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Orphan neuropeptides and receptors: Novel therapeutic targets

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Abstract

Neuropeptides are the largest class of intercellular signaling molecules, contributing to a wide variety of physiological processes. Neuropeptide receptors are therapeutic targets for a broad range of drugs, including medications to treat pain, addiction, sleep disorders, and nausea. In addition to >100 peptides with known functions, many peptides have been identified in mammalian brain for which the cognate receptors have not been identified. Similarly, dozens of “orphan” G protein-coupled receptors have been identified in the mammalian genome. While it would seem straightforward to match the orphan peptides and receptors, this is not always easily accomplished. In this review we focus on peptides named PEN and big LEN, which are among the most abundant neuropeptides in mouse brain, and their recently identified receptors: GPR83 and GPR171. These receptors are co-expressed in some brain regions and are able to interact. Because PEN and big LEN are produced from the same precursor protein and co-secreted, the interaction of GPR83 and GPR171 is physiologically relevant. In addition to interactions of these two peptides/receptors, PEN and LEN are co-localized with neuropeptide Y and Agouti-related peptide in neurons that regulate feeding. In this review, using these peptide receptors as an example, we highlight the multiple modes of regulation of receptors and present the emerging view that neuropeptides function combinatorially to generate a network of signaling messages. The complexity of neuropeptides, receptors, and their signaling pathways is important to consider both in the initial deorphanization of peptides and receptors, and in the subsequent development of therapeutic applications.

Keywords

Deorphanization; orphan peptides; dimers; allosteric modulation; indirect neuropeptides

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1. Introduction

Peptides function as chemical messengers throughout the body as neuropeptides (e.g., enkephalin), as peptide hormones (e.g., insulin), or both hormone and neuropeptide (e.g., oxytocin). Peptides affect a wide range of physiological and behavioral activities, including feeding and body weight regulation, sleep wake cycles and alertness, sexual activity and reproduction, pain, anxiety, fear, stress, depression, and the regulation of homeostasis of many parameters (blood glucose, temperature, etc) (Fricker, 2012; Strand, 2003). Due to the large number of processes controlled by peptides, there has been considerable interest in the development of drugs that target peptide receptors. The most widely used drugs targeting neuropeptide receptors are the opiates, including natural products such as morphine as well as synthetics like fentanyl and oxycodone, all of which are commonly prescribed for the treatment of pain. Antagonists of the mu opioid receptor are used both to treat opioid overdose (naloxone) and to reduce the cravings of opioids, alcohol, nicotine, and food (naltrexone) (Aboujaoude and Salame, 2016; Greig and Keating, 2015; Sudakin, 2016). Other FDA-approved medications that target neuropeptide receptors include aprepitant, which is an antagonist of the substance P/neurokinin receptor NK1 and is used to treat nausea and vomiting, and suvorexant, an antagonist of the orexin receptors OX₁ and OX₂ that is used to treat insomnia (Preskorn, 2014). FDA-approved peptide analogs that target peptide hormone receptors include analogs of oxytocin (carbetocin), vasopressin (desmopressin), and glucagon-like peptide-1 (exenatide and others) (Fosgerau and Hoffmann, 2015; Janzen *et al.*, 2016; Manning *et al.*, 2008). Many more compounds are in development for a variety of indications, including drugs that target corticotropin-releasing factor CRF-1 receptors, neuropeptide Y-1, -2, and -5 receptors, melanocortin MC4R receptors, tachykinin receptors, neurotensin receptors, and others (Barak *et al.*, 2016; Griebel and Beeske, 2012; Griebel and Holsboer, 2012; Jagoda *et al.*, 2011; Kaspar and Reichert, 2013; Zorrilla and Koob, 2010). Most of these targets are G protein-coupled receptors (GPCRs), the largest class of receptors in the mammalian genome.

2. GPCR families and orphan receptors

All known GPCRs can be grouped into five main families based on consideration of amino acid sequence similarities (Fredriksson *et al.*, 2003). The families can be divided into groups and the groups further divided into sub-branches. In most cases these sequence-based divisions correspond to the types of ligands that the receptors recognize. Many of the putative GPCRs identified from analyses of the human genome or other nucleotide sequencing approaches did not have a known function—these were termed “orphan” GPCRs. In order to understand the biological function of orphan receptors, there has been a large effort to identify endogenous receptor ligands and some of these searches were successful (Civelli *et al.*, 2013; Civelli *et al.*, 2006).

Despite considerable efforts, approximately 60 GPCRs in the Family A group (which includes neuropeptide receptors) have not been de-orphanized; their endogenous ligands remain unknown (Khan and He, 2017; Ngo *et al.*, 2016). There are three potential reasons for this. First, it is possible that endogenous ligands have not been detected in the screening assays used because either the screening assay or the conditions used to detect receptor

activation were not correct or sensitive enough. For example, the assays often used to deorphanize receptors involve high throughput methods that are easy to use and give a big signal to noise ratio, but which may not be appropriate for a given receptor-ligand combination. High throughput assays are generally carried out using a single dose of ligand at a single time point. Such assays could easily miss the detection of ligands for receptors that are rapidly desensitized at high doses, or which show a delayed response to ligand activation. Also, the assay might not be appropriate in that the ligand might bind to the receptor but activate a distinct signal transduction pathway (Figure 1); this concept is commonly referred to as biased agonism or functional selectivity. Alternatively, the endogenous ligand might bind to only the dimeric receptor activating a distinct signal transduction pathway as compared to individual receptors (Figure 1). Unless multiple assays are used for deorphanization, the identification of the appropriate ligand for a given receptor can be missed.

Second, an endogenous ligand may not exist. GPCRs often form heterodimers, and some members of the GPCR gene family appear to have lost their ability to directly interact with ligands and instead function only in the modulation of other GPCR proteins (Tao and Conn, 2014). For example, γ -amino butyric acid (GABA) binds to and activates the GPCR named GABA-B R1, but does not bind to the related protein named GABA-B R2. The major function of the R2 protein is to interact with the R1 protein—this interaction is important for cell surface expression of the R1-R2 complex (Pin *et al.*, 2004). Alternatively, some GPCRs are activated by proteases rather than by endogenous ligands (Krumm and Grisshammer, 2015).

The third potential reason that an orphan GPCR has not been de-orphanized is that the appropriate ligand was not tested. Some studies tested brain extracts for the presence of a factor that could activate a receptor, and then fractionated the extract until the compound was pure enough to identify (Civelli *et al.*, 2013; Devane *et al.*, 1992; He *et al.*, 2004; Kojima *et al.*, 1999; Reinscheid *et al.*, 1995; Sakurai *et al.*, 1998; Shimomura *et al.*, 2002). In theory, this approach should be able to deorphanize any receptor for which the endogenous ligand is present at levels sufficient to produce a response above background. However, many endogenous ligands are labile and rapidly degraded in brain extracts. Other high throughput studies avoided the problems with brain or tissue extracts and started with purified molecules (Liu *et al.*, 2003; Meyer *et al.*, 2013; Rezgaoui *et al.*, 2006). However, these studies were limited by the input material and would not have detected novel peptides that were not in their libraries. Furthermore, a general assumption was that receptors would bind molecules similar to the families to which the orphan GPCRs belong. For example, the orphan receptor named GPR171 exhibits highest homology to the P2Y family of receptors and was tested with a variety of P2Y ligands such as nucleotides, dicarboxylic acids and lipids, none of which has been found to exhibit detectable binding to GPR171 (Gomes *et al.*, 2013; Wardman *et al.*, 2016). Because of this prediction, GPR171 was probably not extensively tested for binding to neuropeptides, especially novel “orphan” peptides. Just as there are orphan GPCRs, there are orphan neuropeptides—peptides that appear to be neuropeptides based on their cellular and intracellular location, but which do not have known receptor binding partners.

3. Orphan Neuropeptides

Unlike GPCRs, which are evolutionarily related and have regions with conserved amino acid sequences, there are no sequences conserved among all neuropeptides. Thus, novel neuropeptides cannot be identified from amino acid homology and alternate approaches have to be used. In this context, a biochemical approach was used to identify novel neuropeptide precursors in mice lacking carboxypeptidase E (CPE) activity (Che *et al.*, 2001). This enzyme plays an important role in the biosynthesis of nearly all neuropeptides, removing lysine and/or arginine residues from the C-terminus of the peptide-processing intermediates (Fricker, 2012; Fricker and Snyder, 1982; Fricker and Snyder, 1983). Mice with a mutation in the *Cpe* gene accumulate peptide-processing intermediates (Naggert *et al.*, 1995; Varlamov *et al.*, 1996; Zhang *et al.*, 2008). Together with colleagues, we isolated these intermediates using affinity chromatography selective for peptides with C-terminal lysine or arginine, and sequenced the peptides using mass spectrometry (Che *et al.*, 2001). This led to the identification of a number of novel mouse brain peptides, including peptides we named SAAS, PEN, and LEN (Fricker *et al.*, 2000). These three peptides and several others are derived from the same precursor, proSAAS (Figure 2).

Subsequent studies found proSAAS-derived peptides to be the most commonly detected peptides in mass spectrometry-based peptidomics studies on mouse brain, suggesting that these peptides are among the most abundant brain peptides (Fricker, 2012). All of the proSAAS-derived peptides are highly conserved between mouse and human, with amino acid sequence identities of over 50%; it is likely that high conservation reflects biological activity for these peptides (Table 1). Other orphan peptides that we found many times in peptidomic studies on mouse brain and which are conserved >50% between mouse and human are listed in Table 1. Many other peptides are found in typical peptidomics studies which are not highly conserved. For example, most peptides derived from prothyrotropin releasing hormone (proTRH) detected in mouse brain only show 35-46% sequence identity between mouse and human, and only one proTRH peptide exceeds 50% conservation (Table 1). Peptides with known functions (i.e. thyrotropin releasing hormone, enkephalin) were excluded from Table 1, although fragments of peptides known to be bioactive are included on the list. One example is dynorphin A10-17, which is the C-terminal portion of dynorphin A17 that is released after cleavage into dynorphin A8. Similarly, several highly conserved fragments of proenkephalin that we detected in peptidomic studies are also present as larger forms with N-terminal enkephalin sequences attached (not shown). In these cases, the high conservation may reflect the function of the larger peptides, which bind to opioid receptors. Alternatively, the cleaved peptides may have distinct functions from their precursors. Because the N-termini of the opioid peptides are essential for binding to the opioid receptors, if the cleaved C-terminal fragments have biological activities it is presumably through interaction with other proteins.

The list of peptides shown in Table 1 is not a complete list of likely orphan peptides. For example, the list does not include the well-studied orphan peptide named CART (cocaine and amphetamine-regulated transcript) which is a 41-48 residue peptide that has been reported to function through an unidentified GPCR (Lakatos *et al.*, 2005; Vicentic *et al.*, 2006; Vicentic *et al.*, 2005; Yermolaieva *et al.*, 2001). Although CART was not detected in

our peptidomics studies, possibly due to its large size and/or disulfide bonds, several N-terminal fragments of the CART precursor protein (CARTP) were detected (Table 1). Some of the peptides in Table 1 have been reported to have biological activities, such as the peptide derived from cerebellin 1 precursor protein. This cerebellin peptide, and a shorter form missing the N-terminal residue which was also commonly found in peptidomics, have been reported to stimulate the secretion of catecholamines from cultured adrenal cells and to modulate nociception in an *in vivo* assay (Albertin *et al.*, 2000; Su *et al.*, 2014). While some of these orphan neuropeptides may function as endogenous ligands by directly activating GPCRs, others may function as indirect neuropeptides (described below in section 5).

3. 1. ProSAAS-derived peptides as neuropeptides

ProSAAS mRNA is abundant in brain and is highest in regions that control feeding such as arcuate nucleus, suprachiasmatic nucleus, and several other nuclei in the hypothalamus (Fricker *et al.*, 2000). Using an antibody-based assay to quantify levels, PEN was found to be present in brain at levels comparable to other abundant peptides such as enkephalin (Chakraborty *et al.*, 2006; Lindberg and Yang, 1984). Thus, proSAAS-derived peptides are among the most abundant peptides in mouse brain. Like many neuropeptide precursors, proSAAS is partially processed at some cleavage sites, leading to the generation of peptides of different sizes (Fricker *et al.*, 2000; Mzhavia *et al.*, 2001; Mzhavia *et al.*, 2002). Several of these proSAAS-derived peptides co-localize with other neuropeptides such as neuropeptide Y (NPY) in the arcuate nucleus of the hypothalamus (Wardman *et al.*, 2011). Specifically, PEN and big LEN (b-LEN) show considerable overlap with NPY, but not with α MSH which is present in a distinct neuronal population. Consistent with a role similar to that of NPY, transgenic animals overexpressing proSAAS are slightly overweight, and mice lacking proSAAS are leaner than wild-type littermates (Morgan *et al.*, 2010; Wei *et al.*, 2004). In addition, antibody-mediated depletion of b-LEN or PEN greatly reduced acute feeding (Wardman *et al.*, 2011) while injection of b-LEN into the nucleus accumbens led to elevated acute feeding (Ye *et al.*, 2017). Application of b-LEN to brain slices produced a rapid and reversible inhibition of synaptic glutamate release from parvocellular neurons in the hypothalamic paraventricular nucleus, a target of arcuate NPY projections (Wardman *et al.*, 2011). The b-LEN-induced release of glutamate was abolished by blocking G protein activity (Wardman *et al.*, 2011) suggesting that the peptide activated a G protein-coupled receptor.

4. Matching orphan peptides and orphan receptors

4.1 GPR171 binds b-LEN

To identify receptors for proSAAS-derived peptides, we used a bioinformatics approach to select orphan GPCRs that were highly expressed in brain regions with high levels of proSAAS expression, such as the arcuate nucleus of the hypothalamus. Then, these receptors were individually tested for activation by the major proSAAS-derived peptides. These studies led to the identification of GPR171 as a receptor for b-LEN (Gomes *et al.*, 2013). GPR171 (also known as H963) is highly conserved among mouse, rat and human and belongs to the P2Y-like family of receptors, with highest amino acid sequence similarity to the free fatty acid receptors and dicarboxylic acid receptors. Despite being in the P2Y-like

family, GPR171 is not activated by a variety of carboxylic acids (Gomes *et al.*, 2013). GPR171 does not bind little LEN but does bind the tetrapeptide Leu-Leu-Pro-Pro albeit with lower affinity than it binds b-LEN (Gomes *et al.*, 2013). The peptide Leu-Leu-Pro-Pro is released from b-LEN during its endoproteolytic conversion into little LEN. Hence this enzymatic step regulates the relative potency of ligands that activate GPR171.

GPR171 protein is relatively abundant in mouse brain hypothalamus, including the arcuate nucleus, paraventricular nucleus, and dorsomedial hypothalamus (Gomes *et al.*, 2013) suggesting a broad role for this receptor. Decreasing the expression of GPR171 in the mouse ventral hypothalamus using lentiviral shRNA-mediated knockdown caused a complex affect on food intake (Gomes *et al.*, 2013). Decreasing the expression of GPR171 in the basolateral amygdala by ~60% leads to reduction in anxiety-like behaviors and fear conditioning (Bobeck *et al.*, 2017). Taken together, these results suggest a high endogenous tone for GPR171/b-LEN system under normal conditions that is likely to be subject to intricate regulation during complex behaviors and/or pathologies.

4.2. GPR83 binds PEN

Like GPR171, GPR83 is also relatively abundant in hypothalamus. Although GPR83 was initially considered an orphan receptor, it belongs to the NPY peptide receptor family (Wang *et al.*, 2001) and one previous report claimed that GPR83 bound NPY (Sah *et al.*, 2007). We found that GPR83 bound NPY only at very high concentrations that are not likely to be physiologically relevant (Gomes *et al.*, 2016b). GPR83 has also been reported to be activated by zinc and proteases, but it is not clear if any of these are physiologically important (Muller *et al.*, 2013a; Muller *et al.*, 2013b). Using a strategy similar to that used for deorphanization of GPR171, we identified GPR83 as a high affinity receptor for PEN (Gomes *et al.*, 2016b). PEN binding and signaling exhibit unexpected characteristics. For example, binding of PEN leads to Gq-mediated activation of PLC activity as well as the Gi-mediated inhibition of adenylate cyclase activity in a cell type specific or tissue specific manner (Gomes *et al.*, 2016b) suggesting multiple modes of regulation of signaling by this receptor.

In the mouse brain, GPR83 is present at relatively high levels in multiple regions (Gomes *et al.*, 2016b) and can be regulated by a variety of drugs (Adams *et al.*, 2003; Wang *et al.*, 2001). Animals that lack GPR83 have normal body weight when on a regular diet but are resistant to obesity when on a high-fat diet (Muller *et al.*, 2013b). Interestingly, in these mice the energy metabolism is differentially modulated (compared to wild-type mice) by ghrelin, another biologically active peptide that has been reported to play an important role in body weight regulation (Muller *et al.*, 2013b). Together these results suggest a role for receptor-receptor interactions and signaling cross talk in modifying GPR83 function.

4.3. Interaction of GPR83, GPR171, and other receptors

A concept that has emerged in recent years is that direct receptor-receptor interactions lead to signaling cross-talk. This idea posits that the association between two different receptor protomers leads to generation of dimers that are able to activate a signal transduction pathway distinct from those activated by protomers and this is primarily due to one protomer functioning as the allosteric modulator of the other (Ferre *et al.*, 2014; Fujita *et al.*, 2014).

There are three generally-accepted criteria that must be fulfilled to establish modulation of receptor function by dimerization between two receptors (Gomes *et al.*, 2016a). First, both of the interacting receptors should exhibit cellular and subcellular co-localization. This criterion does not require that the two receptors to be co-localized in all cells, as it is possible for receptors to interact in only a subset of cell types in which they are expressed. Second, activation of the dimer should lead to signaling that is distinct from signaling mediated by activation of individual receptors. Third, the disruption the dimer (or at least the loss of one of the receptors) should lead to the loss of the unique signaling properties found for the dimer.

GPR83 and GPR171 satisfy these three criteria. These two receptors co-localize in some brain regions, such as the paraventricular nucleus (PVN); in this region the two receptors are in close enough proximity to directly interact as determined by proximity ligation assays (Gomes *et al.*, 2016b). PEN binding leads to initiation of PLC signaling in cells and tissues expressing both receptors; this signaling is not seen in cells expressing only GPR83 (Gomes *et al.*, 2016b). Finally, in the absence of GPR83 signaling by b-LEN (i.e GPR171) is also compromised (Gomes *et al.*, 2016b). This last criterion is best tested using agents that specifically block or disrupt dimerization—such studies have yet to be reported. Nonetheless, the data thus far is consistent with the idea that GPR83 associates with GPR171 and this leads to changes in PEN signaling.

GPR83 appears to interact with other receptors such as the ghrelin receptor and these interactions lead to changes in the cell surface expression of GPR83 (Muller *et al.*, 2014) as well as energy metabolism in response to ghrelin (Muller *et al.*, 2013b). Studies carried out with other neuropeptide receptors in feeding circuits suggest that such receptor-receptor interactions might be a general mechanism for regulation of receptor activity in this behavior (Muller *et al.*, 2013b; Rediger *et al.*, 2011). For example, MC4R has been shown to interact with Ghrelin receptors and this has been reported to lead to changes in receptor pharmacological, signaling and trafficking properties (Muller *et al.*, 2013b; Rediger *et al.*, 2011). Thus one way of regulating signaling by neuropeptide receptors is through direct receptor-receptor interactions.

5. Complexity of intercellular signaling

The modulation of neuropeptide signaling can occur via multiple mechanisms. One such mechanism is that of biased agonism. Some endogenous neuropeptides have been recently shown to function as biased agonists at GPCRs, differentially activating G protein and non-G protein coupled pathways (Wisler *et al.*, 2014). For example, while Agouti-related protein (AgRP) functions as an MC4R antagonist when examining G protein signaling, it functions as a potent MC4R agonist activating the extracellular signal-regulated kinase pathway (Tao, 2014) and opening the Kir7.1 potassium channel (Ghamari-Langroudi *et al.*, 2015). As discussed above, another mechanism of modulation of receptor signaling is by receptor-receptor interactions i.e. dimerization (Figure 1).

Most peptide precursors are cleaved into a variety of peptides that are co-stored and co-released, and in some cases each of these peptides can activate distinct receptors and

signaling pathways (Figure 3). For example, proopiomelanocortin (POMC) is cleaved into α MSH, β -endorphin, and a number of other peptides such as joining peptide. ProSAAS also gives rise to a number of distinct peptides (Figure 2). ProNPY is cleaved to produce NPY and a highly conserved C-terminal fragment (Table 1). ProCART and AgRP are also cleaved to produce multiple peptides (Fricker, 2010; Secher *et al.*, 2016). Since all of these peptides are presumably co-secreted by neurons that express the peptide's precursor protein, it is likely that they contribute to complexity in the signaling (Figure 3).

When multiple peptides are co-secreted, there are two ways the peptides can interact. For those peptides which bind to receptors, the different signaling cascades can produce positive or negative interactions. Another completely different mechanism is through competition for the extracellular peptidases that cleave the peptides into smaller fragments. In some cases, the smaller fragments have biological activities that differ from the parent peptide, and in other cases the fragments are completely inactive (Figure 4). None of the extracellular peptidases that have been identified are specific for a particular peptide, and all are able to bind a range of different peptides (Cudic and Fields, 2009; Turner, 2003). Thus, the multiple peptides secreted by a neuron will compete for the limited extracellular peptidases, thereby limiting degradation and leading to local increases in their levels (Figure 4). One bioactive peptide that is known to target extracellular peptidases, rather than receptors, is opiorphin (Thanawala *et al.*, 2008; Wisner *et al.*, 2006), and many more examples are likely. This class of peptide has been termed "indirect neuropeptides" (Fricker, 2012). It is interesting to note that AgRP folds into a structure with 5 pairs of cysteine disulfide bridges; this structure is referred to as a "knottin" and has homology to protease inhibitors. CART contains three disulfide bridges and also folds into a compact structure with similarity to cystine knots (Ludvigsen *et al.*, 2001). However, the idea that AgRP and/or CART function in part as protease inhibitors does not appear to have been tested. It is likely that more examples will be found of indirect neuropeptides, in which secreted peptides interfere with the degradation of neuropeptides that directly bind to receptors. It is possible that some of the highly conserved peptides listed in Table 1 as potential orphan neuropeptides are functional in this alternative capacity rather than through directly binding to GPCRs. Conservation of amino acid sequence suggests a function, but this function is not necessarily through direct binding to receptors.

6. Summary and Future Directions

The recent advances in detection of peptides and proteins in various brain regions have led to the identification of a number of peptides and GPCRs. Using a combination of bioinformatics approaches to identify likely targets, followed by medium-throughput screening, we identified GPR83 and GPR171 as receptors for peptides PEN and LEN. Characterization of these receptor systems as well as other receptor systems in the hypothalamus has begun to reveal some of the intricacies of neuropeptide receptor signaling. However, a complication is the redundancy inherent to many biological systems. This is clearly the case for the AgRP neurons that secrete AgRP, NPY, PEN, LEN, and GABA as well as other peptides derived from proAgRP, proNPY, and proSAAS. Deletion of any individual signaling molecule (i.e. knock-out mice) does not show severe phenotypes of body weight alteration, even though these neurons are clearly of fundamental importance in

the control of feeding (Gropp *et al.*, 2005; Luquet *et al.*, 2005; Qian *et al.*, 2002). But rather than simply being a case of redundancy of signal pathways, each of the signaling molecules can contribute meaning to the message. Optogenetics and chemogenetic techniques in combination with temporal, tissue or cell-type specific expression are likely to help characterize the contribution of neuropeptides from individual neurons since they allow for specific stimulation of selective neurons. Because the total effect of the multitude of secreted molecules is likely to be greater than the sum of the parts, these approaches can complement studies exploring the function of individual signaling molecules. An important area for future studies is to examine the release of neuropeptides from neurons *in vivo*, although techniques to accomplish this are not yet able to detect the vast majority of peptides. Another important area is to understand the ways by which the receptor activity at the postsynaptic neuron is regulated and the hierarchy of this regulation. The effect of extracellular proteolytic activity on peptides, and the competition of peptides for these peptidases is another area for further studies. By developing strategies to explore and delineate the complexity of neuropeptide receptor signaling, we will be better equipped to develop pharmacological agents specifically targeting those receptor systems, circuits, behaviors and pathologies.

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Abbreviations

AgRP	Agouti-related protein
b-LEN	big LEN
CART	Cocaine- and Amphetamine-Regulated Transcript
CARTP	Cocaine- and Amphetamine-Regulated Transcript Protein
CPE	carboxypeptidase E
ERK	extracellular signal-regulated kinase
GABA	γ -amino butyric acid
GPCR	G protein-coupled receptors
NPY	neuropeptide Y
PLC	phospholipase C
ProCCK	procholecystokinin
ProNPY	proneuropeptide Y
ProTRH	prothyrotropin releasing hormone
PVN	paraventricular nucleus

POMC Proopiomelanocortin

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Figure 1.

Different modes of peptide-receptor interaction that lead to differential signaling. **A.** In the classic view, a neuropeptide is thought to bind to its cognate receptor and activate a G protein pathway. **B.** Many receptors can signal through G protein-coupled and G protein-independent pathways (such as arrestin-mediated signaling pathways). The amount of signaling through each pathway depends on the ligand, with some stimulating both pathways while other ligands predominantly stimulating only one of the pathways (and sometimes acting as an antagonist at the other pathway); this process is commonly referred to as biased agonism. **C.** Dimerization of two distinct G protein-coupled receptors can lead to activation of a signal transduction pathway (Signal B) that is different from those activated by individual receptors (Signals A and C).

Figure 2.

Schematic of the ProSAAS precursor and peptides derived from processing. Abbreviations: R, arginine; L, Leucine; V, Valine; K, Lysine; L-SAAS, Little SAAS. Note that SAAS, KEP, GAV, PEN, and LEN are names, not abbreviations.

Figure 3.

multiple neuropeptides and receptors regulate the activity of neurons in feeding circuits. Activation of the NPY/AgRP neuron (pink) or POMC neuron (blue) leads to the release of a number of neuropeptides. NPY functions as an agonist of Y1-Y5 receptors (Moreno-Herrera *et al.*, 2014). ProSAAS-derived b-LEN is an agonist of GPR171 (Gomes *et al.*, 2013) and PEN is an agonist of GPR83 (Gomes *et al.*, 2016b). AgRP is an antagonist/inverse agonist of MC4R signaling through G proteins but is an agonist at MC4R receptors through the ERK1/2 pathway (Tao, 2014). POMC-derived αMSH is an agonist of MC4R, as well as other melanocortin receptors (Sobrino Crespo *et al.*, 2014). Another POMC-derived peptide, β-endorphin 1-31 (β-End) is an agonist at mu opioid receptors (MOR) and also delta opioid receptors. β-endorphin peptides can be N-terminally acetylated, which eliminates their ability to bind to opioid receptors (Deakin *et al.*, 1980). CART is thought to mediate its effects through interaction with a G protein-coupled receptor, but the receptor has not been identified (Lakatos *et al.*, 2005; Lau and Herzog, 2014; Vicentic *et al.*, 2006; Vicentic *et al.*, 2005). Other peptides derived from proNPY, AgRP, proSAAS, POMC, and CART (or additional precursors) can potentially activate a distinct set of receptors indicated here by “?”. In this schematic, peptides derived from the same neuropeptide precursor are shown to be released from the same nerve terminal although this may not always be the case; recently it was reported that αMSH and β-End are differentially expressed and released (Koch *et al.*, 2015), and N-terminally-derived proSAAS peptides (i.e. SAAS) are sorted into distinct vesicles from PEN and b-LEN (Wardman and Fricker, 2014). In this diagram, multiple receptors are shown on the same post-synaptic cell, with activation of the various receptors leading to activation/inhibition of the various signaling cascades resulting in cross-talk among the second messenger systems in the postsynaptic neuron (green). In feeding circuits,

specific neurons are likely to express only a subset of the indicated receptors, as well as receptors for classical neurotransmitters like GABA (not shown).

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Figure 4.

Extracellular peptide processing and ‘indirect neuropeptides’. **(A)** Following secretion, peptides are cleaved by a variety of peptidases present in the extracellular environment (a single peptidase is shown for simplicity). In some cases, these cleavages serve to inactivate the peptide. However, some peptides can be converted into forms that are still active, either at the same receptor (not shown) or at distinct receptors, thereby producing a different response. Such cleavages serve a modulatory role. **(B)** Multiple peptides secreted into the extracellular space can compete for the peptidases. In this example, peptide 2 does not directly bind to a receptor but it produces a biological response by blocking the cleavage of peptide 1, thereby increasing the stability of peptide 1 and allowing for enhanced signaling through peptide 1’s receptor. Peptides that have biological activity by blocking the degradation of other peptides, without directly binding to receptors, are appropriately termed “indirect neuropeptides.”

Table 1

ProSAAS-derived peptides and other predicted orphan peptides identified in mouse brain peptidomic studies

Precursor	Gene	Peptide	Name or position	Times found	Human - Mouse % identity
ProSAAS	Pcsk1n	ARPVKEP(R)	KEP	30	100%
		ARPVKEPRSLAASAPLVETSTPL(RL)	Big SAAS	118	73%
		SLSAASAPLVETSTPL(RL)	Little SAAS	418	61%
		AVPRGEAAGAVQELARALAHLLAEERQE	Big GAV	152	96%
		GAAAGAVQELARALAHLLAEERQE	Little GAV	41	100%
		SVDQDLGPEVPPENVLGAALL(RV)	PEN	226	68%
		LENPSQAPA	Little LEN	25	70%
		LENPSQAPARLLPP	Big LEN	162	81%
Prodyn	Pdyn	SSEMARDEGGQDQVGHEGLY	177-199	14	61%
	Pdyn	PKLKWDNQ	Dynorphin A(10-17)	46	100%
Procnk	Penk	MDELYPMEPEEEANGGEIL/A	114-133	21	95%
	Penk	SPQLEDEAKELQ	197-208	103	100%
	Penk	VGRPEWWMIDYQ FAESLPSEEGENYSKEVPEIE +/- phosph on Ser7	218-228	93	100%
	Penk		238-261	100	86%
ProTRH	Trh	FIDPELQRSWEETEGEGGLMPE	178-200	27	56%
ProTach 1	Tac1	DADSSVEKQVALLKALYGHGQISH	72-95 C-terminal flanking peptide	28	28 96%
	Tac1	ALNSVAYERSAMQNYE		92	100%
ProCCK	Cck	AVLRTDGEPRARLGALLA	46-63	86	72%
	Cck	(K)APSGRMSVLKNLQSLDPSHRISD	72-94	142	88%
ProNPY	Npy	SSPETLISDLLMKESTENAPRTRLEDPSMW	C-terminal peptide	119	90%
CARTP	Cartpt	APGAMLQIEALQEV(LKKLKS)	60-79 (long splice form)	60	65%
Cerebellin 1	Cbln1	(S)GSAAKVAFAIRSTN(H)	Cerebellin peptide	350	100%
Cerebellin 2	Cbln2	(S)GSAAKVAFASTRSTN(H)	88-103	76	100%

Precursor	Gene	Peptide	Name or position	Times found	Human - Mouse % identity
Cerebellin 4	Cbln4	Cbln4 (AAN)SKVAFSAVRSTN	63-77	183	100%

The indicated sequences are for mouse, and residues in parentheses are present in some but not all forms of the peptides as a result of alternative cleavage by endopeptidases and/or exopeptidases. The number in the column labeled “Times Found” indicates the number of times the peptide or related peptides were identified in ~300 peptidomic studies performed in the Fricker laboratory on either whole mouse brain or brain subregions from 2003-present, many of which were previously published [see (Fricker, 2010) for references to the older publications]. Abbreviations: proCCK, procholecystokinin; proNPY, proneuropeptide Y; CARTP, cocaine- and amphetamine-regulated transcript protein; prodyn, prodynorphin; proenk, proenkephalin; proTRH, prothyrotropin releasing hormone; proTach 1, protachykinin 1; phosph, phosphorylation. Since the receptors for PEN and Big LEN have been identified (i.e. they are not orphan peptides) they are shown in blue for the sake of comparison with other proSAAAS peptides.