CHAPTER 2

CNIFERS: CELL-BASED BIOSENSORS WITH NANOMOLAR SENSITIVITY TO *IN VIVO* CHANGES IN NEUROMODULATION

Daniel M. Kircher*,§, Macit Emre Lacin*,§, David Kleinfeld†,‡ and Paul A. Slesinger*,¶

*Department of Neuroscience, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
†Department of Physics, University of California, San Diego, La Jolla, California, USA
‡Section of Neurobiology, University of California, San Diego, La Jolla, California, USA
¶paul.slesinger@mssm.edu

2.1 Introduction

Neuromodulators, such as dopamine, norepinephrine, serotonin, endocannabinoids and neuropeptides, all produce profound effects on circuits that control animal behavior. Perturbation of neuromodulation is implicated in several different brain disorders that include substance abuse and dependence, depression, and severe anxiety. A major obstacle to studying the effect of neuromodulators in the brain has been the inability to detect their release *in vivo* with sufficient sensitivity, chemical specificity, and spatial and temporal resolution. Here, we describe the development of new tools for detecting neuropeptides and other neuromodulators.

§Equal contribution.

2.2 Neuropeptides

Neuropeptides are small proteins that signal like neurotransmitters in the nervous system. Except for a few exceptions, such as FMRFamide-dependent opening of Na^+-channels, neuropeptides typically signal through G protein-coupled receptors (GPCRs) (Lingueglia *et al.*, 1995), have a slower time course than ionotropic receptors, and produce persistent signaling. Peptide signaling through the GPCRs is amplified by G proteins, which stimulate/inhibit enzymes that generate second messengers like cAMP, IP_3, and Ca^{2+} that in turn modulate downstream kinases, e.g., protein kinase A (PKA), protein kinase C (PKC) (Sakurai *et al.*, 1998; Thibonnier *et al.*, 1998). These signaling pathways can also modulate the response of neurons through activation or inhibition of multiple ion channels, including voltage-gated calcium channels and potassium channels (Borgland *et al.*, 2008; Li and van den Pol, 2008).

Neuropeptides are synthesized as an inactive precursor on the rough endoplasmic reticulum in the soma, transferred to the Golgi apparatus for packaging, and then transported down the axon (Sossin *et al.*, 1989) (see Figure 2.1). The active peptide is generated by proteolytic cleavage of the precursor in large dense core

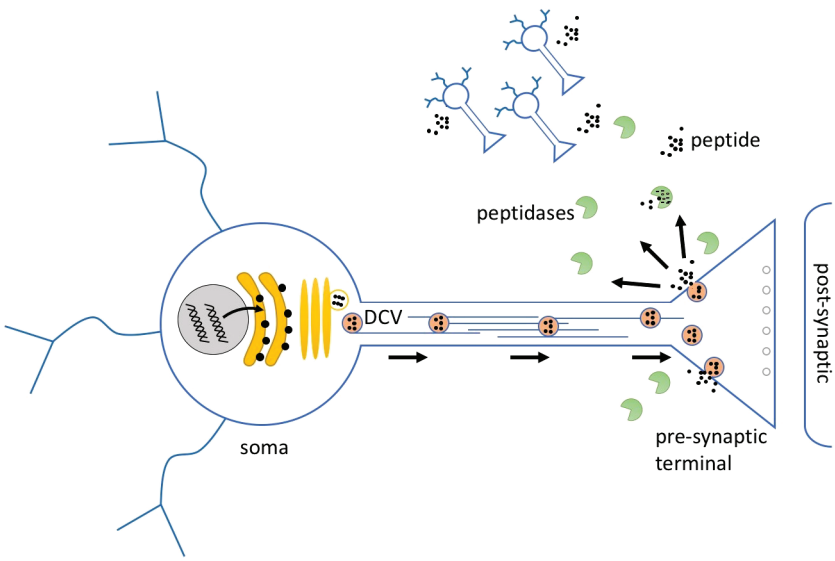

Figure 2.1 Cartoon of peptide synthesis and release in neurons. Neuropeptides are synthesized from mRNA in the soma on the rough ER as an inactive precursor, transferred to the Golgi apparatus for sorting, and transported down the axon in large dense core vesicles (DCVs). Active neuropeptide is generated by the proteolytic cleavage of the precursor peptide in DCVs. Neuropeptides act on receptors located on neurons several hundred micrometers away from release sites. Peptide half-life and diffusion are controlled, in part, by extrasynaptic peptidases, which catabolize the released peptides. In contrast to primary neurotransmitters, peptides are not recycled for re-use.

vesicles (DCVs). In general, DCVs contain multiple peptides and coexist with small vesicles housing small molecule transmitters (Chou *et al.*, 2001; Goodman *et al.*, 2007). In contrast to primary neurotransmitters, peptide-containing DCVs are mostly excluded from the active zone and found at greater distances from the synapse (Kreiner *et al.*, 1986; Fisher *et al.*, 1988). As most voltage-gated Ca^{2+} channels are localized to the active zone, peptide release is thought to require longer and higher frequency firing compared to small vesicle release (Sudhof, 2012).

The extrasynaptic localization of DCVs raises questions on the nature of neuropeptide signaling, particularly how far neuropeptides travel through the extracellular space. Minute-long half-lives of the neuropeptides and high-affinity of the neuropeptide receptors to the ligands (K_d ~ nM) have led researchers to postulate that peptides signal through volume transmission and act on extra-synaptic targets located several hundred micrometers away (Mens *et al.*, 1983; Solano *et al.*, 1996; Schloos *et al.*, 1997; Ludwig and Leng, 2006).

Perturbations in neuropeptide signaling have been implicated in several brain disorders. For example, alterations in neuropeptides appear to play a role in schizophrenia (Caceda *et al.*, 2007; LaCrosse and Olive, 2013). The hypothalamic neuropeptides oxytocin and vasopressin appear to be critically important for the regulation of social and cognitive processes that are abnormal in schizophrenia. The genes that encode oxytocin and vasopressin are associated with schizophrenia, with reduced pituitary and brain levels for both peptides found in individuals with the disease (Frank *et al.*, 2005). In contrast, oxytocin and vasopressin in the hypothalamus appear to be upregulated in individuals who suffer from major depressive disorder (Purba *et al.*, 1996).

Both of these neuropeptides can potentiate the effects of the corticotropin releasing factor (CRF) peptide and thus the activation of the hypothalamic pituitary adrenal (HPA) axis (Raadsheer *et al.*, 1994). Disruption in the stress related peptide CRF has also been implicated in drug addiction (Koob, 1999; Logrip *et al.*, 2011). Many drugs with abuse potential produce potentiation of the CRF system and likely drive atypical activity in response to stressors and contribute to relapse of drug abuse (Kreek and Koob, 1998; Roberto *et al.*, 2017).

2.3 Challenges of studying neuromodulators

Contemporary techniques for studying neuromodulators, such as microdialysis, have provided information on the extracellular concentration of neuromodulators *in vivo* (Day *et al.*, 2001). Microdialysis has been used to measure dopamine release in the medial prefrontal cortex (mPFC) in response to alcohol administration (Schier *et al.*, 2013), where alcohol administration produced significant peak increases in dopamine in the dialysate from mPFC. Microdialysis has also been

used to study other neuromodulators such as the neuropeptide oxytocin. Oxytocin is released in response to social stimuli in the dorso-lateral septum, and is blocked by social fear conditioning (Zoicas *et al.*, 2014). Although microdialysis is suitable for the accurate identification of different neuromodulators (Ji *et al.*, 2008; Greco *et al.*, 2013), there are some disadvantages. First, the collection of large CSF samples and the insertion of the microdialysis probe can disrupt neuromodulator activity near the probe track (Wang and Michael, 2012). Second, the temporal resolution is poor, with the sample collection typically occurring at 10 min intervals or longer, and not in real-time (Mingote *et al.*, 2004).

An alternative method for measuring the extracellular concentration of neuromodulators is fast-scan cyclic voltammetry (FSCV) (Robinson and Wightman, 2007). Compared to microdialysis, FSCV improves the spatial and temporal resolution as well as having nanomolar sensitivity. However, the number of neuromodulators that have been successfully examined with FSCV is limited (e.g., DA, serotonin, and NE). This technique is further limited by the inability to distinguish chemically similar compounds. For example, DA and NE differ by only one hydroxyl, and produce similar cyclic voltammetry signatures (Robinson *et al.*, 2003), making examinations of neuromodulator concentrations in brain regions where both are expressed a challenge.

Measuring changes in neuronal activity with single-cell electrophysiology is another technique for studying the effect of neuropeptides in the brain. Exposure to neuropeptides induces changes in excitatory or inhibitory post synaptic currents (amplitude or frequency), action potential firing, cell excitability, or current/voltage relationships (Radhakrishnan and Henry, 1995; Raggenbass, 2001; Jobst *et al.*, 2004; Gilpin and Roberto, 2012). However, electrophysiological measurements of neuropeptide release are hampered by the small amount of neuropeptide released by a single axonal bouton in the CNS (Dreifuss, 1975; Nordmann and Morris, 1984), which is likely due to a requirement for elevations in cytoplasmic Ca^{2+} that are higher than needed for fast, synaptic release (Tallent, 2007). In addition, the response of receptive cells is relatively slow (Rosenbaum *et al.*, 2009; Hazell *et al.*, 2012), taking many seconds to minutes, making it a challenge to correlate peptide release with a post synaptic response, or to use quantal analysis of single or multiple exocytotic events (Hazell *et al.*, 2012). Unlike fast neurotransmitters, such as glutamate or GABA, the rise time for neuromodulators is much slower making it harder to segregate individual events (van den Pol, 2012).

2.4 Cell-based neurotransmitter fluorescent engineered reporters

To overcome limitations with detecting release of neuromodulators *in vivo*, we have developed a new optical approach for detecting the release of neuromodulators. This

new technology is termed **c**ell-based **n**eurotransm**i**tter **f**luorescent **e**ngineered **r**eporters (CNiFERs).

CNiFERs leverage florescence resonance energy transfer (FRET) to translate transmitter binding into an optical signal. CNiFER cells are engineered from HEK293 cells by stably expressing a specific GPCR for the neuromodulator of interest and a FRET-based calcium sensor, e.g., TN-XXL (Nguyen *et al.*, 2010; Muller *et al.*, 2014; Lacin *et al.*, 2016). For Gq-coupled GPCRs, binding of the neuromodulator of interest to the GPCR expressed on the surface of HEK293 cells leads to an increase in intracellular Ca^{2+} which is detected by the FRET-based Ca^{2+} sensor, i.e. decrease in CFP (FRET donor) emission and an increase of YFP (FRET acceptor) emission (Figure 2.2). CNiFERs can be implanted directly into target brain regions where they can detect release of physiological concentrations of modulators with a temporal resolution of seconds and spatial resolution of less than 100 μm (Nguyen *et al.*, 2010; Muller *et al.*, 2014) (Figures 2.3 B–D). The dual wavelength measurement of the

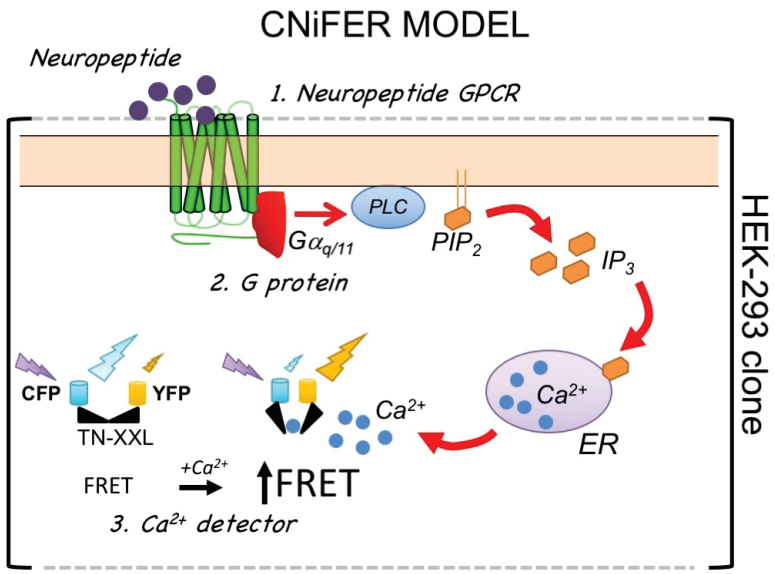

Figure 2.2 Design of peptide CNiFERs. (1) The GPCR for the neuropeptide of interest is expressed on the plasma membrane of HEK-293 cells. (2) Neuropeptide binding produces a conformational change and activation of the G_q G protein, which in turn activates phospholipase C (PLC). A chimeric protein (G_{qi5} or G_{qs5}) can also be expressed to redirect $G_{i/o}$ or G_s-coupled receptors, respectively, to the PLC pathway. Cleavage of PIP_2 into diacyl glycerol and IP_3 by PLC leads to the release of intracellular Ca^{2+} from the endoplasmic reticulum. (3) Ca^{2+} binds to the genetically encoded Ca^{2+} sensor which undergoes FRET. Two-photon excitation of the CNiFER in the absence of the neuropeptide agonist exhibits high CFP and low YFP fluorescence. Neuropeptide binding increases intracellular Ca^{2+} and produces an increase in FRET, detected as a decrease in CFP emission and increase in the YFP emission with CFP excitation.

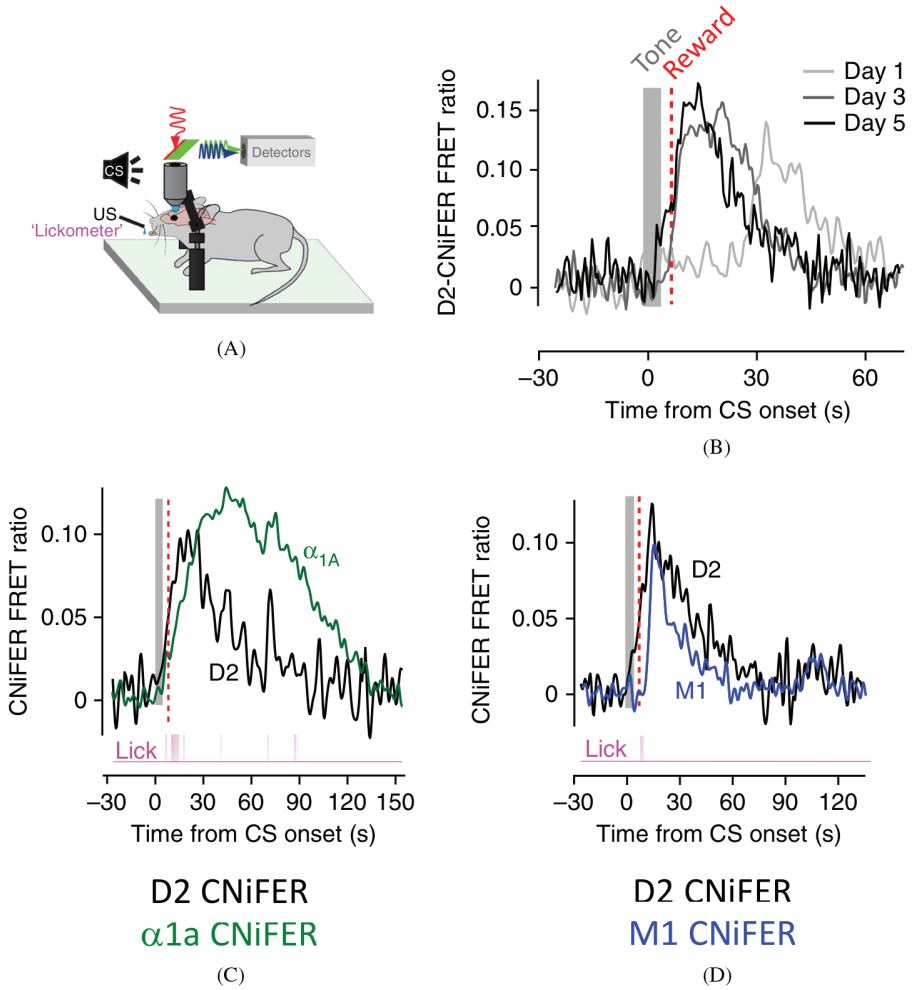

Figure 2.3 Example of CNiFER detection of DA, NE and ACh *in vivo*. (A) Procedure to measure licking behavior and CNIFER fluorescence in head-restrained mice during classical conditioning. (B) Single trial examples of the D2-CNiFER response in the same animal at days 1, 3 and 5 of training. (C) Simultaneous measurement of D2- and α1A-CNiFER FRET, or D2- and M1-CNiFER FRET, in conjunction with bouts of licking, during a single conditioning trial (modified from Muller *et al.*, 2014).

The FRET-based Ca^{2+} sensor provides an intrinsic signal for correcting possible motion-induced artifacts.

2.4.1 Creation of cell-based neurotransmitter fluorescent engineered reporters (CNiFERs)

For each neuromodulator, an appropriate GPCR is stably expressed in a clonal HEK293 cell with a Ca^{2+} sensor, i.e., TN-XXL or equivalent Ca^{2+} sensor

(Figure 2.2). For $G_{i/o}$- or G_s-coupled receptors, a chimeric G protein G_{qi5} or G_{qs5}, respectively, is also expressed in the HEK293 cell to redirect GPCR signaling to the G_q-Ca^{2+} pathway. The calculated fractional change in fluorescence, $\Delta F/F$, for each signal is used to obtain the FRET ratio, denoted $\Delta R/R$. Each clonal CNiFER is assessed for potency (e.g. EC_{50}), efficacy (i.e., largest increase in FRET ratio), and lowest background responses to other neurotransmitter/neuromodulators (Nguyen *et al.*, 2010; Muller *et al.*, 2014; Lacin *et al.*, 2016). The control CNiFER contains the same Ca^{2+} detection components but lacks the specific GPCR. The CNiFER approach is easily adapted for any neurotransmitter, neuromodulator or neuropeptide that signals through a specific GPCR. Thus, a library of CNiFERs can be created for assessing the release of a large number of different neuromodulators. In addition, implanting two different CNiFER types in the brain affords the opportunity for multiplexing, and simultaneous detection of the release of two different neuromodulators (Figures 2.3C and 2.3D) (Nguyen *et al.*, 2010; Muller *et al.*, 2014; Lacin *et al.*, 2016).

The creation of dopamine and norepinephrine CNiFERs provides a good example of simultaneous detection of chemically similar neurotransmitters (Muller *et al.*, 2014). The dopamine CNiFER was created with the D2 dopamine receptor (D2) and norepinephrine CNiFER with the α_{1A} receptor. The D2 and α_{1A} CNiFERs have an EC_{50} of 2.5 nM and 19 nM, respectively, suitable for detection of endogenously released transmitter (Muller *et al.*, 2014). We injected the D2 CNiFER and α_{1A} CNiFER into the cortex, approximately 200 μm apart, and measured the release of DA and NE in awake mice undergoing Pavlovian conditioning, i.e. repeated pairing of a tone (CS) with a sugar reward (Figures 2.3A and 2.3B). We also compared the release of DA with ACh, using a muscaranic M1 CNiFER (Nguyen *et al.*, 2010) and D2 CNiFER for detecting DA (Figure 2.3C). This multiplexing of two different CNiFERs in the brain allows for monitoring the release of two different neuromodulators simultaneously. As predicted, the timing of DA release shifts from the reward to the CS with training. Interestingly, the timing of NE release is highly variable whereas ACh release is tightly correlated with licking (Muller *et al.*, 2014).

Multiplexing of CNiFERs could also be used to compare a single transmitter interacting with two different receptors. For example, dopamine signals through both D1 and D2 receptors, which have different affinities for DA (Lobo and Nestler, 2011). Endogenous dopamine release in a particular region may preferentially activate one CNiFER (D2 *versus* D1) over the other. Another example of multiplexing CNiFERs is to co-implant one CNiFER for a traditional neuromodulator, such as DA, with another CNiFER that expresses a GPCR for a neuropeptide, such as for the kappa opioid receptor to measure the neuropeptide dynorphin (Dyn). DA and Dyn CNiFERs could be implanted into the striatum

of a mouse to measure release dynamics in response to exposure to a drug of abuse such as alcohol (Resendez *et al.*, 2016).

The CNiFER design allows for measuring neuromodulator release on the time scale of seconds, mimicking the activation of natively expressed GPCRs. This temporal response of CNiFERs is a significant improvement over the response time of minutes to hours for microdialysis. The fast response time, high affinity, and the ability to generate a library of sensors capable of measuring any modulator that signals through a GPCR, makes CNiFERs well suited for examining the temporal dynamics of local neuropeptide release. A limitation with CNiFERs, on the other hand, is the inability to directly capture the activity of neuromodulators released within the synapse.

2.5 Future for CNiFER-based research

CNiFERs were first created with HEK293 cells because HEK293 cells typically have low expression of endogenous GPCRs and are easily transduced to create clonal lines. Although HEK293 cells are human cells, implantation into mice does not appear to produce a significant inflammatory response (Muller *et al.*, 2014) when mice are injected with an immunosuppressant (e.g., cyclosporine). Under these conditions, CNiFERs can remain functional for up to a week. However, monitoring neuromodulator release over longer periods might require repeated CNiFER injections. An alternative approach would be to create CNiFERs using rodent cells lines, such as mouse embryonic fibroblasts or chinese hamster ovary (CHO) cells. We have tested CHO cells and measured FRET responses comparable to HEK293 cells (unpublished data).

Currently, CNiFERs are implanted into cortex and monitored *in vivo* using two-photon laser scanning microscopy (TPLSM). However, neuropeptide-producing cells and terminals are widely distributed in the brain, including subcortical regions (Lein *et al.*, 2007; Sakurai, 2014; Acevedo-Rodriguez *et al.*, 2015). Standard TPLSM allows for imaging up to 1 mm in depth and cannot easily image areas below the cortex. In addition, animals are head-fixed for most TPLSM setups, limiting the range of behaviors that can be studied during imaging. Recent advances in imaging technologies have made it possible to monitor subcortical activity in freely moving animals (Gosh *et al.*, 2011; Cai *et al.*, 2016). For example, miniature microscopes (e.g. endoscopes or Miniscopes) contain all the electronic and optical components necessary for imaging and are directly mounted to a mouse skull and tethered to a cable. The head-mounted scopes are usually coupled with a gradient-refractive-index (GRIN) lens that is implanted near the target and relays the fluorescent signal to the scope (Girven and Sparta,

2017). Although currently available Miniscopes are designed for single-wavelength measurements, it should be possible to create a two-channel Miniscope without adding too much weight. Another approach for subcortical measurements of CNiFERs is to use fiber photometry. Fiber photometry is commonly used to measure Ca^{2+} transients using genetically-encoded Ca^{2+} sensors (e.g., GCaMPs) and involves insertion of an optical fiber and coupling to photomultiplier tubes (PMTs) (Girven and Sparta, 2017). Fibers have the advantage of producing less tissue damage because of the small size, ~0.2 mm, compared to GRIN lenses which typically are 0.5–1 mm in diameter (Girven and Sparta, 2017). Fiber photometry measurements lack spatial information, due to summation of the light signal from all cells. However, the average FRET activity is measured in multiple CNiFER cells within an implant, making fiber photometry well suited for CNiFER imaging.

An alternate approach to cell-based CNiFERs is the development of genetically encoded neuromodulator sensors that are capable of capturing the activity of neuromodulators within synapses. The first generation of genetically encoded neurotransmitter detectors were based on mutations of periplasmic binding proteins and detected glutamate release (iGluSnFR (Marvin *et al*., 2013), EOS (Namiki *et al*., 2007), Snifit (Brun *et al*., 2011), FLIPE (Okumoto *et al*., 2005), albeit with microMolar sensitivity. Recently, two groups have developed sensors for detecting DA based on coupling steric changes in a GPCR for DA, which occur upon binding of DA, into producing changes in the optical properties of a closely coupled fluorescent protein (FP). Patriarchi *et al*. (2018) created a fusion protein that directly couples dopamine binding induced conformational changes in an inert human dopamine receptor to changes in the fluorescence intensity of a circularly permuted green fluorescent protein (cpGFP) (termed dLight1). Sun *et al.* (2018) used a similar strategy but inserted cpEGFP in the intracellular loop of the human dopamine receptor (termed $GRAB_{DA}$). One advantage of these sensors is the ability to express the sensor in a particular neuronal population of interest and capturing the temporal dynamics of neuromodulator activity within the synapse (Patriarchi *et al*., 2018; Sun *et al*., 2018). Additionally, the wide expression enables detection of neuromodulator concentrations across the cortical mantle.

An advantage of using CNiFERs is the ability to multiplex and study the simultaneous release of multiple neuromodulators. Currently, the simultaneous imaging of two different transmitters is accomplished by injecting two types of CNiFERs within a 100 μm of each other. Because the two sensors are spatially distinct, it is possible to measure FRET in two implants simultaneously. Another approach is to mix CNiFERs of different colors in the same implant. For example, choosing a calcium sensor that emits at a longer wavelength than the CFP and

YFP of TN-XXL, such as with a red Ca^{2+} sensor (Akerboom *et al.*, 2013). A CNiFER with a longer wavelength calcium sensor also has the advantage of imaging deeper in the brain (Akerboom *et al.*, 2013; Tischbirek *et al.*, 2015) and the longer wavelength excitation two-photon light necessary to excite a red or near-infrared fluorophore scatters less, produces less phototoxic damage, and penetrates deeper (Yaroslavsky *et al.*, 2002; Tischbirek *et al.*, 2015). Moreover, when CNiFER imaging is coupled to optogenetics, it enables continuous imaging of CNiFERs during blue light stimulation of channel-rhodopsin.

In summary, the methods for optically detecting the release of neurotransmitters or neuromodulators *in vivo* in awake animals are rapidly advancing, providing neuroscientists with state-of-the-art tools for probing the function of neuromodulators on brain circuits.

Acknowledgments

Work presented in this chapter was supported by research grants through the US National Institute of Mental Health BRAIN Initiative R01 (MH111499), National Institute on Drug Abuse (NIDA) (DA029706; DA037170), National Institute of Biomedical Imaging and Bioengineering (NIBIB) (EB003832), Hoffman-La Roche (88610A), and the "Neuroscience Related to Drugs of Abuse" training grant through NIDA (DA007315).

References

Acevedo-Rodriguez A, Mani SK, Handa RJ (2015) Oxytocin and estrogen receptor beta in the brain: An overview. *Front Endocrinol (Lausanne)*, 6: 160.

Akerboom J *et al.* (2013) Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. *Front Mol Neurosci*, 6: 2.

Borgland SL, Storm E, Bonci A (2008) Orexin B/hypocretin 2 increases glutamatergic transmission to ventral tegmental area neurons. *Eur J Neurosci*, 28: 1545–1556.

Brun MA, Griss R, Reymond L, Tan KT, Piguet J, Peters RJ, Vogel H, Johnsson K (2011) Semisynthesis of fluorescent metabolite sensors on cell surfaces. *J Am Chem Soc*, 133: 16235–16242.

Caceda R, Kinkead B, Nemeroff CB (2007) Involvement of neuropeptide systems in schizophrenia: Human studies. *Int Rev Neurobiol*, 78: 327–376.

Cai DJ *et al.* (2016) A shared neural ensemble links distinct contextual memories encoded close in time. *Nature*, 534: 115–118.

Chou TC, Lee CE, Lu J, Elmquist JK, Hara J, Willie JT, Beuckmann CT, Chemelli RM, Sakurai T, Yanagisawa M, Saper CB, Scammell TE (2001) Orexin (hypocretin) neurons contain dynorphin. *J Neurosci*, 21: RC168.

Day JC, Kornecook TJ, Quirion R (2001) Application of *in vivo* microdialysis to the study of cholinergic systems. *Methods*, 23: 21–39.

Dreifuss JJ (1975) A review on neurosecretory granules: Their content and mechanisms of release. *AnnNY Acad Sci*, 248: 184–201.

Fisher JM, Sossin W, Newcomb R, Scheller RH (1988) Multiple neuropeptides derived from a common precursor are differentially packaged and transported. *Cell*, 54: 813–822.

Frank M, Thumer L, Lohse MJ, Bunemann M (2005) G Protein activation without subunit dissociation depends on a Gαi-specific region. *J Biol Chem*, 280: 24584–24590.

Ghosh KK, Burns LD, Cocker ED, Nimmerjahn A, Ziv Y, Gamal AE, Schnitzer MJ (2011) Miniaturized integration of a fluorescence microscope. *Nat Methods*, 8(10): 871–878.

Gilpin NW, Roberto M (2012) Neuropeptide modulation of central amygdala neuroplasticity is a key mediator of alcohol dependence. *Neurosci Biobehav Rev*, 36: 873–888.

Girven KS, Sparta DR (2017) Probing deep brain circuitry: New advances in *in vivo* calcium measurement strategies. *ACS Chem Neurosci*, 8: 243–251.

Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology*, 148: 5752–5760.

Greco S, Danysz W, Zivkovic A, Gross R, Stark H (2013) Microdialysate analysis of monoamine neurotransmitters — A versatile and sensitive LC–MS/MS method. *Analy Chim Acta*, 771: 65–72.

Hazell GGJ, Hindmarch CC, Pope GR, Roper JA, Lightman SL, Murphy D, O'Carroll A-M, Lolait SJ (2012) G protein-coupled receptors in the hypothalamic paraventricular and supraoptic nuclei — serpentine gateways to neuroendocrine homeostasis. *Front Neuroendocrin*, 33: 45–66.

Ji C, Li W, Ren X-d, El-Kattan AF, Kozak R, Fountain S, Lepsy C (2008) Diethylation labeling combined with UPLC/MS/MS for simultaneous determination of a panel of monoamine neurotransmitters in rat prefrontal cortex microdialysates. *Anal Chem*, 80: 9195–9203.

Jobst EE, Enriori PJ, Cowley MA (2004) The electrophysiology of feeding circuits. *Trends Endocrinol Metab*, 15: 488–499.

Koob GF (1999) Stress, corticotropin-releasing factor, and drug addiction. *Ann NY Acad Sci*, 897: 27–45.

Kreek MJ, Koob GF (1998) Drug dependence: Stress and dysregulation of brain reward pathways. *Drug Alcohol Depend*, 51: 23–47.

Kreiner T, Sossin W, Scheller RH (1986) Localization of Aplysia neurosecretory peptides to multiple populations of dense core vesicles. *J Cell Biol*, 102: 769–782.

Lacin E, Muller A, Fernando M, Kleinfeld D, Slesinger PA (2016) Construction of cell-based neurotransmitter fluorescent engineered reporters (CNiFERs) for optical detection of neurotransmitters *in vivo*. *J Vis Exp*, May 12; (111).

LaCrosse AL, Olive MF (2013) Neuropeptide systems and schizophrenia. *CNS Neurol Disord Drug Targets*, 12: 619–632.

Lein ES *et al.* (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature*, 445: 168–176.

Li Y, van den Pol AN (2008) Mu-opioid receptor-mediated depression of the hypothalamic hypocretin/orexin arousal system. *J Neurosci*, 28: 2814–2819.

Linguelia E, Champigny G, Lazdunski M, Barbry P (1995) Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel. *Nature*, 378: 730–733.

Lobo MK, Nestler EJ (2011) The striatal balancing act in drug addiction: Distinct roles of direct and indirect pathway medium spiny neurons. *Front Neuroanat*, 5: 41.

Logrip ML, Koob GF, Zorrilla EP (2011) Role of corticotropin-releasing factor in drug addiction: Potential for pharmacological intervention. *CNS Drugs*, 25: 271–287.

Ludwig M, Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci*, 7: 126–136.

Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen TW, Bargmann CI, Orger MB, Schreiter ER, Demb JB, Gan WB, Hires SA, Looger LL (2013) An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nat Methods*, 10: 162–170.

Mens WB, Witter A, van Wimersma Greidanus TB (1983) Penetration of neurohypophyseal hormones from plasma into cerebrospinal fluid (CSF): Half-times of disappearance of these neuropeptides from CSF. *Brain Res*, 262: 143–149.

Mingote S, de Bruin JPC, Feenstra MG (2004) Noradrenaline and dopamine efflux in the prefrontal cortex in relation to appetitive classical conditioning. *J Neurosci*, 24: 2475–2480.

Muller A, Joseph V, Slesinger PA, Kleinfeld D (2014) Cell-based reporters reveal *in vivo* dynamics of dopamine and norepinephrine release in murine cortex. *Nat Methods*, 11: 1245–1252.

Namiki S, Sakamoto H, Iinuma S, Iino M, Hirose K (2007) Optical glutamate sensor for spatiotemporal analysis of synaptic transmission. *Eur J Neurosci*, 25: 2249–2259.

Nguyen QT, Schroeder LF, Mank M, Muller A, Taylor P, Griesbeck O, Kleinfeld D (2010) An *in vivo* biosensor for neurotransmitter release and in situ receptor activity. *Nat Neurosci*, 13: 127–132.

Nordmann JJ, Morris JF (1984) Method for quantitating the molecular content of a subcellular organelle: Hormone and neurophysin content of newly formed and aged neurosecretory granules. *Proc Natl Acad Sci USA*, 81: 180–184.

Okumoto S, Looger LL, Micheva KD, Reimer RJ, Smith SJ, Frommer WB (2005) Detection of glutamate release from neurons by genetically encoded surface-displayed FRET nanosensors. *Proc Natl Acad Sci USA*, 102: 8740–8745.

Patriarchi T, Cho JR, Merten K, Howe MW, Marley A, Xiong WH, Folk RW, Broussard GJ, Liang R, Jang MJ, Zhong H, Dombeck D, von Zastrow M, Nimmerjahn A, Gradinaru V, Williams JT, Tian L (2018) Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded sensors. *Science*, 360: pii: eaat4422.

Purba JS, Hoogendijk WJ, Hofman MA, Swaab DF (1996) Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. *Arch Gen Psychiatry*, 53: 137–143.

Raadsheer FC, Hoogendijk WJ, Stam FC, Tilders FJ, Swaab DF (1994) Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. *Neuroendocrinology*, 60: 436–444.

Radhakrishnan V, Henry JL (1995) Electrophysiology of neuropeptides in the sensory spinal cord. *Prog Brain Res*, 104: 175–195.

Raggenbass M (2001) Vasopressin- and oxytocin-induced activity in the central nervous system: Electrophysiological studies using *in-vitro* systems. *Prog Neurobiol*, 64: 307–326.

Resendez SL, Jennings JH, Ung RL, Namboodiri VM, Zhou ZC, Otis JM, Nomura H, McHenry JA, Kosyk O, Stuber GD (2016) Visualization of cortical, subcortical and deep brain neural circuit dynamics during naturalistic mammalian behavior with head-mounted microscopes and chronically implanted lenses. *Nat Protoc*, 11: 566–597.

Roberto M, Spierling SR, Kirson D, Zorrilla EP (2017) Corticotropin-releasing factor (CRF) and addictive behaviors. *Int Rev Neurobiol*, 136: 5–51.

Robinson DL, Venton BJ, Heien ML, Wightman RM (2003) Detecting subsecond dopamine release with fast-scan cyclic voltammetry *in vivo*. *Clin Chem*, 49: 1763–1773.

Robinson DL, Wightman RM (2007) Rapid Dopamine Release in Freely Moving Rats. In: Electrochemical Methods for Neuroscience (Michael AC, Borland LM, eds). Boca Raton (FL).

Rosenbaum DM, Rasmussen SGF, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature*, 459: 356–363.

Sakurai T (2014) The role of orexin in motivated behaviours. *Nat Rev Neurosci*, 15: 719–731.

Sakurai T *et al*. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*, 92: 573–585.

Schier CJ, Dilly GA, Gonzales RA (2013) Intravenous ethanol increases extracellular dopamine in the medial prefrontal cortex of the long-evans rat. *Alcohol Clin Exp Res*, 37: 740–747.

Schloos J, Raulf F, Hoyer D, Bruns C (1997) Identification and pharmacological characterization of somatostatin receptors in rat lung. Br *J Pharmacol*, 121: 963–971.

Solano RM, Carmena MJ, Carrero I, Cavallaro S, Roman F, Hueso C, Travali S, Lopez-Fraile N, Guijarro LG, Prieto JC (1996) Characterization of vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptors in human benign hyperplastic prostate. *Endocrinology*, 137: 2815–2822.

Sossin WS, Fisher JM, Scheller RH (1989) Cellular and molecular biology of neuropeptide processing and packaging. *Neuron*, 2: 1407–1417.

Sudhof TC (2012) The presynaptic active zone. *Neuron*, 75: 11–25.

Sun F *et al*. (2018) A genetically encoded fluorescent sensor enables rapid and specific detection of dopamine in flies, fish, and mice. *Cell*, 174: 481–496.

Tallent MK (2007) Presynaptic inhibition of glutamate release by neuropeptides: Use-dependent synaptic modification. In: *Inhibitory Regulation of Excitatory Neurotransmission*, pp. 177–200. Berlin: Springer Berlin Heidelberg.

Thibonnier M, Berti-Mattera LN, Dulin N, Conarty DM, Mattera R (1998) Signal transduction pathways of the human V1-vascular, V2-renal, V3-pituitary vasopressin and oxytocin receptors. *Prog Brain Res*, 119: 147–161.

Tischbirek C, Birkner A, Jia H, Sakmann B, Konnerth A (2015) Deep two-photon brain imaging with a red-shifted fluorometric Ca^{2+} indicator. *Proc Natl Acad Sci USA*, 112: 11377–11382.

van den Pol AN (2012) Neuropeptide transmission in brain circuits. *Neuron*, 76: 98–115.

Wang Y, Michael AC (2012) Microdialysis probes alter presynaptic regulation of dopamine terminals in rat striatum. *J Neurosci Methods*, 208: 34–39.

Yaroslavsky AN, Schulze PC, Yaroslavsky IV, Schober R, Ulrich F, Schwarzmaier HJ (2002) Optical properties of selected native and coagulated human brain tissues *in vitro* in the visible and near infrared spectral range. *Phys Med Biol*, 47: 2059–2073.

Zoicas I, Slattery DA, Neumann ID (2014) Brain oxytocin in social fear conditioning and its extinction: Involvement of the lateral septum. *Neuropsychopharm*, 39: 3027–3035.