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REVIEW

The long and the short of it – a perspective on peptidergic regulation of circuits and behaviour

Gáspár Jékely[1,*], Sarah Melzer[2], Isabel Beets[3], Ilona C. Grunwald Kadow[4], Joris Koene[5], Sara Haddad[6] and Lindy Holden-Dye[7,*]

ABSTRACT

Neuropeptides are the most diverse class of chemical modulators in nervous systems. They contribute to extensive modulation of circuit activity and have profound influences on animal physiology. Studies on invertebrate model organisms, including the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, have enabled the genetic manipulation of peptidergic signalling, contributing to an understanding of how neuropeptides pattern the output of neural circuits to underpin behavioural adaptation. Electrophysiological and pharmacological analyses of well-defined microcircuits, such as the crustacean stomatogastric ganglion, have provided detailed insights into neuropeptide functions at a cellular and circuit level. These approaches can be increasingly applied in the mammalian brain by focusing on circuits with a defined and identifiable sub-population of neurons. Functional analyses of neuropeptide systems have been underpinned by systematic studies to map peptidergic networks. Here, we review the general principles and mechanistic insights that have emerged from these studies. We also highlight some of the challenges that remain for furthering our understanding of the functional relevance of peptidergic modulation.

KEY WORDS: Neuropeptide, Invertebrate, Model system, Plasticity, Modulation

Introduction

Neuropeptides are a diverse family of signalling molecules with significant roles in animal physiology and behaviour. They are short chain-length peptides that are synthesized by the enzymatic cleavage of larger polypeptide precursors (Elphick et al., 2018). Peptidergic communication was first recognized in the context of peptide hormones secreted from endocrine glands (Bayliss and Starling, 1902) with the later discovery that peptides may also be synthesized in, and secreted from, neurons (Knowles and Bern, 1966; Olivecrona, 1954; Worthington, 1966; Johnson, 1962; Knowles, 1951) along with 'classical' small-molecule neurotransmitters (Hökfelt et al., 1980). Since then, it has become increasingly clear that neuropeptides add a level of complexity and finesse to neuronal communication that is of key importance for behavioural plasticity (Koh et al., 2003; Stein et al., 2007; Taghert and Nitabach, 2012; van den Pol, 2012).

The aim of this Review is to discuss a selection of recent examples of peptidergic regulation of behaviour from across the animal phyla. Two accompanying review articles focus on other core aspects of neuropeptides. The first focuses on, the evolutionary conservation of neuropeptides families (Elphick et al., 2018). The second provides an update on experimental approaches and emerging techniques to dissect peptidergic networks (DeLaney et al., 2018). Further informative reviews on specific neuropeptide families are available elsewhere (e.g. Beets et al., 2013; Walker et al., 2009).

Complexity of peptidergic signalling in animal nervous systems

Neuropeptide diversity

The genomes of bilaterian animals, on average, encode over a hundred neuropeptide precursors and receptors (Caers et al., 2012; Civelli et al., 2013; Conzelmann et al., 2013; Frooninckx et al., 2012; Mirabeau and Joly, 2013; Zhang et al., 2012). Diversity is further increased by the presence of multiple copies of the same neuropeptide or different types of neuropeptides within one precursor sequence (e.g. the myoinhibitory peptide precursor in the silkworm *Bombyx mori* contains eight different versions of the peptide) (Fig. 1A). Similarly, the mammalian pro-opiomelanocortin (POMC) precursor protein gives rise to adrenocorticotropic hormone as well as opioid (see Glossary), melanotropin and other peptides (Cawley et al., 2016; Wallis, 2010) (Fig. 1B). A single proneuropeptide gene can also generate different isoforms through alternative splicing, producing different peptides that are expressed differentially, as observed for *Drosophila* orcokinin (Fig. 1C), mammalian calcitonin and other peptides (Amara et al., 1982; Chen et al., 2015; Li et al., 2008). Furthermore, the level of post-translational processing can also be modulated in a state-dependent manner. For example, the melanocortin peptide α-MSH derived from POMC regulates body weight: it accumulates during fasting through an increased rate of post-translational processing of POMC, likely underpinned by altered expression of pro-hormone convertases (Perello et al., 2007; Tung et al., 2006).

Neuropeptide receptor diversity

Most neuropeptides signal through seven-transmembrane G-protein-coupled receptors (GPCRs), but they can act through several other classes of receptors. For example, insulin-related peptides signal through insulin receptors, which are receptor tyrosine kinases. The receptors for growth hormone and prolactin are single-pass transmembrane proteins that define a separate family (Boutin et al., 1988). In addition, there are RFamide peptide-gated channels belonging to the degenerin (DEG)/epithelial Na^+ channel

[1]Living Systems Institute, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK. [2]Howard Hughes Medical Institute, Department of Neurobiology, 200 Longwood Avenue, Boston, MA 02115, USA. [3]MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, CB2 0QH, UK. [4]Technical University of Munich, TUM School of Life Sciences, ZIEL – Institute for Food and Health, 85354 Freising, Germany. [5]Vrije Universiteit – Ecological Science, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands. [6]Volen Center for Complex Systems, Brandeis University, Mailstop 013, 415 South Street, Waltham, MA 02454, USA. [7]Biological Sciences, Highfield Campus, University of Southampton, Southampton, SO17 1BJ, UK.

*Authors for correspondence (g.jekely@exeter.ac.uk; l.m.holden-dye@soton.ac.uk)

G.J., 0000-0001-8496-9836; S.M., 0000-0001-6028-9764; I.C.G.K., 0000-0002-9085-4274; J.K., 0000-0001-8188-3439; S.H., 0000-0002-9125-2689; L.H., 0000-0002-9704-1217

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Glossary

Allohormonal
The transfer of a substance from one individual to another member of the same species. The allohormone induces a physiological effect that is typically related to some aspect of sexual selection or reproductive function.

Antidiuretic
An antidiuretic substance is one that reduces the loss of water in the urine by increasing water resorption in the kidney. In mammals, antidiuretic hormone (ADH) is released in response to a drop in blood volume or increase in blood osmolarity.

Axon
A cable-like process that extends from the cell body of a neuron towards its target cell. This may be another neuron or a target tissue (e.g. a muscle cell).

Burstiness
A term used to describe a particular pattern of neuronal activity in which the neuron exhibits short periods of rapid activity in the form of action potentials that are interspersed with periods of quiescence.

Connectomics
This refers to the detailed anatomical mapping of neural networks in which the synaptic connectivity between each and every neuron, a map called the connectome, is defined.

De-orphanization
An orphan receptor is a receptor for which the endogenous, cognate ligand is unknown. A vast number of these have been identified by bioinformatic screening of animal genomes. De-orphanization is the process by which the receptor is paired with its ligand and is an important route to understanding the functional role of orphan receptors.

FMRFamide-like peptide precursors (FLPs)
These are a large family of prepropeptide neuropeptide precursors encoded by *C. elegans flp* genes that give rise to C-terminally amidated neuropeptides (Li and Kim, 2014).

Neuropeptide-like peptide precursors (NLPs)
These are a large family of prepropeptide neuropeptide precursors encoded by *C. elegans nlp* genes (Nathoo et al., 2001).

Nociceptive circuit
Nociception is the detection of a noxious, harmful, potentially tissue damaging stimulus. A nociceptive circuit is a neural pathway that mediates the sensory detection of the stimulus. It is typically a component of the behavioural, affective response of the animal to a harmful stimulus, i.e. pain. However, pain, e.g. neuropathic pain, can occur in the absence of nociception.

Opioid
A drug that binds to opiate receptors, e.g. morphine.

Orexigenic and anorexigenic
An orexigenic substance is one that stimulates appetite, whereas an anorexigenic substance decreases appetite. The neuropeptide orexin was named because of its appetite stimulating action. As with many neuropeptides, its name does not convey its breadth of physiological roles. Orexin is also a key regulator of wakefulness and a lack of orexin signalling in the brain is a cause of narcolepsy.

Pressor effect
A pressor substance is one that leads to an elevation in blood pressure.

RFamide neuropeptide
This is a family of neuropeptides that are characterized by a common carboxy-terminal sequence consisting of arginine followed by phenylalanine which is amidated at the C terminus.

Stomatogastric ganglion
This is a cluster of neurons that are part of the stomatogastric nervous system in arthropods. It has been extensively studied in decapod crustaceans where it controls the activity of the stomach muscles and regulates feeding.

Volume transmission
This is a mechanism whereby a neurotransmitter is released from a neuron into the extracellular space, diluted in the extracellular fluid volume and diffuses to receptors at a distance from the release site. This form of communication may be limited by the stability of the neurotransmitter in the presence of extracellular enzymes and typically the cognate receptor has a high affinity for the neurotransmitter due to the low concentrations that may diffuse to the target site.

(ENaC) family (Cottrell et al., 1990; Lingueglia et al., 1995; Assmann et al., 2014; Dürrnagel et al., 2010; Golubovic et al., 2007; Furukawa et al., 2006; Lingueglia et al., 2006).

Complex regulation of proneuropeptide and receptor gene expression

Differential regulation is possible along every step of the path, from transcription of genes encoding peptides and receptors to the binding and activation of the receptors by their cognate peptide ligands and the resulting downstream effects (Fig. 1D) allowing for regulation that is either immediate and transient, or sustained.

One such sustained effect is provided by the state-dependent transcription of genes encoding neuropeptides and their receptors (Amir-Zilberstein et al., 2012; Fukuchi et al., 2004; Knight et al., 2012; MacArthur and Eiden, 1996; Rojo Romanos et al., 2017; Sonnenberg et al., 1989). For example, fasting increases the expression of agouti-related peptide (AgRP) in the hypothalamus, but decreases POMC expression in the pituitary, two prohormones that regulate homeostasis and have orexigenic and anorexigenic effects (see Glossary), respectively (Varela and Horvath, 2012). In addition, several peptide transcripts have been shown to fluctuate with the circadian clock or the oestrous cycle in the female mouse *Mus musculus* to drive associated behaviours (Aton et al., 2005; Dey et al., 2015; Reghunandanan et al., 1993).

Intricate processing and sorting of neuropeptides

Processing of proneuropeptides involves many distinct enzymes localized to the secretory pathway, with occasional tissue-specific variation (Bicknell, 2008). Following signal peptide cleavage, proneuropeptides are cleaved at mono- or di-basic sites by two types of proteases, cathepsin L and the subtilisin-like prohormone convertases. Further processing of peptide intermediates by amino- or carboxy-peptidases removes the remaining N- or C-terminal basic residues (Funkelstein et al., 2010; Hook et al., 2008; Yasothornsrikul et al., 2003). The peptides often undergo amidation, during which dedicated enzymes convert a C-terminal Gly residue into an α-amide group (–$CONH_2$) (Eipper et al., 1992) (Fig. 1D).

Mature neuropeptides are sorted and stored in dense core vesicles (DCVs), which are larger in diameter (100–200 nm) than the small clear vesicles (SCVs; 40–60 nm) that contain classical small-molecule neurotransmitters. Differential sorting of peptides can also contribute to the fine-tuning of signalling (Sossin et al., 1990). Studies are revealing the detailed mechanisms involved in the allocation of peptides to their designate secretory vesicles (Dikeakos and Reudelhuber, 2007; Zhang et al., 2010). Different neuropeptides expressed in the same cell are often found to colocalize in single DCVs. However, there is evidence that neuropeptides can be sorted into different vesicles, even if they derive from the same precursor (Landry et al., 2003; Perello et al., 2008). The N- and C-terminal-derived peptides from the thyrotropin-releasing hormone (TRH) precursor, for example, are sorted into different secretory vesicles (Perello et al., 2008). Whether and how differential sorting might be regulated in a state-dependent manner and influence synergistic actions of peptides is a remaining question.

Regulation of release

Release of neuropeptides can occur from local projections in the proximity of the neuronal soma, along the length of neurites or from the terminals of long-range projections distant from the soma. Peptides can act at the synapse, diffuse locally to mediate volume transmission (see Glossary; Agnati et al., 2010) or be released into the blood stream at neurohemal sites and act at a distance. The regulation

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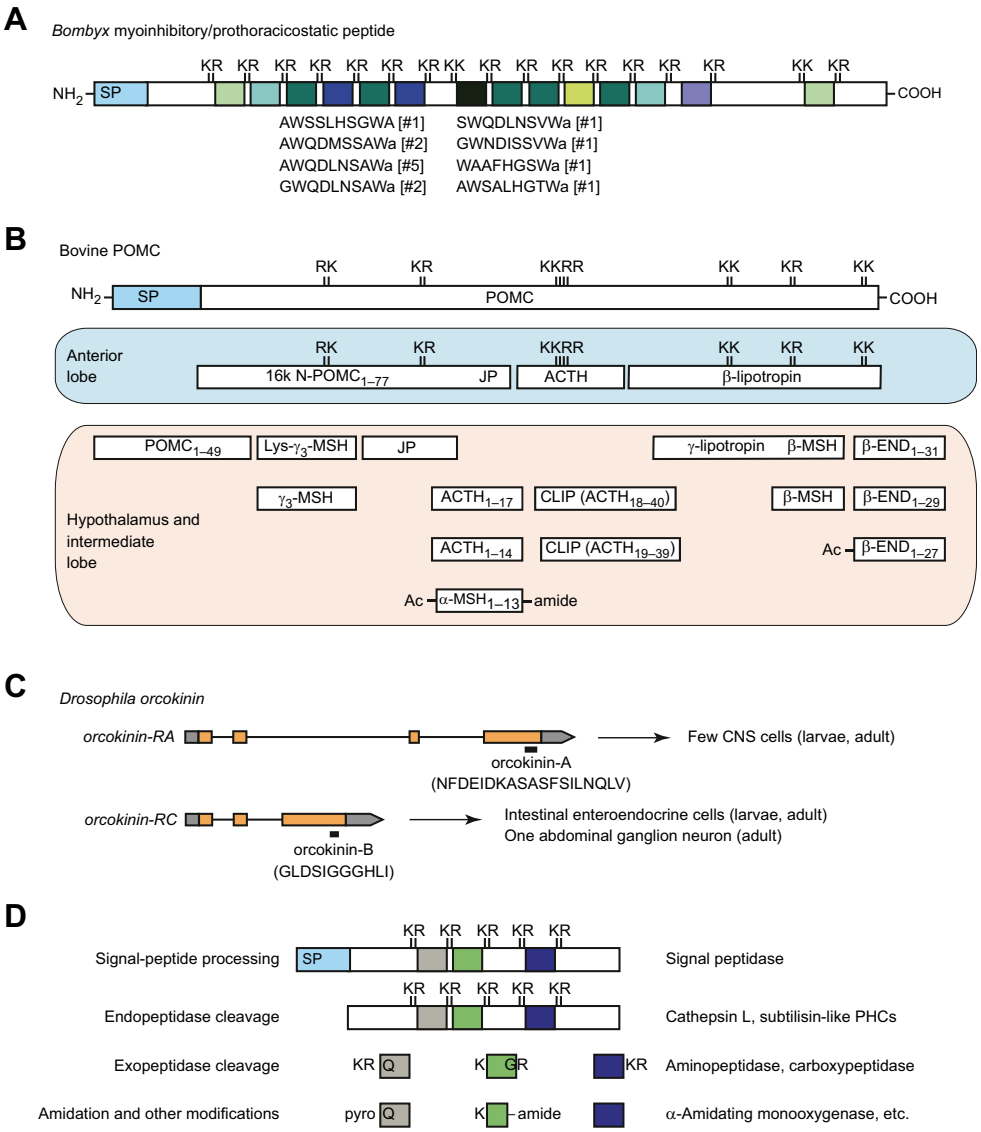

Fig. 1. Proneuropeptides and processing. (A) Structure of the myoinhibitory peptide precursor in *Bombyx mori*. SP, signal peptide; KR or KK, dibasic cleavage sites. The precursor generates the eight neuropeptides listed below the diagram. The number in brackets after each peptide indicates the number of copies produced from one precursor molecule. (B) Tissue-specific processing of the bovine proopiomelanocortin (POMC) precursor. At the top, the organization of the peptide precursor molecule with the SP and dibasic cleavage sites is shown. The two panels underneath show processing in the anterior lobe of the mammalian pituitary and in the intermediate lobe and hypothalamus, which generates neuropeptides as indicated. (C) Alternative splicing of the *Drosophila orcokinin* gene generates two isoforms with tissue-specific expression. (D) Generalized summary of the steps of proneuropeptide processing.

of the timing and site of peptide release opens additional opportunities for activity-dependent regulation of peptide action (Fig. 2). Bursts of action potentials (Bicknell and Leng, 1981) or direct neuropeptide actions can lead to prolonged increases in Ca^{2+} levels at axon (see Glossary) terminals (Iremonger et al., 2017) to stimulate neuropeptide release. Oxytocin demonstrates an interesting case where axonal and dendritic release can be regulated differentially by action potentials and release of Ca^{2+} from intracellular stores (Ludwig et al., 2002).

Receptor-mediated responses on different timescales

Neuropeptide signalling through GPCRs can regulate gene transcription leading to the reprogramming of neuronal metabolism and responsiveness. In addition, suppression of GPCR signalling for up to several hours can be mediated by β-arrestin-dependent desensitization and internalization of receptors (reviewed in Kovacs et al., 2009). Thus peptidergic networks are regulated by the history of their own activation. The opioid system is a classic example of a system that displays differential downstream effects as well as undergoes long-term changes that lead to tolerance and addiction to opioids (reviewed in Christie, 2008). Morphine and the endogenous ligand enkephalin differentially affect ubiquitylation of μ-opiate receptors through the recruitment of distinct isoforms of β-arrestin with morphine recruiting β-arrestin-2 whereas enkephalin engages both β-arrestin-1 and -2 (Groer et al., 2011). This β-arrestin-mediated desensitization underlies the development of tolerance in the use of morphine for pain relief (Bohn et al., 1999).

Distinct synaptic and neuropeptidergic actions

In many cases, peptide receptors are expressed on cell types that are distinct from, or at least only partially overlap with, those that are directly synaptically targeted by a given peptidergic neuron (Fig. 2). For example, vasoactive intestinal peptide (VIP)-expressing neurons in the cerebral cortex of mice do not connect synaptically with pyramidal cells, whereas VIP receptors are widely distributed on different cell types, including pyramidal cells (Pi et al., 2013; Tasic et al., 2016). This uncoupling has important ramifications for the application of conventional ‘connectomics’ (see Glossary) techniques to map peptidergic connections (but see Schlegel et al., 2016; Shahidi et al., 2015 for examples of how to map neuropeptides to connectomes).

Mapping neuropeptide signalling networks

Nematode and annelid networks

The nervous systems of invertebrates, with a small number of identifiable neurons (White et al., 1986), as well as microcircuits

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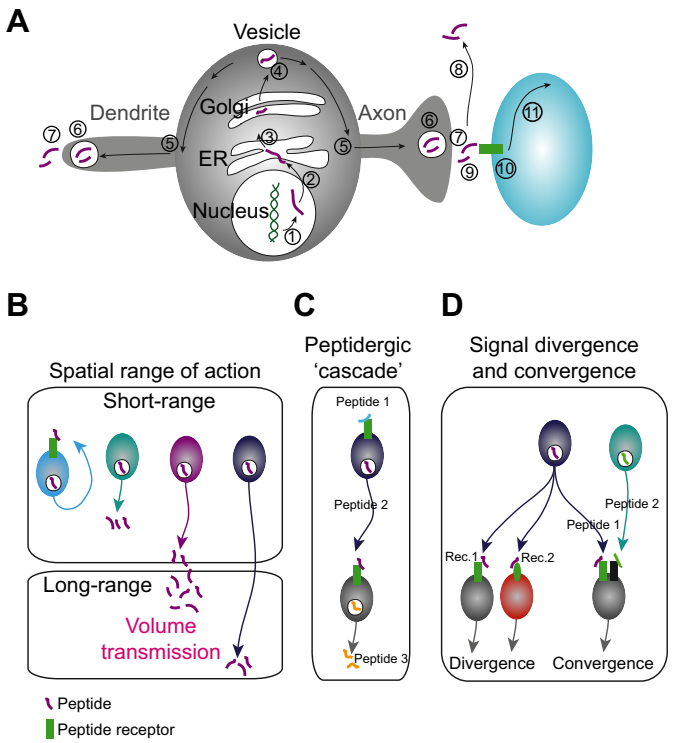

Fig. 2. Regulation of peptidergic signalling. (A) Release and reception of neuropeptide signals can be regulated during (1) transcription of the proneuropeptide gene, (2) translation into a proneuropeptide, (3) post-translational processing in the endoplasmic reticulum (ER), (4) sorting into Golgi vesicles, (5) vesicular transport, (6) localization to the readily releasable pool or priming, (7) release, (8) diffusion and degradation, (9) binding to receptors, (10) owing to expression and regulation of receptors and (11) during regulation of ensuing signalling cascade. (B) Neuropeptides can signal across different ranges. Two short-range examples are shown enabling signalling to self (auto) or neighbouring cells, plus two long-range examples signalling through non-synaptic volume transmission. (C) Neuropeptide signalling can be organized into neuropeptide cascades. (D) Neuropeptide signals can be divergent, in which the same peptide activates different receptors (Rec.1, etc.) on the same or different target cells, leading to different signalling responses, or convergent, in which a number of different peptides must be present to activate associated receptors on the same cell, leading to a single signalling response.

within exceptionally well-defined systems (Nusbaum et al., 2017), are accessible to the cellular level mapping of both extra-synaptic and synaptic peptidergic networks.

This has been played out to great effect in the nematode *C. elegans*. The synaptic connectome of the 302 neurons of hermaphrodites has been completely mapped at the level of electron microscopy and can easily be integrated with gene expression information owing to the stereotypical anatomy of the nematode nervous system (White et al., 1986). A comprehensive analysis of published ligand–receptor interactions and gene expression data recently revealed a draft connectome of monoamine signalling in *C. elegans*, as well as a partial network of neuropeptide signalling (Bentley et al., 2016) (Fig. 3A). A remarkably high fraction of signalling in these modulatory networks seems to be extrasynaptic (Bargmann, 2012; Ludwig and Leng, 2006; Marder, 2012).

The larval nervous system of the marine annelid *Platynereis dumerilii* also provides an excellent platform for revealing peptidergic networks. This has been achieved through large-scale approaches to analyse gene expression through whole-mount *in situ* hybridization and single-cell transcriptomics techniques to facilitate the localization of neuropeptide and receptor gene expression (Achim et al., 2015; Asadulina et al., 2012). In addition, serial-section electron microscopy allows the reconstruction of full-body neural circuits in the small *Platynereis* larva (Randel et al., 2015). The use of serial immunogold labelling with antibodies to neuropeptides led to the direct mapping of several neuropeptides onto the synaptic connectome (Shahidi et al., 2015). These resources facilitate the reconstruction of peptidergic connectivity networks between neurons, where peptide-producing cells represent the source cells, and neurons expressing the corresponding receptor represent the target cells (Williams et al., 2017). Interestingly, the highest expression of neuropeptides and receptors mapped to the anterior neurosecretory region of the larva, known as the 'apical organ'. Single peptidergic neurons co-expressed up to 20 distinct neuropeptide precursor genes. Parallel mapping of the synaptic connectome of this neurosecretory area by serial-section electron microscopy revealed the paucity of chemical synapses in this region of the brain (Fig. 3B). This finding suggests that the apical neurosecretory centre functions as a 'chemical brain', where neuronal communication is defined by peptide and receptor expression, and not by synaptic wiring. In the *Platynereis* larval brain, individual peptide–receptor pathways can be very specific, connecting only a small fraction of all the neurons (Fig. 3B). The majority of neuropeptide receptors in this neurosecretory centre of the larval brain are activated by only one or two related peptides, and, on average, the individual pathways signal between 1% of the neurons in this region (Williams et al., 2017).

A principle that emerges from these mapping studies is the low degree of overlap between peptidergic and synaptic connectomes. Nevertheless, there are crucial interaction points where communication clearly occurs between the different layers of a multiplex neural network, as recently illustrated in the *Platynereis* larval brain, *C. elegans* and *Drosophila* (Bentley et al., 2016; Schlegel et al., 2016; Williams et al., 2017). The neuropeptide and synaptic connectivity maps of these small invertebrate circuits provide a basis to study the role of specific neuropeptides in microcircuits with known connectivity, and represent prototypes for understanding how neuropeptides interact with wired circuitry in larger nervous systems. Single-cell transcriptome datasets of neural tissue represent a rich source of information for the reconstruction of peptidergic signalling networks in the brain. If these datasets are of sufficient quality and depth, they have the potential to reveal the entire neuropeptidome of a neuron, as well as the complement of neuropeptide receptors (Campbell et al., 2017; Romanov et al., 2017; Tasic et al., 2016).

Murine networks

To understand the function of peptidergic connectomes, it is of great importance to supplement the knowledge of putative peptidergic connections between neurons with functional analysis. For example, oxytocin neurons in the mammalian hypothalamus project to several distant brain areas, including the cerebellar cortex, where they exert their actions through oxytocin receptors that are enriched in relatively small subpopulations of interneurons (Li et al., 2016; Tasic et al., 2016). Other examples for ascending long-range peptidergic systems in the rodent brain are the relaxin and orexin systems; the relaxin and orexin peptides are synthetized in a small number of cells in the hypothalamus and brain stem, respectively, but send long-range projections throughout the whole brain (for reviews, see Ebrahim et al., 2002; Smith et al., 2014). Characterization of these pathways has been pursued through optogenetic activation of defined subsets of

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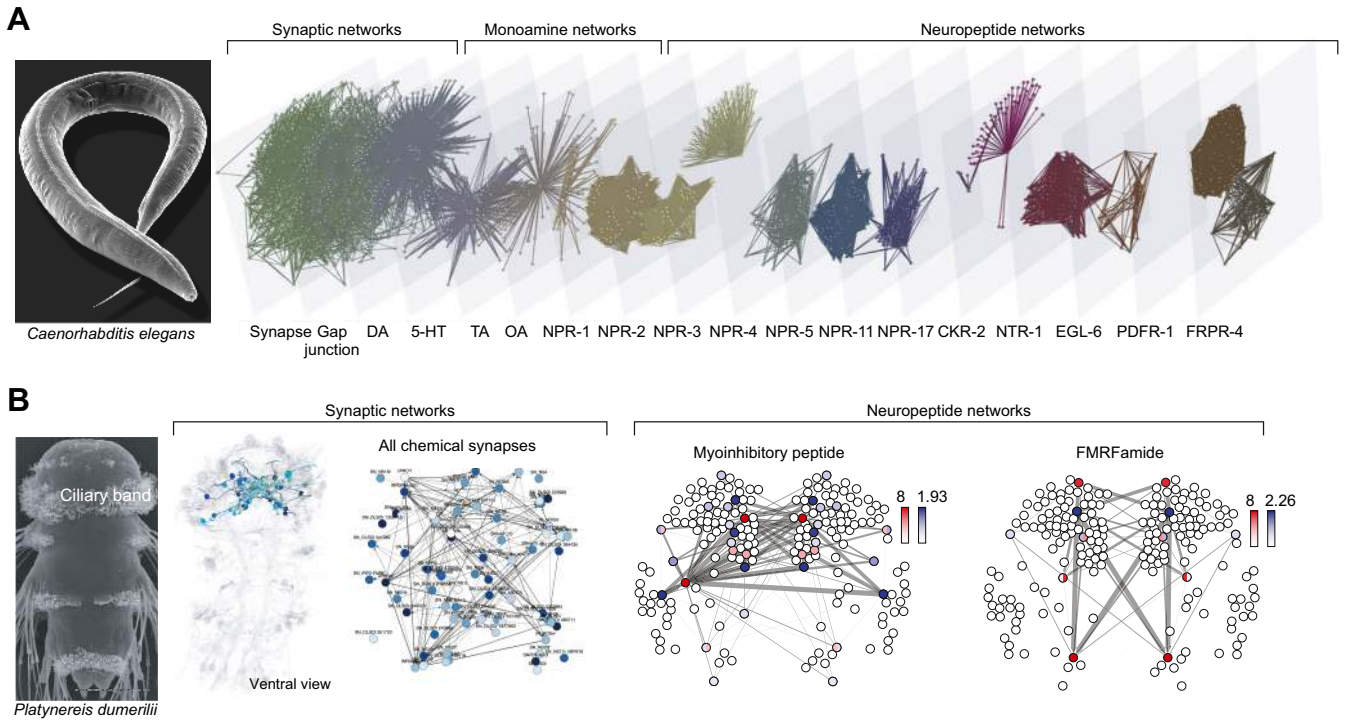

Fig. 3. Analysis of global neuropeptide connectivity in small nervous systems. (A) Multilayer representation of synaptic (chemical synapses and gap junctions), monoamine and neuropeptide networks in the nematode *C. elegans*. Nodes correspond to individual cells. (B) Mapping neuropeptidergic connections in the larval nervous system of the marine annelid *Platynereis dumerilii*. All neuroendocrine cells in the anterior brain and their synaptic connectome were reconstructed by serial electron microscopy (EM). Neuropeptidegic networks can be mapped from single-cell transcriptomic data. Images reproduced from Bentley et al. (2016) and Williams et al. (2017). Scanning EM images courtesy of Jürgen Berger. FMRFamide, Phe-Met-Arg-Phe neuropeptide; DA, dopamine; 5-HT, 5-hydroxytryptamine (serotonin); TA, tyramine; OA, octopamine; CKR2, cholecystokinin; NTR-1, oxytocin/vasopressin; NPR1-5/11/17, neuropeptide F/Y receptors.

peptidergic cells and the study of their postsynaptic effects, as illustrated by the characterization of the extensive axon networks and postsynaptic partners of hypothalamic oxytocin neurons throughout the brain and in the amygdala (Knobloch et al., 2012). Alternatively, the Cre-recombinase-dependent expression of channelrhodopsin allows the specific activation of peptidergic neurons in combination with different Cre-expressing mouse lines (e.g. Somatostatin-Cre, Oxytocin-Cre and Vip-Cre) (Melzer et al., 2012; Sutton et al., 2014; Taniguchi et al., 2011). One caveat of this system is that optogenetic activation can fail to trigger the release of some peptides (Steuer Costa et al., 2017). Specific Cre-driver lines can also be used to express Ca^{2+}-dependent fluorescent proteins enabling the study of the activity of peptidergic neurons or their putative postsynaptic partners (Nakai et al., 2001) or allow the use of optogenetic tagging in electrophysiological recordings (Lima et al., 2009) (Fig. 4A).

Microdialysis and tissue extraction followed by analysis of peptide content through mass spectrometry, enzyme-linked immunosorbent assay (ELISA) or radiolabelling has been used to track the context-dependent release of neuropeptides (Fig. 4B). Novel techniques have also allowed the visualization of neuromodulator release *in vivo*. For example, cell-based neurotransmitter fluorescent engineered reporters (CNiFERs) are receptor-overexpressing cultured cells that track neuromodulator release and binding through increases in Ca^{2+}-dependent fluorescence (Nguyen et al., 2010) (Fig. 4C). Overexpression of modified versions of GPCRs and β-arrestin result in the activation of a reporter gene upon ligand binding and β-arrestin recruitment (Inagaki et al., 2012; Kono et al., 2014). Reporter activation can also be rendered light dependent (iTango), enabling the analysis of certain behavioural states (Lee et al., 2017) (Fig. 4D). Together, these systems present promising tools for the mapping of neuropeptide networks in different animals.

Organization of multi-channel neuropeptide signalling

Organizational motifs

Besides highly specific neuropeptide–receptor pathways, several examples illustrate the existence of complex multichannel signalling networks, cascades and crosstalk among neuropeptides and their receptors. These organizational motifs can provide mechanisms for feedback, coordination or sensory integration to fine-tune the output of neuronal circuits (Komuniecki et al., 2014) (Fig. 2). Several network motifs are possible. For example, a single neuropeptide, or peptides from the same precursor, can act on multiple distinct receptors. Thus, the response to the neuropeptide will be dependent on the receptor with which it interacts, which in turn can be regulated by differential receptor expression. Indeed, a typical feature of peptidergic signalling is the presence of multiple distinct subtypes of GPCR for the same neuropeptide, which often couple to different signal transduction cascades, that are expressed in different tissues and are characterized by distinct pharmacology (Alexander et al., 2015).

Divergent and convergent neuropeptide signalling

A good example of divergent signalling is provided by the neuropeptide vasopressin (VP), also known as antidiuretic hormone (ADH). In mammals, VP is released from the posterior pituitary to maintain blood pressure, through a pressor effect (see Glossary) mediated by V1 receptors on resistance blood vessels, and blood volume, through an antidiuretic effect (see Glossary) requiring V2 receptors in the kidney cells. V1 receptor subtypes are also expressed in the brain and mediate effects on social behaviour (McCall and

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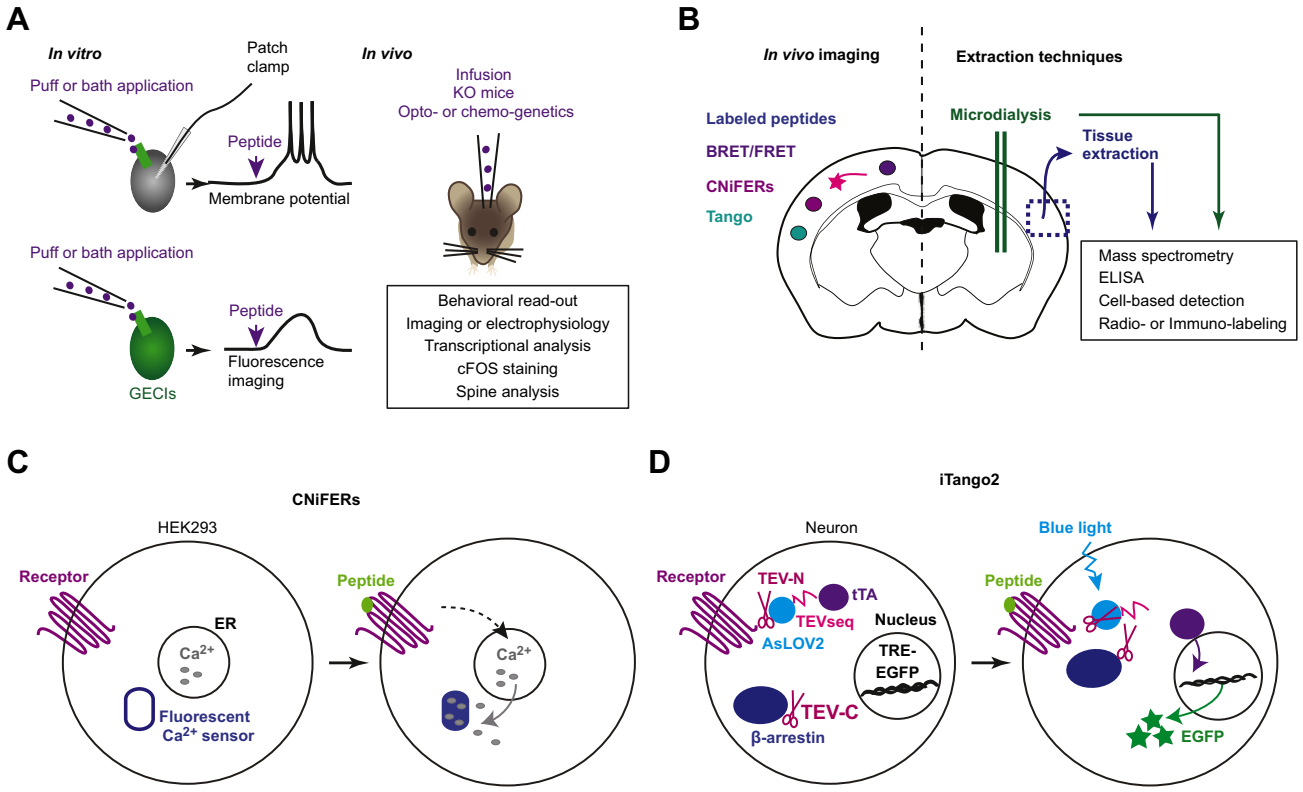

Fig. 4. Methods to study neuropeptide signalling and release in mouse. (A) Bath application of neuropeptides combined with patch-clamp recording or Ca^{2+} imaging with genetically encoded Ca^{2+} indicators (GECIs). (B) The binding of labelled peptides or the release of endogenous peptides can be studied by imaging methods. Released peptides can be recovered by microdialysis followed by mass spectrometric or ELISA analysis. (C) Schematic of the use of CNiFERs to study neuropeptide release. Engineered cells express a receptor and fluoresce upon ligand binding. (D) The use of the iTango2 method to study neuropeptide release. A reporter is activated by a light stimulus allowing precise temporal and spatial analysis.

Singer, 2012; Park and Kwon, 2015; Stoop, 2012). This multifaceted physiological role of VP in mammals resonates with recent studies in *C. elegans*. Here, a vasopressin homologue, nematocin (Elphick and Rowe, 2009), has been shown to regulate reproductive behaviour and behavioural plasticity through distinct receptors (Fig. 5). On the one hand, nematocin promotes gustatory associative learning by activating the nematocin receptor NTR-1 in gustatory neurons (Beets et al., 2012). On the other, it drives male mating through NTR-1 and a second receptor NTR-2 that each modulates partly overlapping aspects of the mating behaviour (Garrison et al., 2012).

The divergent signalling described above, in which a single neuropeptide exerts a repertoire of responses by acting in different tissues expressing distinct receptor subtypes, is paralleled by the occurrence of convergent signalling in which multiple neuropeptides converge on the same neuron (Li and Kim, 2008; van den Pol, 2012; Williams et al., 2017). For example, in the sea hare *Aplysia*, a cholinergic command-like neuron for feeding contains two neuropeptides, feeding circuit-activating peptide (FCAP) and cerebral peptide 2 (CP2). The two peptides are co-released and act synergistically to increase the postsynaptic potential in the same

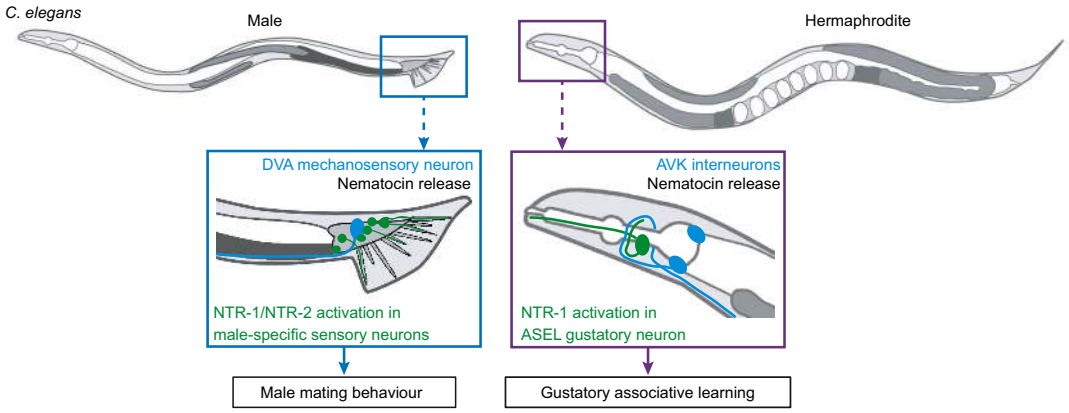

Fig. 5. Studying the role of neuropeptide signalling in *C. elegans*. The vasopressin homologue nematocin signals in distinct cellular contexts to effect mating behaviour in males and gustatory associative learning in hermaphrodites. The male nematocin neurons are shown in blue and the neurons expressing the receptors NTR-1 and NTR-2 are shown in green.

downstream neuron; FCAP increases the quantal size, and CP2 the quantal content of excitatory postsynaptic potentials (Koh et al., 2003).

Multi-channel signalling and crosstalk

The same peptidergic neuron can co-express multiple distinct neuropeptides that can act on different targets. Such 'multi-channel wiring' is not characteristic of synaptic networks and represents a distinct organizational principle for neuropeptides. In the stomatogastric ganglion (see Glossary) of the lobster *Homarus americanus*, red pigment-concentrating hormone and tachykinin are colocalized and co-released, but act on different neurons (Thirumalai and Marder, 2002). The same neuropeptide released from different cells can also have different effects on the same motor circuit, depending on the mixture of co-transmitters (Blitz et al., 1999; Wood et al., 2000). How many of the peptides can be co-released at any one time is unknown, but transcriptome data suggest that multichannel signalling could be a common theme in highly peptidergic neurosecretory brain areas (Campbell et al., 2017; Williams et al., 2017).

Peptide-expressing cells also often express peptide receptors, and they are thus both sources and targets of neuromodulators. There are numerous examples of such peptidergic cascades of intercellular communication in vertebrates, which typically involve homoeostatic feedback from a peripheral tissue to regulate release of a neurohormone. An interesting example is the 'hunger hormone' ghrelin that derives from cells in the gastrointestinal tract and directly activates hypothalamic neurons to trigger the release of growth hormone-releasing hormone (somatoliberin) in a synaptic-transmission-independent manner. This peptidergic multi-neuronal communication is regulated by food deprivation and directly controls energy consumption and body weight (Osterstock et al., 2010).

Neuropeptides derived from different precursors can also crosstalk by acting on the same cognate receptor. Significant evidence for this has come from GPCR de-orphanization (see Glossary) and functional characterization in *C. elegans*. In *C. elegans* hermaphrodites, egg-laying behaviour is regulated by RFamide neuropeptides (FLPs) (see Glossary) from FLP-10 and FLP-17 precursors that all activate a single neuropeptide receptor, EGL-6, in the hermaphrodite-specific neurons (HSNs) of the egg-laying circuit (Ringstad and Horvitz, 2008). Peptides encoded by FLP-17 are expressed in a pair of CO_2 sensory neurons, whereas FLP-10 peptides are synthesized in several other neuronal and non-neuronal tissues. Genetic and neural ablation experiments support a simple model in which relevant sensory cues control FLP-10 and FLP-17 secretion, and thereby directly modulate the activity of the egg-laying motor neurons to suppress egg laying in unfavourable conditions (Ringstad and Horvitz, 2008). In this model, crosstalk of neuropeptides acting on the same receptor integrates multiple inputs for the modulation of behaviours. There is also increasing evidence for crosstalk of RFamide neuropeptides in mammals (Liu and Herbison, 2016; Ma et al., 2009; Oishi et al., 2011). For example, neuropeptide FF receptors (NPFFR1 and NPFFR2) have recently been shown to bind to kisspeptin and other mammalian RFamide neuropeptides and likely mediate the modulatory effects of these peptides in nociceptive circuits (see Glossary) (Elhabazi et al., 2013; Lyubimov et al., 2010; Oishi et al., 2011).

Organization of multi-peptide signalling networks at the circuit level

Peptidergic circuits in *C. elegans* and *Drosophila*

Genetic studies in model organisms are starting to uncover the functional relevance of interacting neuropeptide pathways. For example, in *C. elegans*, a neuropeptide-mediated sensorimotor feedback loop dampens the odour-evoked activity of the olfactory amphid wing 'C' (AWC) neurons (Chalasani et al., 2010). When odour is sensed, AWC neurons release buccalin-related NLP-1 neuropeptides, which activate a neuropeptide receptor (NPR-11) on downstream interneurons to modulate secretion of the insulin-like peptide INS-1. Closing the feedback loop, INS-1 modulates the responsiveness of AWC neurons to olfactory stimuli. In *Drosophila*, the coordination of the stereotypic ecdysis behaviour also depends on crosstalk of multiple neuropeptides (Mena et al., 2016). The behavioural state is initiated by the release of ecdysis-triggering hormone (ETH) (Zitnan and Adams, 2012; Zitnan et al., 1996). Neurons expressing the crustacean cardioactive peptide (CCAP) are one of the key targets of ETH that control the timing and behaviour of the moulting. While the activity of CCAP neurons is directly regulated by ETH, it also depends on the actions of other neuropeptides downstream of ETH, such as bursicon and eclosion hormone (Mena et al., 2016).

Crustacean stomatogastric circuit

The stomatogastric ganglion (STG) is a small central-pattern-generating circuit consisting of 26–30 neurons that is responsible for generating the rhythmic patterns of muscle movements in the crustacean stomach (Fig. 6). The extensive work on this system has recently been reviewed (Nusbaum et al., 2017) and shows that the effects of neuropeptides and monoamines on the STG are different. While dopamine and serotonin modulate many different membrane currents (Kiehn and Harris-Warrick, 1992; Kloppenburg et al., 1999; Krenz et al., 2013, 2015; Peck et al., 2001, 2006; Rodgers et al., 2013; Zhang and Harris-Warrick, 1994, 1995), many neuropeptides converge to activate the same voltage-dependent current (Golowasch and Marder, 1992; Swensen and Marder, 2000, 2001). This current, aptly named the 'modulatory inward current' (I_{MI}), is a voltage-dependent current with characteristics that make it ideally suited to activate rhythmic networks. I_{MI} is a small depolarizing mixed-cation current with similarities to the glutamatergic N-methyl-D-aspartate (NMDA) current (Golowasch and Marder, 1992; Swensen and Marder, 2000). The result of activating I_{MI} is an increase in the so-called 'burstiness' of a neuron (see Glossary), often resulting in more spikes per burst. In the circuit that generates the pyloric rhythm in the STG, comprised of bursting neurons connected with reciprocally inhibitory graded synapses, activating I_{MI} can result in more-prominent bursting of all neurons in the network. This arises from neurons in the network rebounding proportionately to the strength of incoming inhibition.

Endowing a circuit with robustness

The convergence of multiple peptide modulators, each acting through their own specific receptors on I_{MI}, represents one of the examples of degeneracy in the circuit and a mechanism that protects the circuit from over-modulation. The maximal conductance of I_{MI} is an intrinsic property of the neuron and will not be exceeded even in the presence of additional ligands (Swensen and Marder, 2000). In a recent study, modulatory substances were applied exogenously in the absence of all other modulatory input to investigate how neuromodulators affect the pyloric rhythm across different temperatures (Haddad and Marder, 2017, preprint) (Fig. 6). The neuropeptide proctolin or the muscarinic-cholinergic agonist oxotremorine, both of which activate I_{MI} (Swensen and Marder, 2000), protected the networks from temperature perturbation. In contrast, serotonin, which activates multiple conductances on multiple cell types (Kiehn and Harris-Warrick, 1992; Krenz et al., 2015; Zhang and Harris-Warrick, 1994), made the

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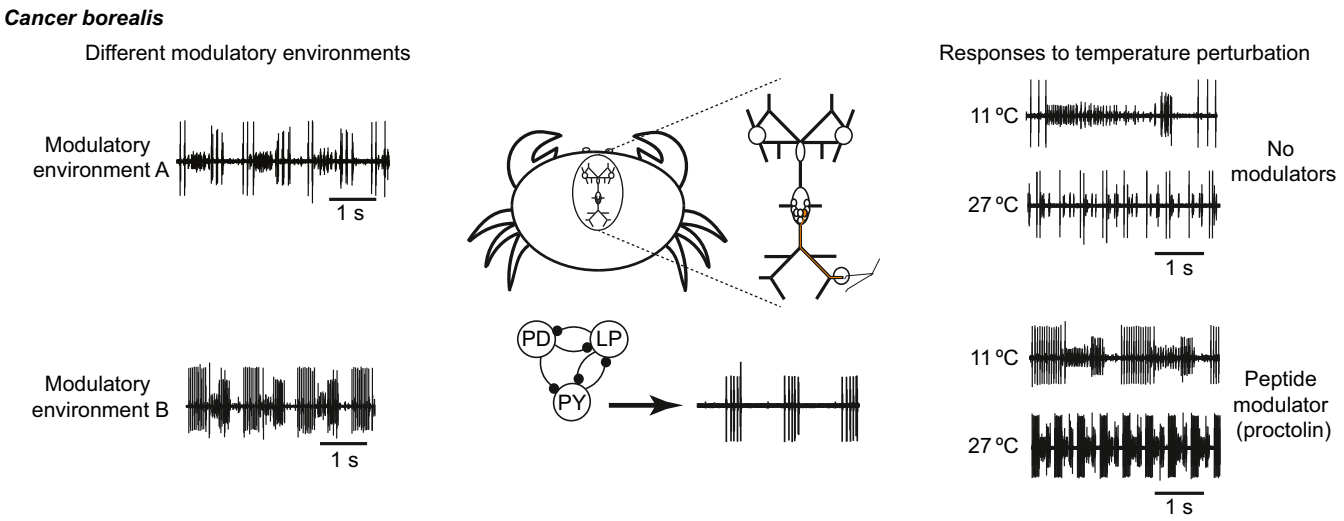

Fig. 6. Studying neuromodulation and circuit robustness in the stomatogastric ganglion of *C. borealis*. The stomatogastric ganglion (STG) is modulated by tens of substances, including peptides, hormonally through the hemolymph and from descending modulatory neural inputs. Different modulatory environments allow for the same structural network (displayed by a simplified wiring diagram of three neurons connected with reciprocal inhibition) to produce multiple behavioural outputs. Peptide neuromodulators can increase the robustness of network output in response to temperature perturbation. The rhythm is altered at 27°C when no modulators are present. The characteristic rhythm is robust to temperature increase in the presence of a peptide modulatory substance.

networks more temperature sensitive than in the complete absence of modulators, and each animal produced different patterns of abnormal activity at high temperature (Haddad and Marder, 2017, preprint). Other mechanisms that protect the circuit from over-modulation include a balance of modulatory substances that act on opposing properties, and of modulators that act at various sites, such as on motor neurons and muscles (Marder, 2012).

Organization of peptidergic networks to provide context dependence

Approaches for studying context dependence

An important challenge is to bridge the cellular to whole-organism level, because this is necessary for the understanding of how neuropeptides contribute to the behavioural flexibility of animals. This can be studied by measuring the effects of increased or decreased levels of peptidergic signalling in a whole-organism context (Fig. 7) (Bargmann and Marder, 2013). Based on work in different model systems, a picture has emerged suggesting that neuropeptides act on many levels of a neural network and can influence sensory perceptions even at the earliest processing level in the brain or periphery. Therefore, peptidergic modulation can impact on cognitive functions by regulating the strength and type of sensory information that passes into the relevant higher brain areas.

Food context and behavioural choice

Several neuropeptides regulate appetite, feeding and food preferences. For example, injection of oxytocin markedly reduces food intake, in particular of sweet foods, in humans (Ott et al., 2013) and oxytocin-knockout mice display an increase in sweet and carbohydrate preference, suggesting that oxytocin modulates sweet gustatory perception and/or sweet taste-predicting reward signals in the central brain (Billings et al., 2006). Although it remains unresolved whether oxytocin modulates gustatory neurons, there is evidence for a role of oxytocin in odour perception, for example social odour (Wacker and Ludwig, 2012). Whether the same is true for food odours is not well understood. In addition, oxytocin receptors (OXTR) are highly expressed in parts of the olfactory system including the anterior olfactory nucleus (AON). A recent study found that oxytocin in rats modulates early olfactory processing through a top-down neuromodulation of OXTR-expressing AON fibres, which increases the glutamatergic synaptic input to interneurons in the olfactory bulb. Removal of OXTR specifically in the AON reduced olfactory exploration and recognition of social odours of conspecifics leading to differences in the behaviour of the animals (Oettl et al., 2016).

Recent work in *Drosophila* highlights the role of neuropeptides in behavioural choice involving food (Itskov and Ribeiro, 2013; Leinwand and Chalasani, 2011; Wang, 2012). Starved flies show higher attraction to food odours and less avoidance of aversive cues. While this behaviour is controlled in part by the mushroom body (Lewis et al., 2015), it is also modulated by neuropeptides acting directly on attractive and repulsive food-odour-detecting chemosensory neurons (Ignell et al., 2009; Root et al., 2011). Short neuropeptide F (sNPF) released by attraction-mediating olfactory sensory neurons enhances, whereas tachykinin reduces, the response of avoidance-mediating olfactory neurons; in both cases, this occurs through GPCRs expressed directly in the olfactory sensory neurons (Ko et al., 2015) (Fig. 7A). An analogous mechanism regulates the strategy of the female fly to find and evaluate egg-laying and feeding sites for her offspring (Hussain et al., 2016a). Mating increases the attraction of females to important and reproductive-success-boosting nutrients, the polyamines (Hussain et al., 2016b), through an increase in the expression of the GPCR sex peptide receptor (SPR) in polyamine-sensing olfactory and gustatory neurons. In this case, myoinhibitory peptides (MIPs), rather than the better known ligand of SPR [sex peptide (SP)], mediate SPR signalling (Fig. 7B). Interestingly, this function of MIP is female specific and does not regulate the attraction of males to polyamines (Hussain et al., 2016a). In both these cases, overexpression of the sNPF receptor or the MIP receptor SPR, respectively, exclusively in peripheral chemosensory neurons is sufficient to switch the fly behavioural or internal state, emphasizing the important role of peripheral modulation in state-dependent behaviour (Leinwand and Chalasani, 2011).

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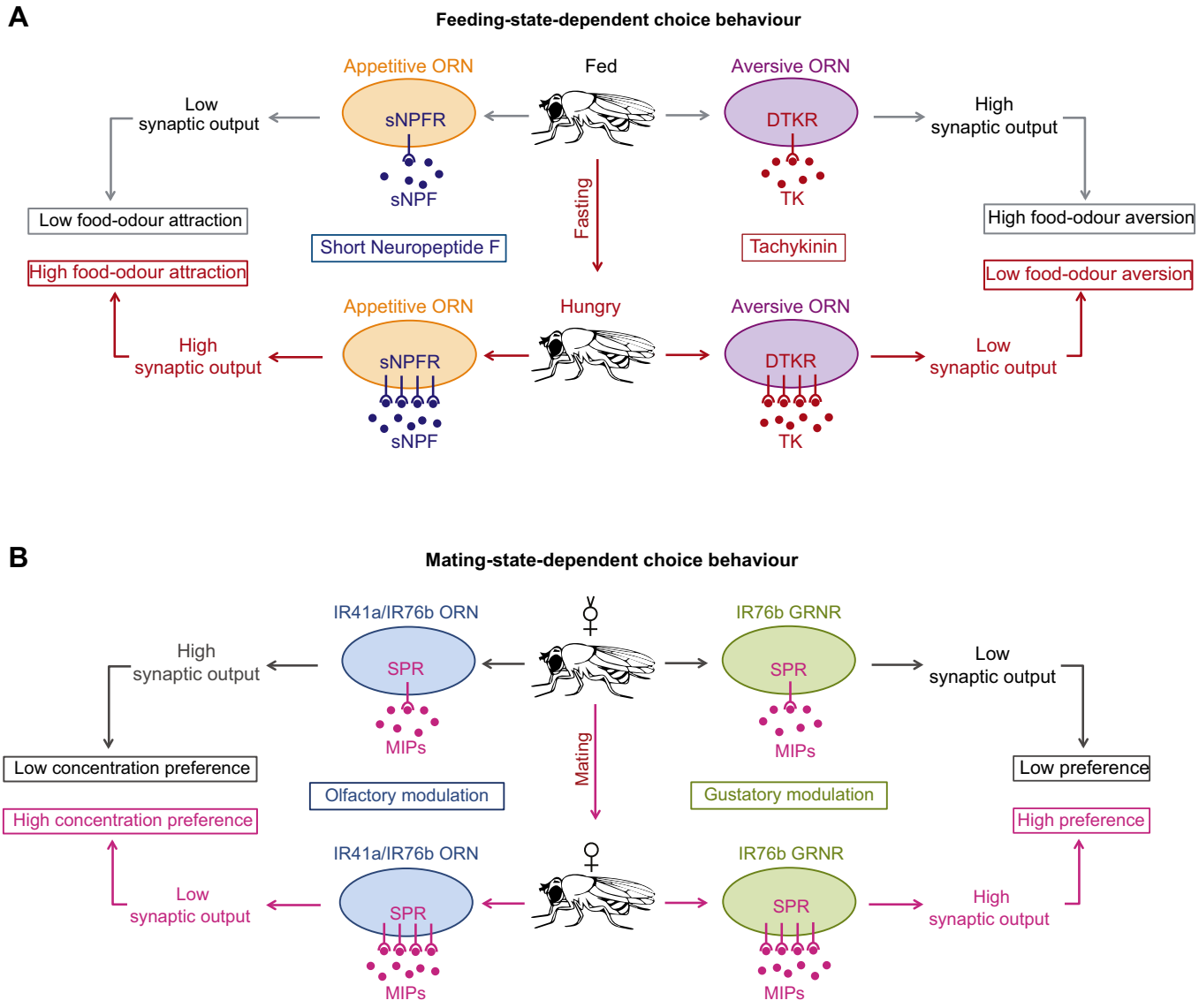

Fig. 7. Studying neuropeptide effects in *Drosophila*. (A) Short neuropeptide F (sNPF) and tachykinin (TK) modulate food odour preference in a feeding-state-dependent manner. Left, starvation upregulates expression of the sNPF receptor in food attraction-mediating olfactory receptor neurons downstream of insulin. sNPF, released from the same neurons, activates the receptor and triggers facilitation at the synapse between the olfactory sensory neuron and the secondary projection neurons. Right, in parallel to sNPF, the TK receptor DTRK is activated in aversion-triggering ORNs by TK, released by local interneurons. This triggers inhibition at ORN–projection neuron synapses. Together, these mechanisms increase attraction to food odors in starved flies. (B) Myoinhibitory peptides (MIPs) regulate mating-state dependent food and oviposition site choice through the sex-peptide receptor in polyamine-sensing olfactory and gustatory neurons. SPR is upregulated in ORNs and gustatory neurons upon mating. MIPs activate SPR signalling and inhibit and activate release from the ORN or GRN, respectively. These modulations induce mated fly-like choice behaviour in virgin females. These examples demonstrate neuropeptidergic modulation at the first level of sensory processing and suggest that internal states lead to information filtering before it can be detected by higher cognitive centres in the brain. DTKR, tachykinin-like peptides receptor; ORN, olfactory receptor neuron; GRNR, gustatory receptor neuron; sNPFR, sNPF receptor; SPR, sex-peptide receptor.

It is likely that MIPs regulate feeding behaviour through additional mechanisms, given their broad expression in the brain and the gut (Veenstra et al., 2008). For instance, a small cluster of MIP-expressing neurons in the central nervous system suppresses feeding and thereby regulates body weight in male and female flies (Min et al., 2016).

Circadian context

The regulation of circadian rhythms by neuropeptides has been the subject of many studies spanning several phyla. MIPs and SPR have been implicated in the control of sleep in *D. melanogaster* (Oh et al., 2014). Another interesting sleep-regulatory peptide in *D. melanogaster* is the so-called pigment-dispersing factor (PDF). PDF-expressing neurons increase arousal during wake states (Sehgal and Mignot, 2011). The PDF neuropeptide also regulates arousal and exploratory behaviour in *C. elegans*. In the nematode, PDF and serotonin function as mutual inhibitors in a neural network that appears to overlay the motor-behaviour-controlling network, acting via an overlapping but not identical circuit, to regulate behavioural state in a slower and potentially more homeostatic manner than that controlling basal locomotor movements (Flavell et al., 2013). In mammals, at least four unrelated neuropeptides, orexin, prokineticin-2, neuropeptide S and vasoactive intestinal peptide (VIP), have similar roles in stimulating arousal in a light-

dependent manner (Chemelli et al., 1999; Cheng et al., 2002; Vosko et al., 2007; Xu et al., 2004).

Social and reproductive context

Neuropeptides also have important roles in complex social, emotional and reproductive behaviours. Interestingly, oxytocin functions in a gender-dependent manner. It is increasingly appreciated that male and female brains differ in certain aspects, and that this is not limited to reproductive control. Differences in cortical oxytocin signalling might explain why men and women show differences in some emotional states and disorders such as anxiety (Li et al., 2016). Specifically, one study has demonstrated that certain OXTR interneurons in male mice regulate anxiety by expressing an antagonist of the stress hormone corticotropin-releasing hormone (CRH), called corticotropin-releasing-hormone-binding protein (CRHBP). CRHBP blocks the CRH-induced potentiation of pyramidal neurons in layer 2/3 of the medial prefrontal cortex selectively in males but not females. This block reduces anxiety in males but not in females. Conversely, the same OXTR interneurons in females modulate social interactions with male mice during the sexually responsive phase of the oestrus cycle (Nakajima et al., 2014).

Inter-organismal neuropeptide signalling

Peptides can have an allohormonal function (see Glossary) (Koene and ter Maat, 2001); for example, accessory gland products that are transferred from one individual to another during the transfer of gametes (Zizzari et al., 2014) and influence the behaviour of the recipient, a classic example being the *Drosophila* sex peptide (Perry et al., 2013). Other species in which this phenomenon has been investigated include species with separate sexes, such as Pletodontid salamanders and seed beetles (e.g. *Callosobruchus maculatus*), but also hermaphroditic species such as flatworms (e.g. *Macrostomum lignano*), land snails (e.g. *Cornu aspersum*) and pond snails (e.g. *Lymnaea stagnalis*) (Yamane et al., 2015; Arbore et al., 2015; Stewart et al., 2016; Watts et al., 2004; Koene et al., 2010). Some of the identified accessory gland products are neuropeptides, including the 'love dart' allohormone, a buccalin-like peptide in the common garden snail *Cornu aspersum* (Stewart et al., 2016).

The type of sexual system can have important implications for the evolution of such substances. For example, simultaneously hermaphroditic species (which use both genders at the same time or in sequence over their lifetime) need to regulate and coordinate their male and female reproductive processes in a largely non-overlapping manner. Hence, the neurobiological wiring and neuroendocrine substances in simultaneous hermaphrodites need to remain separated between the two sexual functions. The performance of conflicting processes or behaviours is avoided by complex excitatory and inhibitory crosstalk between the male and female processes. During mating, the appropriate motor output needs to be initiated, whereas the motor patterns of the opposite sexual role need to be suppressed, that is, when donating sperm to a partner, egg laying should not be initiated at the same time.

This situation in simultaneous hermaphrodites is accompanied by interesting evolutionary processes that do not occur in species with separate sexes. Recent research has revealed that accessory gland products can target the male function of the recipient. In *L. stagnalis*, two accessory gland proteins were identified that cause a snail to transfer half the amount of sperm to its next partner, lowering the paternity of that donor (Nakadera et al., 2014). Thus, in hermaphrodites, a sperm donor not only affects female physiology (Koene et al., 2010), but also male physiology of the recipient (Nakadera et al., 2014) (Fig. 8).

Interestingly, different areas of the central nervous system in hermaphrodites control the execution of male and female reproduction, and these areas express different neuropeptides (Koene, 2010; Koene et al., 2000). However, the neuroendocrine mechanisms that prevent male and female behaviours from being executed at the same time remain to be identified (Koene et al., 2000). Identifying these mechanisms will help us to understand how accessory gland products hijack the reproductive neuroendocrine system of the sperm recipient. The evolutionary importance of these interactions is evidenced by the various injection devices that have evolved for the transfer of accessory gland products (Zizzari et al., 2014). These include the love darts of land snails that inject accessory gland products into the body cavity (Lodi and Koene, 2017), and the stylets of some *Siphopteron* sea slugs that inject their products into the head of the partner and might directly target the central nervous system of their partner (Anthes and Michiels, 2007; Lange et al., 2014).

In the future, the pharmacological characterization of receptor systems for accessory gland products could reveal whether these

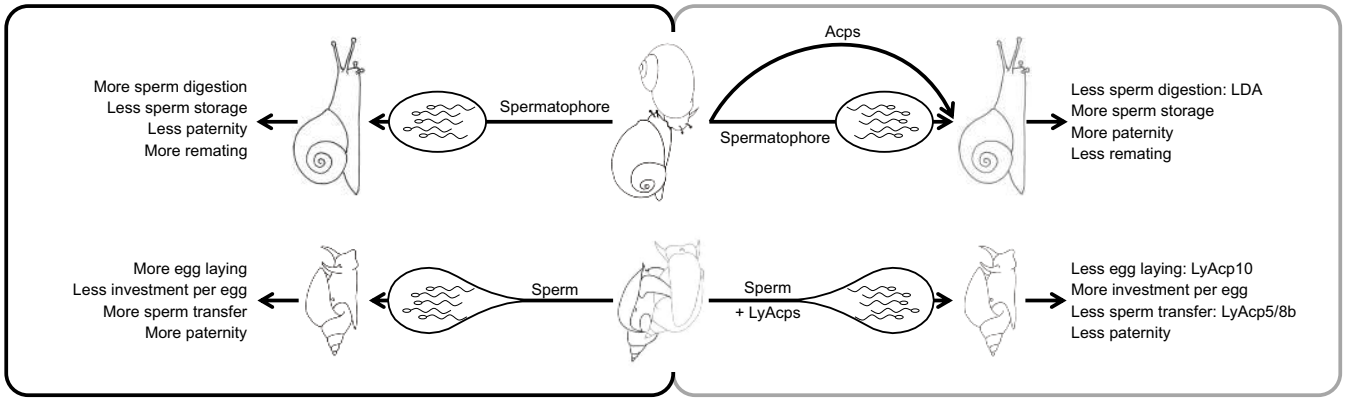

Fig. 8. Modulation of reproductive processes in simultaneous hermaphrodites by accessory gland proteins. Accessory gland proteins (Acps) are transferred during mating in gastropods. Two species are shown, the land snail *C. aspersum* and below the fresh water snail *L. stagnalis*. On the right the effects of Acps on the recipient (in grey) are shown. For *C. aspersum*, Acps are transported, via the love dart, separately from the sperm package (spermatophore; depicted as an oval with sperm inside); for *L. stagnalis* the Acps are transferred via seminal fluid (depicted as a drop shape containing sperm). The identified proteins are indicated along with their demonstrated effect (i.e. LDA, LyAcp). The left side indicates what would happen in the absence of these Acps.

substances are mimics of female regulatory hormones and how these signalling systems evolve. For example, does the female system evolve to counter the effect of accessory gland products (Lodi and Koene, 2017)?

Emerging themes

A common theme for all animals in which peptidergic signalling has been investigated is its remarkable complexity. Why do neural networks deploy such a plethora of neuropeptide transmitters and modulators? Does this reflect a high level of functional redundancy? Or does it reflect a requirement of the animal for multiple routes to behavioural flexibility in the face of a challenging environment or life style? The most likely answer is a combination of the two. Some evidence suggests that signals can be encoded in the mix of neuropeptides rather than in single molecules (Jones et al., 2016; Papaioannou et al., 2005), but precisely how widespread this phenomenon is remains to be determined.

A further notable observation is the apparent dominance of 'wireless' peptide signalling. In the case of the annelid *Platynereis*, there is a distinct paucity of synaptic connections in the neuropeptide-rich region of the brain. It could be that this is a specialization of an anatomically simple system that endows a higher level of complexity within the network that could otherwise be achieved using 'hard-wiring' alone. However, it is equally possible that current knowledge of neuropeptide networks in mammals is still too limited for us to appreciate the extent of non-synaptic communication in more complex brains.

Another emerging theme in peptidergic signalling is the evolutionary conservation of related neuropeptide–receptor pairs in the regulation of similar aspects of animal behaviour across species (Beets et al., 2012; Garrison et al., 2012; Lockard et al., 2017; Scott et al., 2017; Tian et al., 2016; Van Sinay et al., 2017). Thus, comparative investigations have the potential to lead to a more global understanding of fundamental and conserved aspects of peptidergic neural networks, and their roles in behavioural plasticity.

Overall, the capability of neuropeptides to modulate the output of circuits through sensitization or inhibition of target neurons is a common theme to emerge from these studies (Chalasani et al., 2010). A neuropeptide can endow a circuit with flexibility by modulating the response profile of discrete neurons (Chen et al., 2017; Vollmer et al., 2016). Such a mechanism can provide an explanation of how an animal can exhibit two divergent behavioural responses to the same sensory stimulus depending on the context in which the stimulus is perceived.

Future perspectives

Refined neuropeptidomic approaches (DeLaney et al., 2017) are expected to make important contributions in the future. The systematic identification of neuropeptide variants will, for example, facilitate pairing of ligands with their cognate receptors (Bauknecht and Jékely, 2015) and provide the framework for functional interrogation of peptidergic networks. Future studies should also consider how differential sorting of distinct neuropeptides within a given neuron can be regulated in a state-dependent manner, and how this might influence their co-release. A major future challenge is to develop concepts and tools to dissect multi-peptide signalling processes and synergistic actions of peptides in the nervous system

The benefit from understanding peptidergic signalling is under-realized: whereas the success of the opioid analgesics exemplifies the clinical importance of neuropeptides in nociception and pain, there are many other clinical situations where neuropeptides have a role and have yet to be adequately exploited. Neuropeptides also have the potential to provide new tools for pest and parasite control (Holden-Dye and Walker, 2014; McVeigh et al., 2012; Terhzaz et al., 2017).

The investigations reviewed here show that neuropeptides are intimately involved in the capability of animals to exhibit behavioural flexibility. This includes multi-channel convergent and divergent signalling, across both long and short distances, in a transient or sustained manner. These themes appear to be played out across the animal phyla, from the simplest to the most complex.

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