CellPress

Cell Metabolism
Review

An Emerging Technology Framework for the Neurobiology of Appetite

Scott M. Sternson,[1,*] Deniz Atasoy,[2] J. Nicholas Betley,[1,3] Fredrick E. Henry,[1] and Shengjin Xu[1]
[1]Janelia Research Campus, HHMI, 19700 Helix Drive, Ashburn, VA 20147, USA
[2]Department of Physiology, School of Medicine, Istanbul Medipol University, 34810 Istanbul, Turkey
[3]Present address: Department of Biology, University of Pennsylvania, 433 South University Avenue, Philadelphia, PA 19104, USA
*Correspondence: sternsons@janelia.hhmi.org
http://dx.doi.org/10.1016/j.cmet.2015.12.002

Advances in neuro-technology for mapping, manipulating, and monitoring molecularly defined cell types are rapidly advancing insight into neural circuits that regulate appetite. Here, we review these important tools and their applications in circuits that control food seeking and consumption. Technical capabilities provided by these tools establish a rigorous experimental framework for research into the neurobiology of hunger.

> Those that would perfect their work must first sharpen their tools.—Confucius

Introduction

More than 100 years ago, observations that pituitary and hypothalamic tumors resulted in overeating and obesity drew attention to the relationship between brain function and appetite (Babinski, 1900; Bray, 1984; Frohlich, 1901). Consequently, the neurobiology of hunger has focused considerably on how the hypothalamus controls motivated behaviors (Anand and Brobeck, 1951; Delgado and Anand, 1953; Hess, 1957; Margules and Olds, 1962). However, technical challenges posed by this heterogeneous and deep-brain structure have restricted progress. Recently though, neuro-technology advances have emerged as major drivers toward elucidating neural mechanisms by which the hypothalamus regulates appetite.

Investigation of hypothalamic control of appetite has seen three major phases of technology-driven progress. First were classic discoveries that region-specific lesions or electrical stimulation resulted in extreme overeating and obesity (Delgado and Anand, 1953; Hetherington and Ranson, 1940, 1942). From these studies, a "behavioral homunculus" was mapped with discrete hypothalamic areas that were associated with particular motivated behaviors (reviewed in Sternson, 2013). However, different behaviors (e.g., drinking and eating) could sometimes be elicited from perturbations of the same site (Grossman, 1960; Valenstein et al., 1968). When electrophysiological recordings were acquired from appetite-associated brain areas, only some units showed responses correlated with glucose levels, energy deficit, or satiety (Oomura et al., 1964, 1974; Rolls, 1982). It became apparent that manipulations of entire brain regions were insufficiently precise to gain understanding of how the brain controlled complex, motivated appetitive and consummatory responses toward food.

The next major phase of progress was identification of neuropeptides that selectively altered appetite. A tremendous amount of work has been devoted to identifying neuropeptides and their specific behavioral effects. For example, the gut peptide cholestocystokinin and the hypothalamic peptide α-melanocyte stimulating hormone (from the precursor peptide, POMC) both strongly suppress appetite. Identification of neuropeptide Y (NPY), agouti related protein (AGRP), melanin concentrating hormone (MCH), and hypocretin/orexin revealed several different peptides that increased food intake, albeit with differences in the precise appetitive properties. These neuropeptides increase feeding even when applied to the entire brain via the cerebral ventricles, quite unlike small-molecule "chemical" neurotransmitters. This led to the hope that neuropeptide-coded circuits would be sufficient to understand the generation of motivated behaviors such as feeding (Hoebel, 1985). Neuropeptide-expressing neurons that influence appetite are located in brain regions that had been previously identified with lesion and electrical stimulation studies, but they comprise only a small portion of the total neurons in these areas. Moreover, neuropeptide-expressing populations with opposite function, such as orexigenic AGRP neurons and anorexigenic POMC neurons, are intermingled in the same area. These observations highlight the importance of distinguishing molecularly defined neuron populations for deconstructing the neural processes that control appetite. Such considerations were the basis for many studies manipulating gene expression in individual neuron populations using Cre recombinase-based molecular genetic methods, which provide insight into cell-type-specific signaling pathways (Williams et al., 2011). However, genetic alteration is often too slow and indirect to concretely establish the contribution of neurons in short term behaviors such as eating.

Presently, a third phase of technical innovation has the potential to overcome major remaining barriers for a mechanistic understanding of appetite regulation. A rapidly expanding neurobiology toolbox that is tailor-made for molecularly defined cell types and deep-brain circuits is revealing the anatomy, ultrastructure, cell biology, circuit architecture, neuronal dynamics, and effects of neuron activity perturbations with cell-type specificity. Together, these powerful tools have greatly advanced methodology for examining appetite circuits in the hypothalamus (and elsewhere), primarily by adapting the full arsenal of modern neuroscience methods for use with molecularly defined cell types. Here, we review some of the core technologies for cell-type-specific neurobiology that are important for mapping and understanding the function of appetite circuits. In addition, we discuss some critical technical considerations for designing

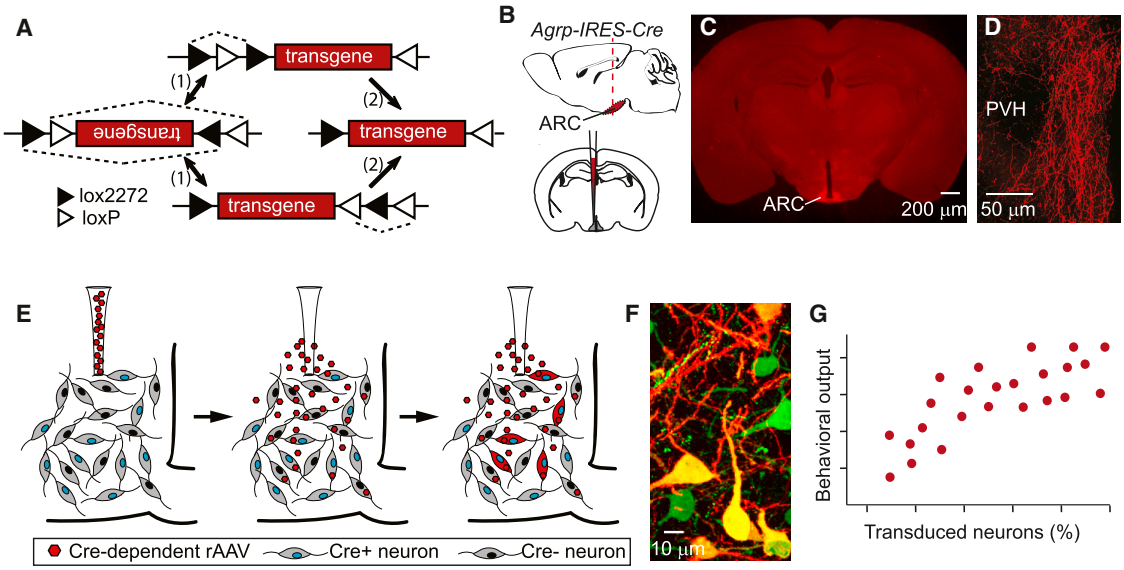


Figure 1. Cell-Type-Selective Transgene Expression

(A) Design of flip-excision (FLEX) switch used in Cre-dependent viruses, also showing recombination events for stable inversion. loxP and lox2272 are heterotypic recombination sites. The inverted orientation of the transgene coding region prevents expression of the transgene in the absence of Cre.

(B–D) Cre-dependent expression of a fluorophore in AGRP neurons. (B) Schematic of viral injection into the hypothalamus of *Agrp*^{Cre} mice. Selective fluorophore expression is limited to ARC^{AGRP} neurons (C) and fluorophore labeling in ARC^{AGRP} → PVH axons (D).

(E) Schematic of cell-type-specific transgene expression. Cre-dependent rAAV transduces neurons in the injected area, but only a subset of Cre-expressing cells (blue nuclei) will show transgene expression.

(F) Micrograph showing transduction of only a subset of ARC^{POMC} neurons (green) following injection into the hypothalamus of rAAV with a Cre-dependent mCherry (red) transegene.

(G) Graph depicting the relationship between viral transduction efficiency and a hypothetical behavioral outcome. Each dot represents a single experimental subject evaluated post hoc.

and interpreting experiments that use these tools, as well as potential pitfalls to keep in mind.

Neuronal Cell Types

Electronic circuits involve wires and nodes that perform computations based on combinations of their inputs. For neural circuits, neuronal subpopulations (here, simply called "cell types") comprise the nodes, and their inter-neuronal axon connections are the wires. Currently, there is little agreement on a precise definition of neuronal cell type. Cell types have been defined primarily based on utilitarian criteria that are important to different groups of experimenters: neuronal morphology (Masland, 2004), gene expression (Lein et al., 2007; Nelson et al., 2006), markers of excitatory or inhibitory neurotransmitter release (Jennings et al., 2013), electrophysiological firing properties (Gupta et al., 2000), axon projection targets (Betley et al., 2013; Brown and Hestrin, 2009), developmental and mature transcription factor codes (Dasen and Jessell, 2009; Michaud et al., 1998), stimulus response sensitivity (Hubel and Wiesel, 1962), information theory (Sharpee, 2014), as well as intersections of these criteria. This diversity of classification regimes reflects a longstanding uncertainty about how to subdivide the brain into fundamental neuronal components. Future improvements that lead to a systematic taxonomy of the neuronal cell types in the brain have the potential to offer improved organizing principles for neuroscience. Ideally, this would offer some predictive insight into computational function of individual nodes in the manner that different electronic circuit elements have predictable computational properties. Improving the definition of cell type is a problem gaining increasing attention, and one new approach involves high-dimensional information from single-cell RNA sequencing methods (Zeisel et al., 2015). This topic is beyond the scope of this review, and here we will continue with current convention, in which individual researchers define neuronal cell type based on the availability of particular tools and their priorities for the problem under investigation.

Cell-Type-Specific Transgene Expression

Molecularly defined cell types are specified by patterns of selective gene expression, which is an especially attractive basis for applying tools to visualize and manipulate neural circuits. A powerful approach in neuroscience makes use of cell-type-specific promoters for selective expression of transgenes, such as neuronal activity indicators or neuronal actuators. However, promoter elements that are selectively expressed in a neuron population typically do not drive expression levels that are sufficient for most anatomical or functional applications (Betley and Sternson, 2011). For example, optogenetic actuators such as channelrhodopsin-2 have small single channel conductances and thus require high expression levels. For this reason, an intersectional approach is often used. This commonly involves using genetically engineered mice in which cell-type-specific promoters drive Cre-recombinase (Cre), and this is coupled to stereotaxic injection of Cre-dependent recombinant adeno-associated viral (rAAV) vectors that use a strong promoter to express a transgene of interest (Figures 1A–1D) (Atasoy et al., 2008). A large number of Cre-dependent viral vectors are readily

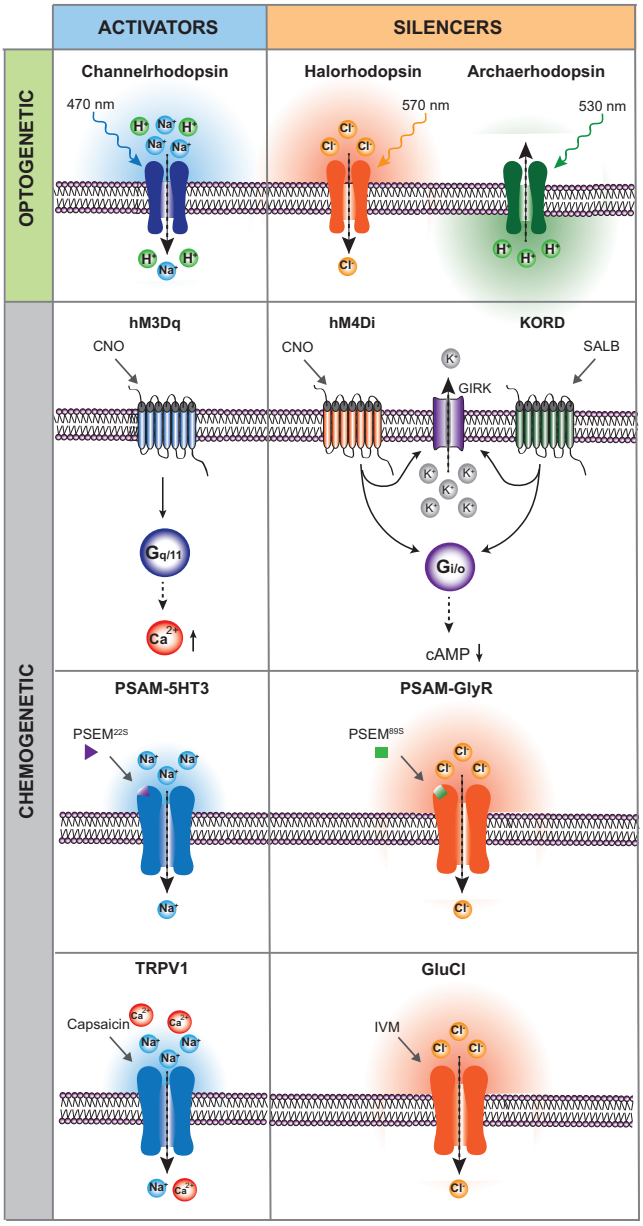


Figure 2. Genetically Encoded Tools for Neuron Activity Manipulation

Optogenetic and chemogenetic neuron activity perturbation systems.

available from nonprofit repositories, as are a growing number of Cre-expressing mouse lines. It is essential for newly developed mouse and viral reagents to be deposited upon publication (preferably, a requirement). Fortunately, institutions like Addgene, Mutant Mouse Regional Resource Center (MMRCC), Jackson Laboratory, and several viral vector cores have removed barriers for making these available.

One caveat of viral transduction approaches is that stereotaxic viral injections can lead to variability in the number of transduced neurons across subjects (Figure 1E). This must be characterized by quantitative post hoc evaluation of brain injection sites for the distribution of transduced cells (Figure 1F). Ideally, the relationship between viral transduction efficiency and a behavioral or physiological outcome should be provided (Figure 1G) (Aponte et al., 2011; Betley et al., 2013, 2015; Lin et al., 2011). Such quantitative post hoc analyses also obviate the questionable practice in some neuron perturbation studies where experimenters simply include or discard subjectively defined "high expressors" and "low expressors," respectively. Unless adequately justified, the absence of transduction quantification should be treated skeptically. These issues surrounding reproducible transgene expression can be mitigated by use of Cre-dependent fluorophore or optogenetic reporter mice (Madisen et al., 2010, 2012), but this requires careful selection of a Cre-expressing mouse line with highly restricted expression pattern and limited off-target developmental expression.

Electrical Activity Manipulations of Molecularly Defined Cell Types

The causal relationship between neuron activity and behavior is important for understanding the function of cell types in the brain. As such, brain lesions and electrical stimulation have been greatly improved by the introduction of cell-type-specific neuron perturbations in living animals. Recently, several genetically targetable tools for rapid and reversible control of neuronal activity have been employed in appetite circuits (Figure 2). These methods vary in terms of temporal kinetics, invasiveness, and ease-of-use, but they provide researchers with a flexible toolbox that can be tailored for a diverse set of physiological questions.

Cell-Type-Specific Neuron Ablation

Regional brain lesion studies have been refined by the development of molecularly mediated cell-type-specific neuron ablation techniques. Selective AGRP neuron ablation was accomplished in gene targeted mice engineered to express diphtheria toxin receptor (DTR) under the *Agrp* promoter (Luquet et al., 2005). DTR is not normally expressed in mice, and activation by its ligand, diphtheria toxin (DT), rapidly kills AGRP neurons expressing the receptor transgene by blocking protein translation (Saito et al., 2001). DT can be injected at any time point, thus allowing temporal control over neuron ablation. For example, AGRP neuron ablation led to anorexia in adult, but not neonatal, mice (Luquet et al., 2005). This technique can be made site specific if DTR is delivered via a viral vector (Zhan et al., 2013), and a similar approach has been reported for DT (although the safety of this reagent may be a concern) (Inutsuka et al., 2014). More recently, caspase expression has been shown to also be an effective cell-type-specific neuronal ablation tool (Yang et al., 2013). This approach was used with Cre-dependent viral vector delivery to ablate LH^{VGAT} neurons, which suppressed food intake (Jennings et al., 2015).

Optogenetics

Traditional neuron electrical stimulation methodology has been transformed by the introduction of genetically targetable microbial opsins, which are light-sensitive ion channels and ion pumps that provide precise control over neuron activity on a millisecond timescale (Fenno et al., 2011). The algal cation channel, ChR2 (Boyden et al., 2005), has been used extensively for gain-of-function studies in hypothalamic circuits. The first demonstration in appetite circuits was optogenetic activation of AGRP neurons, which resulted in food seeking and consumption in sated mice that increased with the proportion of AGRP neurons transduced

with ChR2 (Aponte et al., 2011). Because the optical stimulation pattern is imposed by the experimenter, the relationship between specific activity patterns and behavioral responses can be examined. For AGRP neurons, more food was consumed at higher stimulation frequencies and ceased within a few minutes once stimulation was halted (Aponte et al., 2011). However, the tendency of optogenetic stimulation to elicit potentially non-physiological synchronous entrainment of neuron activity should be kept in mind as a potential caveat. Channelrhodopsin-mediated neuronal activation has since been used for a number of other cell types to evaluate their role in appetite, such as ARC^{POMC} (Aponte et al., 2011; Atasoy et al., 2012), PVH^{SIM1} (Atasoy et al., 2012), $PVH^{Oxytocin}$ (Atasoy et al., 2012; Garfield et al., 2015), PVH^{MC4R} (Garfield et al., 2015), $BNST^{VGAT}$ (Jennings et al., 2013), LH^{VGAT} (Jennings et al., 2015), LH^{VGLUT2} (Jennings et al., 2013), PBN^{CGRP} (Carter et al., 2013), and $CeA^{PKC\delta+}$ (Cai et al., 2014). In most of these studies, optical excitation is achieved by short pulses of blue light (~1–10 ms) and limited to a few minutes to hours of stimulation, although ChR2 activation appears to be effective with 24 hr of continuous stimulation (Aponte et al., 2011). Collectively, these optogenetic studies in several cell types have demonstrated numerous positive and negative neuronal regulators of food intake.

Cell types that are responsible for motivational and rewarding aspects of food seeking and consumption have been investigated by taking advantage of the temporal precision of optogenetic activation. Hypothalamic circuits that relay nutrient value of ingested sugars were studied by optogenetic activation of LH^{MCH} neurons during ingestion of non-nutritive sweetener, which increased its preference over that for nutritive food (Domingos et al., 2013). Pairing cell-type-specific photostimulation with an animal's action is a powerful tool for examining the role of appetite circuits in learning. Activation of $BNST^{VGAT}$ neurons reinforced self-photostimulation behavior, where each lever nose poke delivered pulses of laser light into the brain (Jennings et al., 2013). A related strategy has recently been used to show that mice learned to avoid contextual cues paired with ARC^{AGRP} neuron activation in a closed-loop place preference experiment, and avoidance persisted during a subsequent extinction test (Betley et al., 2015). Conversely, closed-loop place preference indicated that PVH^{MC4R} photostimulation has positive valence (Garfield et al., 2015). These studies and others (Carter et al., 2015) demonstrate that the flexibility and rapidity of photoactivation studies are well suited for appetite-related behavioral conditioning using neuronal stimulation as an unconditional stimulus.

Optogenetic tools that inhibit neuronal activity, such as halorhodopsin and archaerhodopsin, have also been used in appetite circuits. Optogenetic silencing by archaerhodopsin-3 (Arch3.0) in LH^{VGLUT2} neurons increased food intake (Jennings et al., 2013), whereas the same manipulation on the neighboring LH^{VGAT} neurons decreased food intake (Jennings et al., 2015). A microbial chloride pump, halorhodopsin (NpHR), was used to silence $CeA^{PKC\delta}$ neurons, which modestly increased food consumption (Cai et al., 2014). One challenge for using these tools is that physiologically regulated circuits often require inhibition over timescales ranging from minutes to hours or even days, and reports of continuous optogenetic inhibition for an hour or more are rare (Jones et al., 2015). The impact of constant light delivery over these timescales has not been well characterized, and issues arise, such as channel desensitization and tissue heating. In addition, depolarization of the chloride reversal potential has been noted for NpHR, and optogenetic silencers typically result in post-inhibitory rebound spikes, which must be taken into account with optogenetic silencing experiments (Raimondo et al., 2012). Nevertheless, other promising approaches for optical neuronal inhibition have also been reported, which offer several potential improvements such as shunting inhibition and prolonged channel open times that may minimize rebound and further reduce excitability (Berndt et al., 2014; Govorunova et al., 2015; Wietek et al., 2014).

Chemogenetics

Chemogenetic tools present alternatives to optogenetic methods because they can be minimally invasive and do not require specialized equipment (Sternson and Roth, 2014). They also have a longer-lasting effect on neuron activity and can be controlled non-invasively through delivery of ligands for engineered receptors by intraperitoneal injections or, in some cases, in the drinking water (Armbruster et al., 2007; Güler et al., 2012; Lerchner et al., 2007; Magnus et al., 2011). However, these features require trading off precise temporal control offered by optogenetics over the onset and offset of neuron activity perturbations.

A prominent chemogenetic class of tools is the DREADDs (DREADD, designer receptor exclusively activated by designer drug), which are engineered G protein-coupled receptors. DREADD activity manipulation systems have been extensively used in appetite circuits (Atasoy et al., 2012; Betley et al., 2015; Carter et al., 2013; Krashes et al., 2011, 2013, 2014; Pei et al., 2014; Stachniak et al., 2014; Wang et al., 2015c; Yang et al., 2015; Zhan et al., 2013). The DREADD, hM3Dq, is a G_q protein-coupled receptor that is activated by the mostly inert ligand CNO (Armbruster et al., 2007). This receptor mediated ligand-dependent activation of AGRP neurons (Krashes et al., 2011), which resulted in food consumption comparable to optogenetic activation, although feeding was initiated with longer latency and persisted much longer than with optogenetic activation. A corresponding G_i protein-coupled version of this system (hM4Di) (Armbruster et al., 2007) reduced AGRP neuron electrical activity and correspondingly reduced feeding in the dark period after intraperitoneal CNO injection (Krashes et al., 2011). This approach was also used to silence PVH^{SIM1} neurons, which elicited feeding and motivation to work for food (Atasoy et al., 2012). An additional DREADD system based on the engineered κ-opioid receptor and the otherwise inert ligand, salvinorin B, has been introduced that makes it possible to orthogonally silence more than one neuronal population in the same animal using DREADDs (Denis et al., 2015; Vardy et al., 2015).

The effectiveness of hM4Di as a neuronal inhibitor appears to be due largely to a synaptic silencing function (Stachniak et al., 2014). $hM4D^{nrxn}$, a variant that is mostly excluded from somato-dendritic compartments, showed robust synaptic silencing with little effect on somatic conductance or membrane potential, and this tool strongly increased feeding following CNO delivery when expressed in PVH^{SIM1} neurons (Stachniak et al., 2014). Collectively, analyses of feeding behavior using cell-type-specific activity manipulations with DREADDs across various regions have revealed functionally diverse neuronal populations within each area, often with opposite roles.

DREADDs have also been used to understand circuits of energy expenditure and glucose homeostasis. A group of ARC neurons ectopically expressing Cre from a transgenic rat insulin promoter has been activated by hM3Dq. Unlike its neighboring AGRP and POMC neurons, these neurons did not affect feeding, but instead upregulated energy expenditure (Kong et al., 2012). PVH neurons have also been examined for their role in energy expenditure using DREADD perturbations (Sutton et al., 2014). In addition, blood glucose regulation has been analyzed using hM3Dq and hM4Di, which showed that PBN^{CCK} neuron activity is sufficient and necessary for the counter-regulatory response to hypoglycemia (Flak et al., 2014; Garfield et al., 2014).

Because DREADD systems are based on G protein or arrestin pathways that indirectly couple to ion channels, they may cause distinct cellular responses depending on the cell type (Sternson and Roth, 2014). Therefore, experiments must be performed to verify that neuron activity profile is changed in the expected manner once the ligand is applied, ideally using electrophysiological recordings.

An alternative chemogenetic activity perturbation system is based on ion channels (PSAM-IPDs) engineered to selectively respond to synthetic ligands (PSEMs). PSAM/PSEM ion channel/ligand systems directly activate different ion channels and offer remote control of cation or chloride conductances in neurons via blood-brain barrier permeable small molecule agonists (Donato et al., 2013; Esposito et al., 2014; Magnus et al., 2011). These ligands show rapid onset and offset and are suitable for neuron activity manipulations lasting approximately 30 min (for comparison, CNO effects persist for up to 12 hr). PSAM/PSEM inhibitory channels have been used to silence AGRP neurons and correspondingly reduce feeding (Betley et al., 2015). These channels are effective silencers even during strong concurrent optogenetic AGRP neuron photostimulation (Magnus et al., 2011). In addition, AGRP neuron silencing was shown to be sufficient to condition preference to flavor or contextual cues (Betley et al., 2015). The narrow ~30 min window for neuron inhibition (Magnus et al., 2011) was well suited for conditioning experiments by restricting the neuronal perturbation to a timeframe during which the animal was exposed to a conditioned stimulus.

Cre-recombinase-dependent re-expression of a transgene for the Trpv1 ion channel in *Trpv1*$^{-/-}$ mice has also been used in conjunction with injection of the ligand, capsaicin (Güler et al., 2012), for transient activation of AGRP neurons with somewhat faster kinetics than what was observed by optogenetic activation (Dietrich et al., 2015). The origin of slightly faster AGRP neuron-evoked feeding is not clear, but it could be related to the high calcium permeability of Trpv1 channels, which can directly enhance neuropeptide and neurotransmitter release (Fischer et al., 2003) in addition to the action potential-driven release that is associated with neuronal depolarization. This approach was also used to show that mild dopamine neuron activation can overcome starvation following acute AGRP neuron ablation in adult mice (Denis et al., 2015). Further developments using *Trpv1* include new radiogenetic and magnetogenetic methods (Stanley et al., 2012, 2015).

Anatomy and Function of Molecularly Defined Circuits

Circuit Anatomy

Hypothalamic feeding circuits monitor and integrate signals about peripheral energy status, and this information is relayed to multiple brain areas by local and long-range axon projections. Therefore, axon projection anatomy provides a map of potential effector sites. Anterograde mapping techniques have traditionally been applied to investigate the connectivity of groups of neurons (Chamberlin et al., 1998; Gerfen and Sawchenko, 1984). Recently, virally mediated Cre-dependent fluorescent protein expression in discrete cell types (Atasoy et al., 2008) has been coupled with high-speed whole-brain 2-photon microscopy imaging to generate a database showing the projection patterns of molecularly defined cell types from discrete brain regions (Oh et al., 2014). One issue unaddressed by these anatomical maps is the axonal projection pattern of individual neurons. In principle, axonal arborization can be configured such that each neuron of a population projects to all targets of the population (one-to-all), a subset of the targets (one-to-many), or only one of the targets (one-to-one) (Figure 3A). For neurons with a small number of different projection targets, axon terminal uptake and retrograde transport of chemical dyes or fluorescent beads at discrete axon projection fields has been used to measure axon collateralization in the population as the fraction of overlapping labels (Kuypers et al., 1977; Swanson and Kuypers, 1980). For this method, it is important to assess the uptake efficiency of each retrogradely transported dye, which can be done by co-injecting the two dyes in a single target region and determining the co-localized fraction. However, as the number of projection sites increases, the retrograde labeling combinations grow supralinearly. An alternative strategy leverages axonally targeted viral vectors that are taken up by axon terminals and retrogradely transported to the cell body. Subsequent fluorescent protein expression fills the neuron, including its axonal arbor. If the axons of that cell type are independently marked with a fluorescent reporter, then a quantitative measure of collateralization across all axon projection targets can be determined as the fraction of the viral transgene colocalized with the axonal reporter (Figure 3B). To achieve cell-type-specific axonal transduction, neurons can be transduced with the TVA receptor and axonally transduced with the protein G-deleted rabies viral vectors pseudotyped with the TVA ligand, EnvA (Figure 3B). Use of this approach to examine the collateralization of AGRP neurons indicated that this neuron population was comprised of distinct subpopulations each projecting to one of the populations target areas (one-to-one configuration) (Betley et al., 2013), and a similar result was reported for PVH^{MC4R} neurons (Garfield et al., 2015). In addition, other methods for retrograde axonal transduction include: canine adenovirus 2 (CAV2) (Soudais et al., 2001), virus-mediated WGA-Cre expression (Gradinaru et al., 2010), retrograde rAAV vectors (Rothermel et al., 2013), retrograde HSV1 vectors (Lima et al., 2009), and retrograde lentiviral vectors (Mazarakis et al., 2001). In principle, the most comprehensive approach is single-neuron anatomical reconstruction and their axons across the entire brain. Recently, new microscopes have been developed that use extremely rapid 2-photon imaging of molecularly defined neurons throughout the entire brain to enable complete anatomical reconstruction of individual neurons (N. Clack and E. Myers, personal communication). Based on these new microscopes as well as advances in tissue clearing methods (Chung et al., 2013; Yang et al., 2014), the development of databases with the complete anatomy of individual neurons spanning entire brains will be possible.

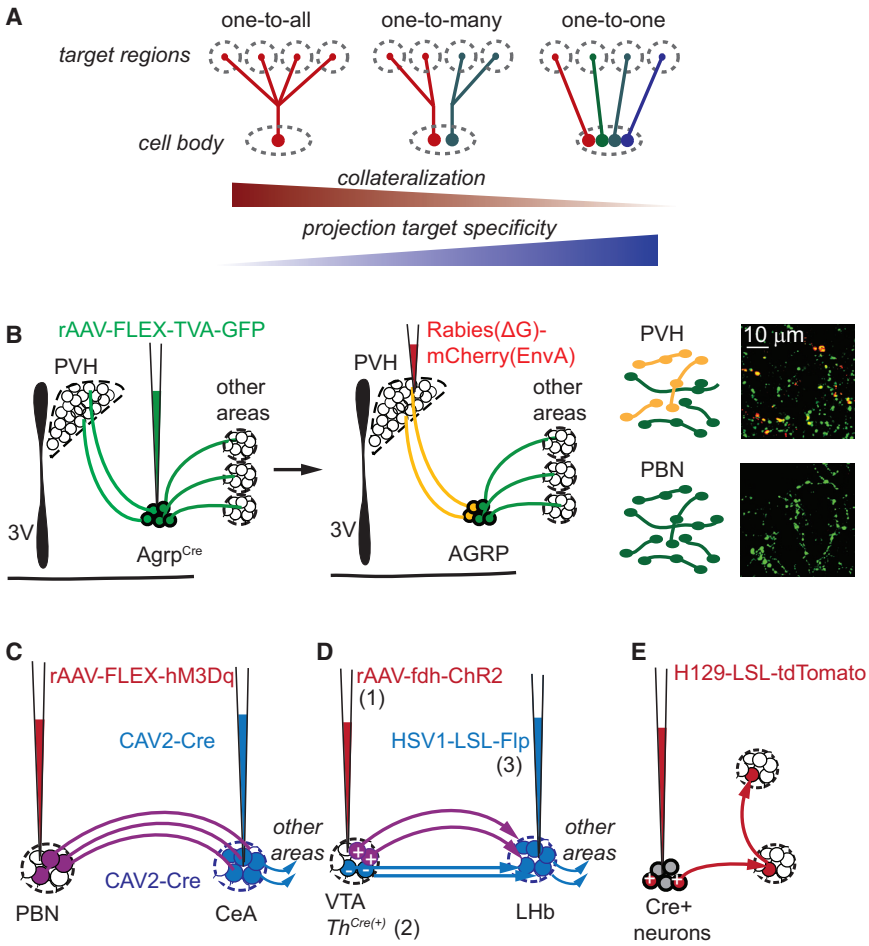


Figure 3. Axon Anatomy

(A) Potential configurations for axon projections from individual neurons within a molecularly defined population.

(B) Schematic of axonal transduction of genetically defined neurons using a G-deleted, EnvA-pseudotyped rabies viral vector. Axonal transduction leads to expression of the mCherry reporter exclusively in a neuronal subset expressing TVA-GFP that project to the stereotaxically targeted injection site. mCherry expression in other target areas is used to assess the axon projection configuration. For example, AGRP neurons projecting to the PVH do not have other detectable axon collaterals as can be seen by the lack of mCherry in other projection targets, such as the PBN (right).

(C) A 2-way intersectional strategy to specifically express hM3Dq in an axon projection-based subpopulation of neurons using axonally targeted CAV2-Cre and somatically targeted rAAV-FLEX-hM3Dq. In this example, only neurons in the PBN that project to the CeA will express hM3Dq.

(D) A 3-way intersectional strategy to specifically express ChR2 in a molecularly defined neuron population with a defined axon projection (Stamatakis and Stuber, 2012). (1) Axon-targeted injection of retrograde competent HSV1 engineered to express Flp recombinase only in the presence of the Cre recombinase (HSV-LSL-Flp), (2) Th^{Cre} mouse, and (3) somatic injection of a Flp recombinase-inducible rAAV virus engineered to express ChR2 (rAAV-fdh-ChR2). In this example, only tyrosine hydroxylase-expressing (Th) neurons in the ventral tegmental area (VTA) that project to the lateral habenula (LHb) will express ChR2.

(E) Cre-dependent activation of an anterograde herpes simplex virus (HSV/H129) engineered to express tdTomato in a Cre-dependent manner (H129-LSL-tdTomato). Upon uptake by Cre-expressing neurons (+), tdTomato expression is enabled. This virus is transported transsynaptically, primarily in the anterograde direction.

Circuit Function

The functional role of discrete axon projections from molecularly defined neurons can be examined using axon projection-specific optogenetic and chemogenetic activity manipulations. Action potentials can be efficiently evoked in distal ChR2-expressing axons (Figure 4). Using this approach, it was shown that AGRP axons that project to the PVH, but not those that project to the PBN, are sufficient to drive acute food intake (Atasoy et al., 2012). This approach has also been used with projections of additional cell types that activate or inhibit feeding: $BNST^{VGAT} \rightarrow LH$ (Jennings et al., 2013), $LH^{PDX} \rightarrow PVH$ (Wu et al., 2015), $PVH^{MC4R} \rightarrow PBN$, $PBN^{CGRP} \rightarrow CeA$ (Carter et al., 2013), $mPFC^{D1R} \rightarrow mBLA$ (Land et al., 2014), and $LH^{VGAT} \rightarrow VTA$ (Nieh et al., 2015). However, distally generated action potentials back-propagate, and if there are collateral branches that terminate elsewhere, then projections to those regions may also be activated (Figure 4). Several studies have attempted to mitigate this issue by injecting a sodium channel blocker into the region where the somata of the cell type of interest resides (Stuber et al., 2011; Wang et al., 2015b), a caveat of which is whether collateralization happens distal to the soma. In addition, axon photostimulation can also stimulate labeled axons-of-passage from the molecularly defined cell type that project to different regions but on a common trajectory (Figure 4). Therefore, axon activation experiments typically necessitate careful evaluation of axon arborization anatomy.

Inhibition of molecularly defined axon projections is also valuable for examining the necessity of circuit projections (Figure 5). Microbial opsin-based neuronal inhibitors are thought to impede propagation of action potentials with high temporal precision (Figure 5A) (Stuber et al., 2011; Tye et al., 2011), although this does not appear to have been demonstrated directly by axon-attached recordings. The axon projection from $BNST^{VGAT} \rightarrow LH$ was suppressed with archaerhodopsin-3 (Arch3.0), which reduced feeding (Jennings et al., 2013). Similarly, halorhodopsin (NpHR) was used to inhibit $LH \rightarrow VTA$ projections to suppress sucrose seeking (Nieh et al., 2015). However, axon projection inhibition is not restricted to blocking local synaptic function and can presumably block action potential transmission through molecularly defined axons of passage as well, which could complicate interpretation of results if the inhibited axons have additional synaptic connections in regions further away from the soma.

These shortcomings can be avoided by direct inhibition of synaptic release. Genetically encodable toxins directly block synaptic transmission (Han et al., 2015; Kim et al., 2009); however, targeting them to a subset of projections could be problematic, and this manipulation is slow and largely irreversible. Light-dependent destruction of synaptic proteins by chromophore-assisted

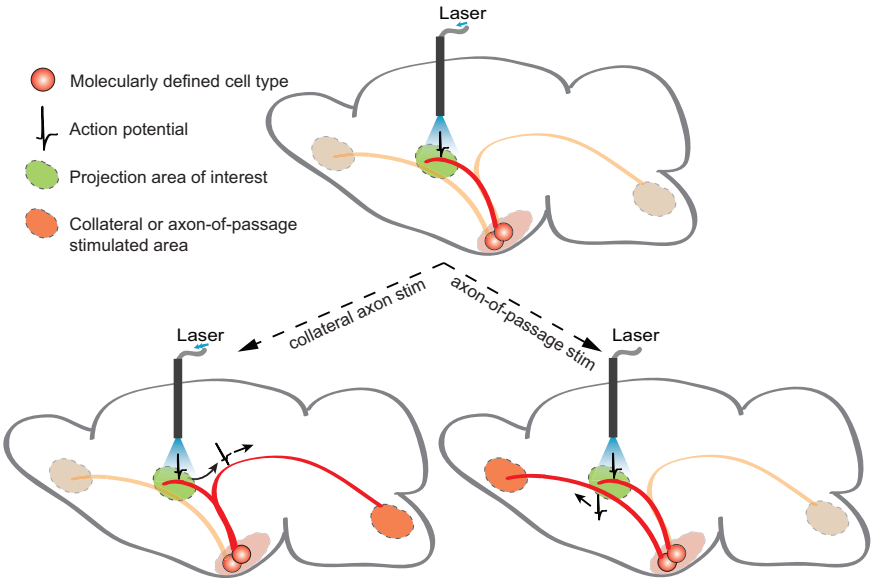


Figure 4. Potential Caveats with Optogenetic Axon Projection Activation
Light delivery through optical fibers placed over an intended axon projection area of interest may activate other projection targets due to collateral branches that terminate elsewhere (left). Light could also activate nearby molecularly defined axons of passage that terminate in a different brain region (right).

light inactivation (CALI) provides a rapid and efficient method for synaptic inhibition (Figure 5B), although the reversibility of this manipulation is very slow due to the requirement for replenishment of degraded synaptic release machinery (Lin et al., 2013). Alternatively, the chemogenetic inhibitor hM4Di^{nrxn} robustly and reversibly impairs synaptic release in targeted projections without blocking action potential propagation (Figure 5C) (Stachniak et al., 2014) (a similar approach using hM3Dq for synaptic activation has been used [Wang et al., 2015c], but it has not yet been demonstrated to elicit selective synaptic release without an action potential that can spread to collateral projections). Using small-volume, localized intracranial CNO injections for selective inhibition of PVH^{SIM1} neuron synaptic connections, functional mapping showed that synaptic inhibition of PVH projections in the vicinity of the ventrolateral periaqueductal gray and dorsal raphe was sufficient to evoke feeding (Stachniak et al., 2014). A limitation of these approaches is the necessity for an invasive approach to deliver light or small molecules. Moreover, the precision of small-molecule intracranial injections is limited by spread of the molecule and must be examined by measuring dose response curves as well as tiling injections at surrounding brain areas (Stachniak et al., 2014).

An alternative projection-specific manipulation approach is to access a cell population based on a specific subset of its axon projections by axonal transduction with a stereotaxically injected retrogradely transported viral vector. Some Cre-dependent rAAV vectors have been reported to have this property (Rothermel et al., 2013). Combination of axonal transduction with a somatically targeted Cre-recombinase has been used to study appetite-suppressing circuits in the PBN with chemogenetic actuators (Carter et al., 2013). The central nucleus of amygdala (CeA) was injected with CAV2-Cre virus, which is taken up by axon terminals and retrogradely transported to the subset of PBN neurons that project to the CeA. Subsequent transduction of the PBN with Cre-dependent rAAV-hM3Dq allowed selective manipulation of only this subset with intraperitoneal CNO injections and confirmed their involvement in appetite suppression (Figure 3C). This projection-specific Cre-tagging approach, however, labeled all PBN→CeA neurons regardless of their cell type. One approach to overcome this type of limitation was used in a different circuit, where axonal transduction with a herpes simplex virus encoding a Cre-dependent flipase (Flp) into *Th*^{Cre} mice in conjunction with Flp-dependent ChR2 rAAV injection in the VTA achieved expression selectively in VTATH neurons that project to the lateral habenula (Stamatakis et al., 2013) (Figure 3D). As always, careful consideration of the collateral projections of neurons labeled with these methods is also needed.

Synaptic Physiology in Molecularly Defined Neural Circuits

Cell-type-selective axon activation is also a powerful technique for rigorously establishing circuit connections with cell-type resolution. Optogenetic axon photostimulation enables detailed investigation of functional synaptic properties (Petreanu et al., 2007) from the long-range axon projections of molecularly defined neurons (Figure 6A) (Atasoy et al., 2008). In appetite regulatory circuits, channelrhodopsin-assisted circuit mapping has been used to establish cell-type-specific connectivity (Atasoy et al., 2012; Garfield et al., 2015; Jennings et al., 2013; Kong et al., 2012; Krashes et al., 2014). This technique also permits synaptic strength, sign, release probability, and other parameters that affect circuit function to be directly measured. In appetite circuits, detailed analysis of the release profile for ARC^{AGRP}→PVH synapses has been reported, which displayed pronounced asynchronous GABA release that increased their inhibitory output (Atasoy et al., 2012). The release probability of these synapses is increased in *Npy*$^{-/-}$ mice, suggesting involvement of a synaptic mechanism for functional compensation (Atasoy et al., 2012). Moreover, optogenetic minimal stimulation methods were used to estimate the average number of ARC^{AGRP}→PVH axons and the corresponding synaptic connections onto individual PVH neurons (Atasoy et al., 2014). Optogenetic methods have also been useful in hypocretin/orexin neurons to dissect the properties of fast neurotransmitter and neuropeptide release (Schöne et al., 2014), as well as to demonstrate Exendin 4-mediated synaptic plasticity in a NTS^{PHOX2B}→VTA circuit (Wang et al., 2015c). There are some caveats where the closure kinetics of ChR2 could artificially depolarize stimulated axonal membranes beyond physiological time frames of an action potential, which can compromise the analysis of release properties (Schoenenberger et al., 2011). These issues may be addressed by development of channelrhodopsin

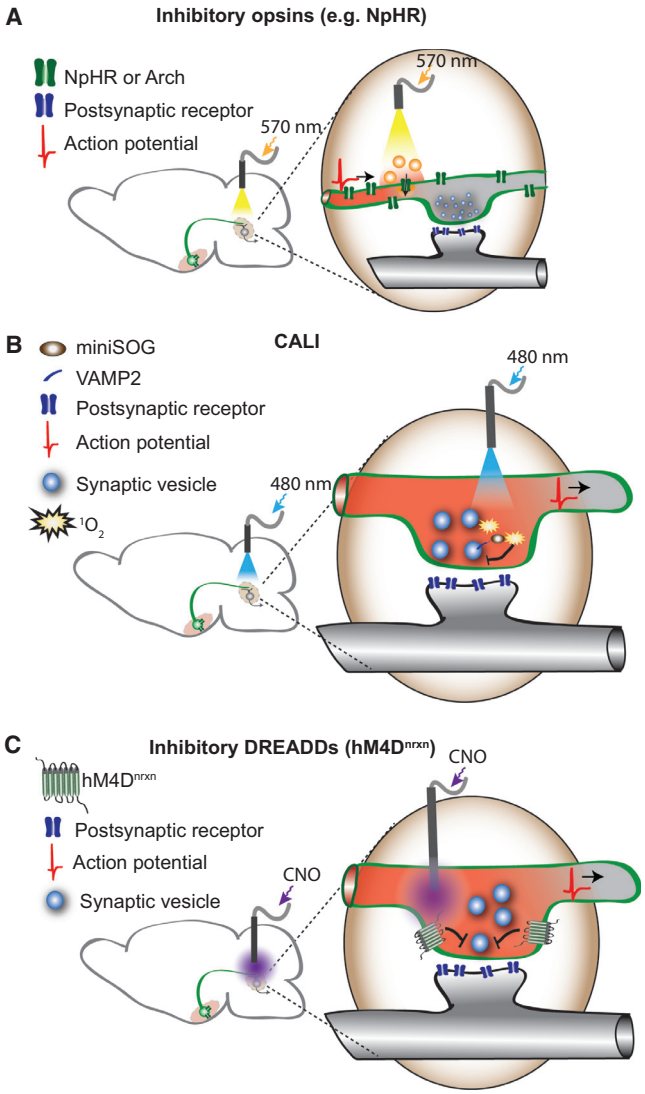


Figure 5. Methods for Axon Projection Inhibition

(A) Optogenetic inhibition of axon projections. Light-dependent activation of inhibitory opsins in axon terminals can inhibit action potential propagation, consequently suppressing synaptic release.

(B) Inhibition of synaptic release by chromophore-assisted light inactivation (CALI). A synaptically targeted miniSOG module can be activated by blue light to drive local production of singlet oxygen, which in turn degrades synaptic proteins required for vesicle release.

(C) Inhibition of synaptic release by GPCRs. Synaptically targeted inhibitory DREADD, hM4D^{nrxn}, can be activated by direct delivery of the CNO ligand to a selected projection field. Synaptic release is inhibited without affecting action potential propagation.

variants with faster kinetics (Berndt et al., 2011) and laser scanning stimulation technologies that target light to the axon and avoid direct optical illumination of the synapse (Atasoy et al., 2008; Petreanu et al., 2007).

Cell-Type-Specific Ultrastructural Circuit Mapping

Evidence of synaptic connectivity can be directly observed using electron microscopy, which provides complementary insights to functional approaches. Traditionally, knowledge of molecularly defined synaptic connectivity required antibody labeling of cell-type-specific antigens (immuno-EM labeling). This method is challenging because many epitopes cannot endure fixation protocols for EM. Moreover, immuno-EM often results in degraded ultrastructure quality, and studies are typically limited to the clearest anecdotal example images of molecularly defined synaptic connections. However, to understand connectivity, statistical analysis of the ultrastructure of molecularly defined synapses in a brain volume is needed, which often requires large datasets.

One approach is to combine cell type information from fluorescence microscopy with high-resolution EM using correlated light and electron microscopy imaging (CLEM), which is achieved by overlaying molecular marker information to ultrastructural images (reviewed in de Boer et al., 2015). Specialized probes allow LM and EM imaging of the same sample (Paez-Segala et al., 2015; Shu et al., 2011) and can be used to determine anatomical origins as well as molecular and genetic composition of the neurons that give rise to imaged connections.

Alternatively, transgenic cell-type-specific markers can be directly observed in electron micrographs without immunolabeling. Enzymes that generate electron-dense end products, such as horseradish peroxidase (HRP) (Atasoy et al., 2014; Li et al., 2010), miniSOG (Shu et al., 2011), and APEX (Lam et al., 2015; Martell et al., 2012), have been used to determine ultrastructure with genetic targeting. This approach was implemented in vivo with long-range hypothalamic ARC^{AGRP} and ARC^{POMC} neuron axonal projections to the PVH (Atasoy et al., 2014). A Genetically Encoded Synaptic marker for Electron Microscopy (GESEM) was developed based on a VAMP2:HRP fusion protein, which oriented HRP into the lumen of synaptic vesicles (Figure 6B). This topology enabled selective labeling in EM micrographs that was restricted to the interior of synaptic vesicles without degrading the ultrastructure of neuronal membranes (Figure 6B). Using this approach, systematic reconstruction of 129 complete AGRP and POMC axonal release sites (Figure 6D) across more than 200,000 μm^3 of tissue (comprised of more than 22,000 transmission electron micrographs) revealed that AGRP and POMC axons in the PVH have distinct subcellular targets. Because of the high-quality ultrastructure with this method, the approach may also be applied, in the future, to dense EM reconstructions within a brain region while labeling molecularly defined axon projections.

In most cases, correspondence is expected between functional connectivity, measured by channelrhodopsin-assisted circuit mapping, and ultrastructural analysis of synaptic connectivity, as was found for ARC^{AGRP}→PVH connections (Atasoy et al., 2012, 2014). However, the inability to detect functional synaptic connections does not prove the absence of synaptic connectivity. For example, GESEM-assisted ultrastructure analysis of ARC^{POMC}→PVH circuits showed morphological evidence of abundant synaptic connections, but functional synaptic connections were only rarely detected using channelrhodopsin-assisted circuit mapping (Atasoy et al., 2014). This was likely due to the fact that ARC^{POMC}→PVH synapses were on small-diameter dendritic processes, indicating that they were far from the cell body and thus more likely to be cut in tissue sections or electrotonically attenuated in somatic recordings. This highlights the importance of ultrastructure studies as a complement to functional synaptic analysis when assessing synaptic connectivity.

Putting It All Together: The Function of Molecularly Defined Circuit Connections

The assorted methods for molecularly defined circuit mapping and manipulation now permit systematic, node-by-node neural circuit analysis. Mapping circuits involves the sequential combination of electrical activity perturbations in molecularly defined cell types, projection-specific perturbation, followed by cell-type-specific synaptic physiology and/or ultrastructure with postsynaptic partners. Based on measured synaptic connection properties, the corresponding activity manipulations can be applied iteratively to second-order circuit nodes and beyond. Functional circuit mapping up to third-order nodes has been reported for a number of circuits: $ARC^{AGRP} \rightarrow PVH \rightarrow vlPAG/DR$, $ARC^{AGRP} \rightarrow PVH^{MC4R} \rightarrow PBN$, $NTS \rightarrow PBN^{CGRP} \rightarrow CeA$ (reviewed in Sternson and Atasoy, 2014). The first multi-node systematic analysis of a feeding circuit was put together for connections involving ARC^{AGRP} neurons that were expected to mediate this population's food seeking and consumption behavioral responses (Figure 7A) (Atasoy et al., 2012). After ARC^{AGRP} neurons were shown to be sufficient to elicit feeding behavior, major projections to ARC^{POMC} neurons, the PVH, and the PBN were evaluated as downstream effectors. Selective optogenetic activation of $ARC^{AGRP} \rightarrow PVH$ projection was sufficient to drive feeding in sated mice, but not projections to the PBN. Channelrhodopsin-assisted circuit mapping as well as ultrastructural studies showed that ARC^{AGRP} connections are strongly inhibitory at local ARC^{POMC} neurons as well as at PVH neurons. Direct chemogenetic inhibition of POMC neurons failed to rapidly stimulate feeding (although chronic silencing did increase appetite). Corresponding chemogenetic inhibition studies of PVH^{SIM1} neurons showed that this population was sufficient to elicit feeding. Therefore, these direct electrical activity perturbations in two of the key molecularly defined postsynaptic targets of AGRP neurons revealed their relative capacity to mediate the acute induction of appetite.

The necessity of a particular circuit connection for a behavioral response elicited by a molecularly defined circuit projection can be evaluated by an experimental approach akin to classical genetic epistasis experiments (Figure 7B). For the inhibitory connections of AGRP neurons, determining functional necessity requires "occlusion" of the input from the inhibitory presynaptic neuron by simultaneously activating the target neuron. This approach was applied to POMC and PVH neurons to show that $ARC^{AGRP} \rightarrow PVH$ projections were necessary and sufficient for AGRP neuron-evoked feeding, while $ARC^{AGRP} \rightarrow ARC^{POMC}$ neurons connections were not (Atasoy et al., 2012). Connections of several other cell types have been evaluated using this approach to dissect the functional necessity of other molecularly defined circuit connections for feeding and glucose homeostasis (Garfield et al., 2014, 2015; Krashes et al., 2014).

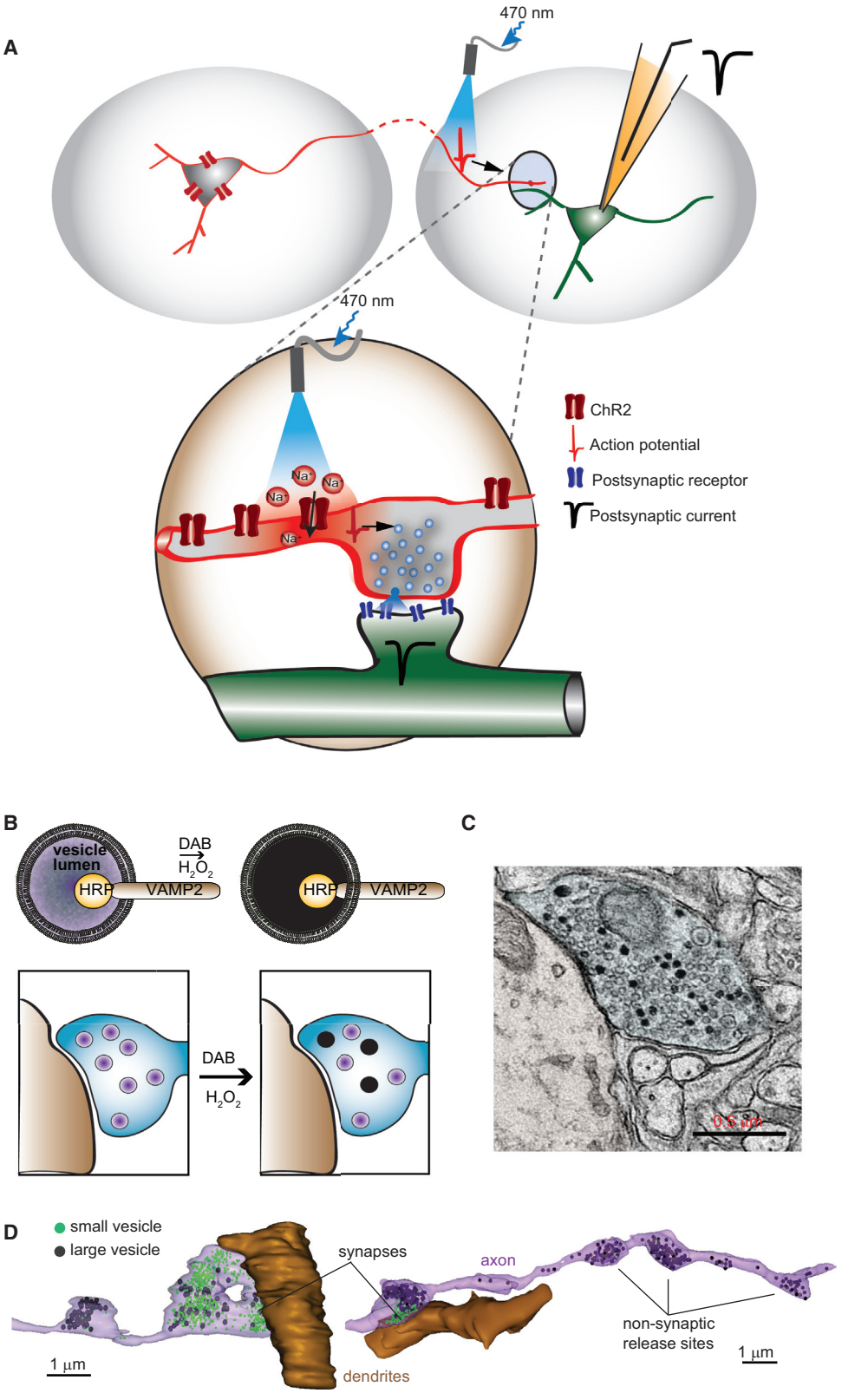
Mapping Synaptic Inputs and Outputs

Additional tools have been developed to facilitate the identification of upstream and downstream circuit nodes. Anatomical analysis of synaptic connectivity in molecularly defined circuits has been enabled by powerful transsynaptic viral methods. Cell-type-specific retrograde and anterograde transsynaptic tracers have been developed based on Cre-dependent excision of a transcriptional stop cassette before an essential viral gene. These include PRV (DeFalco et al., 2001), HSV129 (Atasoy et al., 2014; Lo and Anderson, 2011), and VSV (Beier et al., 2011). After Cre-dependent activation, these viral systems continue to transmit transsynaptically, which makes it difficult to unequivocally identify monosynaptic connections (Figure 3E). For monosynaptic connectivity, the rabies viral vector system has been heavily optimized and is in widespread use. Cell-type-specific expression of rabies glycoprotein G and TVA allow selective uptake of EnvA-pseudotyped, protein G-deleted rabies virus, resulting in monosynaptic transsynaptic retrograde transfer (Wall et al., 2010; Wickersham et al., 2007) (Figure 8). A related approach has been developed toward monosynaptic anterograde VSV vectors (Beier et al., 2011).

Retrograde viral methods have been used for identifying the inputs from upstream brain areas in appetite circuits, which was shown for multiple appetite circuits (Betley et al., 2013; Jennings et al., 2013; Krashes et al., 2014; Wang et al., 2015a). Careful attention must also be paid to the specificity of the starter cells because high-titer viral injections have been shown to lead to low levels of aberrant TVA expression (Wall et al., 2010; Weissbourd et al., 2014), which can confound analysis of the presynaptic neurons. Furthermore, non-synaptic neuromodulatory inputs show less-efficient uptake of the retrogradely transported rabies virus (Wall et al., 2013). With this in mind, it is necessary to confirm these putative connections with functional or ultrastructural methods. An elegant implementation of this used retrograde rabies virus tracing from AGRP neurons, which labeled numerous PVH neurons. A functional role of an excitatory $PVH^{TRH} \rightarrow ARC^{AGRP}$ connection was established and verified by channelrhodopsin circuit mapping. Epistasis analysis of the two connected cell types using simultaneous hM3Dq-dependent activation of PVH^{TRH} and hM4Di-dependent inhibition of ARC^{AGRP} neurons to suppress ARC^{AGRP}-based excitatory input (Figure 8) (Krashes et al., 2014) indicated that $PVH^{TRH} \rightarrow ARC^{AGRP}$ was responsible for the increased food intake observed with PVH^{TRH} activation.

Activity of Molecularly Defined Cell Types In Vivo

To understand neural function in appetite circuits, it is essential to monitor the dynamics of molecularly defined neuron populations in vivo. The deep-brain locations of these molecularly defined neural circuits have posed considerable challenges for traditional neurophysiology techniques, such as in vivo recordings or in vivo imaging. Instead, most studies have relied on immediate early gene expression, especially Fos (Sheng et al., 1990), using post hoc immunohistochemistry or in situ hybridization (Carter et al., 2013; Lin et al., 2011; Poulin and Timofeeva, 2008). Fos gene expression increases with neuron electrical activity, but it should be noted that other cellular signaling pathways can also increase Fos expression (Rivera and Greenberg, 1990). Nevertheless, detection of immediate early gene expression can be a powerful approach for brain-wide mapping of candidate neurons that may change activity under particular physiological or behavioral states. New viral tools and mouse lines have also been developed to integrate changes in immediate early gene expression using a doxycycline-sensitive transactivator or tamoxifen-activated Cre recombinase that can subsequently drive neuron activity actuators in order to perturb the electrical activity of cells that were previously activated during a particular behavior or physiological state (Garner et al., 2012;



(legend on next page)

Guenthner et al., 2013; Reijmers et al., 2007). One limitation of immediate early gene techniques is relatively poor temporal precision, ranging from several minutes to several hours. This can be improved using CaMPARI (calcium-modulated photoactivatable ratiometric integrator), which captures a temporally precise (<1 s) ''activity snapshot'' of activated neurons (Figure 9A) (Fosque et al., 2015). This tool could be used to identify the neuronal cell types that are activated during a specific phase of a behavioral task by post hoc immunohistochemistry, in situ hybridization, or transcriptional profiling of labeled neurons. In the future, such approaches might also be adapted to increase the temporal precision for driving transcription of neuronal actuators in neurons that are activated during particular behavioral epochs.

In Vivo Electrophysiology

Monitoring neuronal dynamics with the highest temporal precision requires in vivo electrophysiological recordings. In vivo recordings of hypothalamic or striatal circuits have measured responses to presentation of food, water, or alteration of circulating blood glucose (Carelli and Wondolowski, 2003; Oomura et al., 1964, 1974; Rolls, 1982). These extracellular recordings usually lack information about the identity of the cell types that are being monitored. Exceptions to this have involved laborious efforts to record from single hypocretin/orexin neurons in the hypothalamus followed by juxtacellular labeling of these neurons and post hoc immunohistochemical identification of their molecular identity (Lee et al., 2005; Mileykovskiy et al., 2005). To improve the efficiency of recordings from molecularly defined neurons in deep-brain structures, optrode recording has been developed by coupling optogenetic methods with multi-electrode in vivo extracellular recording methods (Figure 9B). Optical activation of a specific type of neurons expressing ChR2 during recordings allows ''phototagging'' by identification of spikes from a target population of neurons (Lima et al., 2009). To relate directly light-evoked spikes to those recorded during natural neuronal activity, light-evoked action potentials must be classified using rigorous criteria: (1) having short latency, (2) following high-frequency repetitive light pulses (to help distinguish synaptically driven neurons), and (3) showing high similarity between optically evoked and natural spike waveforms (Figures 9C–9F). These high-stringency criteria have been found to result in acceptance of only 20%–30% extracellularly measured units (Cohen et al., 2012, Cohen et al., 2015). Other reports using subsets of these criteria show a higher acceptance of optically identified units but with reduced confidence about molecular identity. Recently, optrode recordings were used to monitor the activity of AGRP neurons in vivo, in which spikes were assigned to AGRP neurons using template matching, but inclusion was not restricted to only those units that followed trains of light pulses, resulting in a high 41% assignment rate of light-activated responses (Mandelblat-Cerf et al., 2015). Optogenetic methods were also used to identify neurons that have particular projection targets from LH→VTA (Nieh et al., 2015). Two types of responsive LH neurons were classified using phototagging, with the latency of light-evoked responses as a criterion, but a requirement to follow high-frequency light pulses was not used. ''Type-1'' responses with short latency were identified to be LH neurons projecting to VTA; ''Type-2'' responses with long latency were LH neurons receiving feedback input from the VTA (Nieh et al., 2015). LH→VTA projecting neurons were reported to encode conditioned food-seeking responses, which is different from other LH neurons that were identified as receiving input from the VTA.

Cell-Type-Specific Optical Methods

Fully optical approaches can be especially powerful for investigating the neuronal dynamics of molecularly defined neuron populations. Recent advances in genetically encoded fluorescent probes (e.g., Ca^{2+} indicators and voltage sensors) allow researchers to target specific cell types for optical measurement of cellular activity over chronic timescale (Chen et al., 2013; Jin et al., 2012). Optical voltage sensors are promising but still have limitations for accurately signaling voltage in mammalian neurons with good signal-to-noise ratio (St-Pierre et al., 2015). Calcium indicators are useful because intracellular neuronal calcium increases in conjunction with electrical activity, and calcium has low basal levels in most neurons; thus, indicators have high dynamic range as well as good signal-to-noise ratio (Chen et al., 2013). For monitoring neuronal calcium activity in deep-brain structures, such as those that regulate appetite, one useful approach is simply collecting bulk fluorescence from a genetically encoded calcium indicator through an implanted optical fiber (Cui et al., 2013; Gunaydin et al., 2014). Photometry techniques do not create an image of individual neurons, but it allows the population calcium activity of molecularly defined neurons to be monitored with high temporal resolution. This approach has been used to measure the activity of AGRP neurons and POMC neurons in freely moving mice by simply expressing GCaMP6 using a Cre-dependent virus and the appropriate Cre-expressing mouse lines along with an optical fiber implanted in the vicinity of the arcuate nucleus (Chen et al., 2015). Because these indicators are quite bright and have a high signal-to-noise ratio, relatively little precision is required in order to readout the population activity, making this a straightforward method. Similarly, the calcium activity in axon projections can also be measured using this technique, which takes advantage of the substantial calcium fluxes that occur in presynaptic release sites (Chen et al., 2015). Of course, the population activity does not reveal the response properties of individual neurons; for this, imaging methods are required.

Deep-brain in vivo imaging techniques enable individual neurons expressing fluorescent indicators, such as GCaMP6, to be resolved. To acquire deep-brain images of neurons

Figure 6. Functional and Ultrastructural Analysis of Long-Range Synaptic Connections

(A) Synaptic connectivity between molecularly defined neurons can be evaluated through photoactivation of ChR2-expressing axons while simultaneously recording light-evoked postsynaptic currents from neurons in the target area. The ability to drive action potentials in severed ChR2-expressing axonal segments allows functional evaluation of long-distance connections.

(B) A genetically encoded synaptic marker for electron microscopy (GESEM). Diagram of HRP targeted to the synaptic vesicle lumen by C-terminal fusion to VAMP2. Upon exposure to H_2O_2, HRP catalyzes production of DAB polymers that are deposited within the vesicle lumen.

(C) Example electron micrograph illustrating ARC^{POMC}→PVH synaptic contact with labeled vesicles marked by black interior.

(D) Example reconstructions of ARC^{AGRP}→PVH axonal segments (purple) with multiple GESEM-labeled boutons.

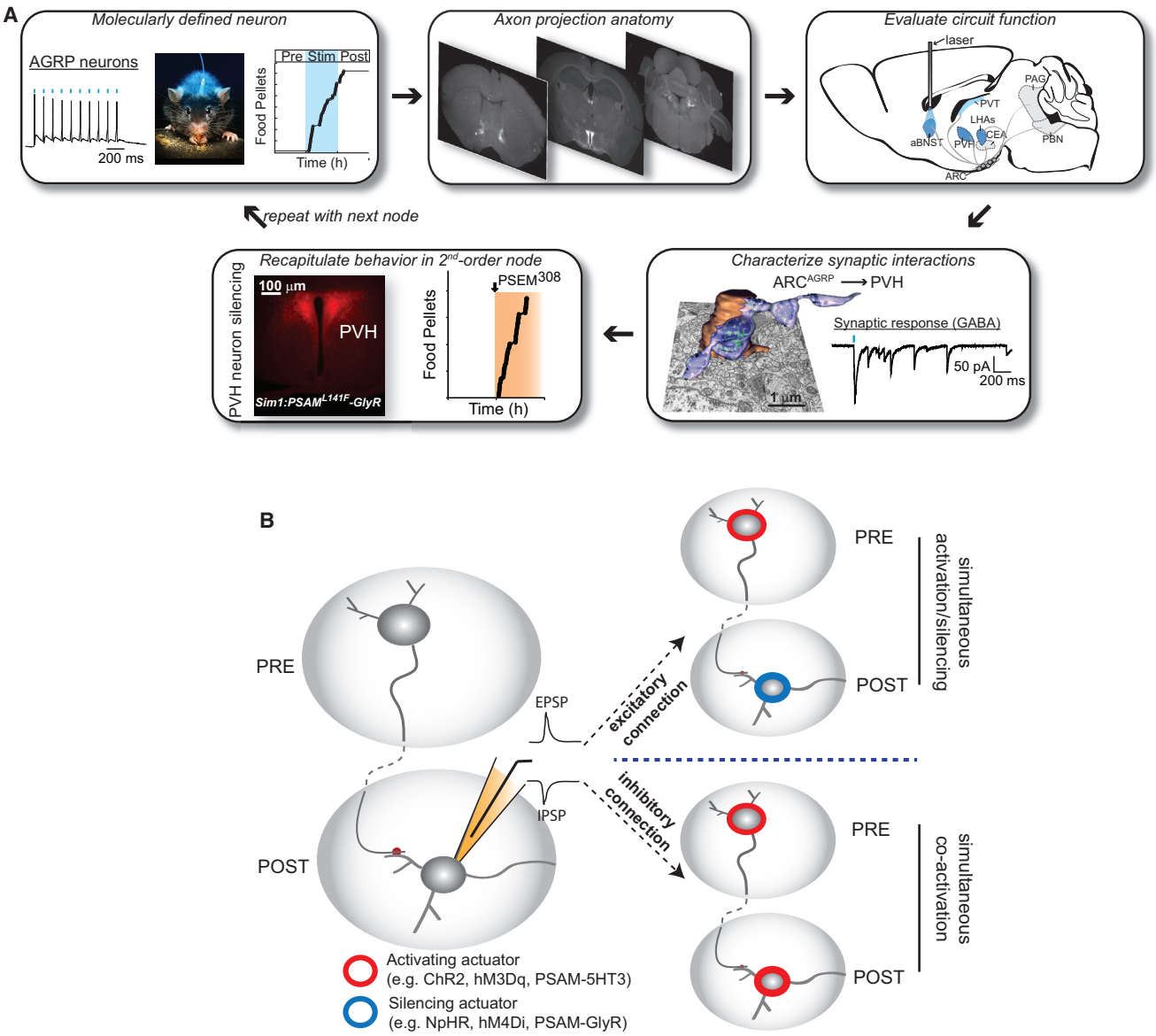


Figure 7. Node-by-Node Circuit Analysis

(A) Experimental sequence suggested for node-by-node evaluation of appetite circuits. Starting with a molecularly defined neuron population, activity manipulations can be used in conjunction with behavioral analysis. The anatomy of the axonal projections of these neurons can be determined and then selectively activated to assess the relative role of individual axon projection target sites (e.g., blue shading in upper right represents capacity of axon projection photostimulation to increase food intake). Functional and ultrastructural evaluation of synaptic connectivity can be used to gain insight into the identity of the second-order neuron as well as how it integrates inputs from the first-order neuron. The sufficiency of second-order neuron to recapitulate a behavioral response can then be directly tested through activity manipulations based on the sign of the synaptic interaction with the starting neuron population.

(B) Neural circuit epistasis analysis. Information gained through functional and anatomical methods can be used to predict how presynaptic neuronal activation affects the postsynaptic neuron activity. Based on the sign of interaction, an occlusion approach can be used to evaluate the necessity of the connection for a behavioral response. The nature of the manipulation in the postsynaptic neuron depends on the sign of the synaptic input.

expressing a calcium indicator, a gradient refractive index (GRIN) lens is implanted directly over those neurons. These neurons can be imaged through the GRIN lens using a traditional microscope if the animal is head fixed, but new miniature microscopes that can be carried mounted on the head of a mouse have allowed imaging deep-brain neuronal calcium activity in freely moving mice (Figures 9G and 9H) (Barretto et al., 2009; Ghosh et al., 2011). This can be applied to large groups of neurons in the same mouse, as was demonstrated by imaging *Slc32a1* (*vgat*)-expressing neurons in the lateral hypothalamus of the mouse, which form a large proportion of total neurons in this brain area. Subsets of these neurons showed elevated responses during food consumption and other subsets that increased their activity while working for food (Jennings et al., 2015). Although molecular definition of the cell type was used (*vgat*), this is a broadly defined population, and therefore it is

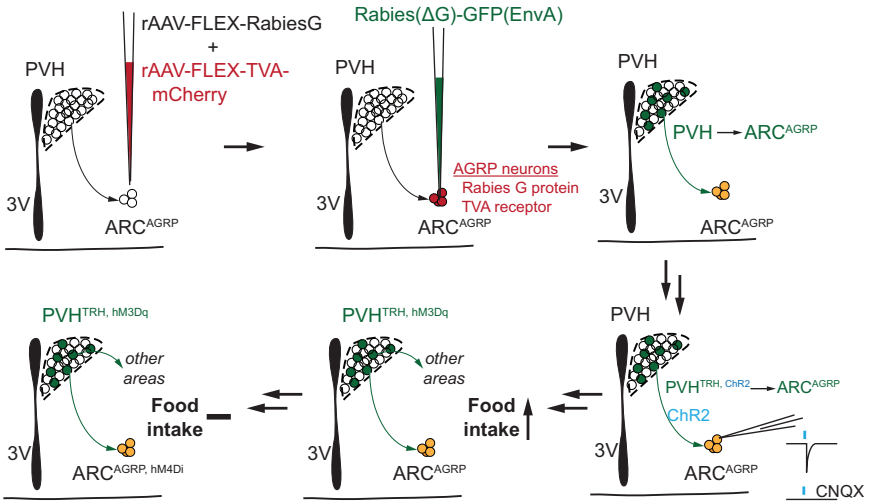


Figure 8. Cell-Type-Specific Retrograde Transsynaptic Rabies Virus Tracing

Cre-dependent expression of TVA allows selective uptake of EnvA-pseudotyped, protein G-deleted rabies virus (Rabies(ΔG)-GFP-(EnvA)) exclusively in AGRP neurons. Selective expression of Rabies G in AGRP neurons provides the rabies virus with the glycoprotein necessary for a single trans-synaptic retrograde transfer. Monosynaptic inputs from the PVH^{TRH} neurons to the ARC^{AGRP} neurons were established by channelrhodopsin-assisted circuit mapping. PVH^{TRH} neuron activation with hM3Dq increased food intake, and using neural circuit epistasis analysis, this was blocked by selective hM4Di-mediated silencing of AGRP neurons.

not surprising that heterogeneous responses were observed. AGRP neurons in the arcuate nucleus are a smaller population, but they have been found to be comprised of separate subpopulations that project to different brain areas. Expressing GCaMP6 in AGRP neurons and imaging with a head-mounted miniature microscope through a GRIN lens revealed that the subpopulations show a remarkably homogeneous response to food (Figures 9I and 9J) (Betley et al., 2015). Most strikingly, 96% of the neurons in food-restricted mice rapidly reduced calcium activity upon presentation of food; only 1% of the neurons increased their activity.

Imaging neuronal calcium indicators is an area with a great deal of promise for investigating neuronal dynamics in deep-brain appetite circuits. However, a number of caveats should be kept in mind. First, it is important to establish the relationship between electrical activity and calcium responsiveness in a neuron population. Some neurons with very high basal calcium activity may have high firing rates that would require specially designed calcium indicators in order to monitor activity. For example, some AGRP neurons with high spontaneous tonic activity did not show calcium transients for individual action potentials; instead, rapid calcium transients reflected bursts of action potentials, and changes in the tonic firing rate were reflected by corresponding slow changes in the baseline calcium indicator fluorescence (Betley et al., 2015). Another issue for deep-brain imaging is movement artifacts. For some brain areas, it is sufficient to apply simple translation of the images to accommodate the movement of the brain that occurs when animals are moving (Ziv et al., 2013). In the arcuate nucleus and likely other brain areas, brain movement cannot be corrected by a simple linear translation because of nonlinear brain flexing and stretching. For this, the nonlinear alignment software ANTS (Avants et al., 2008) was used for deep-brain calcium imaging data (Betley et al., 2015), which is a program that has been developed to register functional MRI signals with a reference brain atlas. In addition, out-of-focus fluorescence is collected with epifluorescence imaging in the brain, the contribution of which needs to be removed computationally, for example using a combination of principal component analysis and independent component analysis (Mukamel et al., 2009). Another issue to anticipate is that brain tissue can move permanently in relationship to the implanted GRIN lens, and if a non-focusing head-mounted microscope is used, then researchers cannot count on images in some brain areas to remain stable after several days. It is also important to consider the use of other neuronal activity indicators, such as voltage indicators (St-Pierre et al., 2015), which will present additional challenges for deep-brain imaging because they require fast detection of fluorescence changes in the membrane of individual cell populations, requiring high-speed photon collection. Despite the technical challenges, the opportunities to monitor neuron dynamics anywhere in the brain provide a tremendous advance for investigating how appetite circuits influence behavior.

Investigating the Circuit Nodes

The neurobiology of molecularly defined circuits that control appetite is also tied to the cell biology of individual circuit nodes. Appetite circuits are highly sensitive to changes in metabolic and hormonal state. The expression and functional role of neuropeptides, receptors, and several intracellular signaling pathways have been examined by a combination of pharmacological and cell-type-specific genetic methods. However, the molecular and cellular mechanisms underlying circuit node function can be more comprehensively evaluated with cell-type-specific transcriptomic methods.

Approaches to achieve cell-type-specific transcriptomic profiling of molecularly defined neuronal populations fall under three major categories (Figure 10): (1) laser capture microdissection, which is used to target specific regions and even individual cells for RNA extraction in the context of a brain tissue section (Bonaventure et al., 2002); (2) genetically targeted, epitope-tagged ribosome immunoprecipitation approaches such as TRAP (Heiman et al., 2008) or Ribotag (Sanz et al., 2009), which allow immunopurification of RNA from a homogenate containing cell types of interest; and (3) neuron purification techniques including fluorescence-activated cell sorting (Lobo et al., 2006), immunopanning (Dugas et al., 2008), and manual sorting of fluorescent neurons (Hempel et al., 2007; Sugino et al., 2006).

In brain regions for which unique molecular markers are not available, laser capture microdissection and transcriptomic profiling have been used to identify candidate marker genes. The ARC, VMH, and DMH have been examined with this

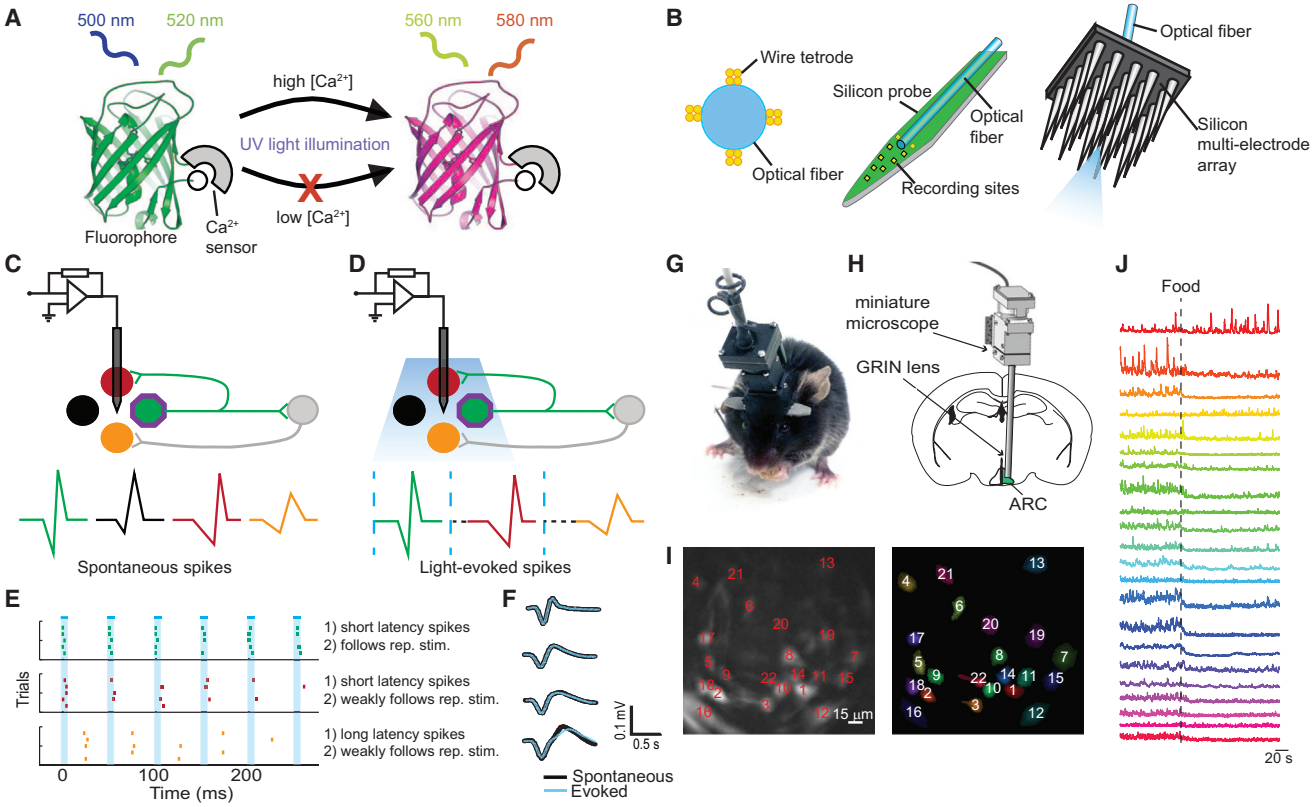


Figure 9. Methods to Monitor Activity of Molecularly Defined Cell Types In Vivo

(A) Schematic of CaMPARI function.

(B) Types of optrodes combining multi-electrode recording methods with optogenetic methods.

(C–F) Diagrams illustrating phototagging for specific neuronal cell types. Spikes from ChR2-positive (green octagon) (C) and ChR2-negative (circles, cell types with different circuit connections) (D) are recorded extracellularly during natural and light-evoked neuronal activity. Note, for inhibitory neuron photoactivation, synaptic driving of neuron activity involves an intervening neuron. (E) ChR2-positive units can be identified on the basis of the latency of their responses to a brief flash of blue light (D), reliability of their responses to repetitive light pulse train, and similarity between spontaneous and light-evoked spike waveforms (F).

(G and H) Configuration for deep-brain imaging of AGRP neurons with a miniature microscope in (G) freely moving mice. (H) Light path for fluorescence excitation and emission of ARC neurons is through a GRIN lens.

(I) Image of AGRP^{GCaMP6f} neurons (left) by deep-brain imaging and their region of interest (ROI) spatial filters (right).

(J) GCaMP6f fluorescence traces from individual neurons in (I) during grain-based food pellet consumption. Black line, food delivery.

approach to identify a number of unique candidate markers of these brain regions (Figure 10A) (Jovanovic et al., 2010; Lee et al., 2012; Segal et al., 2005). The VMH- or DMH-specific expression of each of these marker genes was verified using in situ hybridization (Figure 10A). Laser capture microdissection has also been applied to profile individual cell types (Lovatt et al., 2015).

For large populations of neurons, immunoprecipitation methods such as TRAP (Figure 10B) can be applied effectively, which was first shown for striatal neuron subpopulations. This has also recently been applied to Leptin receptor (Lepr)-expressing neurons in the hypothalamus and hindbrain. These efforts led to the identification of molecular markers specific to Lepr-expressing neurons in each brain region, including *Cck* and *Vip* in brainstem Lepr neurons and *Dyn*, *Ghrh*, and *Crh* in hypothalamic Lepr neurons (Figure 10B) (Allison et al., 2015). The TRAP-seq approach has been extended to specific neuronal classes based on projection targets of molecularly defined cell types (Ekstrand et al., 2014). A related method allows profiling mRNA from translationally activated cells via immunoprecipitation of phosphorylated S6 protein-containing ribosomes (Knight et al., 2012). It should be kept in mind, however, that for small neuron populations, current immunoprecipitation approaches involve combining tissue from several mice in order to immunopurify sufficient amounts of RNA. This can create challenges for generating sufficient sample sizes for statistical comparison between conditions.

Molecularly defined cell types can also be dissociated and purified intact from brain tissue followed by RNA extraction and transcriptional profiling. This approach has been applied to hypothalamic AGRP neurons. Dissociation of neonatal brain tissue with fluorescently labeled AGRP neurons was used for fluorescence-activated cell sorting of AGRP neurons with and without *Foxo1* gene ablation (Ren et al., 2012). This transcriptional profiling effort required combining neurons from 40–50 mice for each group, and each group contained only a single pooled sample, which precluded statistical analysis. A subsequent study used manually sorted AGRP and POMC neurons from the arcuate nucleus expressing fluorescent markers (Figure 10C). These neurons were taken from adult mice where each sample contained

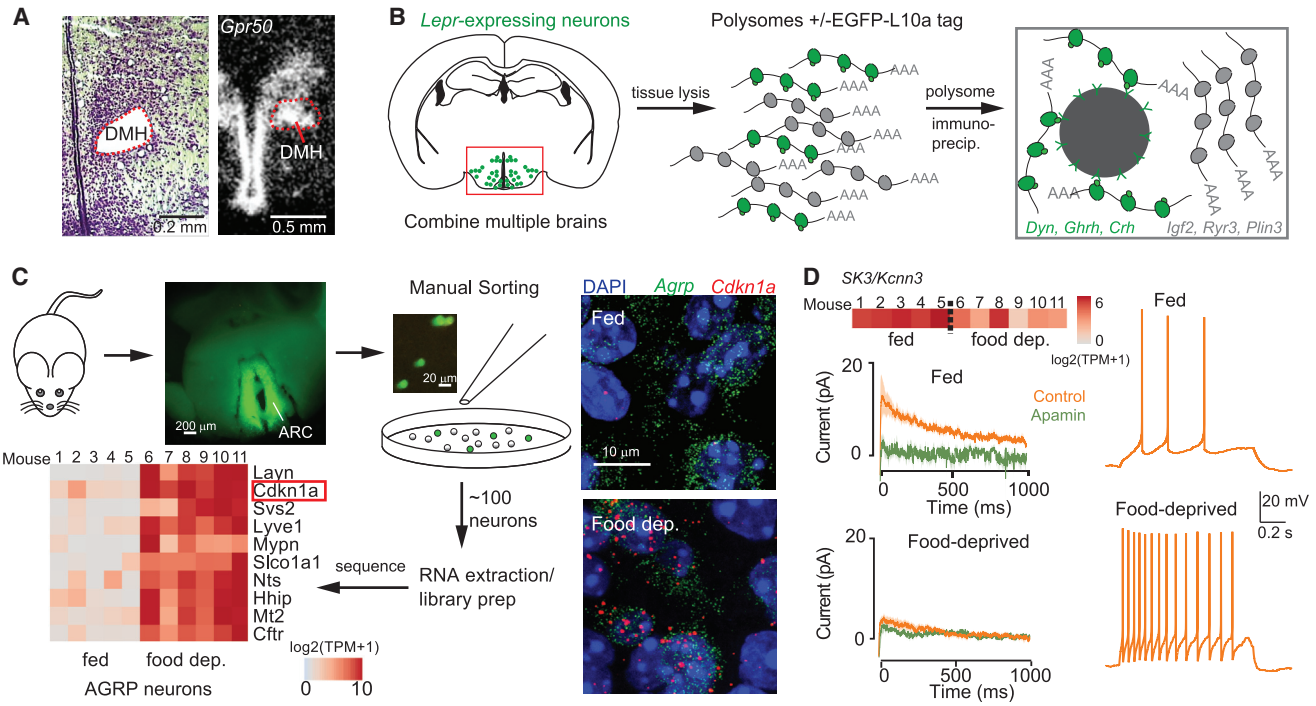


Figure 10. Cell-Type-Specific Transcriptional Profiling

(A) Laser capture microdissection to identify region-specific markers of hypothalamic nuclei with validation by in situ hybridization. Reproduced with permission from Segal et al. (2005).

(B) Translating ribosome affinity purification (TRAP) for cell-type-specific molecular profiling of leptin receptor (Lepr) neurons in the hypothalamus (red box) based on Allison et al. (2015).

(C and D) Transcriptomic analysis of starvation-sensing AGRP neuron responses to fasting using manual sorting of fluorescent neurons. (C) Each mouse provides a replicate sample for either fed or food-deprived conditions. Right: validation of fasting-induced *Cdkn1a* upregulation by double smFISH for *Agrp* and *Cdkn1a*. (D) Downregulation of SK3 channels with food deprivation leads to loss of apamin-sensitive tail currents and increased excitability of AGRP neurons from food-deprived mice. From Henry et al. (2015).

40–250 neurons such that each mouse could be used as an independent sample. This allowed statistical analysis of differential gene expression between AGRP and POMC neurons and each of these cell types in ad libitum-fed versus food-deprived conditions (Figure 10C). Using this method, over 800 statistically significant >2-fold differentially expressed genes were identified in AGRP neurons after food deprivation, and these could be validated by single-molecule fluorescent in situ hybridization (smFISH). In addition, *Kcnn3*, the SK3 ion channel, was shown to be strongly downregulated with food deprivation, and this corresponded to increased AGRP neuron excitability (Figure 10D). Thus, cell-type-specific RNA-seq can reveal molecular components that altered input-ouput characteristics of circuit node function.

Single-cell transcriptomic approaches offer further exciting opportunities for investigating appetite circuits. Harvesting mRNA after electrophysiological recording has been demonstrated in the hypothalamus and used to determine cell-type markers (Eberwine and Bartfai, 2011; Jennings et al., 2013; Qiu et al., 2010). Newly developed microfluidic approaches have enabled automated preparation of single-cell transcriptomic libraries at large scale. Presently, this technology has been applied to classify cell types in brain regions with heterogeneous cellular makeup including the hippocampus (Zeisel et al., 2015), developing cerebral cortex (Pollen et al., 2014), paraventricular hypothalamus (Romanov et al., 2015), and retina (Macosko et al., 2015). Single-cell transcriptomic methods are scalable and have the potential to allow researchers to obtain gene expression profiles from thousands of cells in any brain region of interest. This may potentially allow an unbiased readout of the diversity of cell types in brain structures that regulate appetite, with the possibility of defining a detailed parts list for the underlying circuits.

Conclusions

Neurobiology has undergone rapid evolution over the past 10 years to fully embrace molecularly defined cell types, which have long been prominent in studies of appetite circuits. Sophisticated approaches for manipulating and monitoring neuronal function with cell-type precision can now be applied anywhere in the brain. This permits investigation of specific attributes of appetite circuits with methods that were previously only suitable for more accessible cortical structures. Probably the most important advance is that cell-type-specific neuronal dynamics can be monitored during behavioral experiments, which allows for careful examination of the relationship of sensory inputs and behavioral outputs to neuronal activity. This is an essential first step for evaluating neural circuit function. After measuring endogenous activity patterns, their causal role can then be investigated by perturbation experiments that are relevant to the underlying neuronal dynamics in order to examine the causal

relationships of these activity patterns to behavior and physiology. Neuronal connectivity can be assessed in detail with synaptic physiology, ultrastructure, and viral tools. The full integration of these techniques considerably enhances the possibilities for understanding molecularly defined appetite circuits across all of the length scales of biology: molecules → cells → circuits → animal behavior. Although the techniques will undoubtedly continue to improve rapidly, the core capabilities for mapping, monitoring, and manipulating deep-brain, molecularly defined appetite circuits are now in place. Collectively, these approaches form a comprehensive framework for research into the neural circuits that control the motivation to eat.

There are many remaining challenges associated with implementing this scheme in deep-brain structures involving multiple molecularly defined neurons. For example, neuron activity manipulations of molecularly defined cell types are powerful tools, but the limitations should also be appreciated. First, although optogenetics and chemogenetics have taken us a long way, it is difficult to currently precisely recapitulate endogenous neuron activity patterns. Also, as discussed above, although neuron perturbation tools are essential for evaluating the causal relationship between neuron activity and behavior, the reproducibility of neuron perturbation and circuit mapping experiments is greatly aided by post hoc quantification of the number of transduced neurons. Moreover, even though molecularly defined neuronal cell types can be selectively manipulated, this does not mean that the effects of the perturbations are restricted to that cell type. This is due to potential synaptic coupling between cell types, which must be examined. Finally, even the concept of cell type requires further consideration, and this topic will hopefully be better worked out with new tools to define and test putative cell types.

With these issues in mind, new tools present neurobiologists with a tremendous opportunity to rigorously examine the neuronal processes that regulate appetite. Investigation of these circuits offers the possibility of identifying new principles for motivated behaviors along with conceptual insights as to how behavioral state is controlled.

ACKNOWLEDGMENTS

S.M.S. is funded by the Howard Hughes Medical Institute.

REFERENCES

Allison, M.B., Patterson, C.M., Krashes, M.J., Lowell, B.B., Myers, M.G., Jr., and Olson, D.P. (2015). TRAP-seq defines markers for novel populations of hypothalamic and brainstem LepRb neurons. Mol. Metab. *4*, 299–309.

Anand, B.K., and Brobeck, J.R. (1951). Localization of a "feeding center" in the hypothalamus of the rat. Proc. Soc. Exp. Biol. Med. *77*, 323–324.

Aponte, Y., Atasoy, D., and Sternson, S.M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat. Neurosci. *14*, 351–355.

Armbruster, B.N., Li, X., Pausch, M.H., Herlitze, S., and Roth, B.L. (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc. Natl. Acad. Sci. USA *104*, 5163–5168.

Atasoy, D., Aponte, Y., Su, H.H., and Sternson, S.M. (2008). A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. J. Neurosci. *28*, 7025–7030.

Atasoy, D., Betley, J.N., Su, H.H., and Sternson, S.M. (2012). Deconstruction of a neural circuit for hunger. Nature *488*, 172–177.

Atasoy, D., Betley, J.N., Li, W.P., Su, H.H., Sertel, S.M., Scheffer, L.K., Simpson, J.H., Fetter, R.D., and Sternson, S.M. (2014). A genetically specified connectomics approach applied to long-range feeding regulatory circuits. Nat. Neurosci. *17*, 1830–1839.

Avants, B.B., Epstein, C.L., Grossman, M., and Gee, J.C. (2008). Symmetric diffeomorphic image registration with cross-correlation: evaluating automated labeling of elderly and neurodegenerative brain. Med. Image Anal. *12*, 26–41.

Babinski, M.J. (1900). Tumeur du corps pituitaire san acromegalie et avec arret de developpement des organes genitaux. Rev. Neurol. *8*, 531–533.

Barretto, R.P., Messerschmidt, B., and Schnitzer, M.J. (2009). In vivo fluorescence imaging with high-resolution microlenses. Nat. Methods *6*, 511–512.

Beier, K.T., Saunders, A., Oldenburg, I.A., Miyamichi, K., Akhtar, N., Luo, L., Whelan, S.P., Sabatini, B., and Cepko, C.L. (2011). Anterograde or retrograde transsynaptic labeling of CNS neurons with vesicular stomatitis virus vectors. Proc. Natl. Acad. Sci. USA *108*, 15414–15419.

Berndt, A., Schoenenberger, P., Mattis, J., Tye, K.M., Deisseroth, K., Hegemann, P., and Oertner, T.G. (2011). High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc. Natl. Acad. Sci. USA *108*, 7595–7600.

Berndt, A., Lee, S.Y., Ramakrishnan, C., and Deisseroth, K. (2014). Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. Science *344*, 420–424.

Betley, J.N., and Sternson, S.M. (2011). Adeno-associated viral vectors for mapping, monitoring, and manipulating neural circuits. Hum. Gene Ther. *22*, 669–677.

Betley, J.N., Cao, Z.F., Ritola, K.D., and Sternson, S.M. (2013). Parallel, redundant circuit organization for homeostatic control of feeding behavior. Cell *155*, 1337–1350.

Betley, J.N., Xu, S., Cao, Z.F., Gong, R., Magnus, C.J., Yu, Y., and Sternson, S.M. (2015). Neurons for hunger and thirst transmit a negative-valence teaching signal. Nature *521*, 180–185.

Bonaventure, P., Guo, H., Tian, B., Liu, X., Bittner, A., Roland, B., Salunga, R., Ma, X.-J., Kamme, F., Meurers, B., et al. (2002). Nuclei and subnuclei gene expression profiling in mammalian brain. Brain Res. *943*, 38–47.

Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G., and Deisseroth, K. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. Nat. Neurosci. *8*, 1263–1268.

Bray, G.A. (1984). Syndromes of hypothalamic obesity in man. Pediatr. Ann. *13*, 525–536.

Brown, S.P., and Hestrin, S. (2009). Intracortical circuits of pyramidal neurons reflect their long-range axonal targets. Nature *457*, 1133–1136.

Cai, H., Haubensak, W., Anthony, T.E., and Anderson, D.J. (2014). Central amygdala PKC-δ(+) neurons mediate the influence of multiple anorexigenic signals. Nat. Neurosci. *17*, 1240–1248.

Carelli, R.M., and Wondolowski, J. (2003). Selective encoding of cocaine versus natural rewards by nucleus accumbens neurons is not related to chronic drug exposure. J. Neurosci. *23*, 11214–11223.

Carter, M.E., Soden, M.E., Zweifel, L.S., and Palmiter, R.D. (2013). Genetic identification of a neural circuit that suppresses appetite. Nature *503*, 111–114.

Carter, M.E., Han, S., and Palmiter, R.D. (2015). Parabrachial calcitonin gene-related peptide neurons mediate conditioned taste aversion. J. Neurosci. *35*, 4582–4586.

Chamberlin, N.L., Du, B., de Lacalle, S., and Saper, C.B. (1998). Recombinant adeno-associated virus vector: use for transgene expression and anterograde tract tracing in the CNS. Brain Res. *793*, 169–175.

Chen, T.W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V., et al. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature *499*, 295–300.

Chen, Y., Lin, Y.C., Kuo, T.W., and Knight, Z.A. (2015). Sensory detection of food rapidly modulates arcuate feeding circuits. Cell *160*, 829–841.

Chung, K., Wallace, J., Kim, S.Y., Kalyanasundaram, S., Andalman, A.S., Davidson, T.J., Mirzabekov, J.J., Zalocusky, K.A., Mattis, J., Denisin, A.K., et al. (2013). Structural and molecular interrogation of intact biological systems. Nature *497*, 332–337.

Cohen, J.Y., Haesler, S., Vong, L., Lowell, B.B., and Uchida, N. (2012). Neuron-type-specific signals for reward and punishment in the ventral tegmental area. Nature *482*, 85–88.

Cohen, J.Y., Amoroso, M.W., and Uchida, N. (2015). Serotonergic neurons signal reward and punishment on multiple timescales. eLife *4*, 4.

Cui, G., Jun, S.B., Jin, X., Pham, M.D., Vogel, S.S., Lovinger, D.M., and Costa, R.M. (2013). Concurrent activation of striatal direct and indirect pathways during action initiation. Nature *494*, 238–242.

Dasen, J.S., and Jessell, T.M. (2009). Chapter Six Hox Networks and the Origins of Motor Neuron Diversity. In Curr Top Dev Biol, P. Olivier, ed. (Academic Press), pp. 169–200.

de Boer, P., Hoogenboom, J.P., and Giepmans, B.N.G. (2015). Correlated light and electron microscopy: ultrastructure lights up!. Nat. Methods *12*, 503–513.

DeFalco, J., Tomishima, M., Liu, H., Zhao, C., Cai, X., Marth, J.D., Enquist, L., and Friedman, J.M. (2001). Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. Science *291*, 2608–2613.

Delgado, J.M., and Anand, B.K. (1953). Increase of food intake induced by electrical stimulation of the lateral hypothalamus. Am. J. Physiol. *172*, 162–168.

Denis, R.G., Joly-Amado, A., Webber, E., Langlet, F., Schaeffer, M., Padilla, S.L., Cansell, C., Dehouck, B., Castel, J., Delbès, A.S., et al. (2015). Palatability Can Drive Feeding Independent of AgRP Neurons. Cell Metab. *22*, 646–657.

Dietrich, M.O., Zimmer, M.R., Bober, J., and Horvath, T.L. (2015). Hypothalamic Agrp neurons drive stereotypic behaviors beyond feeding. Cell *160*, 1222–1232.

Domingos, A.I., Sordillo, A., Dietrich, M.O., Liu, Z.W., Tellez, L.A., Vaynshteyn, J., Ferreira, J.G., Ekstrand, M.I., Horvath, T.L., de Araujo, I.E., and Friedman, J.M. (2013). Hypothalamic melanin concentrating hormone neurons communicate the nutrient value of sugar. eLife *2*, e01462.

Donato, F., Rompani, S.B., and Caroni, P. (2013). Parvalbumin-expressing basket-cell network plasticity induced by experience regulates adult learning. Nature *504*, 272–276.

Dugas, J.C., Mandemakers, W., Rogers, M., Ibrahim, A., Daneman, R., and Barres, B.A. (2008). A novel purification method for CNS projection neurons leads to the identification of brain vascular cells as a source of trophic support for corticospinal motor neurons. J. Neurosci. *28*, 8294–8305.

Eberwine, J., and Bartfai, T. (2011). Single cell transcriptomics of hypothalamic warm sensitive neurons that control core body temperature and fever response Signaling asymmetry and an extension of chemical neuroanatomy. Pharmacol. Ther. *129*, 241–259.

Ekstrand, M.I., Nectow, A.R., Knight, Z.A., Latcha, K.N., Pomeranz, L.E., and Friedman, J.M. (2014). Molecular profiling of neurons based on connectivity. Cell *157*, 1230–1242.

Esposito, M.S., Capelli, P., and Arber, S. (2014). Brainstem nucleus MdV mediates skilled forelimb motor tasks. Nature *508*, 351–356.

Fenno, L., Yizhar, O., and Deisseroth, K. (2011). The development and application of optogenetics. Annu. Rev. Neurosci. *34*, 389–412.

Fischer, M.J.M., Reeh, P.W., and Sauer, S.K. (2003). Proton-induced calcitonin gene-related peptide release from rat sciatic nerve axons, in vitro, involving TRPV1. Eur. J. Neurosci. *18*, 803–810.

Flak, J.N., Patterson, C.M., Garfield, A.S., D'Agostino, G., Goforth, P.B., Sutton, A.K., Malec, P.A., Wong, J.-M.T., Germani, M., Jones, J.C., et al. (2014). Leptin-inhibited PBN neurons enhance responses to hypoglycemia in negative energy balance. Nat. Neurosci. *17*, 1744–1750.

Fosque, B.F., Sun, Y., Dana, H., Yang, C.T., Ohyama, T., Tadross, M.R., Patel, R., Zlatic, M., Kim, D.S., Ahrens, M.B., et al. (2015). Neural circuits. Labeling of active neural circuits in vivo with designed calcium integrators. Science *347*, 755–760.

Frohlich, A. (1901). Ein fall von tumor der hypophysis cerebri ohne akromegalie. Wiener Klin *15*, 883–886.

Garfield, A.S., Shah, B.P., Madara, J.C., Burke, L.K., Patterson, C.M., Flak, J., Neve, R.L., Evans, M.L., Lowell, B.B., Myers, M.G., Jr., and Heisler, L.K. (2014). A parabrachial-hypothalamic cholecystokinin neurocircuit controls counterregulatory responses to hypoglycemia. Cell Metab. *20*, 1030–1037.

Garfield, A.S., Li, C., Madara, J.C., Shah, B.P., Webber, E., Steger, J.S., Campbell, J.N., Gavrilova, O., Lee, C.E., Olson, D.P., et al. (2015). A neural basis for melanocortin-4 receptor-regulated appetite. Nat. Neurosci. *18*, 863–871.

Garner, A.R., Rowland, D.C., Hwang, S.Y., Baumgaertel, K., Roth, B.L., Kentros, C., and Mayford, M. (2012). Generation of a synthetic memory trace. Science *335*, 1513–1516.

Gerfen, C.R., and Sawchenko, P.E. (1984). An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: immunohistochemical localization of an axonally transported plant lectin, Phaseolus vulgaris leucoagglutinin (PHA-L). Brain Res. *290*, 219–238.

Ghosh, K.K., Burns, L.D., Cocker, E.D., Nimmerjahn, A., Ziv, Y., Gamal, A.E., and Schnitzer, M.J. (2011). Miniaturized integration of a fluorescence microscope. Nat. Methods *8*, 871–878.

Govorunova, E.G., Sineshchekov, O.A., Janz, R., Liu, X., and Spudich, J.L. (2015). NEUROSCIENCE. Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics. Science *349*, 647–650.

Gradinaru, V., Zhang, F., Ramakrishnan, C., Mattis, J., Prakash, R., Diester, I., Goshen, I., Thompson, K.R., and Deisseroth, K. (2010). Molecular and cellular approaches for diversifying and extending optogenetics. Cell *141*, 154–165.

Grossman, S.P. (1960). Eating or drinking elicited by direct adrenergic or cholinergic stimulation of hypothalamus. Science *132*, 301–302.

Guenthner, C.J., Miyamichi, K., Yang, H.H., Heller, H.C., and Luo, L. (2013). Permanent genetic access to transiently active neurons via TRAP: targeted recombination in active populations. Neuron *78*, 773–784.

Güler, A.D., Rainwater, A., Parker, J.G., Jones, G.L., Argilli, E., Arenkiel, B.R., Ehlers, M.D., Bonci, A., Zweifel, L.S., and Palmiter, R.D. (2012). Transient activation of specific neurons in mice by selective expression of the capsaicin receptor. Nat. Commun. *3*, 746.

Gunaydin, L.A., Grosenick, L., Finkelstein, J.C., Kauvar, I.V., Fenno, L.E., Adhikari, A., Lammel, S., Mirzabekov, J.J., Airan, R.D., Zalocusky, K.A., et al. (2014). Natural neural projection dynamics underlying social behavior. Cell *157*, 1535–1551.

Gupta, A., Wang, Y., and Markram, H. (2000). Organizing principles for a diversity of GABAergic interneurons and synapses in the neocortex. Science *287*, 273–278.

Han, S., Soleiman, M.T., Soden, M.E., Zweifel, L.S., and Palmiter, R.D. (2015). Elucidating an Affective Pain Circuit that Creates a Threat Memory. Cell *162*, 363–374.

Heiman, M., Schaefer, A., Gong, S., Peterson, J.D., Day, M., Ramsey, K.E., Suárez-Fariñas, M., Schwarz, C., Stephan, D.A., Surmeier, D.J., et al. (2008). A translational profiling approach for the molecular characterization of CNS cell types. Cell *135*, 738–748.

Hempel, C.M., Sugino, K., and Nelson, S.B. (2007). A manual method for the purification of fluorescently labeled neurons from the mammalian brain. Nat. Protoc. *2*, 2924–2929.

Henry, F.E., Sugino, K., Tozer, A., Branco, T., and Sternson, S.M. (2015). Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. eLife *4*, e09800.

Hess, W.R. (1957). The functional organization of the diencephalon (New York: Grune & Stratton).

Hetherington, A.W., and Ranson, S.W. (1940). Hypothalamic lesions and adiposity in the rat. Anat. Rec. *78*, 149–172.

Hetherington, A.W., and Ranson, S.W. (1942). The relation of various hypothalamic lesions to adiposity in the rat. J. Comp. Neurol. *76*, 475–499.

Hoebel, B.G. (1985). Brain neurotransmitters in food and drug reward. Am. J. Clin. Nutr. *42* (5, Suppl), 1133–1150.

Hubel, D.H., and Wiesel, T.N. (1962). Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. J. Physiol. *160*, 106–154, 102.

Inutsuka, A., Inui, A., Tabuchi, S., Tsunematsu, T., Lazarus, M., and Yamanaka, A. (2014). Concurrent and robust regulation of feeding behaviors and metabolism by orexin neurons. Neuropharmacology *85*, 451–460.

Jennings, J.H., Rizzi, G., Stamatakis, A.M., Ung, R.L., and Stuber, G.D. (2013). The inhibitory circuit architecture of the lateral hypothalamus orchestrates feeding. Science *341*, 1517–1521.

Jennings, J.H., Ung, R.L., Resendez, S.L., Stamatakis, A.M., Taylor, J.G., Huang, J., Veleta, K., Kantak, P.A., Aita, M., Shilling-Scrivo, K., et al. (2015). Visualizing hypothalamic network dynamics for appetitive and consummatory behaviors. Cell *160*, 516–527.

Jin, L., Han, Z., Platisa, J., Wooltorton, J.R., Cohen, L.B., and Pieribone, V.A. (2012). Single action potentials and subthreshold electrical events imaged in neurons with a fluorescent protein voltage probe. Neuron *75*, 779–785.

Jones, J.R., Tackenberg, M.C., and McMahon, D.G. (2015). Manipulating circadian clock neuron firing rate resets molecular circadian rhythms and behavior. Nat. Neurosci. *18*, 373–375.

Jovanovic, Z., Tung, Y.C., Lam, B.Y., O'Rahilly, S., and Yeo, G.S. (2010). Identification of the global transcriptomic response of the hypothalamic arcuate nucleus to fasting and leptin. J. Neuroendocrinol. *22*, 915–925.

Kim, J.C., Cook, M.N., Carey, M.R., Shen, C., Regehr, W.G., and Dymecki, S.M. (2009). Linking genetically defined neurons to behavior through a broadly applicable silencing allele. Neuron *63*, 305–315.

Knight, Z.A., Tan, K., Birsoy, K., Schmidt, S., Garrison, J.L., Wysocki, R.W., Emiliano, A., Ekstrand, M.I., and Friedman, J.M. (2012). Molecular profiling of activated neurons by phosphorylated ribosome capture. Cell *151*, 1126–1137.

Kong, D., Tong, Q., Ye, C., Koda, S., Fuller, P.M., Krashes, M.J., Vong, L., Ray, R.S., Olson, D.P., and Lowell, B.B. (2012). GABAergic RIP-Cre neurons in the arcuate nucleus selectively regulate energy expenditure. Cell *151*, 645–657.

Krashes, M.J., Koda, S., Ye, C., Rogan, S.C., Adams, A.C., Cusher, D.S., Maratos-Flier, E., Roth, B.L., and Lowell, B.B. (2011). Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J. Clin. Invest. *121*, 1424–1428.

Krashes, M.J., Shah, B.P., Koda, S., and Lowell, B.B. (2013). Rapid versus delayed stimulation of feeding by the endogenously released AgRP neuron mediators GABA, NPY, and AgRP. Cell Metab. *18*, 588–595.

Krashes, M.J., Shah, B.P., Madara, J.C., Olson, D.P., Strochlic, D.E., Garfield, A.S., Vong, L., Pei, H., Watabe-Uchida, M., Uchida, N., et al. (2014). An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature *507*, 238–242.

Kuypers, H.G., Catsman-Berrevoets, C.E., and Padt, R.E. (1977). Retrograde anoxal transport of fluorescent substances in the rat's forebrain. Neurosci. Lett. *6*, 127–133.

Lam, S.S., Martell, J.D., Kamer, K.J., Deerinck, T.J., Ellisman, M.H., Mootha, V.K., and Ting, A.Y. (2015). Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat. Methods *12*, 51–54.

Land, B.B., Narayanan, N.S., Liu, R.J., Gianessi, C.A., Brayton, C.E., Grimaldi, D.M., Sarhan, M., Guarnieri, D.J., Deisseroth, K., Aghajanian, G.K., and DiLeone, R.J. (2014). Medial prefrontal D1 dopamine neurons control food intake. Nat. Neurosci. *17*, 248–253.

Lee, M.G., Hassani, O.K., and Jones, B.E. (2005). Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. J. Neurosci. *25*, 6716–6720.

Lee, S., Bookout, A.L., Lee, C.E., Gautron, L., Harper, M.J., Elias, C.F., Lowell, B.B., and Elmquist, J.K. (2012). Laser-capture microdissection and transcriptional profiling of the dorsomedial nucleus of the hypothalamus. J. Comp. Neurol. *520*, 3617–3632.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J., et al. (2007). Genome-wide atlas of gene expression in the adult mouse brain. Nature *445*, 168–176.

Lerchner, W., Xiao, C., Nashmi, R., Slimko, E.M., van Trigt, L., Lester, H.A., and Anderson, D.J. (2007). Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated Cl- channel. Neuron *54*, 35–49.

Li, J., Wang, Y., Chiu, S.-L., and Cline, H.T. (2010). Membrane targeted horseradish peroxidase as a marker for correlative fluorescence and electron microscopy studies. Front. Neural Circuits *4*, 6.

Lima, S.Q., Hromádka, T., Znamenskiy, P., and Zador, A.M. (2009). PINP: a new method of tagging neuronal populations for identification during in vivo electrophysiological recording. PLoS ONE *4*, e6099.

Lin, D., Boyle, M.P., Dollar, P., Lee, H., Lein, E.S., Perona, P., and Anderson, D.J. (2011). Functional identification of an aggression locus in the mouse hypothalamus. Nature *470*, 221–226.

Lin, J.Y., Sann, S.B., Zhou, K., Nabavi, S., Proulx, C.D., Malinow, R., Jin, Y., and Tsien, R.Y. (2013). Optogenetic inhibition of synaptic release with chromophore-assisted light inactivation (CALI). Neuron *79*, 241–253.

Lo, L., and Anderson, D.J. (2011). A Cre-dependent, anterograde transsynaptic viral tracer for mapping output pathways of genetically marked neurons. Neuron *72*, 938–950.

Lobo, M.K., Karsten, S.L., Gray, M., Geschwind, D.H., and Yang, X.W. (2006). FACS-array profiling of striatal projection neuron subtypes in juvenile and adult mouse brains. Nat. Neurosci. *9*, 443–452.

Lovatt, D., Bell, T., and Eberwine, J. (2015). Single-neuron isolation for RNA analysis using pipette capture and laser capture microdissection. Cold Spring Harb. Protoc. *2015*, t072439.

Luquet, S., Perez, F.A., Hnasko, T.S., and Palmiter, R.D. (2005). NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. Science *310*, 683–685.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell *161*, 1202–1214.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. *13*, 133–140.

Madisen, L., Mao, T., Koch, H., Zhuo, J.M., Berenyi, A., Fujisawa, S., Hsu, Y.W., Garcia, A.J., 3rd, Gu, X., Zanella, S., et al. (2012). A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. Nat. Neurosci. *15*, 793–802.

Magnus, C.J., Lee, P.H., Atasoy, D., Su, H.H., Looger, L.L., and Sternson, S.M. (2011). Chemical and genetic engineering of selective ion channel-ligand interactions. Science *333*, 1292–1296.

Mandelblat-Cerf, Y., Ramesh, R.N., Burgess, C.R., Patella, P., Yang, Z., Lowell, B.B., and Andermann, M.L. (2015). Arcuate hypothalamic AgRP and putative POMC neurons show opposite changes in spiking across multiple timescales. eLife *4*, 4.

Margules, D.L., and Olds, J. (1962). Identical "feeding" and "rewarding" systems in the lateral hypothalamus of rats. Science *135*, 374–375.

Martell, J.D., Deerinck, T.J., Sancak, Y., Poulos, T.L., Mootha, V.K., Sosinsky, G.E., Ellisman, M.H., and Ting, A.Y. (2012). Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat. Biotechnol. *30*, 1143–1148.

Masland, R.H. (2004). Neuronal cell types. Curr. Biol. *14*, R497–R500.

Mazarakis, N.D., Azzouz, M., Rohll, J.B., Ellard, F.M., Wilkes, F.J., Olsen, A.L., Carter, E.E., Barber, R.D., Baban, D.F., Kingsman, S.M., et al. (2001). Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. Hum. Mol. Genet. *10*, 2109–2121.

Michaud, J.L., Rosenquist, T., May, N.R., and Fan, C.M. (1998). Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. Genes Dev. *12*, 3264–3275.

Mileykovskiy, B.Y., Kiyashchenko, L.I., and Siegel, J.M. (2005). Behavioral correlates of activity in identified hypocretin/orexin neurons. Neuron *46*, 787–798.

Mukamel, E.A., Nimmerjahn, A., and Schnitzer, M.J. (2009). Automated analysis of cellular signals from large-scale calcium imaging data. Neuron *63*, 747–760.

Nelson, S.B., Sugino, K., and Hempel, C.M. (2006). The problem of neuronal cell types: a physiological genomics approach. Trends Neurosci. *29*, 339–345.

Nieh, E.H., Matthews, G.A., Allsop, S.A., Presbrey, K.N., Leppla, C.A., Wichmann, R., Neve, R., Wildes, C.P., and Tye, K.M. (2015). Decoding neural circuits that control compulsive sucrose seeking. Cell *160*, 528–541.

Oh, S.W., Harris, J.A., Ng, L., Winslow, B., Cain, N., Mihalas, S., Wang, Q., Lau, C., Kuan, L., Henry, A.M., et al. (2014). A mesoscale connectome of the mouse brain. Nature *508*, 207–214.

Oomura, Y., Kimura, K., Ooyama, H., Maeno, T., Iki, M., and Kuniyoshi, M. (1964). Reciprocal Activities of the Ventromedial and Lateral Hypothalamic Areas of Cats. Science *143*, 484–485.

Oomura, Y., Ooyama, H., Sugimori, M., Nakamura, T., and Yamada, Y. (1974). Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. Nature *247*, 284–286.

Paez-Segala, M.G., Sun, M.G., Shtengel, G., Viswanathan, S., Baird, M.A., Macklin, J.J., Patel, R., Allen, J.R., Howe, E.S., Piszczek, G., et al. (2015). Fixation-resistant photoactivatable fluorescent proteins for CLEM. Nat. Methods *12*, 215–218, 4, 218.

Pei, H., Sutton, A.K., Burnett, K.H., Fuller, P.M., and Olson, D.P. (2014). AVP neurons in the paraventricular nucleus of the hypothalamus regulate feeding. Mol. Metab. *3*, 209–215.

Petreanu, L., Huber, D., Sobczyk, A., and Svoboda, K. (2007). Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. Nat. Neurosci. *10*, 663–668.

Pollen, A.A., Nowakowski, T.J., Shuga, J., Wang, X., Leyrat, A.A., Lui, J.H., Li, N., Szpankowski, L., Fowler, B., Chen, P., et al. (2014). Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. Nat. Biotechnol. *32*, 1053–1058.

Poulin, A.M., and Timofeeva, E. (2008). The dynamics of neuronal activation during food anticipation and feeding in the brain of food-entrained rats. Brain Res. *1227*, 128–141.

Qiu, J., Fang, Y., Rønnekleiv, O.K., and Kelly, M.J. (2010). Leptin excites proopiomelanocortin neurons via activation of TRPC channels. J. Neurosci. *30*, 1560–1565.

Raimondo, J.V., Kay, L., Ellender, T.J., and Akerman, C.J. (2012). Optogenetic silencing strategies differ in their effects on inhibitory synaptic transmission. Nat. Neurosci. *15*, 1102–1104.

Reijmers, L.G., Perkins, B.L., Matsuo, N., and Mayford, M. (2007). Localization of a stable neural correlate of associative memory. Science *317*, 1230–1233.

Ren, H., Orozco, I.J., Su, Y., Suyama, S., Gutiérrez-Juárez, R., Horvath, T.L., Wardlaw, S.L., Plum, L., Arancio, O., and Accili, D. (2012). FoxO1 target Gpr17 activates AgRP neurons to regulate food intake. Cell *149*, 1314–1326.

Rivera, V.M., and Greenberg, M.E. (1990). Growth factor-induced gene expression: the ups and downs of c-fos regulation. New Biol. *2*, 751–758.

Rolls, E.T. (1982). Feeding and reward. In The neural basis of feeding and reward, B.G. Hoebel and D. Novin, eds. (Brunswick, ME: Haer Institute), pp. 323–338.

Romanov, R.A., Alpár, A., Zhang, M.D., Zeisel, A., Calas, A., Landry, M., Fuszard, M., Shirran, S.L., Schnell, R., Dobolyi, Á., et al. (2015). A secretagogin locus of the mammalian hypothalamus controls stress hormone release. EMBO J. *34*, 36–54.

Rothermel, M., Brunert, D., Zabawa, C., Díaz-Quesada, M., and Wachowiak, M. (2013). Transgene expression in target-defined neuron populations mediated by retrograde infection with adeno-associated viral vectors. J. Neurosci. *33*, 15195–15206.

Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Mekada, E., Kimata, Y., Tsuru, A., and Kohno, K. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. Nat. Biotechnol. *19*, 746–750.

Sanz, E., Yang, L., Su, T., Morris, D.R., McKnight, G.S., and Amieux, P.S. (2009). Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. Proc. Natl. Acad. Sci. USA *106*, 13939–13944.

Schoenenberger, P., Schärer, Y.P., and Oertner, T.G. (2011). Channelrhodopsin as a tool to investigate synaptic transmission and plasticity. Exp. Physiol. *96*, 34–39.

Schöne, C., Apergis-Schoute, J., Sakurai, T., Adamantidis, A., and Burdakov, D. (2014). Coreleased orexin and glutamate evoke nonredundant spike outputs and computations in histamine neurons. Cell Rep. *7*, 697–704.

Segal, J.P., Stallings, N.R., Lee, C.E., Zhao, L., Socci, N., Viale, A., Harris, T.M., Soares, M.B., Childs, G., Elmquist, J.K., et al. (2005). Use of laser-capture microdissection for the identification of marker genes for the ventromedial hypothalamic nucleus. J. Neurosci. *25*, 4181–4188.

Sharpee, T.O. (2014). Toward functional classification of neuronal types. Neuron *83*, 1329–1334.

Sheng, M., McFadden, G., and Greenberg, M.E. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571–582.

Shu, X., Lev-Ram, V., Deerinck, T.J., Qi, Y., Ramko, E.B., Davidson, M.W., Jin, Y., Ellisman, M.H., and Tsien, R.Y. (2011). A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol. *9*, e1001041.

Soudais, C., Laplace-Builhe, C., Kissa, K., and Kremer, E.J. (2001). Preferential transduction of neurons by canine adenovirus vectors and their efficient retrograde transport in vivo. FASEB J. *15*, 2283–2285.

St-Pierre, F., Chavarha, M., and Lin, M.Z. (2015). Designs and sensing mechanisms of genetically encoded fluorescent voltage indicators. Curr. Opin. Chem. Biol. *27*, 31–38.

Stachniak, T.J., Ghosh, A., and Sternson, S.M. (2014). Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus→midbrain pathway for feeding behavior. Neuron *82*, 797–808.

Stamatakis, A.M., and Stuber, G.D. (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. Nat. Neurosci. *15*, 1105–1107.

Stamatakis, A.M., Jennings, J.H., Ung, R.L., Blair, G.A., Weinberg, R.J., Neve, R.L., Boyce, F., Mattis, J., Ramakrishnan, C., Deisseroth, K., and Stuber, G.D. (2013). A unique population of ventral tegmental area neurons inhibits the lateral habenula to promote reward. Neuron *80*, 1039–1053.

Stanley, S.A., Gagner, J.E., Damanpour, S., Yoshida, M., Dordick, J.S., and Friedman, J.M. (2012). Radio-wave heating of iron oxide nanoparticles can regulate plasma glucose in mice. Science *336*, 604–608.

Stanley, S.A., Sauer, J., Kane, R.S., Dordick, J.S., and Friedman, J.M. (2015). Remote regulation of glucose homeostasis in mice using genetically encoded nanoparticles. Nat. Med. *21*, 92–98.

Sternson, S.M. (2013). Hypothalamic survival circuits: blueprints for purposive behaviors. Neuron *77*, 810–824.

Sternson, S.M., and Atasoy, D. (2014). Agouti-related protein neuron circuits that regulate appetite. Neuroendocrinology *100*, 95–102.

Sternson, S.M., and Roth, B.L. (2014). Chemogenetic tools to interrogate brain functions. Annu. Rev. Neurosci. *37*, 387–407.

Stuber, G.D., Sparta, D.R., Stamatakis, A.M., van Leeuwen, W.A., Hardjopra-jitno, J.E., Cho, S., Tye, K.M., Kempadoo, K.A., Zhang, F., Deisseroth, K., and Bonci, A. (2011). Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. Nature *475*, 377–380.

Sugino, K., Hempel, C.M., Miller, M.N., Hattox, A.M., Shapiro, P., Wu, C., Huang, Z.J., and Nelson, S.B. (2006). Molecular taxonomy of major neuronal classes in the adult mouse forebrain. Nat. Neurosci. *9*, 99–107.

Sutton, A.K., Pei, H., Burnett, K.H., Myers, M.G., Jr., Rhodes, C.J., and Olson, D.P. (2014). Control of food intake and energy expenditure by Nos1 neurons of the paraventricular hypothalamus. J. Neurosci. *34*, 15306–15318.

Swanson, L.W., and Kuypers, H.G. (1980). The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. J. Comp. Neurol. *194*, 555–570.

Tye, K.M., Prakash, R., Kim, S.Y., Fenno, L.E., Grosenick, L., Zarabi, H., Thompson, K.R., Gradinaru, V., Ramakrishnan, C., and Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature *471*, 358–362.

Valenstein, E.S., Cox, V.C., and Kakolewski, J.W. (1968). Modification of motivated behavior elicited by electrical stimulation of the hypothalamus. Science *159*, 1119–1121.

Vardy, E., Robinson, J.E., Li, C., Olsen, R.H., DiBerto, J.F., Giguere, P.M., Sassano, F.M., Huang, X.P., Zhu, H., Urban, D.J., et al. (2015). A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior. Neuron *86*, 936–946.

Wall, N.R., Wickersham, I.R., Cetin, A., De La Parra, M., and Callaway, E.M. (2010). Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus. Proc. Natl. Acad. Sci. USA *107*, 21848–21853.

Wall, N.R., De La Parra, M., Callaway, E.M., and Kreitzer, A.C. (2013). Differential innervation of direct- and indirect-pathway striatal projection neurons. Neuron *79*, 347–360.

Wang, D., He, X., Zhao, Z., Feng, Q., Lin, R., Sun, Y., Ding, T., Xu, F., Luo, M., and Zhan, C. (2015a). Whole-brain mapping of the direct inputs and axonal projections of POMC and AgRP neurons. Front. Neuroanat. *9*, 40.

Wang, L., Chen, I.Z., and Lin, D. (2015b). Collateral pathways from the ventromedial hypothalamus mediate defensive behaviors. Neuron *85*, 1344–1358.

Wang, X.F., Liu, J.J., Xia, J., Liu, J., Mirabella, V., and Pang, Z.P. (2015c). Endogenous Glucagon-like Peptide-1 Suppresses High-Fat Food Intake by Reducing Synaptic Drive onto Mesolimbic Dopamine Neurons. Cell Rep. *12*, 726–733.

Weissbourd, B., Ren, J., DeLoach, K.E., Guenthner, C.J., Miyamichi, K., and Luo, L. (2014). Presynaptic partners of dorsal raphe serotonergic and GABAergic neurons. Neuron *83*, 645–662.

Wickersham, I.R., Lyon, D.C., Barnard, R.J., Mori, T., Finke, S., Conzelmann, K.K., Young, J.A., and Callaway, E.M. (2007). Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. Neuron *53*, 639–647.

Wietek, J., Wiegert, J.S., Adeishvili, N., Schneider, F., Watanabe, H., Tsunoda, S.P., Vogt, A., Elstner, M., Oertner, T.G., and Hegemann, P. (2014). Conversion of channelrhodopsin into a light-gated chloride channel. Science *344*, 409–412.

Williams, K.W., Scott, M.M., and Elmquist, J.K. (2011). Modulation of the central melanocortin system by leptin, insulin, and serotonin: co-ordinated actions in a dispersed neuronal network. Eur. J. Pharmacol. *660*, 2–12.

Wu, Z., Kim, E.R., Sun, H., Xu, Y., Mangieri, L.R., Li, D.P., Pan, H.L., Xu, Y., Arenkiel, B.R., and Tong, Q. (2015). GABAergic projections from lateral hypothalamus to paraventricular hypothalamic nucleus promote feeding. J. Neurosci. *35*, 3312–3318.

Yang, C.F., Chiang, M.C., Gray, D.C., Prabhakaran, M., Alvarado, M., Juntti, S.A., Unger, E.K., Wells, J.A., and Shah, N.M. (2013). Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males. Cell *153*, 896–909.

Yang, B., Treweek, J.B., Kulkarni, R.P., Deverman, B.E., Chen, C.K., Lubeck, E., Shah, S., Cai, L., and Gradinaru, V. (2014). Single-cell phenotyping within transparent intact tissue through whole-body clearing. Cell *158*, 945–958.

Yang, L., Qi, Y., and Yang, Y. (2015). Astrocytes control food intake by inhibiting AGRP neuron activity via adenosine A1 receptors. Cell Rep. *11*, 798–807.

Zeisel, A., Muñoz-Manchado, A.B., Codeluppi, S., Lönnerberg, P., La Manno, G., Juréus, A., Marques, S., Munguba, H., He, L., Betsholtz, C., et al. (2015). Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. Science *347*, 1138–1142.

Zhan, C., Zhou, J., Feng, Q., Zhang, J.E., Lin, S., Bao, J., Wu, P., and Luo, M. (2013). Acute and long-term suppression of feeding behavior by POMC neurons in the brainstem and hypothalamus, respectively. J. Neurosci. *33*, 3624–3632.

Ziv, Y., Burns, L.D., Cocker, E.D., Hamel, E.O., Ghosh, K.K., Kitch, L.J., El Gamal, A., and Schnitzer, M.J. (2013). Long-term dynamics of CA1 hippocampal place codes. Nat. Neurosci. *16*, 264–266.