z, (m) unknowns 2481.26/2481.77 m/z, (n) galanin, and (o) thymosin β-4. ACN, acetonitrile, EBSS, Earle's Essential Balanced Salt Solution, HCCA, alpha-cyano-4-hydroxycinnamic acid, MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, S, stimulator, SPE, solid phase extraction, stim, stimulation. Work adapted with permission (79).

What does the future hold? Although such MS-based peptidomics measurements allow peptides from well-defined brain regions to be probed without analyte pre-selection, technology has yet to evolve to allow their associated receptors to be characterised using similar analytical techniques. Perhaps the combination of multiplexed biosensors with antibodies tailored to specific peptide receptors, transcriptomics to measure their expression, and MS-based peptidomics will allow peptide signalling to be followed from peptide formation, cellular release, to receptor binding. Such combined approaches should provide an unprecedented view of the intricacies of peptide signalling within the hypothalamus.

The projects described were supported by the National Institute on Drug Abuse (NIDA) through P30 DA018310 and the National Heart, Lung and Blood Institute (NHLBI) through RO1HL092571 Z ARRA.

Accessing the HNS with genetic tools

Having characterised global patterns of transcript and protein expression in the HNS, it is now necessary to put all of this information into physiological context. For this, we need genetic access to HNS cells. Germline gene transfer techniques have been used to study HNS regulation and function for close to three decades. Starting in mice (82,83), and moving to rats (84,85), a number of groups sought to define the genome sequences required to drive the appropriate cell-specific and physiological regulation of the AVP and OXT genes. These studies progressed to the point where genomic sequences could be used to very accurately target reporter molecules to AVP and OXT neurones. The generation and use of germline transgenic rats expressing fluorescent reporter genes in the HNS is described in below.

As subsequently described, viral vectors are now widely used to introduce new genes into specific cell populations of the HNS, and promise to rapidly accelerate the acquisition of physiological understanding.

Fluorescent visualisation of AVP- and OXT-containing neurones in the hypothalamus and extra-hypothalamic areas under physiological and pathophysiological conditions

Recently, modern imaging techniques using fluorescent proteins such as green fluorescent protein (GFP) and related proteins have become one of the most powerful tools to study the dynamics of molecules in living cells in real time (Fig. 1). Here, we demonstrate that specific target molecules, AVP and OXT, can be readily visualised by fluorescent proteins, using transgenic techniques in rats.

We have generated separate transgenic rat lines that express an AVP-enhanced GFP (eGFP) fusion transgene (85) and an OXT-monomeric red fluorescent protein (mRFP) fusion gene (86). AVP-eGFP fluorescence was observed in the AVP neurones of the hypothalamus, as previously described by immunohistochemistry (87), such as the SON, the PVN, the SCN, and the posterior pituitary (85,88). In extra-hypothalamic areas, eGFP fluorescence was induced in the locus coeruleus after i.c.v. administration of colchicine (89), and consequential to s.c. injection of kainic acid that induced seizure. OXT-mRFP fluorescence was also observed in the SON, the PVN and the posterior pituitary of transgenic rats (86). AVP-eGFP fluorescence in the SON and the PVN was dramatically increased after chronic salt loading (90), endotoxin shock (91) and nociceptive stimulation (92). OXT-mRFP fluorescence in the SON and the PVN was also markedly increased after chronic salt loading (86).

Freshly dissociated neurones from the hypothalamus of transgenic rats were easily identified by eGFP fluorescence and mRFP fluorescence under fluorescent microscopy (93). Whole-cell patch-clamp recordings were performed from these isolated eGFP and mRFP positive neurones (93).

Next, we generated a double transgenic rat that expressed the AVP-eGFP gene and the OXT-mRFP gene. The eGFP positive neurones and mRFP positive neurones were clearly observed in the same section of the SON and the PVN (86). Thus, in the same animal, we can readily identify AVP and OXT neurones in the hypothalamus by virtue of their different fluorescent characteristics. This is a major boon to scientists studying the neuroanatomy and electrophysiology of these neurones.

Immediate early genes such as the *c-fos* gene are transcriptional regulators that have been used routinely as markers of neuronal activity (35). We have generated transgenic rat lines that express either a c-fos-eGFP fusion gene or a c-fos- mRFP fusion gene. In these transgenic rats, c-fos-eGFP and c-fos-mRFP fluorescence was observed in the nuclei of neurones in the SON and the PVN after an appropriate stimulus, such as acute osmotic challenge. A double transgenic rat that expresses both the AVP-eGFP gene and the c-fos-mRFP gene showed that eGFP positive (AVP) neurones expressed nuclear red fluorescence 90 min after i.p. injection of hypertonic saline (36). Thus, activated neurones with a specific neurochemical identity can be readily identified.

Now, we have four transgenic lines that express fusion transgenes (AVP-eGFP, OXT-mRFP, c-fos-eGFP and c-fos-mRFP) (Fig. 3). These animals can be interbred to produce double transgenic rats that are very useful in the study AVP and OXT in both *in vivo* and *in vitro* preparations. These animals will find utility in studies on gene expression, neuronal activity and the dynamic changes in protein level and distribution. Astute use of different fluorescent transgenes will enable the temporal pathway of a physiological response to a stimulus to be readily monitored in identified neuronal populations of the HNS (Fig. 4).

Use of adenoviruses to assess the role of PVN AVP receptors in baroreflex control

Neuroanatomical studies reveal a wide distribution of AVP V1a receptors in the brain: along the axonal terminals, within the nuclei containing neuronal cell bodies and at remote brain areas distant of vasopressinergic wiring (94). The widespread distribution of brain V1a receptors suggests that AVP acts at multiple sites in the brain to control the cardiovascular system. The best-known neuroendocrine pathway involves the axonal release of AVP from the neurohypophysis directly into the circulation. From there, AVP acts as a hormone at target organs, the kidney, resistance blood vessels and

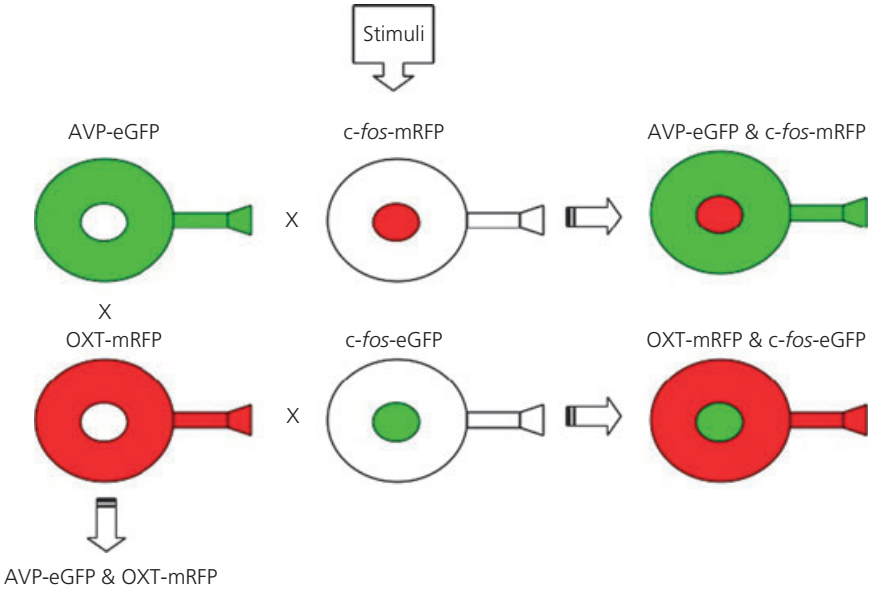

Fig. 3. Four transgenic rats that express vasopressin (AVP)-enhanced green fluorescent protein (eGFP), oxytocin (OXT)-monomeric red fluorescent protein (mRFP), c-fos-eGFP and c-fos-mRFP were generated and can be bred to create informative double transgenic rats. AVP-eGFP and OXT-mRFP are cytosolic fusion proteins, whereas c-fos-eGFP and c-fos-mRFP are nuclear. AVP, arginine-vasopressin, OXT, oxytocin.

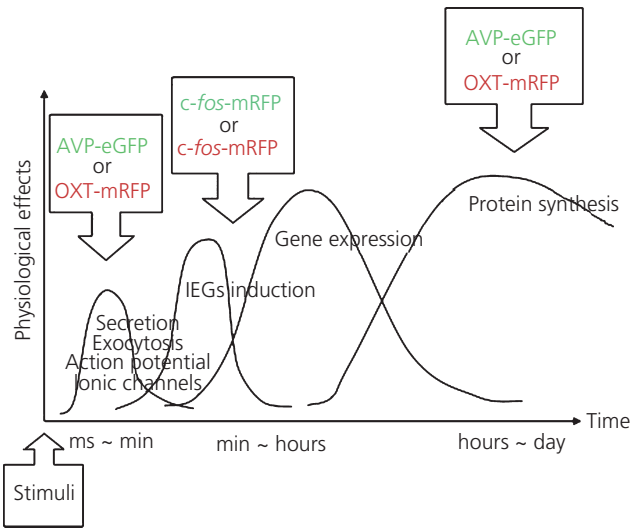

Fig. 4. By utilising the facile monitoring of different fluorescent proteins under the control of different promoters, the temporal pathway of a physiological response to a stimulus can be readily monitored in identified neuronal populations of the HNS. IEG, immediate early gene.

area postrema, to regulate blood volume, peripheral resistance and the functioning of the baroreceptors reflex (95). In the second neuronal pathway AVP is released from axonal projections of PVN neurones into the rostral ventro-lateral medulla, nucleus tractus solitarius and intermediolateral column of the spinal cord, which are areas responsible for integration of peripheral sympathetic and vagal outflow (96). A third source of AVP is the neuronal cell bodies and dendrites of vasopressinergic neurones. The peptide is released into the nearby tissue and diffuses into the cerebrospinal fluid, whereby it may diffuse to remote target sites and exert long-lasting neuro-modulatory action (97).

We hypothesised that somato-dendritically released AVP in PVN binds to V1a receptors on somata and dendrites, and could therefore play an important role in cardiovascular homeostasis. To test this hypothesis, we developed a model of somatic transgenic rat using an adenoviral vector to increase expression of V1a receptors on neurones in the PVN. The somatic transgenic model of the rat has important advantages over the wild-type rat with i.c. cannulation used for drug injection (Fig. 5).

The main advantage is more selective and longer-lasting changes of receptor function than drugs induce. Rats are fully recovered, freely moving, without i.c. cannulation, and are devoid of the confounding effects of anaesthesia on cardiovascular regulation. Finally the effect of up-regulating receptors reflects the action of naturally occurring agonist at physiological concentration, whereas the action of a drug agonist reflects effects of structurally different molecules at nonphysiological (i.e. pharmacological) concentrations. Transfection of PVN neurones was induced by nonreplicant adenoviruses (98). Host neurones in PVN of rats were simultaneously transfected with V1a receptor gene and a gene for eGFP to mark their transduction; this was confirmed by eGFP immunostaining (not shown).

Adenoviruses were microinjected into the PVN of male adult Wistar rats equipped with radiotelemetric probe in abdominal aorta for blood pressure (BP) recording. Four to 7 days post-transfection, when gene expression was found to be maximal, rats were submitted to a simple experimental protocol consisting of BP recordings under baseline physiological condition followed by exposure to 15-min long air-jet stress, and recovery. Systolic BP,

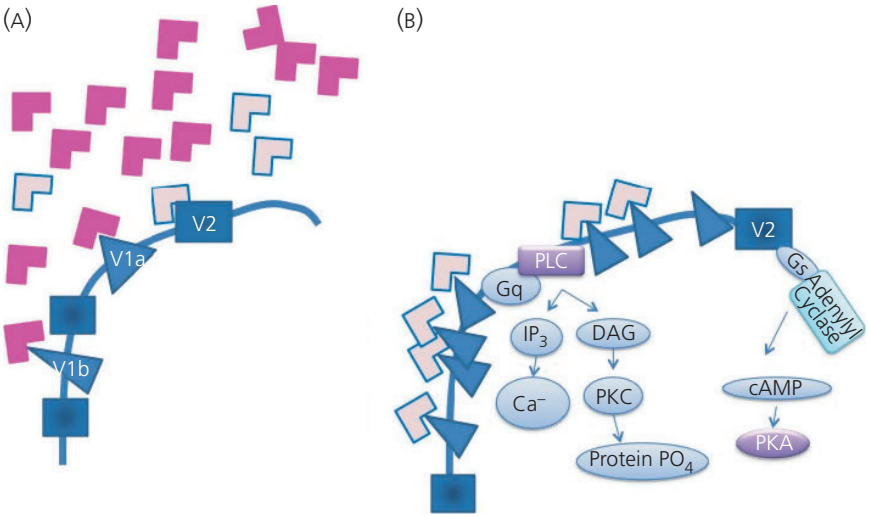

Fig. 5. Schematic illustration of pharmacological (A) and transgenic (B) approach for studying receptor function. Adenoviral transfection in PVN induces more selective and longer-lasting changes of V1a receptor function than the microinjection of drugs. cAMP, cyclic 3',5'-adenosine monophosphate, DAG, diacylglycerol, IP3, inositol 3-phosphate, PLC, phospholipase C, PKA, protein kinase A, PKC, protein kinase C, V1a, Vasopressin V1a receptor, V1b, Vasopressin V1b receptor, V2, Vasopressin V2. receptor.

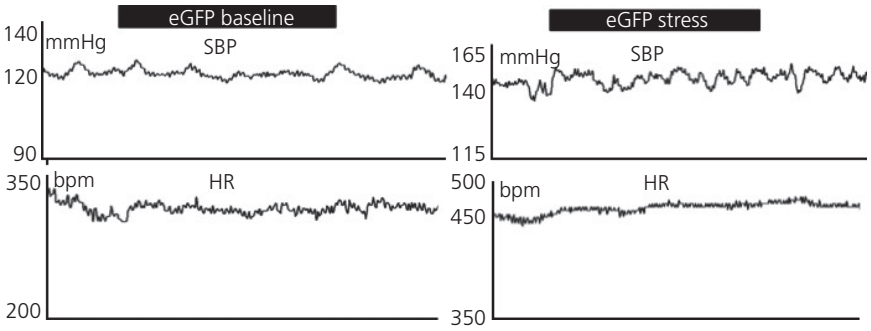

Fig. 6. Typical recording of systolic blood pressure and heart rate using radiotelemetry of one enhanced green fluorescent protein (eGFP) transfected rat under baseline conditions (left) and during exposure to air-jet stress (right). SBP, systolic blood pressure; HR, heart rate; eGFP, rats transfected with enhanced green fluorescent protein.

pulse interval (PI) and heart rate (HR) were derived from the recorded BP signal as maximum, inter-beat interval between two maxima and its inverse, respectively. Evaluation of the baroreflex was carried out using the sequence method, established both in humans and rats (99–101).

Figure 6 shows a 10 min long continuous recording of systolic blood pressure (SBP) and HR of one eGFP transfected rat under basal (A) and stressful (B) physiological conditions.

Blood pressure and the HR of eGFP transfected rats exhibit normal values of hemodynamic and baroreflex parameters under basal conditions (Table 2). Exposure to stress induced an increase in BP and HR and a decrease in baroreflex sensitivity and re-setting of baroreflex toward higher SBP and lower PI values comparable to wild-type rats (102,103). Under basal physiological conditions, rats over-expressing V1a receptors in PVN have comparable values of BP and HR to eGFP transfected rats. However, V1a transfected rats exhibit reduced sensitivity and operating range of the baroreflex under basal physiological conditions. When exposed to air-jet stress, V1a transfected rats exhibited similar increase of SBP, HR, suppression of baroreflex sensitivity and re-setting towards higher SBP and lower PI values (Table 2). However, the operating range of the baroreflex of these rats during exposure to stress was significantly increased (Table 2).

Our findings show that desensitisation of the baroreflex can be induced by somato-dendritic release of AVP and the stimulation of V1a receptors in PVN, possibly by inducing priming of magnocellular neurones. These findings implicate a possible role of somato-dendritic release of AVP in PVN and of V1a receptors in cardiovascular pathology, especially hypertension and heart failure whose poor prognosis is associated with baroreflex desensitisation (104,105).

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Table 2. Mean Values of Systolic Blood Pressure (SBP), Heart Rate (HR) and Baroreflex Sensitivity (BRS) Parameters in Enhanced Green Fluorescent Protein (eGFP) and V1a Transfected Rats.

	SBP (mmHg)	HR (b.p.m.)	BRS (ms/mmHg)	Operating range (mmHg ms)	SBP set point (mmHg)	PI set point (ms)
eGFP – baseline	118 ± 5.1	341 ± 16.6	2.1 ± 0.2	263.3 ± 55	117.2 ± 5.3	180.4 ± 9.2
eGFP – stress	138 ± 5.4**	448 ± 16.6***	1.3 ± 0.4**	647.5 ± 262	137.5 ± 5.7***	133.8 ± 5.2**
V1a – baseline	113 ± 1.2	323 ± 13.2	1 ± 0.1†	153.7 ± 19.8†	112.6 ± 1.1	188.2 ± 5.2
V1a – stress	135 ± 2.6**	400 ± 11.6**	1.4 ± 0.3	458.9 ± 100.8***	134.3 ± 2.8**	154 ± 6.9***

**P < 0.01, ***P < 0.001 versus baseline (ANOVA multiple measures follow by Bonferroni post-hoc test); †P < 0.05 versus eGFP-transfected group of rats (Student's t-test). PI, pulse interval.

Using viral vector technology in probing axonal oxytocin release in the forebrain

During the last decade, viral vectors were extensively used in the field of neuroscience. This approach allowed manipulation of neurones *in vitro* and *in vivo* to study different brain functions. The use of viruses is a relatively fast and cheaper technique, compared to conventional transgenic animal models, and provides a great opportunity to introduce or down-regulate different genes of interest in the brain of different mammalian and non-mammalian species (106). Although virus-based techniques primarily focused on various higher brain regions, such as the cortex, hippocampus and amygdala, the use of this technique in the hypothalamus was rather rare. In our work, we achieved the cell type specific targeting of OXT and AVP neurones, using short evolutionarily conserved sequences of promoters of the respective genes, which were introduced into recombinant adeno-associated virus (rAAV) backbones (unpublished data). The rAAV-based genetic transfer allowed us to discriminate projections of these two populations of magnocellular neurones and analyse forebrain regions innervated by fibres originating either from OXT or AVP neurones (unpublished data). Furthermore, expressing axonal markers in these neurones helped us to discriminate axonal versus dendritic processes at light and electron microscopic levels (unpublished data). These anatomical data provided a basis for further evaluation of functional significance of these axons in various brain structures in relation to the region-specific behaviour. This can be done, for example, by the expression of light-sensitive channel channelrhodopsin-2 in OXT cell bodies, local dendrites and distant axons with subsequent *in vitro* and *in vivo* blue-light stimulation of endogenous neuropeptide release within the hypothalamus or/and extrahypothalamic brain regions. The virally-mediated optogenetics appears as a new powerful tool for dissecting the role of the endogenous neuropeptide (released either intrahypothalamically from dendrites or extrahypothalamically from axons) in the modulation of a wide variety of behaviours, including fear, anxiety, aggression and social interactions, in which the modulatory effects of OXT and AVP are very well described. For example, our initial experiments with blue-light stimulation of endogenous OXT release in the central amygdala suggest the efficiency of this approach in inducing changes in the activity of the central amygdala microcircuit, leading to the prominent modulation of fear responses in fear-conditioned rats (unpublished data). Moreover, the application of virus-based targeting serves as an excellent approach for the evaluation of possible alterations in wiring of magnocellular neurones and their capacity to release endogenous neuropeptides in the brain of animal models of human psychosocial diseases, such as autism and schizophrenia.

The projects described were supported by the German Research Foundation (DFG) grants GR3619/2-1 and GR3619/3-1.

Conclusions

Recent deep exploration of the mammalian genome and its expression has revealed a daunting complexity that is only just starting to be understood in terms of molecular mechanics of regulation and function. The subsequent task will be to place this complexity in physiological context. It is to be expected that the HNS will play its customary role as the physiological model of choice as this endeavour progresses.

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