Chapter 1

INTRODUCTION

Neuropeptides and the "Chemical Connectome"

A common metaphor to describe the brain is that it is like a supercomputer. Consequently, current efforts at improving technologies for large-scale recording of brain function are primarily focused on measuring its electrical activity. However, unlike a supercomputer, the brain is an electrochemical machine: its function is dependent on both electrical and chemical (neuromodulatory) signaling. Superimposed upon the brain's physical connectome is a "chemical connectome," a largely invisible network of neuromodulators, including biogenic amines and neuropeptides, that exert a profound influence on brain function (Bargmann & Marder, 2013). These neuromodulators influence brain states in a manner that changes the computations performed by neural circuits (Marder et al., 2014). For example, the ~25 neurons comprising the crustacean stomatogastric ganglion can produce close to half a dozen different motor outputs, depending on their pattern of neuromodulation (Marder & Bucher, 2007). Neuromodulators influence brain states that alter the computations performed by neural circuits, and are central to emotion, mood, and affect (Pert et al., 1985; Wang & Pereira, 2016). An understanding of neuromodulatory influences is particularly important because of their relevance to psychiatric disorders in humans (Kramer et al., 1998; Rotzinger et al., 2010). Without the ability to measure and perturb the release of specific neuromodulators with high spatio-temporal resolution. our understanding of neuronal circuit function will be fundamentally incomplete.

Surprisingly, despite the fundamental importance of neuromodulation, techniques for measuring the release of specific neuromodulators especially neuropeptides (NPs), at large scale and with high spatio-temporal resolution, have lagged far behind those for recording or imaging electrical activity. Available methods, such as microdialysis (Benveniste & Hüttemeier, 1990; Ernberg & Alstergren, 2004; Frost et al., 2008; Lee & Kwon, 2022) or fast-scanning cyclic voltammetry (Makos, Kim, et al., 2009; Makos, Kuklinski, et al., 2009) are useful primarily for measuring "volume transmission," but are invasive, have poor spatial resolution and limited general applicability. There is no generally applicable method for measuring, with millisecond time resolution, the release of specific neuropeptides from individual neurons or nerve terminals.

Our long-term goal is to develop new methods for visualizing, detecting, and inhibiting neuropeptide release *in vivo*, and to apply these methods to understanding the dynamics of neuromodulation of specific, behaviorally relevant neural circuits. The rationale for this research is that the development of new tools for imaging neuropeptide release *in vivo* could have a transformative impact on our ability to characterize and analyze neural circuit function, as well as facilitate the development of technologies for selectively perturbing release.

Over 100 neuropeptides have been identified, which collectively regulate a variety of developmental, physiological, and behavioral functions (Russo, 2017). While each neuropeptide is idiosyncratic in regard to its molecular structure, chemical properties, and anatomical distribution, they impinge on the nervous system in a similar fashion (Agrawal

et al., 2019)): peptidergic (i.e., neuropeptide-producing) neurons and the neuroendocrine cells synthesize and package a massive amount of neuropeptide molecules within a subcellular compartment called the Dense Core Vesicle (DCV), where they are stored and released to the extracellular space upon strong stimulation (electrical or hormonal) of the cells. The released neuropeptides undergo diffusion to bind a group of proteins named "receptors," which are membrane-embedded proteins, typically in the G protein-coupled receptor (GPCR) family on other cells (van den Pol, 2012). These receptors, once peptide-bound, activate downstream biochemical signaling cascades, to regulate many other genes (Zhang et al., 2010) and proteins that control neuronal excitability. These neuropeptide-induced changes in cell physiology can last for a long time, in contrast to the effects of "classical" neurotransmitters like glutamate or GABA, which typically last only milliseconds. In summary, a neuropeptide signaling pathway defines a "neuropeptide information flow" that enables cell-cell communications (Nusbaum et al., 2017) .

Imaging Neuropeptide Release and Localization with a Genetically Engineered Reporter

The central objective is to tag components of large dense core vesicles (LDCVs) and/or specific neuropeptides and to determine whether these reporters can be used to image neurosecretory granule release. In invertebrate systems, there is genetic evidence in *C. elegans* that mutating a neuropeptide precursor processing enzyme (UNC-31) can inhibit the release of some neuropeptides *in vivo* (X. G. Lin et al., 2010; Speese et al., 2007). The composition of neuropeptide processing machinery is well characterized in mammalian

chromaffin cells (Hook et al., 2010; Podvin et al., 2015; Wegrzyn et al., 2010). In bovine adrenal chromaffin cells for instance, 23 different proteases are found in DCVs. However, the catalytic specificity of each protease remains unknown—we have no idea which protease(s) processes which neuropeptide(s). In comparison to chromaffin cells, the understanding of mammalian neurons is even thinner, as neither the composition or specificity in DCVs is known. Therefore, tagging a neuropeptide per se to a fluorescent protein is a more practical way of constructing peptide-specific reporters. Neuropeptide precursor proteins, also called prepropeptides, are cleaved and matured into multiple neuropeptide isoforms. The cleavage sites are di-/tribasic amino acid sequences, whose variety is buttressed by distinct permutations of arginine, lysine, glycine and phenylalanine residues.

We reasoned that an optimal *in vivo* real-time NP release reporter should include (1) a reporter domain that reflects the physico-chemical contrast between the intravesicular milieu and the extracellular space and (2) a sorting domain that ensures its selective trafficking into DCVs. The NP precursor may function as the sorting domain. The sorting domain candidates will be various truncates of neuropeptide prepropeptides, and the reporter domain candidates will include a collection of previously reported fluorescent proteins whose biophysical properties provide contrast to reflect differences between intravesicular and extracellular microenvironments, such as pH, free calcium, and potentially others. The configurations of reporter domains in relation to the sorting domain, as well as the presence or absence of cleavage sites, are also considered in the design of these reporters.

Neuropeptides and their processing enzymes are evolutionarily conserved (Hoyle, 1998). It is highly likely that the development and engineering of NP reporters can be done in multiple model organisms in a similar fashion. Our lab has a long term interest in investigating neuropeptides and their behavioral relevance in fruit flies (Asahina et al., 2014; Hergarden et al., 2012; Tayler et al., 2012) and mice (Zelikowsky et al., 2018). Therefore, we selected our neuropeptides of interest based on the current understanding of biological process and the research relevance to our lab for prototypical studies. In Chapter 2, I will introduce a neuropeptide release reporter for *Drosophila* tachykinin (dTK) in flies. In Chapters 3-4, I included clinical significance as another dimension for the selection of neuropeptide in mammalian cell lines, which are heavily used and hold huge potential for large-scale drug screening that targets neuropeptide signaling (Figure 1A) (Hökfelt et al., 2003).

Exploring Novel Therapeutics with Genetically Engineered Reporters

A variety of psychiatric and metabolic disorders are associated with the dysfunction of neuropeptide signaling pathways (Griebel & Holsboer, 2012). For example, it is widely believed that disrupted cholecystokinin (CCK), neurokinin (NK), and corticotropin-release factor (CRF) pathways cause depression and anxiety (Bowers et al., 2012; Schank et al., 2012); abnormal neuropeptide Y (NPY) and Agouti-Related Peptide (AGRP) signaling results in feeding disorders which can potentially lead to obesity (Arora & Anubhuti, 2006; Dhillo & Bloom, 2001), Calcitonin gene-related peptide (CGRP) and substance P are thought to be related to the transmission of pain (Hökfelt et al., 2001; Russell et al., 2014). The list goes on. A huge battery of drugs has been developed in the hopes that targeting neuropeptide pathways will lead to novel therapies for neuropsychiatric, neurodegenerative, or

neurometabolic disorders. These drugs primarily function by competitively binding to a specific neuropeptide receptor to antagonize the binding of the endogenous peptide. Drugs that survived clinical trials can prove to be a big success. For example, Aimovig (erenumab), a potent CGRP receptor blocker (to CGRP-R1, specifically) generated by Amgen, is a highly acclaimed, novel therapy for the prevention of migraine (King et al., 2019) .

Many potential neuropeptide receptor antagonists, however, fail in the clinical trials. For example, one of the pharma industry's most notable failures was MK-869, a Substance P receptor (NK1) antagonist, which was developed by Merck as a novel therapy for depression (Argyropoulos & Nutt, 2000; Kramer et al., 1998). One potential reason that receptor antagonists may fail in the clinical phase is that each neuropeptide often exerts its function via multiple, functionally redundant receptors, instead of through one-to-one ligand/receptor correspondence. Therefore, inhibiting just one receptor may not suffice to have any effect. While combining multiple receptor antagonists for a given neuropeptide is possible, in theory, the potential for unwanted side- and off-target effects increases with each additional drug.

The complementary approach to blocking neuropeptide receptors is to block the synthesis, release, or function of the neuropeptide itself. Indeed, eptinezumab, a blocking monoclonal antibody to CGRP, has also been FDA-approved for migraine treatment (Edvinsson et al., 2018). An advantage of blocking the neuropeptide, rather than its receptor, is that receptor-binding antibodies, by inducing conformational changes in their targets, could cause unwanted signaling events in the receptor-expressing neurons, whereas neuropeptide-

binding antibodies would not. A problem with using monoclonal antibodies to treat neuropsychiatric or neurodegenerative disorders, however, is that they are macromolecules that do not cross the blood-brain barrier (BBB). While small molecule compounds that cross the BBB can be effective neuropeptide receptor antagonists, there is no rational pathway to design small-molecule inhibitors that bind to the neuropeptide itself.

The advent and iteration of cutting-edge technologies, such as CRISPR-Cas9 (Hsu et al., 2014), recombinant antibody (Holliger & Hudson, 2005; Hoogenboom, 2005), genetically-encoded biosensors (Lin & Schnitzer, 2016), and viral delivery (Berns & Muzyczka, 2017; Hudry & Vandenberghe, 2019), enabled us to explore the uncharted path to targeting neuropeptide signaling for treating human diseases. In the long term, we aim to establish and streamline an imaging platform that combines optimal neuropeptide reporters, cell lines, and imaging techniques. The platform potentially enables us to integrate modern biotechnologies, and collectively constitute a therapeutic ecosystem (Figure 1B).

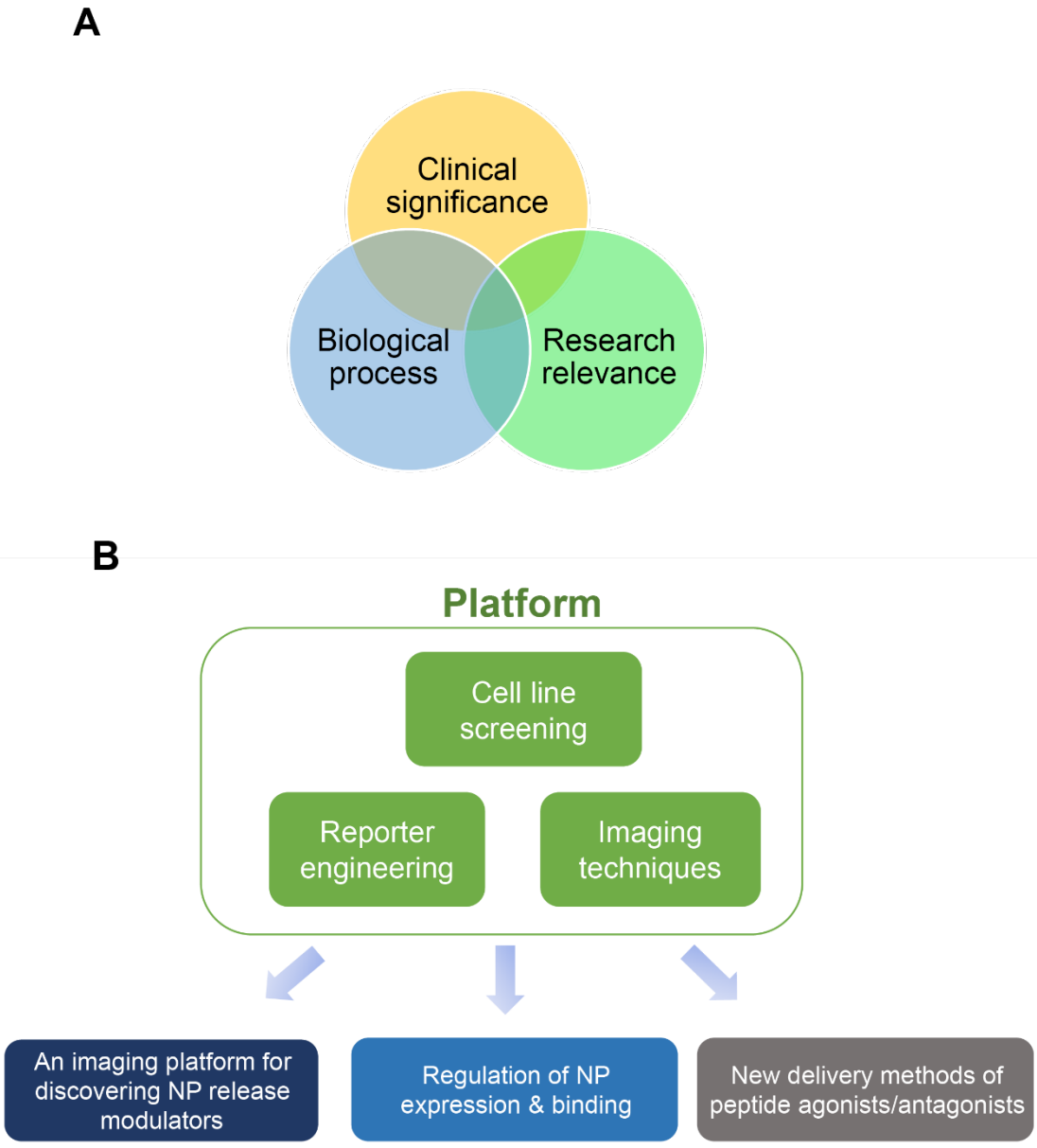

Figure 1: Rationales and visions of imaging neuropeptides

(A) Over 100 neuropeptides are identified. To shortlist our neuropeptide of interest, we consider three dimensions: understanding of biological process, relevance to current research, and clinical significance. (B) The neuropeptide imaging ecosystem. The long-term plan is to establish a platform that contains optimal reporters, cell lines and proper imaging techniques. With it we will further branch out to three arms: the discovery of neuropeptide release modulators, means to regulate neuropeptide expression and binding, and new delivery methods of peptide agonists and antagonists.

References

Agrawal, P., Kumar, S., Singh, A., Raghava, G. P. S., & Singh, I. K. (2019). NeuroPIpred: a tool to predict, design and scan insect neuropeptides. *Scientific Reports*. https://doi.org/10.1038/s41598-019-41538-x

Argyropoulos, S. V, & Nutt, D. J. (2000). Substance P antagonists: Novel agents in the treatment of depression. *Expert Opinion on Investigational Drugs*. https://doi.org/10.1517/13543784.9.8.1871

Arora, S., & Anubhuti. (2006). Role of neuropeptides in appetite regulation and obesity - A review. *Neuropeptides*. https://doi.org/10.1016/j.npep.2006.07.001

Asahina, K., Watanabe, K., Duistermars, B. J., Hoopfer, E., González, C. R., Eyjólfsdóttir, E. A., Perona, P., & Anderson, D. J. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in drosophila. *Cell*, *156*(1–2), 221–235. https://doi.org/10.1016/j.cell.2013.11.045

Bargmann, C. I., & Marder, E. (2013). From the connectome to brain function. *Nature Methods*. https://doi.org/10.1038/nmeth.2451

Benveniste, H., & Hüttemeier, P. C. (1990). Microdialysis-Theory and application. *Progress in Neurobiology*. Prog Neurobiol. https://doi.org/10.1016/0301-0082(90)90027-E

Berns, K. I., & Muzyczka, N. (2017). AAV: An Overview of Unanswered Questions. *Human Gene Therapy*. https://doi.org/10.1089/hum.2017.048

Bowers, M. E., Choi, D. C., & Ressler, K. J. (2012). Neuropeptide regulation of fear and anxiety: Implications of cholecystokinin, endogenous opioids, and neuropeptide Y. *Physiology and Behavior*. https://doi.org/10.1016/j.physbeh.2012.03.004

Dhillo, W. S., & Bloom, S. R. (2001). Hypothalamic peptides as drug targets for obesity. *Current Opinion in Pharmacology*. https://doi.org/10.1016/S1471-4892(01)00110-2

Edvinsson, L., Haanes, K. A., Warfvinge, K., & Krause, Di. N. (2018). CGRP as the target of new migraine therapies - Successful translation from bench to clinic. *Nature Reviews Neurology*. https://doi.org/10.1038/s41582-018-0003-1

Ernberg, M. M., & Alstergren, P. J. (2004). Microdialysis of neuropeptide Y in human muscle tissue. *Journal of Neuroscience Methods*, *132*(2), 185–190. https://doi.org/10.1016/j.jneumeth.2003.09.009

Frost, S. I., Keen, K. L., Levine, J. E., & Terasawa, E. (2008). Microdialysis methods for in vivo neuropeptide measurement in the Stalk-median eminence in the Rhesus monkey. *Journal of Neuroscience Methods*, *168*(1), 26–34. https://doi.org/10.1016/j.jneumeth.2007.09.001

Griebel, G., & Holsboer, F. (2012). Neuropeptide receptor ligands as drugs for psychiatric diseases: The end of the beginning? *Nature Reviews Drug Discovery*. https://doi.org/10.1038/nrd3702

Hergarden, A. C., Tayler, T. D., & Anderson, D. J. (2012). Allatostatin-A neurons inhibit feeding behavior in adult Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(10), 3967–3972. https://doi.org/10.1073/pnas.1200778109

Hökfelt, T., Bartfai, T., & Bloom, F. (2003). Neuropeptides: Opportunities for drug discovery. *Lancet Neurology*. https://doi.org/10.1016/S1474-4422(03)00482-4

Hökfelt, T., Pernow, B., & Wahren, J. (2001). Substance P: A pioneer amongst neuropeptides. *Journal of Internal Medicine*. https://doi.org/10.1046/j.0954-6820.2000.00773.x

Holliger, P., & Hudson, P. J. (2005). Engineered antibody fragments and the rise of single domains. *Nature Biotechnology*. https://doi.org/10.1038/nbt1142

Hoogenboom, H. R. (2005). Selecting and screening recombinant antibody libraries. *Nature Biotechnology*. https://doi.org/10.1038/nbt1126

Hook, V., Bark, S., Gupta, N., Lortie, M., Lu, W. D., Bandeira, N., Funkelstein, L., Wegrzyn, J., O'Connor, D. T., & Pevzner, P. (2010). Neuropeptidomic components generated by proteomic functions in secretory vesicles for cell-cell communication. *AAPS Journal*. https://doi.org/10.1208/s12248-010-9223-z

Hoyle, C. H. V. (1998). Neuropeptide families: Evolutionary perspectives. *Regulatory Peptides*. https://doi.org/10.1016/S0167-0115(97)01073-2

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. https://doi.org/10.1016/j.cell.2014.05.010

Hudry, E., & Vandenberghe, L. H. (2019). Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality. *Neuron*. https://doi.org/10.1016/j.neuron.2019.02.017

King, C. T., Gegg, C. V, Hu, S. N.-Y., Sen Lu, H., Chan, B. M., Berry, K. A., Brankow, D. W., Boone, T. J., Kezunovic, N., Kelley, M. R., Shi, L., & Xu, C. (2019). Discovery of the Migraine Prevention Therapeutic Aimovig (Erenumab), the First FDA-Approved Antibody against a G-Protein-Coupled Receptor. *ACS Pharmacology & Translational Science*, *2*(6), 485–490. https://doi.org/10.1021/acsptsci.9b00061

Kramer, M. S., Cutler, N., Feighner, J., Shrivastava, R., Carman, J., Sramek, J. J., Reines, S. A., Liu, G., Snavely, D., Wyatt-Knowles, E., Hale, J. J., Mills, S. G., MacCoss, M., Swain, C. J., Harrison, T., Hill, R. G., Hefti, F., Scolnick, E. M., Cascieri, M. A., … Rupniak, N. M. J. (1998). Distinct mechanism for antidepressant activity by blockade of central substance P receptors. *Science*. https://doi.org/10.1126/science.281.5383.1640

Lee, D., & Kwon, H. B. (2022). Current and future techniques for detecting oxytocin: Focusing on genetically-encoded GPCR sensors. *Journal of Neuroscience Methods*, *366*, 109407. https://doi.org/10.1016/j.jneumeth.2021.109407

Lin, M. Z., & Schnitzer, M. J. (2016). Genetically encoded indicators of neuronal activity. *Nature Neuroscience*. https://doi.org/10.1038/nn.4359

Lin, X. G., Ming, M., Chen, M. R., Niu, W. P., Zhang, Y. D., Liu, B., Jiu, Y. M., Yu, J. W., Xu, T., & Wu, Z. X. (2010). UNC-31/CAPS docks and primes dense core vesicles in C. elegans neurons. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2010.05.148

Makos, M. A., Kim, Y. C., Han, K. A., Heien, M. L., & Ewing, A. G. (2009). In vivo electrochemical measurements of exogenously applied dopamine in Drosophila melanogaster. *Analytical Chemistry*, *81*(5), 1848–1854. https://doi.org/10.1021/ac802297b

Makos, M. A., Kuklinski, N. J., Berglund, E. C., Heien, M. L., & Ewing, A. G. (2009). Chemical measurements in Drosophila. *Trends in Analytical Chemistry : TRAC*, *28*(11), 1223–1234. https://doi.org/10.1016/j.trac.2009.08.005

Marder, E., & Bucher, D. (2007). Understanding circuit dynamics using the stomatogastric

nervous system of lobsters and crabs. *Annual Review of Physiology*. https://doi.org/10.1146/annurev.physiol.69.031905.161516

Marder, E., O'Leary, T., & Shruti, S. (2014). Neuromodulation of Circuits with Variable Parameters: Single Neurons and Small Circuits Reveal Principles of State-Dependent and Robust Neuromodulation. *Annual Review of Neuroscience*, *37*(1), 329–346. https://doi.org/10.1146/annurev-neuro-071013-013958

Nusbaum, M. P., Blitz, D. M., & Marder, E. (2017). Functional consequences of neuropeptide and small-molecule co-transmission. *Nature Reviews Neuroscience*. https://doi.org/10.1038/nrn.2017.56

Pert, C., Ruff, M., Weber, R., & Herkenham, M. (1985). Neuropeptides and their receptors: a psychosomatic network. *The Journal of Immunology*, *135*(2).

Podvin, S., Bundey, R., Toneff, T., Ziegler, M., & Hook, V. (2015). Profiles of secreted neuropeptides and catecholamines illustrate similarities and differences in response to stimulation by distinct secretagogues. *Molecular and Cellular Neuroscience*, *68*, 177–185. https://doi.org/10.1016/j.mcn.2015.06.008

Rotzinger, S., Lovejoy, D. A., & Tan, L. A. (2010). Behavioral effects of neuropeptides in rodent models of depression and anxiety. *Peptides*. https://doi.org/10.1016/j.peptides.2009.12.015

Russell, F. A., King, R., Smillie, S. J., Kodji, X., & Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiological reviews*. https://doi.org/10.1152/physrev.00034.2013

Russo, A. F. (2017). Overview of Neuropeptides: Awakening the Senses? *Headache*, *57*(Suppl 2), 37–46. https://doi.org/10.1111/head.13084

Schank, J. R., Ryabinin, A. E., Giardino, W. J., Ciccocioppo, R., & Heilig, M. (2012). Stress-Related Neuropeptides and Addictive Behaviors: Beyond the Usual Suspects. *Neuron*. https://doi.org/10.1016/j.neuron.2012.09.026

Speese, S., Petrie, M., Schuske, K., Ailion, M., Ann, K., Iwasaki, K., Jorgensen, E. M., & Martin, T. F. J. (2007). UNC-31 (CAPS) is required for dense-core vesicle but not synaptic vesicle exocytosis in Caenorhabditis elegans. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.1466-07.2007

Tayler, T. D., Pacheco, D. A., Hergarden, A. C., Murthy, M., & Anderson, D. J. (2012). A neuropeptide circuit that coordinates sperm transfer and copulation duration in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(50), 20697–20702. https://doi.org/10.1073/pnas.1218246109

van den Pol, A. N. (2012). Neuropeptide Transmission in Brain Circuits. *Neuron*. https://doi.org/10.1016/j.neuron.2012.09.014

Wang, F., & Pereira, A. (2016). Neuromodulation, Emotional Feelings and Affective Disorders. *Mens Sana Monographs*. https://doi.org/10.4103/0973-1229.154533

Wegrzyn, J. L., Bark, S. J., Funkelstein, L., Mosier, C., Yap, A., Kazemi-Esfarjani, P., La Spada, A. R., Sigurdson, C., Oconnor, D. T., & Hook, V. (2010). Proteomics of dense core secretory vesicles reveal distinct protein categories for secretion of neuroeffectors for cell-cell communication. *Journal of Proteome Research*. https://doi.org/10.1021/pr1003104

Zelikowsky, M., Ding, K., & Anderson, D. J. (2018). Neuropeptidergic Control of an Internal Brain State Produced by Prolonged Social Isolation Stress. *Cold Spring*

Harbor Symposia on Quantitative Biology, *83*, 97–103. https://doi.org/10.1101/SQB.2018.83.038109

Zhang, X., Bao, L., & Ma, G.-Q. (2010). Sorting of neuropeptides and neuropeptide receptors into secretory pathways. *Progress in Neurobiology*, *90*(2), 276–283. https://doi.org/10.1016/J.PNEUROBIO.2009.10.011