Imaging neuropeptide release and localization with genetically engineered reporters

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ABSTRACT

Neuropeptides are a class of neural signaling molecules that play a pivotal role in brain function and human health through neuromodulatory influences. There are over 100 types of neuropeptides identified and characterized, yet genomic analysis suggests that it is only the tip of the iceberg, with extra hundreds of putative neuropeptides awaiting further investigation. Neuropeptides collectively regulate a variety of developmental, physiological, and behavioral functions. 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.

Surprisingly, despite their fundamental importance, techniques for measuring the localization, expression and release of neuropeptides, at large scale and with high spatio-temporal resolution, have lagged far behind. Microdialysis and fast-scanning cyclic voltammetry are useful primarily for measuring "volume transmission," but are invasive, and have poor spatial resolution and limited general applicability. FP-tagged vesicle reporters are mainly tested and used in limited cell types. Little is characterized about their functional universality and specificity. GPCR-based sensors are designed to visualize the binding, instead of expression and release, of a neuropeptide.

Therefore, I aim to develop new methods for visualizing, detecting, and inhibiting NP expression and release *in vivo*. The long-term goal is to apply these methods to understanding the dynamics of neuromodulation of specific, behaviorally relevant neural circuits, and to providing a dynamic, high-resolution view of chemical modulation of circuit function.

In Chapter 2, I will describe the design, screening, and proof-of-concept validation of novel genetically engineered neuropeptide release reporters (NPRR) in *Drosophila*. I further demonstrated the idiosyncrasy of neuropeptide release dynamics, as well as cell-type specific release properties of a neuropeptide. In Chapter 3, I conceived and constructed a neuropeptide imaging platform that exploits the discoveries and strategies from *Drosophila* NPRRs. Besides a series of redesign of mammalian NPRRs, a collection of sister reporters to visualize localization and expression (Neuropeptide Localization and Expression Reporter, NPLER) were built in parallel. I also established a prototypical pipeline to systematically screen for appropriate cell lines for the purpose of NPRR/NPLER applications.

Malfunctioning of neuropeptide pathways can potentially result in a variety of mental illnesses triggered by stress, and metabolic disorders including obesity. Drugs targeting neuropeptide signaling have received heavy investment, but most have failed in the clinical trials. We therefore propose alternative strategies to target the processing/release of the neuropeptide from neurons, rather than blocking its receptor. In Chapter 4, I describe the ongoing process of adapting modern biotechnologies to the imaging platform to explore novel therapeutic strategies for neuropeptide-relevant disorders and abnormalities.

The Appendix includes a serendipitous finding from our attempt to generalize NPRR to *Caenorhabditis elegans*.

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K. D. contributed to: Data curation, Formal analysis, Methodology.

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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).

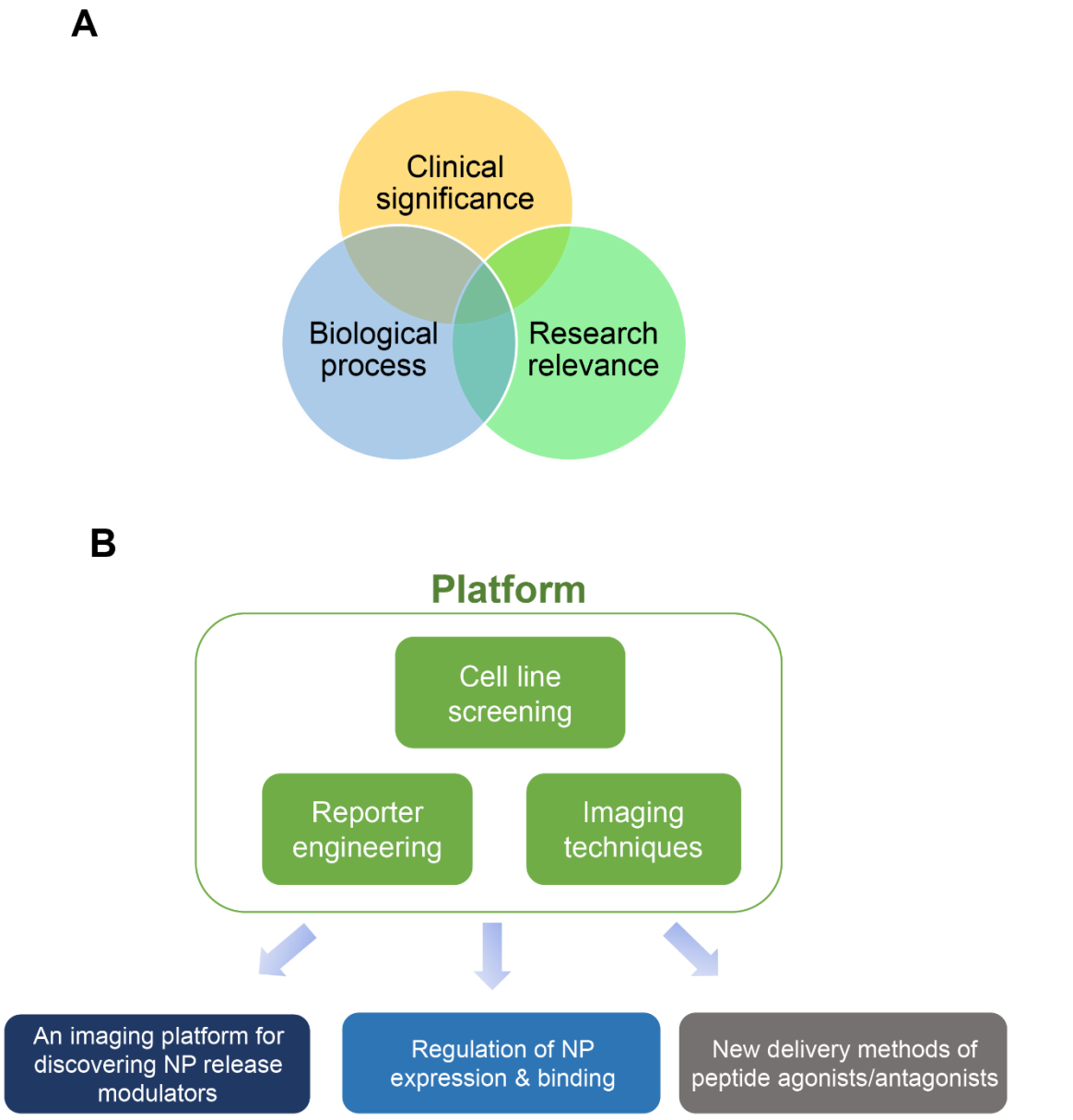

Figure 1: Rationales and visions of imaging neuropeptides

(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.

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Chapter 2

IMAGING NEUROPEPTIDE RELEASE AT *DROSOPHILA* SYNAPSES WITH A GENETICALLY ENGINEERED REPORTER

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Summary

Research on neuropeptide function has advanced rapidly, yet there is still no spatio-temporally resolved method to measure the release of neuropeptides *in vivo*. Here we introduce Neuropeptide Release Reporters (NPRRs): novel genetically-encoded sensors with high temporal resolution and genetic specificity. Using the *Drosophila* larval neuromuscular junction (NMJ) as a model, we provide evidence that NPRRs recapitulate the trafficking and packaging of native neuropeptides, and report stimulation-evoked neuropeptide release events as real-time changes in fluorescence intensity, with sub-second temporal resolution.

Introduction

Neuropeptides (NPs) exert an important but complex influence on neural function and behavior (Bargmann & Marder, 2013; Hokfelt et al., 2000; Insel & Young, 2000; Nassel & Winther, 2010). A major lacuna in the study of NPs is the lack of a method for imaging NP release *in vivo*, with subcellular spatial resolution and subsecond temporal resolution. Available techniques for measuring NP release include microdialysis (Kendrick, 1990), antibody-coated microprobes (Schaible, Jarrott, Hope, & Duggan, 1990), and GFP-tagged propeptides visualized either by standard fluorescence microscopy (van den Pol, 2012), or by TIRF imaging of cultured neurons (Xia, Lessmann, & Martin, 2009). In *Drosophila*, a fusion between rat Atrial Natriuretic Peptide/Factor (ANP/F) and GFP was used to investigate neuropeptide trafficking at the fly neuromuscular junction (NMJ) (Rao, Lang, Levitan, & Deitcher, 2001). Release was measured indirectly, as a decrease in ANP-GFP fluorescence intensity at nerve terminals reporting residual unreleased peptide, on a time-scale of seconds (Wong, Cavolo, & Levitan, 2015). None of these methods combined NP specificity, genetically addressable cell type-specificity, high temporal resolution and applicability to *in vivo* preparations (Supplementary Table 1). A major challenge is to develop a tool that encompasses all these features for direct, robust measurement of NP release *in vivo*.

Results

Neuropeptides are synthesized as precursors, sorted into dense core vesicles (DCVs), post-translationally modified and cleaved into active forms prior to release (Taghert & Veenstra, 2003). 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 (Figure 1—figure supplement 1A); and (2) a sorting domain that ensures its

selective trafficking into DCVs (Figure 1—figure supplement 1b). The NP precursor may function as the sorting domain, suggested by studies of DCV fusion using pIAPP-EGFP (Barg et al., 2002) and NPY-pHluorin (Zhu et al., 2007) in cultured neurons, or ANP-GFP in *Drosophila* (Rao et al., 2001). We therefore developed a pipeline to screen various transgenes comprising NP precursors fused at different sites to fluorescent reporters, in adult flies (Figure 1—figure supplement 1B-C). A total of 54 constructs were tested. We found that optimal trafficking was achieved by substituting the reporter for the NP precursor C-terminal domain that follows the final peptide (Figure 1—figure supplement 1B). In order to maintain covalent linkage with the reporter domain, we removed the dibasic cleavage site C-terminal to the final peptide.

The DCV lumen has lower pH and free calcium (pH = 5.5-6.75, $[Ca^{2+}]$ ~30 µM) compared to the extracellular space (pH =7.3, $[Ca^{2+}]$ ~2 mM) (Mitchell et al., 2001; Sturman, Shakiryanova, Hewes, Deitcher, & Levitan, 2006). These differences prompted us to test validated sorting domains in a functional *ex vivo* screen using either pH-sensitive fluorescent proteins (Miesenbock, De Angelis, & Rothman, 1998) or genetically-encoded calcium indicators (GECIs) (Lin & Schnitzer, 2016; Tian, Hires, & Looger, 2012) (Figure 1—figure supplement 1A-D). Reporters based on pHluorins (Miesenbock et al., 1998) did not perform well in our hands, therefore we focused on GCaMP6s (Chen et al., 2013). The calcium sensitivity threshold of GCaMP6s is below the calcium concentration in both DCVs and the extracellular space. However, GCaMP6s fluorescence is quenched in the acidic DCV lumen (Barykina et al., 2016), enabling it to function as a dual calcium/pH indicator (Figure 1A). These key properties should boost the contrast between GCaMP6s fluorescence in unreleased vs. released DCVs, potentially allowing us to trace NP release at the cellular level *in vivo*.

We sought to test several NP precursor-GCaMP6s fusion proteins, called NPRRs (NeuroPeptide Release Reporters; unless otherwise indicated all NPRRs refer to fusions with GCaMP6s), in an intact preparation using electrical stimulation to evoke release. Initially for proof-of-principle experiments, we used the Dro*sophila* larval NMJ to test $NPRR^{ANP}$, a GCaMP6s fusion with rat ANP (Burke et al., 1997). NMJ terminals are large, individually identifiable, and easy to image and record. In particular, boutons on muscle 12/13 are diverse—Type Ib and Type Is boutons contain mostly synaptic vesicles and few DCVs, while Type III boutons contain an abundance of DCVs but no synaptic vesicles (Menon, Carrillo, & Zinn, 2013); moreover, Type III-specific GAL4 drivers are available (Koon & Budnik, 2012) (Figure 1B).

Expression of $NPRR^{ANP}$ pan-neuronally (under the control of nsyb-GAL4) followed by double immuno-staining for ANP and GCaMP (anti-GFP) indicated that the sorting domain and the reporter domains showed a similar localization in Type III neurons (Figure 1—figure supplement 2). Moreover, the distribution of $NPRR^{ANP}$ overlapped that of Bursicon (Figure 1—figure supplement 3D), an NP that is endogenously expressed in Type III neurons (Loveall & Deitcher, 2010). Both GCaMP and Bursicon immunoreactivity were strongest within boutons, consistent with the known subcellular localization of DCVs (Gorczyca & Budnik, 2006).

Glutamate is the only known canonical neurotransmitter used at the larval NMJ (Menon et al., 2013). This allowed visualization of the subcellular localization of small synaptic vesicles (SV) by immuno-staining for vGluT, a vesicular glutamate transporter (Fremeau et al., 2001; Kempf et al., 2013). In Type Ib neurons (which contain relatively few DCVs relative to SVs (Menon et al., 2013)), vGluT

staining was observed as patches with dim center, which may reflect clustered SVs, while $NPRR^{ANP}$ immunoreactivity was seen in dispersed, non-overlapping punctae (Figure 1C, α-GFP, inset). In Type III neurons, NPRRs were strongly expressed but no vGluT immunoreactivity was detected (Figure 1C). The subcellular distribution of this NPRR in larval NMJ neurons, therefore, is similar to that of other DCV-targeted markers previously used in this system (Rao et al., 2001; Shakiryanova, Tully, & Levitan, 2006), and appears to reflect exclusion from SVs.

The diffraction limit of light microscopy precluded definitive co-localization of NPRRs in DCVs. Therefore, we employed Immuno-Electron microscopy (Immuno-EM) to investigate the subcellular localization of NPRRs at the nanometer scale. To maximize antigenicity for Immuno-EM, we generated constructs that replaced GCaMP6s with GFP ($NPRR^{ANP\text{-}GFP}$;). $NPRR^{ANP\text{-}GFP}$ showed dense labeling in association with DCVs (Figure 1D, arrows), where the average number of gold particles/μm^2 was substantially and significantly higher than in neighboring bouton cytoplasm (DCV/Bouton ~14.26) (Figure 1E, Supplementary Table 2). Taken together, these data indicate that $NPRR^{ANP\text{-}GFP}$ is localized to DCVs. By extension, they suggest that $NPRR^{ANP\text{-}GCaMP6s}$ (which has an identical structure to $NPRR^{ANP\text{-}GFP}$ except for the modifications that confer calcium sensitivity) is similarly packaged in DCVs. While these two reporters show indistinguishable distributions by immunofluorescence, we cannot formally exclude that the substitution of GCaMP for GFP may subtly alter subcellular localization of the NPRR in a manner undetectable by light microscopy.

To measure the release of NPRRs from DCVs, we next expressed $NPRR^{ANP}$ in Type III neurons using a specific GAL4 driver for these cells (Koon & Budnik, 2012) (Figure 2E and Figure 1—figure supplement 3D). We delivered 4 trials of 70 Hz electrical stimulation to the nerve bundle, a

frequency reported to trigger NP release as measured by ANF-GFP fluorescence decrease (Rao et al., 2001; Shakiryanova et al., 2006), and used an extracellular calcium concentration that promotes full fusion mode (Ales et al., 1999). This stimulation paradigm produced a relative increase in $NPRR^{ANP}$ fluorescence intensity (ΔF/F), whose peak magnitude increased across successive trials (Figure 2A, red bars and 2D; Video 1; Figure 2—figure supplement 1, A_1 vs. A_7). Responses in each trial showed a tri-phasic temporal pattern: (1) In the "rising" phase, $NPRR^{ANP}$ ΔF/F peaked 0.5-5 secs after stimulation onset, in contrast to the virtually instantaneous peak seen in positive control specimens expressing conventional GCaMP6s in Type III neurons (Figure 2A-B). The $NPRR^{ANP}$ latency to peak was similar to the reported DCV fusion latency following depolarization in hippocampal neurons (Xia et al., 2009). This delay is thought to reflect the kinetic difference between calcium influx and DCV exocytosis due to the loose association between DCVs and calcium channels (Xia et al., 2009). (2) In the "falling" phase, $NPRR^{ANP}$ ΔF/F began to decline 1-5 seconds before the termination of each stimulation trial, presumably reflecting depletion of the available pool of releasable vesicles. In contrast, GCaMP6s fluorescence did not return to baseline until after stimulation offset (Figure 2A-B). (3) Finally, unlike GCaMP6s, $NPRR^{ANP}$ exhibited an "undershoot" (ΔF/F below baseline) during the post-stimulation intervals, followed by a "recovery" phase (Figure 2A; Figure 2C, I1-4). This undershoot may reflect dilution of released fluorescent NPRR molecules by diffusion into the synaptic cleft (van den Pol, 2012), while recovery may reflect DCV replenishment in the boutons from vesicles proximal to the imaged release site.

Because $NPRR^{ANP}$ fluorescence was preferentially accumulated within boutons, we asked whether these regions contributed to ΔF/F peaks more significantly than the inter-bouton intervals (IBIs). To do this, we partitioned the processes into boutons and IBI fields (Figure 2—figure supplement 2A), and compared the ΔF/F in these regions during stimulation trials. The time-averaged ratio of

bouton/IBI ΔF/F (see Materials and Methods) was significantly higher for $NPRR^{ANP}$ than for GCaMP6s, particularly during later stimulation trials (Figure 2—figure supplement 2B, green bars, S2-4). This contrast indicates that $NPRR^{ANP}$ signals are preferentially observed in boutons, where DCVs are located, and do not reflect differences in cytoplasmic free Ca^{2+} levels between these regions as detected by GCaMP6s.

To test definitively if $NPRR^{ANP}$ ΔF/F signals are dependent upon NP release, we blocked vesicle fusion at terminals of Type III neurons using expression of tetanus toxin light chain (TNT) (Sweeney, Broadie, Keane, Niemann, & O'Kane, 1995), a protease that cleaves n-synaptobrevin, a v-snare required for DCV fusion (Figure 2—figure supplement 3) (T. Xu, Binz, Niemann, & Neher, 1998). As a control, we used impotent TNT (TNT^{imp}), a reduced activity variant (Sweeney et al., 1995). TNT expression completely abolished stimulation-induced ΔF/F increases from $NPRR^{ANP}$, while TNT^{imp} did not (Figure 2F). Further analysis revealed that both the ΔF/F peaks and inter-stimulation undershoots were diminished by TNT (Figure 2G-H). In contrast, neither TNT nor TNT^{imp} affected the kinetics of GCaMP6s signals in Type III neurons (Figure 2—figure supplement 2C), which report cytosolic Ca^{2+} influx. Taken together, these data support the idea that $NPRR^{ANP}$ signals specifically reflect DCV release.

ANP is a rat NP that lacks a *Drosophila* homolog (Rao et al., 2001). To determine whether our method could be applied to detect the release of a specific, endogenous fly NP, we tested $NPRR^{dTK}$, one of 6 different reporter variants we initially generated from the *Drosophila* neuropeptide precursor, DTK (Figure 1—figure supplement 1B). In contrast to ANP which encodes a single peptide, DTK yields multiple NP derivatives (Winther, Siviter, Isaac, Predel, & Nassel, 2003). Light

microscopy (Figure 3A) and Immuno-EM (Figure 3B, arrows) confirmed that $NPRR^{dTK}$, like $NPRR^{ANP}$, was localized to DCVs (DCV/bouton ~22.19, Figure 3C). Using the Type III-specific GAL4 driver to express $NPRR^{dTK}$ and the same stimulation protocol as used for $NPRR^{ANP}$, the basic tri-phasic response profile was also observed (Figure 3D). However, peak heights and baseline fluorescence fell progressively with successive stimulation trials (Figure 3E), in contrast to $NPRR^{ANP}$ where the first peak and undershoot were lower (Figure 2C-D). The reason for this difference is currently unclear.

We next investigated the relationship between NPRR signal and stimulation intensity, by delivering to the Type III neurons a series of low to high frequency electrical stimuli (1-70 Hz; (Levitan, Lanni, & Shakiryanova, 2007)) while imaging the nerve terminals. For direct comparison of NPRR responses across different preparations, we applied *a posteriori normalization of fluorescent peaks in each trial to the highest response obtained among all trials.* For both $NPRR^{ANP}$ and $NPRR^{dTK}$ (Figure 4A-B), the peak responses showed a positive correlation with stimulation frequency, analogous to that observed using cytosolic GCaMP6s (Figure 4C). In Type III neurons, the responses of both NPRRs to stimulation frequencies<30 Hz (1,5,10,20 Hz) were not statistically significant from zero. $NPRR^{ANP}$ showed a higher sensitivity to high stimulation frequencies (30 Hz: 18.14%, 50 Hz: 82.40% Normalized peak $\Delta F/F$), while $NPRR^{dTK}$ showed a higher stimulation threshold and lower sensitivity (30Hz: 3.57%, 50Hz: 24.67% Normalized peak $\Delta F/F$).

We next investigated whether the relatively high stimulation frequency required to observe significant responses with NPRRs was a function of the reporters, or rather of the cell class in which they were tested. To do this, we expressed both NPRRs in Type Ib neurons, a class of motor neurons

that contains both SVs and DCVs (Figure 1B, Figure 4D-F), and performed stimulation frequency titration experiments. Strikingly, in Type Ib neurons, significant increases in ΔF/F could be observed at frequencies as low as 10 Hz (Figure 4D, E; $NPRR^{ANP}$ @ 20 Hz: 12.50%, $NPRR^{dTK}$ @ 20 Hz: 17.67% normalized peak ΔF/F). The reason for the difference in NPRR threshold between Type III and Type Ib neurons is unknown, but parallels their difference in GCaMP6s response to electrical stimulation (Figure. 4C vs. 4F).

Notably, although $NPRR^{ANP}$ and $NPRR^{dTK}$ presented distinct response profiles in Type III neurons, their performance in Type Ib neurons was more similar (Fig. 4A vs. 4B; cf. 4D vs. 4E). In summary, the differences in performance we observed between the two NPRRs appeared to be specific to Type III neurons, and were minor in comparison to the differences in performance of both reporters between the two cell classes in. The reason for the differences between $NPRR^{ANP}$ and $NPRR^{dTK}$ sensitivity and kinetics in Type III neurons is unknown but may reflect differences in how well these reporters compete with the high levels of endogenous neuropeptide (Bursicon) for packaging, transport or release.

Discussion

Here we present proof-of-principle for a method to detect the release of different neuropeptides in intact neural tissue, with subcellular spatial and sub-second temporal resolution. By exploiting the fluorescent change of GCaMP in response to a shift in pH and $[Ca^{2+}]$, we visualized the release of neuropeptides by capturing the difference between the intravesicular and extracellular microenvironment. NPRR responses exhibited triphasic kinetics, including rising, falling and recovery phases. In the falling phase, a post-stimulus "undershoot," was observed in which the

fluorescent intensity fell below pre-stimulation baseline. This undershoot presumably reflects the slow kinetics of DCV replenishment relative to release.

The molecular mechanisms of NP release are incompletely understood (Tao Xu & Xu, 2008). It is possible that individual DCVs only unload part of their cargo during stimulation, in which case many DCVs that underwent fusion may still contain unreleased NPRR molecules following a stimulus pulse. Although we are convinced that NPRR signals do indeed reflect NP release, due to the presence of the recovery phase, we cannot formally exclude that unreleased NPRRs may contribute to the signal change due to their experience of intravesicular $[Ca^{2+}]$/pH changes that occur during stimulation. To resolve this issue in the future, an ideal experiment would be to co-express an NPRR together with a $[Ca^{2+}]$/pH-invariant NP-reporter fusion. Multiple attempts to generate such fusions with RFP were unsuccessful, due to cryptic proteolytic cleavage sites in the protein which presumably result in degradation by DCV proteases during packaging.

To test if $NPRR^{ANP}$ $\Delta F/F$ signals are dependent on NP release, we expressed the light chain of tetanus toxin (TNT), a reagent shown to effectively block NP release in many (Hentze, Carlsson, Kondo, Nassel, & Rewitz, 2015; McNabb & Truman, 2008; Zandawala et al., 2018), if not all (Umezaki, Yasuyama, Nakagoshi, & Tomioka, 2011), systems. We observed a striking difference in NPRR kinetics in flies co-expressing TNT vs. its proteolytically inactive "impotent" control form TNT^{imp} (Figure 2F). The strong reduction of NPRR signals by TNT-mediated n-syb cleavage is consistent with the idea that these signals reflect the release of NPRRs from DCVs.

We have tested the generalizability of the principles used to generate NPRRs by (1) constructing a surrogate NP reporter $NPRR^{ANP}$ as well as a multi-peptide-producing endogenous *Drosophila* NP reporter $NPRR^{dTK}$ (Figure 2-3); (2) characterized NPRR signals in response to varying intensities of electrical stimulation; and (3) recorded NPRR signals in two different classes of NMJ motor neurons containing DCVs with or without SVs, respectively (Figure 4). These experiments revealed, to our surprise, that NPRR responses exhibit cell-type specific characteristics (Figure 4). As NPRRs are applied to other neuropeptides and cell types, a systematic characterization of neuropeptide release properties in different peptidergic neurons should become possible, furthering our understanding of neuropeptide biology.

The method described here can, in principle, be extended to an *in vivo* setting. This would open the possibility of addressing several important unresolved issues in the study of NP function *in vivo*. These include the "which" problem (which neuron(s) release(s) NPs under particular behavioral conditions?); the "when" problem (when do these neurons release NPs relative to a particular behavior or physiological event?); the "where" problem (are NPs released from axons, dendrites or both?); and the "how" problem (how is NP release regulated?). The application of NPRRs to measuring NP release dynamics in awake, freely behaving animals may yield answers to these important long-standing questions.

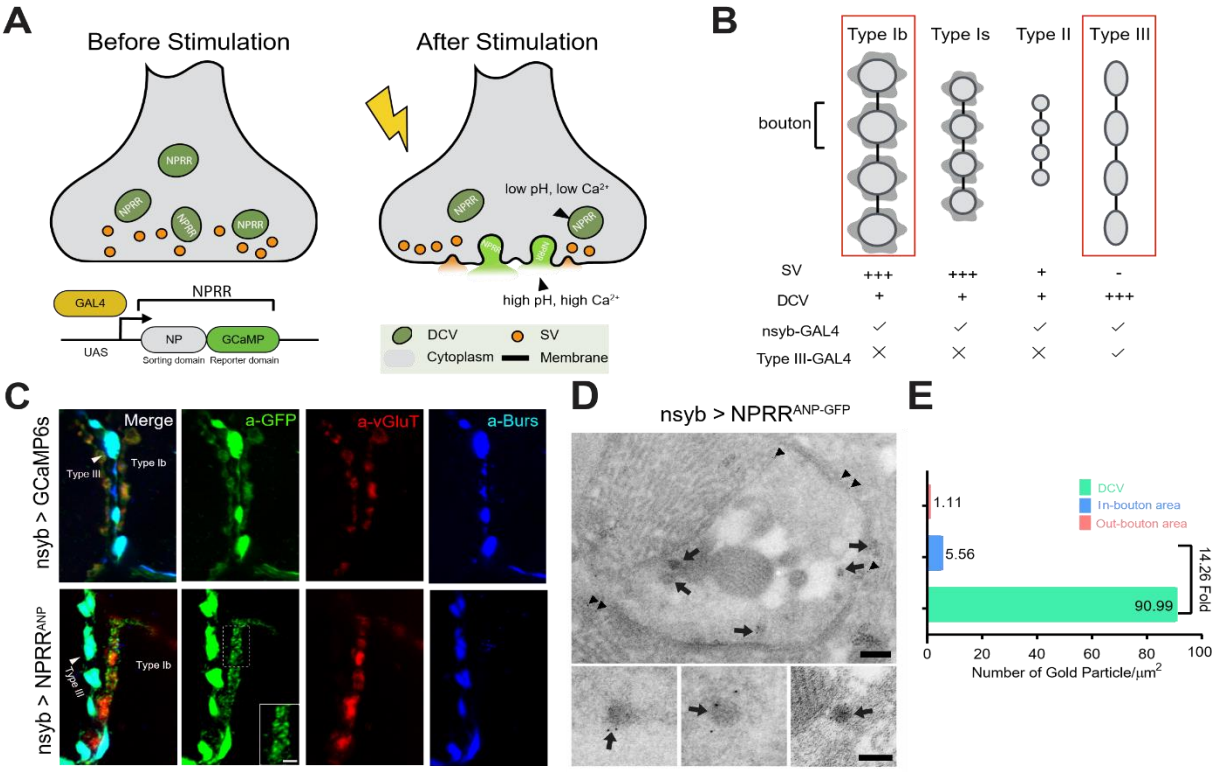

Figure 1: **Localization of an NPRR.**

(A) Schematic illustrating the principle of NPRRs (**N**euro**p**eptide **R**elease **R**eporters). NPRR molecules in the DCV lumen (low pH/low calcium, *left*) exhibit increased fluorescence when released by fusion into the extracellular space (neutral pH/high calcium, *right*). NPRR fluorescent signal is expected to decay following diffusion into the synaptic cleft. New NPRR-containing DCVs are produced by synthesis and transport from the soma, not by recycling. NP: Neuropeptide. DCV: Dense Core Vesicle. SV: Synaptic Vesicle. **(B)** Distinct motor neuron subtypes at the *Drosophila* NMJ (muscle 12/13) have different proportions of DCVs vs. SVs. The GAL4 driver R57C10-Gal4 (nsyb-GAL4) labels all subtypes, while R20C11-GAL4 selectively labels only Type III neurons, which lack SVs ("Type III-GAL4"). Light grey circles, black lines and dark grey shading represent boutons, inter-bouton intervals and subsynaptic reticulum, respectively. The studies in this paper focus on Type Ib neurons and Type III neurons (in red rectangles). **(C)** Triple immunolabeling for GFP (green), Bursicon (blue) and vGluT (red), in flies containing nsyb-GAL4 driving UAS-GCaMP6s *(upper)*, or $NPRR^{ANP}$ (*lower*). Type Ib and Type III boutons are indicated. Scale bar, 5 µm. Inset image ($NPRR^{ANP}$, a-GFP channel) shows details of puncta distribution of $NPRR^{ANP}$ in Type Ib neuron. Scale bar, 2 µm. **(D)** TEM images of boutons immunolabeled with anti-GFP (5 nm gold particle-conjugated) to detect nsyb>$NPRR^{ANP-GFP}$, which has an identical structure to $NPRR^{ANP}$, but is a GFP rather than GCaMP6s fusion to improve antigenicity. Note strong labeling in DCVs (arrows) and the neuronal plasma membrane (arrowheads). Scale bar, 200 nm. Lower panel shows representative images of labeled DCVs. Scale bar,100 nm. **(E)** Quantification for TEM images in **(D)**.

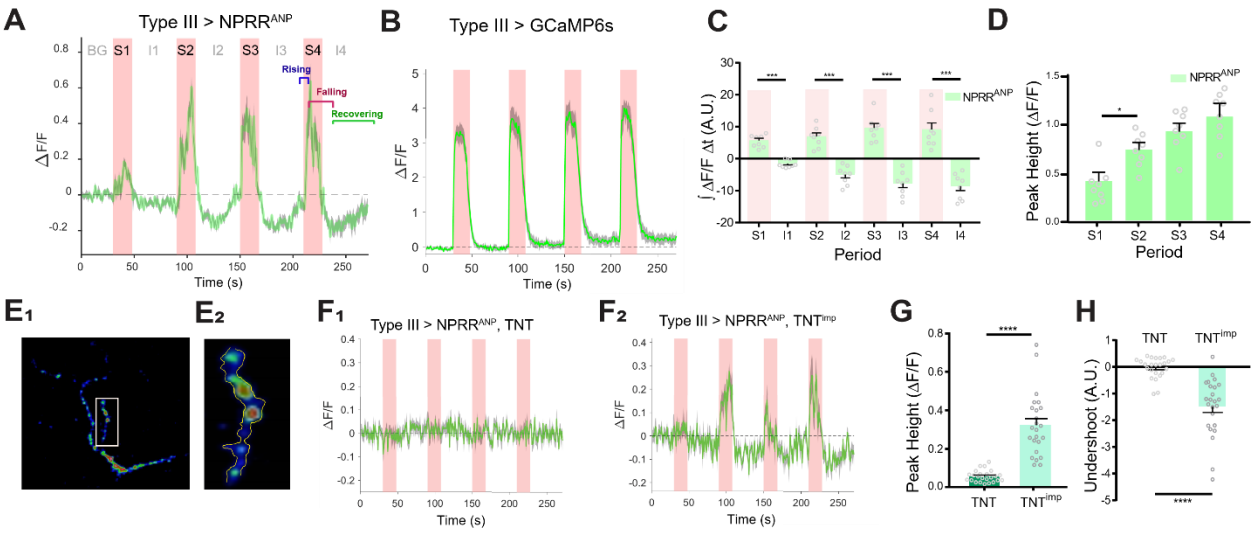

Figure 2: NPRR specifically reports neuropeptide release.

(A) Trace from a representative experiment showing changes in NPRR^{ANP} fluorescence intensity (ΔF/F) in Type III motor neurons at the larval NMJ evoked by electrical stimulation. BG: background. S1-S4: Stimulation trials 1-4. I1-I4: Inter-stimulation Intervals (ISIs) 1-4. Green line: ΔF/F averaged across all boutons in the field of view. Grey shading: s.e.m envelope. Red bar: electrical stimulation trials (70 Hz). The three typical phases of the response are indicated in S4. The peak height of the response on the first trial is characteristically lower (see also **(D)**), and may reflect competition with unlabeled DCVs in the readily releasable pool. **(B)** ΔF/F traces in control flies expressing cytoplasmic GCaMP6s in Type III neurons. **(C)** Integrated NPRR^{ANP} ΔF/F values during trials S1-4 and intervals I1-4. A.U.: arbitrary units. $n = 8$. ***, $P<0.001$. **(D)** Average NPRR^{ANP} ΔF/F peak heights for trials S1-4. $n = 8$. *, $P<0.05$. Plotted values in (**C-D**) are mean±s.e.m. **(E$_1$-E$_2$)** Representative selection of ROIs (yellow). Details see **Materials and methods**. Scale bar, 5 µm. **(F)** NPRR^{ANP} ΔF/F response are abolished in Type III GAL4>UAS-NPRR^{ANP} flies bearing UAS-TNT (**F$_1$**) but not UAS-TNT^{imp} (**F$_2$**). **(G)** Average peak heights of NPRR^{ANP} ΔF/F in combined stimulation trials (S1-4) from (**F**). ****, $P<0.0001$. **(H)** Average "undershoot," defined as the integrated ΔF/F during ISIs I1-4 (see (**C**)). In **C-D** and **G-H**.

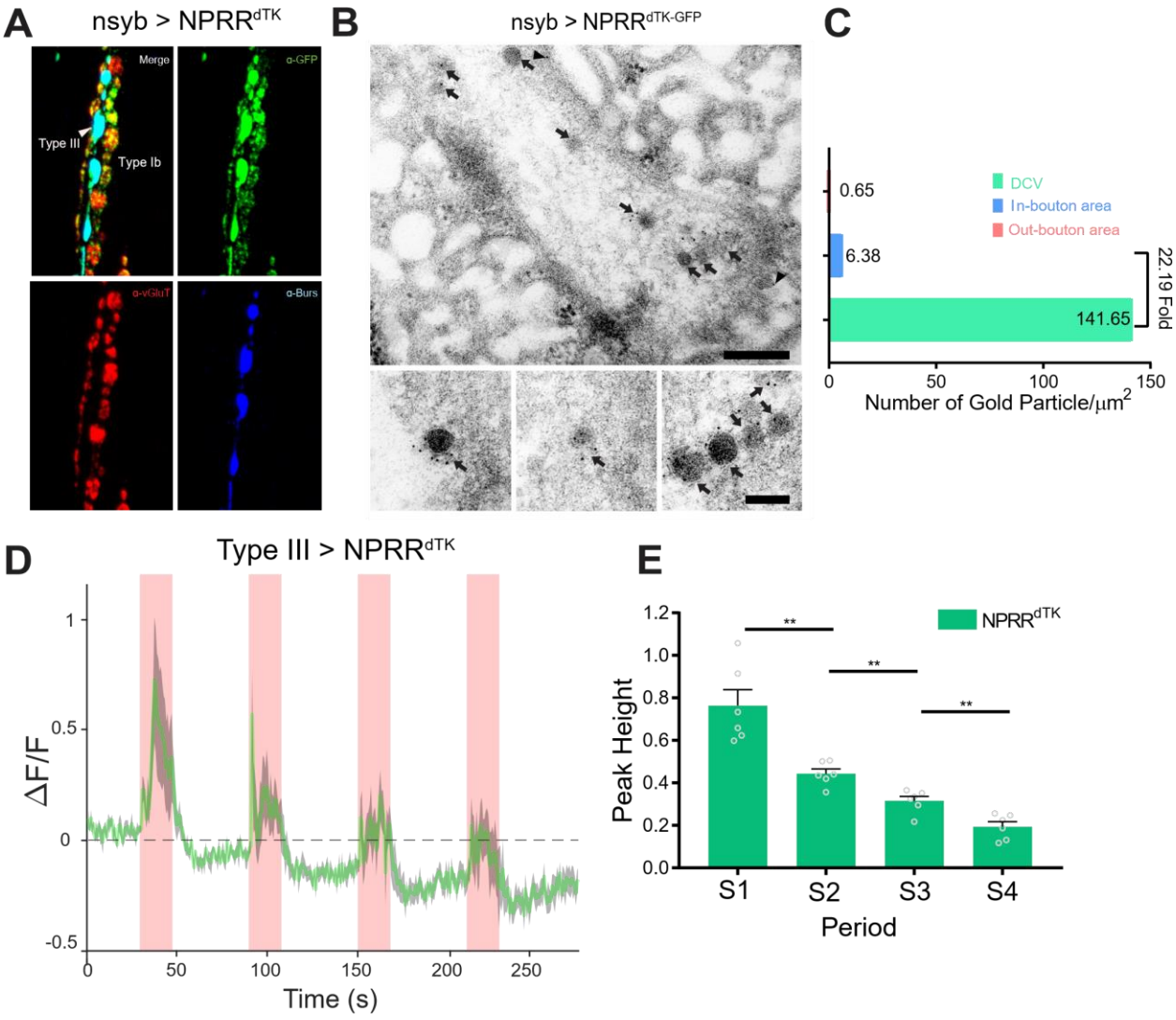

Figure 3: Application of the NPRR approach to a *Drosophila* neuropeptide.

(A) Triple immunolabeling for GFP (green), Bursicon (blue) and vGluT (red) in Type III-GAL4>UAS-NPRR^{dTK} flies. Scale bar, 5 µm. **(B)** TEM images of boutons immunolabeled against GFP (5 nm gold) in nsyb-GAL4>UAS-NPRR$^{dTK\text{-}GFP}$ flies. Note strong labeling in DCVs (arrows) and bouton plasma membrane (arrowheads). Scale bar, 200 nm. Lower panel shows representative images of labeled DCVs. Scale bar,100 nm. **(C)** Quantification of TEM images in **(B)**. **(D)** NPRR^{dTK} ΔF/F curve; stimulation conditions as in **Figure 2A**. **(E)** Average NPRR^{dTK} ΔF/F peak height above pre-stimulation baseline (corrected; see **Materials and methods**) for stimulation trials S1-4. n = 6. **, P<0.01.

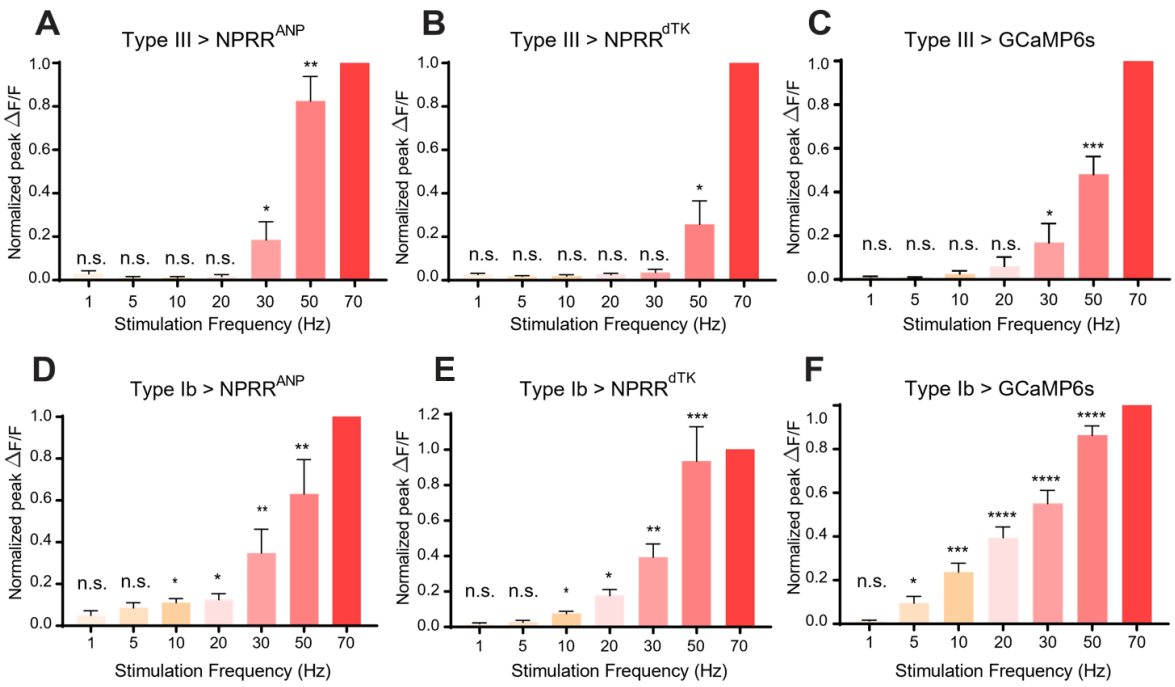

Figure 4: NPRR reveals distinct cell-type specific peptide release properties.

For each preparation, a series of stimulation trials were delivered at frequencies from 1 Hz to 70 Hz, as indicated. In-stimulation response peaks were normalized to 70 Hz. The normalized peaks of NPRRs or calcium responses (measured with cytosolic GCaMP6s) were pooled and plotted for both Type III **(Figure 4A-C)** and Type Ib **(Figure 4D-F)** neurons. Responses were compared to zero. *n* = 6-12. n.s., not significant. *, *P*<0.05. **, *P*< 0.01. ***, *P*< 0.001. ****, *P*<0.0001.

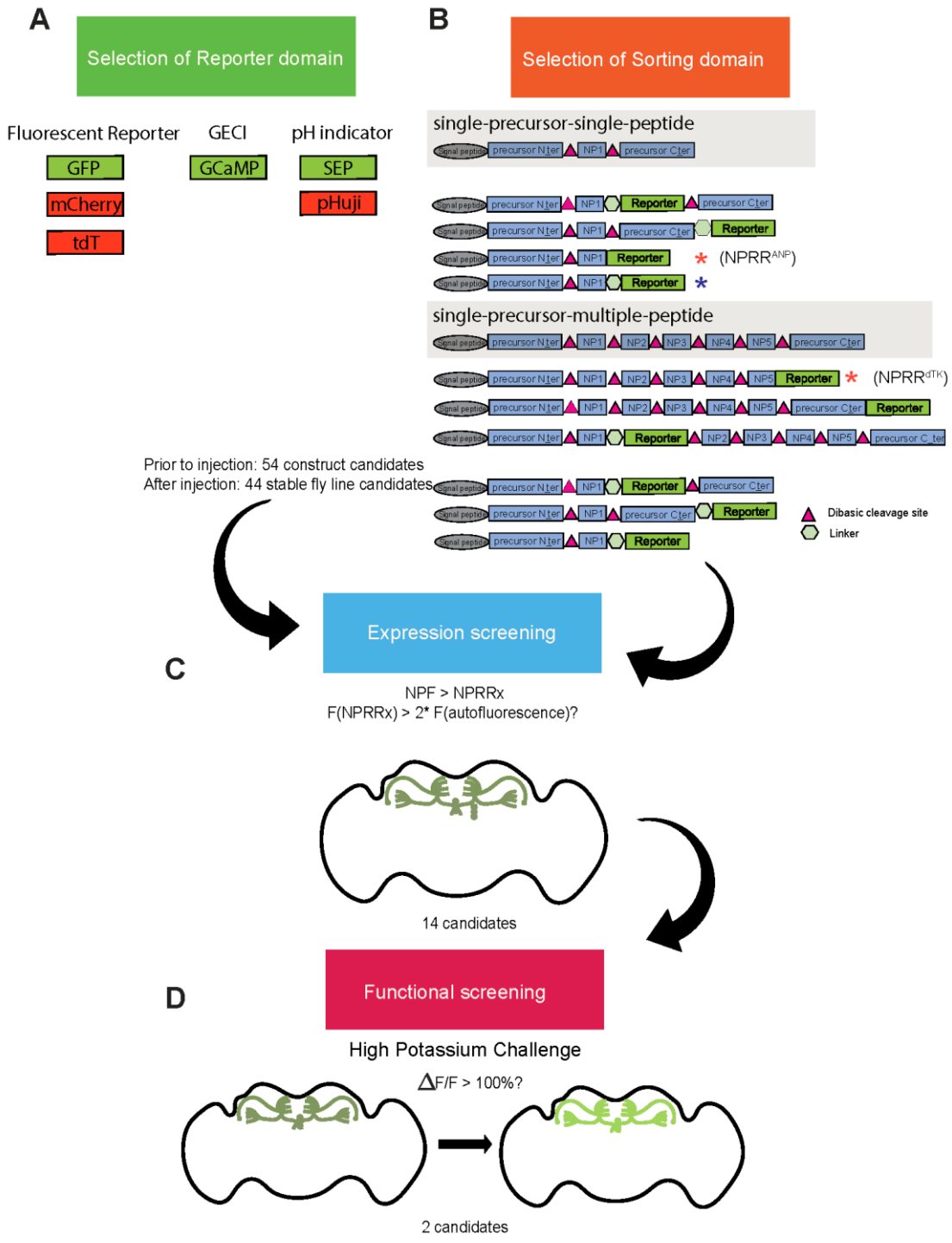
A
Selection of Reporter domain
Fluorescent Reporter
GFP
mCherry
tdT
GECI
GCaMP
pH indicator
SEP
pHuji
B
Selection of Sorting domain
single-precursor-single-peptide
single-precursor-multiple-peptide
(NPRR^ANP)
(NPRR^dTK)
Dibasic cleavage site
Linker
Prior to injection: 54 construct candidates
After injection: 44 stable fly line candidates
C
Expression screening
NPF > NPRRx
F(NPRRx) > 2* F(autofluorescence)?
14 candidates
D
Functional screening
High Potassium Challenge
ΔF/F > 100%?
2 candidates

Figure 1—figure supplement 1: NPRR screening pipeline.
A series of reporter-neuropeptide precursor fusions were designed, codon-optimized for *Drosophila*, cloned into expression vectors under the control of the GAL4 upstream activator sequence (UAS), and used to generate transgenic flies. **(A)** Candidate reporters interrogated included (constitutive) fluorescent reporters, genetically encoded calcium indicators (GECI) and pH indicators (pHluorins). **(B)** Sorting domain candidates included different truncated versions of rat Atrial Natriuretic Peptide (ANP; single-precursor-single-peptide) and *Drosophila* tachykinin (dTK; single-precursor-multiple-peptide) precursors. 52 constructs were built and injected. 44 of 54 were successfully integrated as transgenic lines, while 8 were excluded due to lethality or unstable expression. **(C-D)** Candidate UAS-NPRR lines were crossed with an NPF-Gal4 driver line and selected based on their expression in NPF terminals in the adult fly brain. The raw fluorescence intensity of each NPRR candidate was measured using the same microscope parameters (laser power, HV, offset value). 14 candidates passed this screening. (**C**) We screened the performance of difference NPRRs (signal-to-noise contrast) by measuring fluorescence before and immediately after 70mM high-potassium challenge in an *ex vivo* explant preparation of adult fly brains. The post/pre KCl fluorescence ratio is defined as $\Delta F/F$. We arbitrarily set the threshold as 100%. 2 NPRRs with highest $\Delta F/F$ passed the final round of screening.
Red asterisks indicate the candidates selected for the studies in **Figure 2** and **Figure 3**. Blue asterisk indicates original ANP-GFP fusion)(Burke et al., 1997; Rao et al., 2001).

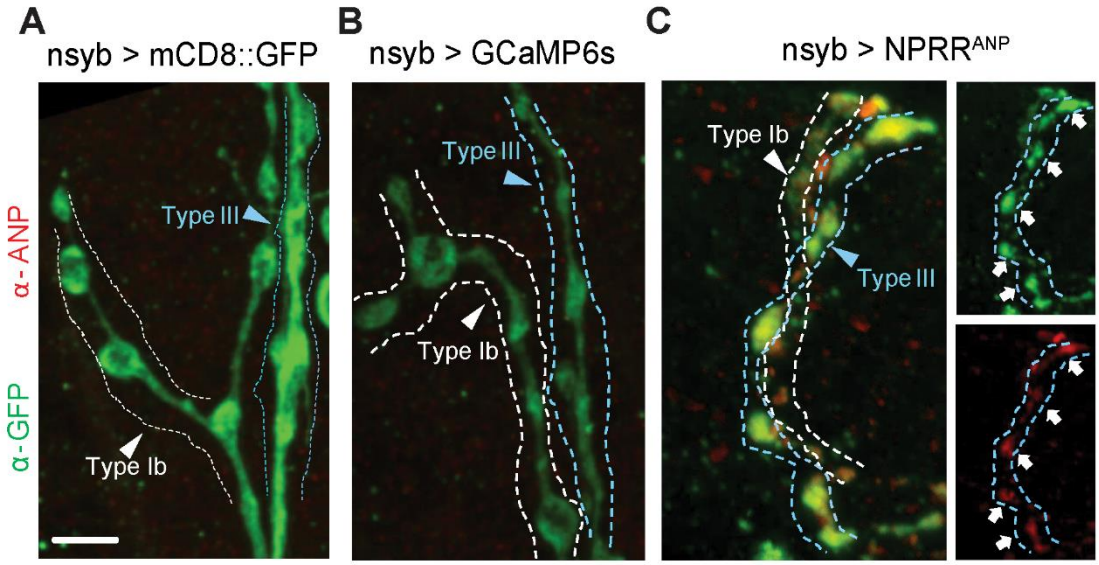

Figure 1—figure supplement 2: Exogeneous neuropeptide ANP dictates the expression pattern of NPRR^{ANP}.

Membrane-bound mCD8::GFP fusion **(A)**, cytosolic GCaMP6s (**B**) and NPRR^{ANP} (**C**) were expressed pan-neuronally in the larval NMJ and stained for both ANP (red) and NPRR (green, anti-GFP). **(C)** Note co-localization of ANP and GFP. Scale bar, 5 μm.

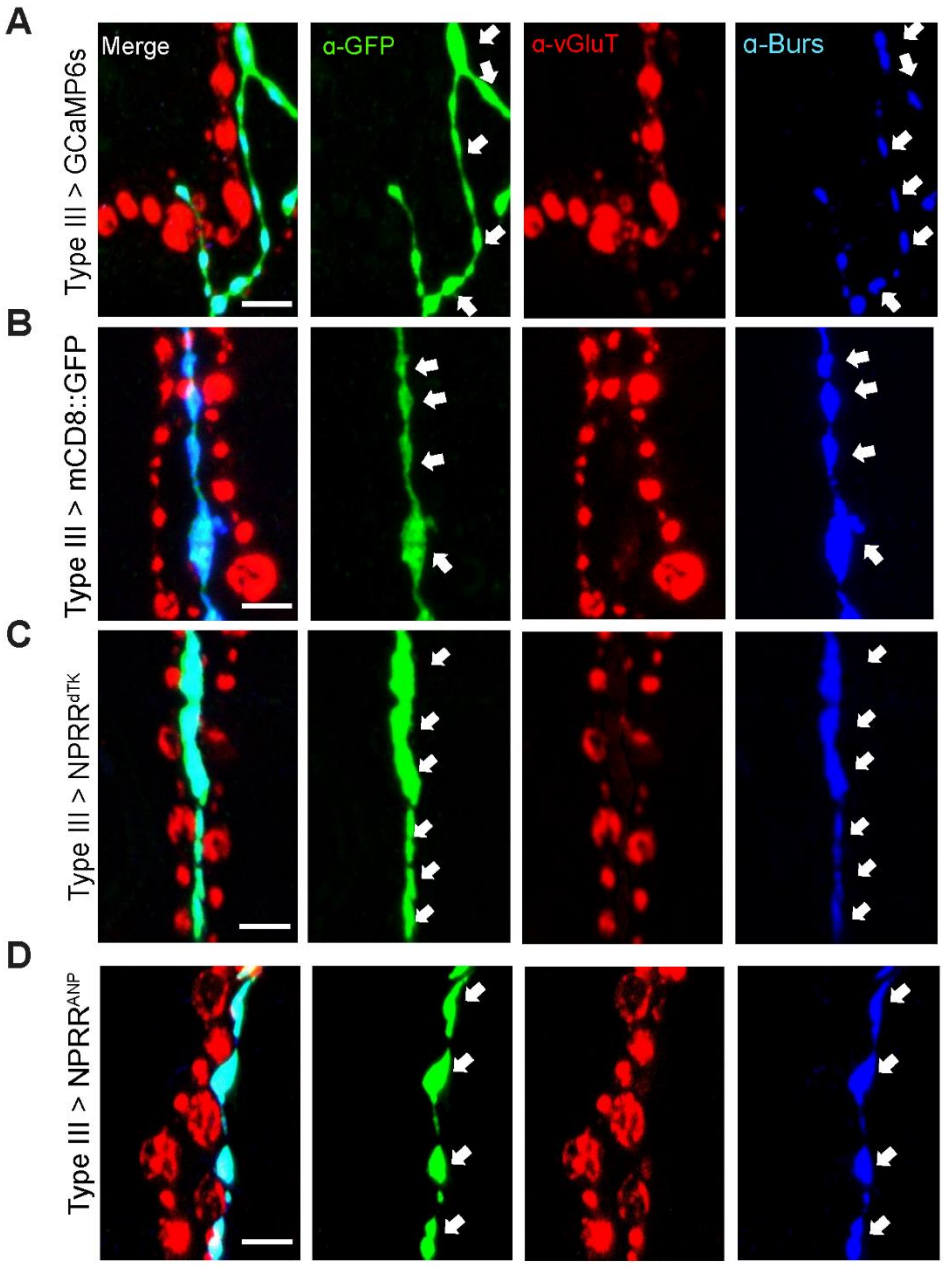

Figure 1—figure supplement 3: Expression of different reporters in Type III neurons in the larval NMJ.

A GAL4 line (R20C11-GAL4, named Type III-GAL4 in this report) allows specific expression in Type III neurons. Expression patterns of **(A)** conventional GCaMP, **(B)** membrane-bound GFP, **(C)** NPRR^{dTK} and **(D)** NPRR^{ANP} using Type III-GAL4. Arrows indicate boutons in Type III neurons, which contain the neuropeptide Bursicon. Note that anti-vGluT stains other types of motor neurons, which are not labeled by the Type III-specific driver used in this experiment. Scale bar, 5 μm.

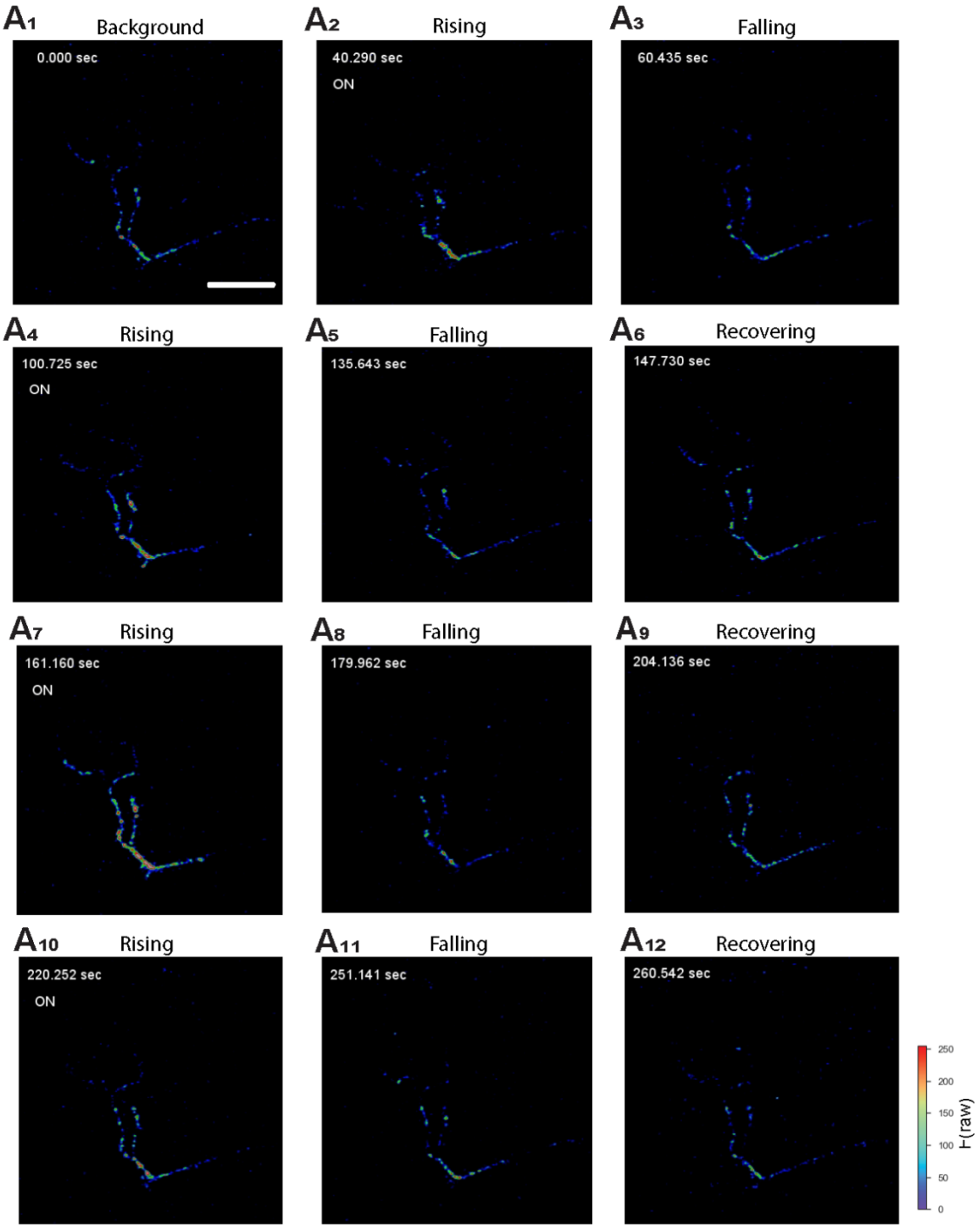

Figure 2—figure supplement 1: Activation of NPRR^{ANP} *in situ*.
Representative still frames (**A$_1$-A$_{12}$**) from video recordings of NPRR^{ANP}-expressing Type III neurons at the larval NMJ. "On" (**A$_{2,4,7,10}$**) represents the onset of electrical pulses. Color bar: Raw fluorescence intensity. Scale bar, 50 µm.

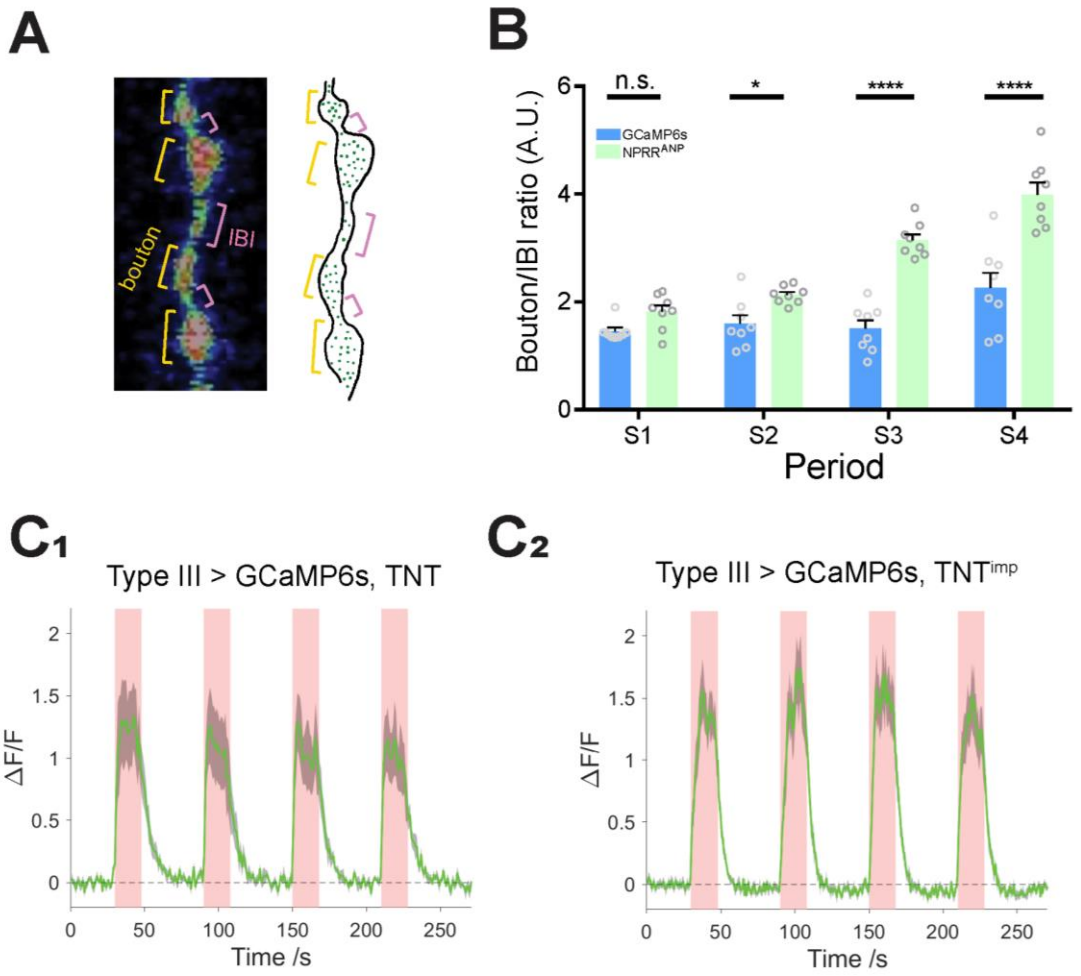

Figure 2—figure supplement 2: NPRR specifically reports neuropeptide release.
(A) Left: Segmentation of Type III neurons into boutons (orange) and inter-bouton intervals (IBIs, red). Right: Schematic illustrating DCV distribution in Type III neurons, based on photomicrograph to the left. Green dots, DCVs. **(B)** Average time-integrated ratio of ΔF/F in boutons/IBIs (**Materials and Methods**), within each stimulation periods. n.s., not significant. *, $P<0.05$. ***, $P< 0.001$. ****, $P<0.0001$.**(C)** TNT does not affect GCaMP6s ΔF/F kinetics. n = 6-7. GCaMP6s peak magnitudes were reduced slightly in TNT **(C_1)** in comparison to TNT^{imp} **(C_2)** preparations, perhaps reflecting partial vulnerability of the cytosolic GCaMP6s reporter to TNT-mediated cleavage and degradation. NPRRs are expected to be protected from TNT by the DCV membrane.

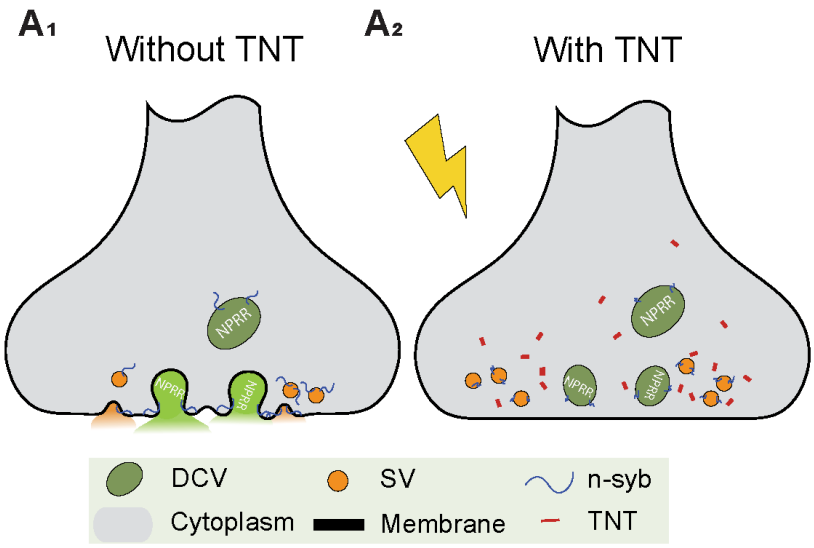

Figure 2—figure supplement 3: Blocking DCV fusion using Tetanus Toxin.
(A1, A2) Tetanus toxin (TNT) blocks vesicle fusion by cleavage of n-synaptobrevin (n-syb).

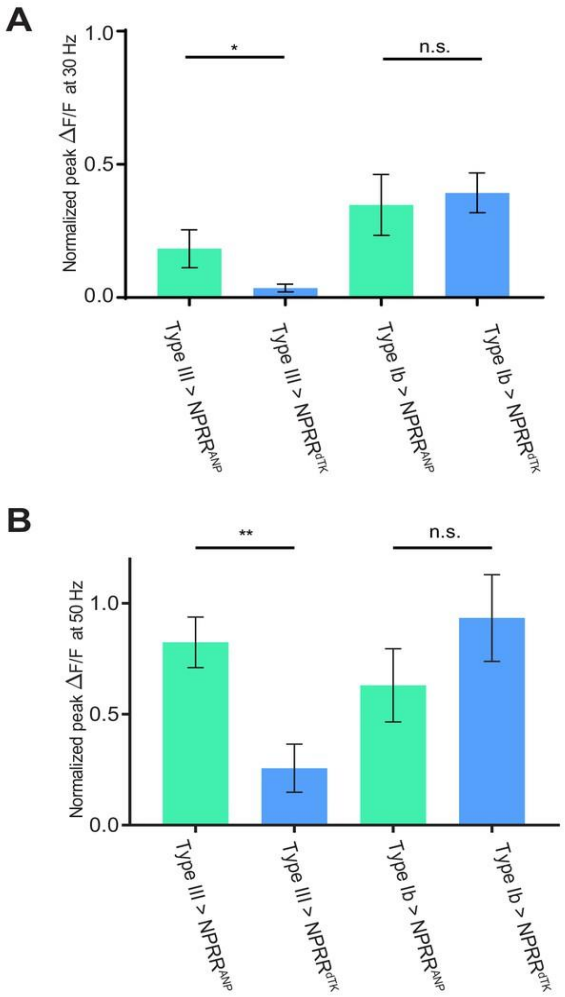

Figure 4—figure supplement 1: Comparison of NPRR response at 30 and 50 Hz.
Normalized ΔF/F peaks in at 30 Hz **(A) and** 50 Hz **(B)** electrical stimulation in Figure 4A, B, D, E are replotted and compared. *n* = 6-7. *, *P*<0.05. **, *P*<0.01. n.s., not significant.

Current techniques for neuropeptide release measurement

	Chemical specificity	Genetic specificity	Spatial specificity	Temporal dynamics	Signal relative to background
Microdialysis	Low	NA	Brain region	minutes-days	High
Antibody-coated microprobe	High	NA	Brain region	minutes-days	High
GFP-tagged propeptide imaging	High	Yes	Neuron of interest	~seconds	Low
NPRR	High	Yes	Neuron of interest	~subseconds	High

Supplementary Table. 1: Current techniques for neuropeptide release measurement.
Summary of current techniques used for neuropeptide release, including microdialysis, antibody-coated microprobes, GFP-tagged propeptide imaging and NPRR. NA, "not applicable"

	NPRR$^{ANP\text{-}GFP}$ (Ib)	**NPRR$^{dTK\text{-}GFP}$**
average gold particles per DCV	0.71	1.11
gold within DCV area [μm^{-2}]	90.99	141.65
gold within bouton area [μm^{-2}]	5.56	6.38
gold outside bouton area [μm^{-2}]	1.11	0.65
controls		
gold per imaged area [μm^{-2}]	2.18	2.47
background (internal control) [μm^{-2}]	0.57	0.52
background (biological control) [μm^{-2}]	0.25	-
SNR (gold/DCV area vs. background)	159.6	272.4

Supplementary Table. 2: Stereological labeling estimates
Stereological labeling estimations of NPRR$^{ANP\text{-}GFP}$ and NPRR$^{dTK\text{-}GFP}$, respectively, in Type Ib neurons, or in Type Ib and Type III neurons. Biological controls and internal controls are described in Materials and methods. SNR: Signal-to-Noise Ratio.

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (*D. melanogaster*)	UAS-NPRR^{ANP} (attp2)	this paper		See Materials and methods, subsection Construction of transgenic animals.
Genetic reagent (*D. melanogaster*)	UAS-NPRR^{dTK} (attp2)	this paper		Same as above.
Genetic reagent (*D. melanogaster*)	UAS-TNT^{imp}	Bloomington Drosophila Stock Center	BDSC:28840; FLYB:FBti0038575; RRID:BDSC_28840	Flybase symbol: w[*]; P{w[+mC]=UAS TeTxLC. (-)V}A2
Genetic reagent (*D. melanogaster*)	UAS-TNT	Bloomington Drosophila Stock Center	BDSC:28838; FLYB:FBti0038527; RRID:BDSC_28838	Flybase symbol: w[*]; P{w[+mC]=UAS TeTxLC.tnt}G2
Genetic reagent (*D. melanogaster*)	w; +; UAS-GCaMP6s (su(Hw)attp1)	Hoopfer et al., 2015		
Antibody	anti-GFP (chicken polyclonal)	Aveslab	Aveslab: GFP-1020; RRID:AB_2307313	(1:250:Immuno-EM, 1:1000: IHC)
Antibody	anti-ANP (rabbit polyclonal)	abcam	abcam #14348	(1:500)

Materials and Methods

Fly strains

All experimental flies were reared on a 12/12-hour day-night cycle at 25°C. Standard chromosomal balancers and genetic strategies were used for all crosses and for maintaining mutant lines. The following strains were obtained from Bloomington Stock Center (Indiana University): R20C11-Gal4 (#48887), R57C10-Gal4 (#39171), UAS-mCD8::GFP (#32185), UAS-TNT (#28838), UAS-TNT^{imp} (#28840). UAS-opGCaMP6s was made by Barret Pfeiffer (Gerald Rubin's lab, Janelia Farm) (Hoopfer, Jung, Inagaki, Rubin, & Anderson, 2015).

Construction of transgenic animals

All PCR reactions were performed using PrimeSTAR HS DNA polymerase (Takara #R045Q). All constructs were verified via DNA sequencing (Laragen).

To construct UAS-NPRR^{ANP}, Drosophila codon-optimized ANP and GCaMP6s were synthesized using gBlocks service (Integrated DNA Technologies), and subcloned into pJFRC7 vector (from Addgene #26220)(Pfeiffer et al., 2010) using Gibson cloning. UAS-dTK-NPRR is built in a similar way except the dTK fragment was cloned from the *Drosophila* brain cDNA. NPRRdTK-GFP and NPRRANP-GFP were built similarly except *Drosophila* codon-optimized GFP was used for the subcloning. All the vectors were injected and integrated into attP2 or attp40 sites (Bestgene Inc).

Expression screening of NPRR candidates

Adult fly brains were dissected in chilled PBS and fixed in 4 % formaldehyde for 55 min at room temperature. After three 10 min rinses with PBS, the brains were cleared with Vectashield (#1000, Vectorlabs), mounted, and used for native fluorescence measurements. We trace the NPF neuron somata and arborization as ROIs. We selected regions next to NPF neurons and measured its fluorescent intensity as a reference, which represents background autofluorescence. Candidates whose fluorescence reached at least 2-fold higher than reference were selected for functional screening.

Functional screening of NPRR candidates

For the baseline fluorescence measurement, we crossed NPF-Gal4 to the candidate lines and generated NPF-Gal4 > NPRRx (x = candidate label) flies for tests. The dissected adult fly brains were mounted on a petri dish and immersed in *Drosophila* imaging saline (108 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM trehalose, 10 mM sucrose, 5 mM HEPES, pH 7.5). To deliver high potassium challenge, High-K imaging saline was perfused (43 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM trehalose, 10 mM sucrose, 5 mM HEPES, pH 7.5). Live imaging series were acquired using a Fluoview FV3000 Confocal laser scanning biological microscope (Olympus) with a 40×, 0.8 N.A. (Numerical Aperture) water immersion objective (Olympus). Candidates whose post-stimulation fluorescence reached at least 2-fold of baseline fluorescence (measured as averaged pre-stimulation fluorescence) were selected for *in vivo* tests at NMJ. For each candidate line, at least 3 brains were tested and fold-change of each was averaged.

Immunocytochemistry (ICC)

Cells were fixed in 4 % formaldehyde or Bouin's solution for 30 min at room temperature. After three 15 min rinses with PBS, tissues were incubated with primary antibodies overnight at 4 °C. Following three 15 min rinses with PBS, tissues were incubated with secondary antibody for 2 hours at room temperature. Following three 15 min rinses, tissues were cleared with Vectashield (#1000, Vectorlabs) and mounted. Confocal serial optical sections were acquired using a Fluoview FV3000 Confocal laser scanning biological microscope (Olympus) with a 60×, 1.30 N.A. silicone oil objective (Olympus). All image processing and analyses were done using ImageJ (National Institute of Health).

The following primary antibodies were used: Chicken anti-GFP (1:250-1:1000, Aveslab #1020), Rabbit anti-ANP (1:500, abcam #14348), Guinea pig anti-vGluT(Goel & Dickman, 2018) (1:1500), Rabbit anti-syt1(Littleton, Bellen, & Perin, 1993) (1:500) and Rabbit anti-Bursicon (1:2000, a gift from Dr. Benjamin White).

The following secondary antibodies were used: Alexa Fluor 488 Goat anti-Chicken IgY (#A11039, Invitrogen), Alexa Fluor 488 Goat anti-Rabbit IgG (#A11008, Invitrogen), Alexa Fluor 568 Goat anti-Rabbit IgG(H+L) (#A11011, Invitrogen), Alexa Fluor 633 Goat anti-Rabbit IgG(H+L) (#A21070, Invitrogen), Alexa Fluor 488 Goat anti-Guinea Pig IgG(H+L) (#A11073, Invitrogen), Alexa Fluor 568 Goat anti-Guinea Pig IgG(H+L) (#A11075, Invitrogen), Alexa Fluor 568 Goat anti-Mouse IgG(H+L) (#A11004, Invitrogen) and Alexa Fluor 633 Goat anti-Mouse IgG(H+L) (#A21050, Invitrogen).

Electron microscopy

Drosophila tissues were fixed in 4% formaldehyde in PBS and stored at 4°C until preparation by high-pressure freezing (HPF) and freeze-substitution (FS) (Buser & Drubin, 2013; Buser & Walther, 2008). Tissues were cryoprotected in 2.3 M sucrose for 45 minutes, transferred to 200 µm deep planchettes and high-pressure frozen in an EMPact2 with RTS (Leica, Vienna, Austria). FS was carried out in an AFS2 (Leica, Vienna, Austria) in methanol containing 5% water, 0.05% glutaraldehyde and 0.1% uranyl acetate (-90 °C, 3 h; -90 to -80 °C, 10 h; -80 °C, 4 h; -80 to 4 °C, 24 h). Samples were washed once in methanol containing 5% water, infiltrated with hard grade LR White (Electron Microscopy Sciences, Hatfield, PA, USA) at 4 °C ([LR White] : [methanol containing 5% water] 1:1, 24 h; 100% LR White, 3x 24 h) and polymerized in a fresh change of LR

White using a Pelco BioWave (Ted Pella, Inc., Redding, CA, USA) set to 750 W, 95 °C for 45 minutes.

60 nm thin sections (UCT ultramicrotome, Leica, Vienna, Austria) were picked up on formvar-coated 50 mesh copper grids. The sections were blocked for 3 minutes in blocking buffer (PBS with 0.5% bovine serum albumin, which was used for all antibody dilutions), incubated in anti-GFP antibody (1:500, Aveslab #1020) for 5 minutes, washed 3 times in blocking buffer, incubated in rabbit anti chicken antibody (1:50, MP Biomedicals #55302) for 5 minutes, washed 3 times on blocking buffer, incubated on protein A - 5nm gold (1:50, Utrecht, Netherlands), and washed 3 times in PBS and 3 times in distilled water. The sections were stained in uranyl acetate or uranyl acetate and Reynolds lead citrate depending on the desired contrast and imaged at 80 kV in a Zeiss EM10C (Zeiss, Oberkochen, Germany) using a CCD camera (Gatan, Pleasanton, CA, USA).

Labeling density was estimated using stereological methods(Griffiths & Hoppeler, 1986). Cross-sections through boutons were recorded and the following parameters were measured: total image area, total number of gold particles, number of visible dense core vesicles (DCV), number of gold particles within a 50 nm radius of the DCV center, bouton area (grid intersection estimate), gold within the bouton cytoplasm, gold within 20 nm of the bouton plasma membrane, gold outside of the bouton (mainly sER). Background labeling was estimated using internal controls (labeling on blank resin and on muscle fibers) and a biological control (non-GFP expressing genotype). Occasional obvious, large gold aggregates were disregarded. Background was consistently below 0.6 gold/μm^2 in independently repeated labeling experiments.

Electrical Stimulation

The dissection of third-instar larvae was performed in zero-calcium HL3 saline. The CNS was removed to avoid spontaneous motor neuron activity. To minimize muscle contraction induced by electrical stimulation of motor neurons, the larval body walls were slightly stretched and incubated in HL3 saline supplemented with 10 mM glutamate for 5 mins after dissection to desensitize postsynaptic glutamate receptors. Samples were then shifted to HL3 saline containing 1mM glutamate and 1.5mM Ca^{2+}. Motor nerves were sucked into a glass micropipette with a stimulation electrode. In Figure 2 and Figure 3, to induce maximum dense core vesicle release at type III motor neuron terminals, 4 repetitive bursts (70 Hz stimulation for 18-20s with pulse width of 1ms) with intervals of 40-42s were programmed and triggered with a Master-9 stimulator (A.M.P.I., Israel) connected to an iso-flex pulse stimulator (A.M.P.I., Israel). The stimulation intensity was tested and set to double the intensity required to induce muscle contraction by a single pulse stimulation.

In Figure 4, stimulation trials were delivered with the same duration, but with a series of frequencies spanning 1 Hz to 70 Hz.

Calcium imaging

A Nikon A1R confocal microscope with resonant scanner and NIS Element software were used to acquire live Ca^{2+} imaging on third instar larvae, bathed with 1 mM glutamate added in 1.5 mM Ca^{2+} HL3 saline. Type III motor neuron terminals in abdominal segments from A2 to A5 were imaged using a 60x APO 1.4 N.A. water immersion objective with 488 nm excitation laser. A 5-min period was used for time-lapse imaging at a resonance frequency of 1 fps (512 x 512 pixels or 1024 x 1024 pixels), with z-stacks (step length varying from 1 to 1.5 μm) covering the depth of entire type III motor neuron terminals. The repetitive electrical stimulation of 70 Hz was delivered during the

imaging session. Samples with severe muscle contractions were abandoned due to imaging difficulties. Maximum intensity projection (MIP) and image registration were conducted using Image J. Plugins including Image Stabilizer (K. Li, CMU) and Template Matching (Q. Tseng) were used for compensating drifting and correcting movement induced by electrical stimulations. ROIs were manually selected by tracing the outer edge of each neuron based on the baseline fluorescence. If the fluorescence was too weak to trace, we established a reference stack by empirically adjusting the contrast on a duplicate of the raw image stack. We used the reference stack for ROI selection and projected the selected ROIs back onto to the raw image stack for measurement. For frames in which the sample movement could not be automatically corrected, we manually outlined the ROIs used for measurements. Preparations with severe movement or deformation artifacts were abandoned to avoid unreliable measurements. Each ROI represent a traceable neuronal branch except Figure 2—figure supplement 2B, in which the ROIs were further manually partitioned into boutons and IBIs (Inter-Bouton Intervals) based on morphology. Fluorescence change were normalized to the pre-stimulation background except for Figure 3E, for which the data in each trial was normalized to the average $\Delta F/F$ during a 5 seconds period just before stimulation was initiated. No sample size is predetermined based on statistics. Ca^{2+} imaging data were acquired from at least 6 independent NMJs from at least 5 animals.

Statistical Analysis

Data are presented as mean ± s.e.m. All data analysis was performed with Graphpad Prism 6, Microsoft Excel and custom Matlab codes. Mann-Whitney U test was used for comparison except in Figure 4, where One-sample T test was used for comparison with a specified value (0).

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Chapter 3

IMAGING NEUROPEPTIDE LOCALIZATION AND RELEASE IN MAMMALIAN CELLS WITH NOVEL GENETICALLY ENGINEERED REPORTERS

[This chapter is temporarily embargoed.]

Chapter 4

EXPLORING NOVEL THERAPEUTICS WITH GENETICALLY ENGINEERED REPORTERS

[This chapter is temporarily embargoed.]

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.

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A P P E N D I X

FLUORESCENCE DYNAMICS OF LYSOSOMAL-RELATED ORGANELLE FLASHING IN THE INTESTINAL CELLS OF *CAENORHABDITIS ELEGANS*

C. Tan., Ding, K., Anderson, D. J., Sternberg, P.W. Fluorescence dynamics of lysosomal-related organelle dissipation in the intestinal cells of *Caenorhabditis elegans* (In preparation)

Summary

Autofluorescent lysosome-related organelles (or gut granules) in the intestinal cells of *Caenorhabditis elegans* have been shown to play an important role in metabolic and signaling processes, but they have not been fully characterized. Using a preparation comprising live worms with intestinal tissue exposed, we report here a previously undescribed phenomenon in which gut granule autofluorescence is quenched in a rapid and dynamic manner. We show that at least two types of fluorophores are present in the gut granules. One displayed a "flashing" phenomenon, in which quenching is preceded by a sharp increase in fluorescence intensity that expands into the surrounding area. The flashing phenomenon is strongly correlated with food availability, suggesting that the underlying activities are likely to be physiological and may be part of a metabolic process.

Introduction

The intestinal cells of *Caenorhabditis elegans* and related nematodes are known to contain a type of organelle known as gut granules or rhabditin granules (Chitwood & Chitwood, 1950; Laufer, Bazzicalupo, & Wood, 1980). These birefringent and autofluorescent granules are robustly present in intestinal and intestinal precursor cells and thus serve as a useful marker for the intestinal linage

(Hermann et al., 2005; Laufer et al., 1980). Based on morphological, biochemical, and genetic evidence, these granules are considered to be lysosome-related organelles (Clokey & Jacobson, 1986; Hermann et al., 2005; Kostich, Fire, & Fambrough, 2000). As with lysosome-related organelles in other organisms, the biological roles of gut granules are not fully understood. There is evidence, however, that these organelles are likely to be involved in metabolic and homeostatic processes such as the storage of fat and cholesterol (Lee et al., 2015; Schroeder et al., 2007) and trace metal storage and detoxification (Chun et al., 2017; Roh, Collier, Guthrie, Robertson, & Kornfeld, 2012). In addition, gut granules are also known to play a signaling role through the biogenesis of ascarosides (Le et al., 2020).

In this study, we show that in partially exposed *C. elegans* intestinal tissue, some autofluorescent granules underwent a rapid and dynamic change in fluorescence intensity. Since the spatio-temporal pattern of the dynamic changes in green and red fluorescence channels are different, we concluded that at least two different fluorophores are present in the gut granule and were involved in this process. Prior to fluorescence dissipation, there was a sharp and significant increase in green fluorescence intensities that extended to the surrounding areas, a phenomenon that we describe as "flashing." Gut granule flashing was strongly dependent on food availability at the time of the experiments, being almost entirely absent in preparations without added food. Finally, we show that the worm ortholog of human Rab32/38, *glo-1* (Gut granule LOss) (Hermann et al., 2005; Morris et al., 2018), which has been shown to be required for gut granules biogenesis, was necessary for the flashing phenotype, suggesting that the source of the autofluorescent flashing signals was indeed gut granules. We have yet to identify the fluorophores responsible for the phenomenon, the underlying

biochemical processes or its biological significance. However, we found this to be an intriguing phenomenon providing insights into the functions and mechanisms of lysosome-related organelles.

Results

Some autofluorescent granules in intestinal cells displayed dynamic changes in fluorescent intensities

During our observational analysis of exposed *Caenorhabditis elegans* intestine with fluorescence confocal microscopy, in which the worm is cut open near the anus to expose the intestine (Fig 1B), we observed that some of the fluorescent granules in the intestinal cells displayed dynamic changes in fluorescence intensity (Fig 1A, C, S1 Movie). In these granules, the green fluorescence, illuminated with the 473nm laser of the confocal microscope, rises sharply and significantly (Fig 1A, C, D, S1 Movie). The intensity increase is not only limited to the original "core" area (the granule proper), as identified by the high level of fluorescence at a steady-state prior to the rapid intensity changes, but also to the surrounding areas (which we refer to as the "cloud." Details of how the area is identified are in the Methods; Fig 1E). The fluorescence intensity in both the "core" and the surrounding areas subsequently dropped off, with the intensity at the "core" dropping to a level that is lower than the previous steady-state, and the intensity in the surrounding areas ("clouds") decreasing to a level that is similar to the previous steady-state (Fig 1E). We estimated that the rapid changes in fluorescence intensity occur in less than 10 seconds, in a way that resembles the bursting of fireworks or the flashing of a light (Fig 1E). In contrast, the red fluorescent signal, illuminated with the 561nm laser of the confocal microscope, lacked the initial sharp increase observed within the green fluorescence channel, but fell concurrently (Fig 1F). The dynamic differences observed in the green and red fluorescent channels suggest that they may represent at least two distinct types of

fluorophores presented in the autofluorescent granules. This phenomenon is rare but is consistently observed in a portion of the animals in this preparation through over two years of study. The fluorophore is not exogenous, as the phenomena can be observed in wild-type animals, which were used exclusively in this study.

The autofluorescent granules flashing phenomena are strongly correlated with food availability

Since the primary function of the intestine is food digestion, we reasoned that the autofluorescent granules flashing phenomena could be associated with nutrient uptake. In *C. elegans*, food availability significantly influences the rhythmic defecation cycle (Thomas, 1990), which is controlled by calcium oscillations in the intestinal cells (Dal Santo, Logan, Chisholm, & Jorgensen, 1999). To test whether granule flashing phenomena are associated with food availability, we provided food (*Escherichia coli* OP50) to the experimental animal on the microscopic slides. We found that in worms with a significant amount of food near the head region, the occurrence of the granule flashing phenomena increased dramatically (Fig. 2A-D, S1 Movie). All seven worms provided with food displayed the phenomena (Fig. 2C, D, H), while only 1 of 7 without food did at a low frequency (Fig. 2A, B, H). It is worth noting that a small amount of food is still present in the "no food" group, which may explain the occasional occurrence of the phenomena.

The flashing autofluorescent granules are lysosome-related organelles

The worm intestinal cells are known to contain numerous autofluorescent granules known as "gut granules" (Hermann et al., 2005; Laufer et al., 1980). Gut granules are lysosome-related organelles that have been shown to play an important physiological role in both nutrient homeostasis and signal

transduction (Chun et al., 2017; Le et al., 2020; Roh et al., 2012; Schroeder et al., 2007). To characterize the nature of the observed autofluorescent granules flashing phenomena, we analyzed *glo-1(lf)* mutant animals that are defective in gut granules biogenesis. *glo-1* (Gut granule LOss) encodes an ortholog of Rab32/38 that localize to gut granules (Hermann et al., 2005; Morris et al., 2018). *glo-1(lf)* animals displayed a large reduction in the number of autofluorescent granules in the intestine, similar to what was described in Hermann et al. (2005), and also completely eliminated the granules flashing phenomena (Fig. 2E, F, H). Even with food provided, none of the seven *glo-1(lf)* animals observed displayed the phenomena, as compared with seven of seven in wild-type animals as previously described (Fig. 2H), suggesting that the flashing autofluorescent granules are lysosome-related organelles.

Discussion

Some lysosome-related organelles in the worm intestine exhibit dynamic flashing autofluorescence

In the process of establishing a baseline for fluorescence dynamics in ex vivo intestines, we characterize an intriguing dynamic of lysosome-related organelles. When provided with food, some of the lysosome-related organelles in intestinal cells underwent a rapid change in fluorescence intensities. We observed two distinctive fluorescence dynamics in green and red fluorescence. The dynamic of the green fluorescence is characterized by a sharp and rapid increase in intensity that diffuses into the surrounding area, followed by a rapid decrease and dissipation of the autofluorescence. On the other hand, the dynamic of the red fluorescence is only that of decreases in intensity (Fig. 1). The green and red fluorescence dynamic combination coincidentally resemble the predicted outcome of our original experiment, in which a green pH-sensitive encoded vesicle release

reporter is paired with a red non-pH-sensitive control. The very rare occurrence of the event under conditions without food being present initially misled us as to the source of the fluorescence. Only after the discovery of the effect of food presence did we realize what we were studying was likely a pair of naturally occurring worm fluorophores with an intriguing dynamic.

The gut granule flashing phenomena may be part of a metabolic or signaling process

In the worm intestine, gut granules coexist with more conventional lysosomes (Campbell & Fares, 2010; Kostich et al., 2000; Morris et al., 2018), and although prevailing and distinctive (Chitwood & Chitwood, 1950; Clokey & Jacobson, 1986; Hermann et al., 2005) are not thought to be the major site of intracellular digestion as with lysosome-related organelles in many other spices (Delevoye, Marks, & Raposo, 2019). In *C. elegans*, gut granules have been shown to have both metabolic and signaling functions. The lysosome-related organelles are a site for fat and cholesterol storage (Lee et al., 2015; Schroeder et al., 2007) as well as functioning both as a storage and a sequestering site for micronutrient metals (Chun et al., 2017; Roh et al., 2012). Lysosome-related organelles are also the site of biosynthesis for signaling molecules such as ascarosides (Le et al., 2020). Other than being static sites of storage and metabolic processes, gut granules have been shown to undergo structural and morphological changes responding to changes in dietary conditions. For example, in response to high dietary zinc, gut granules are remodeled from the typical round sphere to bilobed granules with asymmetrical distribution of both internal content and membrane proteins (Roh et al., 2012). The mechanism of such a structural rearrangement remains largely uncharacterized.

The gut granule flashing phenomena were highly associated with the immediate presence of food (Fig. 2). This observation implies that the phenomena could be a part of or a consequence of a

metabolic or signaling process in food intake. One possibility is that this phenomenon is associated with a vesicle content release of the gut granules, and the fluorophores or the content that it is released with plays metabolic or signaling roles in food uptake. Another possibility is that the phenomenon is associated with the breakdown of gut granules, either as part of a physiological process or as a result of our *ex-vivo* experimental conditions. Two reasons argue that the second possibility is less likely than the first. First of all, we have, on rare instances, observed similar events in intact worms, albeit we have not been able to record such events. We have also observed multiple flashing events in some of the granules. Secondly, the phenomenon is visually different from that of the visualized fluorescently labeled gut granules disruption in osmotic sensitive mutants under hypotonic shock (Luke et al., 2007), although there is some similarity in the diffusion of the fluorescence. Whatever the case, it would be interesting to know the identity of the at least two fluorophores involved.

C. elegans are long known to be autofluorescent (Babu, 1974), with most of the autofluorescence from the gut granules (Clokey & Jacobson, 1986). Particular interest has been paid to the increasing level of autofluorescence as the worms age (Clokey & Jacobson, 1986; Davis, Anderson, & Dusenbery, 1982; Forge & Macguidwin, 1989; Klass, 1977; Pincus, Mazer, & Slack, 2016). However, the fluorophores responsible for gut granule autofluorescence remain largely undefined. One of the fluorophores emitting blue fluorescence has been identified as anthranilic acid (Babu, 1974; Coburn et al., 2013), and changes in the blue fluorescence near the death of the animals (death fluorescence, or DF) has been described (Coburn et al., 2013; Pincus et al., 2016). Although both green and red fluorescence also increase with age and green fluorescence intensifies near the death of the animals (Coburn et al., 2013; Pincus et al., 2016), the patterns of the fluorescence changes are different, and they are also likely emitted by different fluorophores. It is also important to note that

the fluorescence dynamic of DF is also distinct from the phenomenon that we are describing in this research, both spatially and temporally.

A major caveat of this study is its *ex-vivo* nature. One could argue that the fluorescence dynamic we observed may not be physiological. It is important to stress, however, that besides the fact that we have been able to observe the phenomenon in live worms on a few occasions, the phenomenon is dependent upon a potential environmental cue- the presence of food; and is genetically dependent on the gene *glo-1*. It is also worth noting that conditions such as oxidative stress does not appear to increase autofluorescence in *C. elegans* (Pincus et al., 2016). Regardless, even if the phenomenon is not itself naturally occurring, it is a beautiful experimental phenotype that could also be informative.

Further understanding of the phenomenon would likely require the characterization of the green and red fluorophores. It is unclear how to best identify these fluorophores, although we would predict that the green fluorophores emit stronger fluorescence under low pH conditions, while the red fluorophores are likely pH neutral. Nonetheless, we described a visually spectacular fluorescence dynamic phenomenon that involves a type of lysosome-related organelles known as gut granules in nematode intestines. It is possible that what we observed is part of a common cellular process but only visualized through the strong autofluorescence of the fluorophores. If that is the case, the gut granules of *C. elegans*, with its naturally occurring fluorophores, may be a potential platform for the understanding of lysosome-related organelles as well as cellular vesicle membrane dynamics.

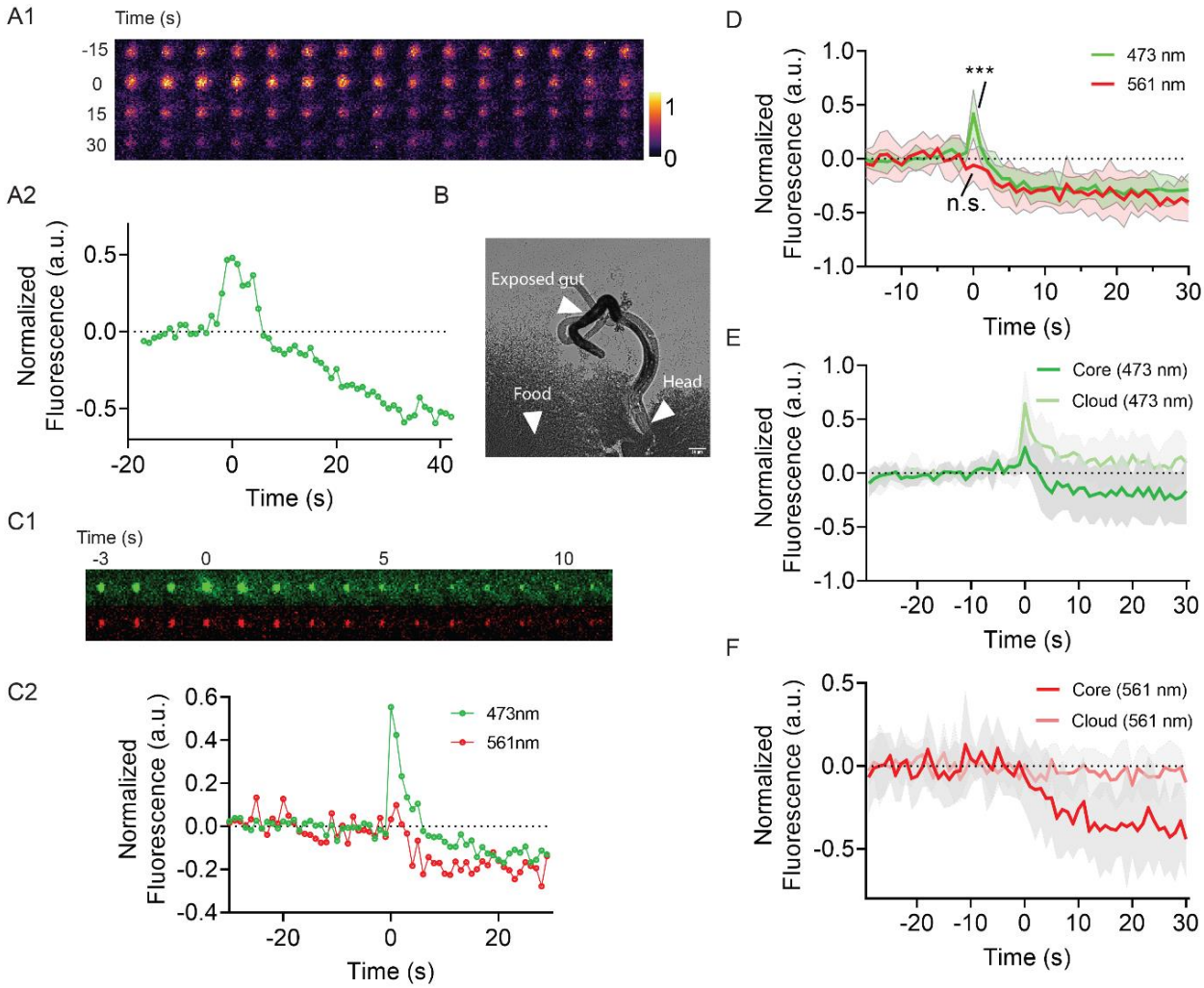

Figure 1: The dynamics of gut granule dissipation.

(A) The green fluorescence in the granule increases and falls sharply. Representative example of changes in green fluorescence intensity during gut granule dissipation. (A1) A 1-minute pseudo-color time series of a gut granule dissipation event. Imaging rate: 1Hz. The time axis is zeroed at the dissipation onset (see Methods). (A2) Normalized green fluorescent intensity plot of (A1). (B) An example of the ex-vivo experimental setting. L4 stage nematodes were carefully incised near the anus to expose the gut. In the "with food" conditions, nematodes were positioned to embed their heads in food. (C-D) The green fluorescence in the granule increases and falls sharply, while the red lack the initial sharp increase. (C) A representative example of two-color time-lapse imaging (C1) and normalized quantifications (C2). The fluorescence intensity is normalized by the baseline fluorescence calculated as the mean of fluorescence in the ROIs prior to the dissipation onset. (D) The average fluorescence intensity of all recorded dissipations in one sample. (E-F) Gut granule flashes during dissipation. Two-color imaging of all recorded gut granule dissipation events in a representative sample. (E-F) The granular area ("core"), as well as the surrounding ring area that presents diffusion of fluorescence ("cloud"), were measured separately in both 473nm (E) and 561nm (F) illumination. (E). The intensity increase in green fluorescence is not only limited to the original "core" area but also to the surrounding areas "cloud ."(F) No intensity increase was observed with the red fluorescence, neither in the "core" nor the "cloud ." Raw images for analysis were

identical to (D), except a longer pre-onset timeframe was selected for normalization. In (D-F), pooled values are presented as mean ± s.d (N=11 in D, N=8 in E and F). The time axis is zeroed at the dissipation onset. Scale bar, 50 μm.

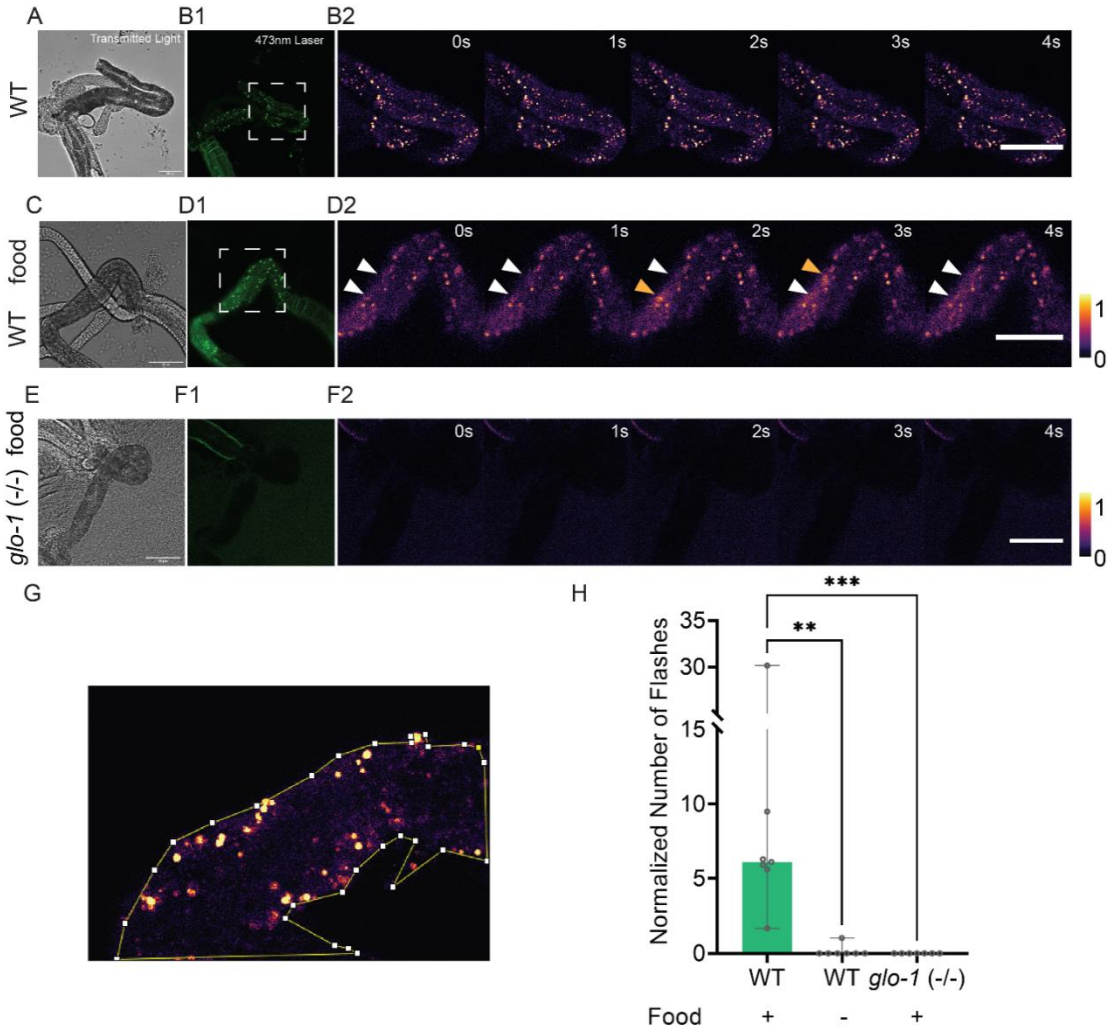

Figure 2: The gut granule dissipation phenomena are dependent on the presence of food.

The field of view was illuminated and recorded with transmitted light (A, C, E), epifluorescence (B1, D1, F1), and 473nm laser illumination (B2, D2, F2). In (B2, D2, F2) 5-second clips of time-series with 473nm laser illumination were pseudo-colored and shown. (A-B) In wild-type worms not provided with food on slide, the phenomena were rarely observed. (C-D) In wild-type worms provided with food on slide, the occurrences of phenomena were increased dramatically. Orange arrows in (D2) indicate the onset of gut granule dissipation events. White arrows point to the same region of interest before and after onset. (E-F) There is no detection of events in *glo-1(lf)* worm even with the presence of food on slide. (G-H) Gut granule dissipation events were manually identified and scored. To normalize the number of events for comparison, the counts for each sample were divided by (1) the area of exposed intestine within the field of view as shown in (G) and (2) by the length of time series (details in Materials and Methods). (H) Normalized data from all conditions were plotted (N=6-7), and each group was compared with the WT + food group (**P<0.01, ***P<0.001, Mann-Whitney *U* test). Scale bar, 50 µm.

Methods

Nematode strains maintenance and general methods.

The culture and maintenance of *C. elegans* w were done similarly to the standard procedure as described in Brenner (1974). Briefly, worms were cultured on Nematode Growth Medium (NGM) dishes with a lawn of *Escherichia coli* strain OP50 at 20°C. The Bristol N2 strain (Brenner, 1974) was used as the wild-type reference strain, and from which the mutant strain was derived. The five times out-crossed *glo-1(zu391 lf)* X (Hermann et al., 2005) mutant strain OJ1347 (Wang et al., 2013) was a gift from Dr. Derek Sieburth of the University of Southern California.

Sample preparation for *ex-vivo* imaging.

L4 stage hermaphrodite worms were transferred to a drop of Iwasaki–Teramoto (I–T) solution (Teramoto & Iwasaki, 2006) [136mMNaCl, 9mMKCl, 1mM $CaCl_2$, 3mM $MgCl_2$, 77mM glucose, and 5mM HEPES (pH 7.4)] and immobilized with 1mM levamisole on a microscopic slide. To expose the intestine for imaging, worms were incised with a pair of 30G 5/8" needles (PrecisionGlide, BD, Franklin Lakes, NJ) near the anus. For experiments with food added on the slide, a glob of OP50 collected by scrapping the lawn off an NGM plate using a cell scraper was transferred into the droplet via the platinum wire worm pick. An insulation spacer was drawn using a PAP pen (RPI, #195506) around the solution droplet, and a piece of cover-glass was mounted atop subsequently.

Microscopy

Confocal time series were acquired using a Fluoview FV3000 Confocal laser scanning biological microscope (Olympus) with a 60×, 1.30 N.A. silicone oil objective (Olympus). Frame rate was

calibrated to 1Hz by adjusting the size of the imaging window and the line averaging multiplier. 5-min time lapse imaging was acquired for each sample. All image processing and analyses were done using ImageJ (National Institute of Health). The transmitted light channel was illuminated simultaneously with the imaging laser (473nm) for positional imaging. Panel A1 in Figures 1, as well as Panels B2, D2, F2 and G in Figure 2, were pseudo-colored with mpl-inferno LUT in FIJI.

Imaging data processing

ROIs of granules were manually selected in FIJI for all data. For Figure 2(G-H), ROIs of peri-granule "cloud" were defined based on the identification of fluorescent pattern of dissipation events. The time series data were zeroed at the flashing onsets of each granule and pooled into metadata.

ROI selection: ROIs were defined based on visual identification of granules in the intestine region, fluorescence normalization baseline was set accordingly as the average of pre-dissipation period. Standard deviation envelopes were visualized in the time series. For Figure 2(H), the number of gut granule dissipation events was normalized as per minute *10-4 μm^2 of the intestinal surface area from single optical sections, which are manually delineated (Figure 2(G)). Nonparametric Mann-Whitney U test was used for comparison between WT food vs. no-food, and WT food vs. *glo-1(lf)*.

Data and Statistical analysis

Data were plotted as mean± s.d, except in Figure 2(H), as raw data failed to passed all normality tests (Kolmogorov-Smirnov test, Shapiro-Wilk test, D'Agostino & Pearson test). Data in Figure 2(H) were plotted as median ± 95% CI instead. All data analysis was performed with Graphpad Prism 9 and Microsoft Excel.

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