Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The major conclusions and findings are summarized as the following:

Part A. *Drosophila*

- Invention of Neuropeptide Release Reporter (NPRR), a novel method to detect the release of different neuropeptides in intact neural tissue in *Drosophila*.
- NPRR has subcellular spatial and sub-second temporal resolution.
- NPRR responses exhibit triphasic kinetics, including rising, falling, and recovering phases, possibly reflect the slow kinetics of DCV replenishment relative to release.
- NPRR responses exhibit cell type-specific characteristics.
- NPRR exhibit peptide-specific expression pattern. Each NP deserves its own NPRR.

Part B. Mammalian cell lines

- Pilot establishment of an NPLER/NPRR imaging platform which consists of choice of neuropeptide of interest, proper cell lines, and imaging reporters.
- Pioneering evidence of different neuropeptides in the same cell undergo different subcellular trafficking process.
- Harnessing Next-gen pHluorin based Neuropeptide Release Reporters (NPRRs) for cell line imaging
- An NPLER-based RNAi screening platform.
- Engineered PYY outperforms native PYY in tissue distribution and serum concentration.

Future directions

Neuropeptide Imaging Zootopia: Generalization of NPLER/NPRR to other model organisms

Neuropeptides are evolutionarily conserved (Hoyle, 1998) and widely believed to be closely associated with the emergence of nervous systems (Grimmelikhuijzen & Hauser, 2012). Major progress in understanding neuropeptides were done in many well-characterized model organisms, such as *Mus musculus* (mice) (Arora & Anubhuti, 2006; Hökfelt et al., 2000; Kormos & Gaszner, 2013; Nusbaum et al., 2017; Russo, 2017), *Drosophila Melanogaster* (fruit flies) (Nässel & Winther, 2010), *Caenorhabditis elegans* (nematodes) (Bargmann & Marder, 2013), *Danio Rerio* (zebrafish) (Löhr & Hammerschmidt, 2011; Volkoff, 2006) and others. NPRRs were successfully developed and applied in fruit flies (Chapter 2) and mice (Chapter 3). An important future direction is to generalize NPRR and NPLER to the other model organisms.

In the course of NPRR engineering for fruit flies and mice, parallel efforts were made in other species. Nevertheless, generating and validation of NPRRs in *C.elegans* did not yield convincing results due to the complications of previously unknown source of fluorescence. Details are included in this thesis as an appendix chapter. Several NPRR/NPLER constructs were redesigned for zebrafish and jellyfish, yet still in the preliminary screening phase as of the drafting of this thesis.

How to migrate the design of NPRR from one model organism to another? Here are some thoughts and aspects to consider:

(1) Tuning of reporter expression. An ideal NPRR should follow the route of synthesis, sorting, transportation, and release as an endogenous neuropeptide. On the one hand, overly strong expression may lead to the accumulation of transgene products in ER and/or Golgi apparatus, which

in turn can potentially activate the protein degradation signaling pathways. Cells are likely to suffer toxicity by the protein overload or to experience changes of expression/release profiles. Neither are unfavorable for the application of NPRRs. On the other hand, weakly expressed reporters are less identifiable and trackable. To overcome or to alleviate this issue, either highly sensitive imaging technique, or DCV-enriched cells or subcellular regions are required. All these conditions entail heavy investment in extra labors and costs. Therefore, the choice of codon optimization, gene loci, expression vectors and regulatory elements needs prudent design and investigations.

(2) Deep understanding of neuropeptide of interest. The latest discoveries regarding neuropeptides often involve identifying new neuropeptides, uncovering previously unknown functions of a neuropeptide, and a mix of both in new animal models. However, NPRRs were not conceptualized for the research of such kinds. Alternatively, NPRRs take advantage of the understanding of neuropeptides and assist the exploration of means to regulate neuropeptide expression, sorting, trafficking, and release (Chapter 4, Figure 1). Understanding of neuropeptide structures and domains is helpful to optimize the sorting domain designs; whilst the information of cellular organelle markers provides a reference framework to characterize the expression and sorting semi-quantitatively.

(3) The research advance of fluorescent proteins (FPs). NPRRs and NPLERs rely heavily on the development of fluorescent proteins, which are iterated and optimized amazingly fast. Thanks to the generosity of these protein engineers, DNA sequences encoding the new FPs are made public almost immediately. One should pay close attention to the advance of the FP engineering.

Outside the box: Alternative strategies to image neuropeptide localization and release

All the imaging reporters made and discussed in this thesis are genetically encoded. Other genetically encoded strategies are mostly GPCR-based that mimic the expression of neuropeptide receptors to detect binding, notably the GRAB sensors for oxytocin, vasopressin and CCK (Dong et al., 2022; Qian et al., 2022; Wang et al., 2022) . These recently developed sensors are complementary to our reporters. An ideal yet very difficult experiment is to simultaneously image the release of neuropeptide from the upstream cells, and the detection of neuropeptide from the downstream cells. Unfortunately, the excitation and emission spectra of efficient GRAB sensors and NPRR sensors cannot be separated optically. A more red-shifted GRAB or NPRR will be particularly useful for the proposed imaging experiment.

The rapid advance in nucleotide deliveries, as well as the availability of NPY-specific short nucleotide aptamers, lead to the possibility of transferring DCV-targeting aptamers to directly bind intravesicular NPY (Mendonsa & Bowser, 2005; Proske et al., 2002). This idea is similar to the recombinant antibody techniques described in Chapter 4. Neuropeptide-binding, pH-sensitive organic dyes also deserve further investigations, though many technical difficulties are foreseeable.

The holy grail: *In vivo* imaging of neuropeptide release in behaving animals

Calcium imaging experiments in behaving animals are enabled by an optimized genetically engineered calcium indicator, a sensitive detection technique, and several cutting-edge algorithms to process and perfect the collected images. The holy grail is to make *in vivo* imaging of neuropeptide release in behaving animals possible. With prudent genetic manipulation and image registration, we will be able to identify both correlations and causalities between neuropeptide release and behaviors.

More specific sub-questions include (1) what cell/neuron releases neuropeptide; (2) when and how many neuropeptides are released; (3) the characterization of release dynamics and its relation to behavioral phenotypes and intensities; (4) genes/proteins involved in the regulation of neuropeptides. The list goes on. I hope that the prototypical reporters described in this thesis get us a little bit closer to the holy grail.

References

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

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

Dong, C., Zheng, Y., Long-Iyer, K., Wright, E. C., Li, Y., & Tian, L. (2022). Fluorescence Imaging of Neural Activity, Neurochemical Dynamics, and Drug-Specific Receptor Conformation with Genetically Encoded Sensors. *Annual Review of Neuroscience*, *45*(1). https://doi.org/10.1146/annurev-neuro-110520-031137

Grimmelikhuijzen, C. J. P., & Hauser, F. (2012). Mini-review: The evolution of neuropeptide signaling. *Regulatory Peptides*. https://doi.org/10.1016/j.regpep.2012.05.001

Hökfelt, T., Broberger, C., Xu, Z. Q. D., Sergeyev, V., Ubink, R., & Diez, M. (2000). Neuropeptides - An overview. *Neuropharmacology*. https://doi.org/10.1016/S0028-3908(00)00010-1

Hoyle, C. H. V. (1998). Neuropeptide families: Evolutionary perspectives. In *Regulatory Peptides* (Vol. 73, Issue 1, pp. 1–33). Elsevier. https://doi.org/10.1016/S0167-0115(97)01073-2

Kormos, V., & Gaszner, B. (2013). Role of neuropeptides in anxiety, stress, and depression: From animals to humans. *Neuropeptides*. https://doi.org/10.1016/j.npep.2013.10.014

Löhr, H., & Hammerschmidt, M. (2011). Zebrafish in Endocrine Systems: Recent Advances and Implications for Human Disease. *Annual Review of Physiology*, *73*(1), 183–211. https://doi.org/10.1146/annurev-physiol-012110-142320

Mendonsa, S. D., & Bowser, M. T. (2005). In vitro selection of aptamers with affinity for neuropeptide Y using capillary electrophoresis. *Journal of the American Chemical Society*, *127*(26), 9382–9383. https://doi.org/10.1021/ja052406n

Nässel, D. R., & Winther, Å. M. E. (2010). Drosophila neuropeptides in regulation of physiology and behavior. *Progress in Neurobiology*. https://doi.org/10.1016/j.pneurobio.2010.04.010

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

Proske, D., Fliger, M. H., Sö Ll, R. M., Beck-Sickinger, A. G., & Famulok, M. (2002). A Y2 receptor mimetic aptamer directed against neuropeptide Y. *Journal of Biological Chemistry*, *277*(13), 11416–11422. https://doi.org/10.1074/jbc.M109752200

Qian, T., Wang, H., Wang, P., Geng, L., Mei, L., Osakada, T., Tang, Y., Kania, A., Grinevich, V., Stoop, R., Lin, D., Luo, M., & Li, Y. (2022). Compartmental Neuropeptide Release Measured Using a New Oxytocin Sensor. *BioRxiv*, 2022.02.10.480016. https://doi.org/10.1101/2022.02.10.480016

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

Volkoff, H. (2006). The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*. https://doi.org/10.1016/j.cbpa.2005.10.026

Wang, H., Qian, T., Zhao, Y., Zhuo, Y., Wu, C., Osakada, T., Chen, P., Ren, H., Yan, Y., Geng, L., Fu, S., Mei, L., Li, G., Wu, L., Jiang, Y., Qian, W., Peng, W., Xu, M., Hu, J., … Li, Y. (2022). A toolkit of highly selective and sensitive genetically encoded neuropeptide sensors.

BioRxiv, 2022.03.26.485911. https://doi.org/10.1101/2022.03.26.485911