Communication in Neural Circuits: Tools, Opportunities, and Challenges

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Communication, the effective delivery of information, is fundamental to life across all scales and species. Nervous systems (by necessity) may be most specifically adapted among biological tissues for high rate and complexity of information transmitted, and thus, the properties of neural tissue and principles of its organization into circuits may illuminate capabilities and limitations of biological communication. Here, we consider recent developments in tools for studying neural circuits with particular attention to defining neuronal cell types by input and output information streams—i.e., by how they communicate. Complementing approaches that define cell types by virtue of genetic promoter/enhancer properties, this communication-based approach to defining cell types operationally by input/output (I/O) relationships links structure and function, resolves difficulties associated with single-genetic-feature definitions, leverages technology for observing and testing significance of precisely these I/O relationships in intact brains, and maps onto processes through which behavior may be adapted during development, experience, and evolution.

Introduction

Nervous systems are designed for communication over many scales (Figure 1), beginning at the most fundamental level shared by all cellular systems in biology, in which communication occurs via protein-protein interactions, movement of second messengers within cells, and local release and detection of diffusible transmitters between cells. Nervous systems become clearly unique in their communication properties only at the tissue and organ level, in which billions of cells may work together as an intricately organized, interconnected circuit. It is through the organization of cells into these neural circuits that the brain supports the vast diversity of animal behavior, up to and including human consciousness, cognition, and emotion.

Neural circuits are both extremely complex and exquisitely specific, and the connectivity motifs used to build these circuits vary widely even within a single organism. Contrast the mammalian cerebellar granule neuron, which may receive only five mossy fiber inputs (Llinas et al., 2004) with the mammalian cortical pyramidal neuron, which receives thousands of inputs from a broad array of cortical and subcortical brain regions (Ballesteros-Yáñez et al., 2006). As with inputs, output structuring of neuronal types is also highly diverse, with a broad range of numbers and distributions of both local downstream neurons and distant postsynaptic partners across the nervous system. Indeed, each neuron type might be viewed as a distinct elemental device, definable in part by how it communicates via receiving, processing, and disseminating information. Understanding communication in the nervous system will require analyzing the input/output organization of these elements within larger neural circuits, observing the actual operation of these elements during behavior, and testing hypotheses built on this knowledge with model-guided perturbations targeted to these elements to determine the behaviorally relevant dynamics of information flow and processing.

Given the fundamental necessity of cell-cell communication for brain function, neuroscientists have long devoted substantial effort to developing and deploying technologies for exploring the structure and function of brain communication networks. Although many decades of neuroanatomical research have provided foundational principles underlying neural circuit organization, much remains to be discovered, and opportunities for discovery are particularly abundant at the borders between communication scales (Figure 1). Recent technological developments are indeed beginning to allow neuroscientists to connect neuronal circuit architecture and activity information across different scales and modalities. These methods are advancing the understanding of circuits in behaviorally relevant contexts, while at the same time heightening the need for cell typology that is more tightly linked to function, in order to define the cellular properties that are most relevant for nervous system operation. In this primer, we focus on currently available and rapidly evolving technologies for such structural and functional circuit-level analysis—with attention to both opportunities and limitations—and highlight the concept of the input/output (I/O)-defined circuit element (IODE) as a basic and recent experimentally tractable building block for the study and understanding of nervous system communication across scales.

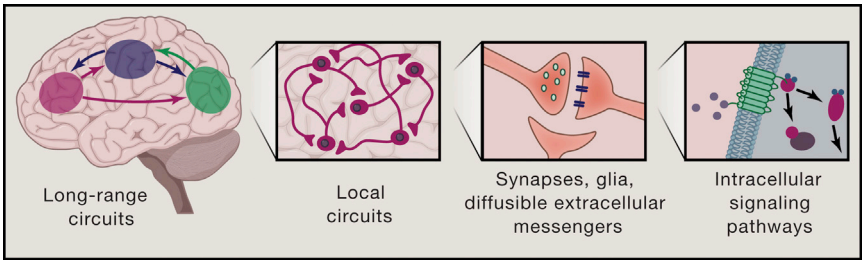

Figure 1. Nervous Systems Are Designed for Communication over Many Scales
Nervous systems communicate at the brainwide level, the circuit level, the intercellular (synaptic) level, and the intracellular level (shown left to right). While the latter levels are fundamental to all biological systems, the more complex brainwide and circuit levels of communication distinguish the nervous system and support the unique function of this highly specialized tissue. Opportunities for new discovery in neural communication are abundant across these scales of analysis.

Structural Definition of Communicating Circuit Elements: Molecules and Wiring

Which neuron types communicate with which other neuron types, and how is this relevant to behavior? For more than 100 years, dating back to the first elegant and prescient hand-drawn arrows depicting putative information flow between specific kinds of neurons (defined by shape and location) from Santiago Ramon y Cajal and his students, neuroscientists have presumed that the study of brain function will depend in part on the identification of cellular connections that mediate information transfer. Ramon y Cajal was able to combine a simple and robust neuronal visualization technique (the Golgi stain) with his keen observer's eye and a systematic workflow to infer a great deal about neuronal communication despite the many limitations of the methodology.

Since Cajal's era, many other anatomical tracing methods, which to various extents address some of the limitations of Golgi staining, have become widely utilized in the field (Table S1). Established techniques include dyes such as FluoroGold and other injectable markers taken up by cells that can give rise to fluorescent, pigmented, or electron-dense signals suitable for examination of long-range projections across the brain (Honig and Hume, 1989; Katz and Iarovici, 1990; Katz et al., 1984; Naumann et al., 2000; Reiner et al., 2000). Several proteins have also been adapted for neuronal tracing (Table S1; Conte et al., 2009; Gerfen and Sawchenko, 1984; Kissa et al. 2002; LaVail and LaVail, 1972; Schwab et al., 1978). Although these protein tracers are not solely retrograde or anterograde in all systems, they can be effective when used in the context of separately validated circuit anatomy (e.g., Gradinaru et al., 2010; Gunaydin et al., 2014; Xu et al., 2012). Some of these tracers (e.g., wheat germ agglutinin [WGA]) provide the additional leverage of *trans*-synaptic labeling, in which the cell bodies of neurons synaptically connected to a "starter-cell" population (those cells which initially contained the marker) are labeled, although restriction of the label only to monosynaptic connections cannot in principle be guaranteed by these proteins alone without further engineering and genetic targeting (e.g., using the GAL4-UAS system in *Drosophila* or zebrafish or recombinase-driver lines and engineered viral vectors in mammals as discussed below).

The relatively recent availability of engineered viral vectors for circuit tracing has driven rapid and substantial progress in the investigation of neural circuits, particularly in mammals. One of the most commonly used vector types is derived from adeno-associated viruses (AAVs). AAVs are now a workhorse tool for circuit mapping since they can be engineered to safely deliver genes encoding protein markers to neurons within a practical size limit set by the viral capsid capacity (~5 kB). Moreover, they are relatively cheap, can be concentrated to high titers (~10^{13} viral genomes/mL), and are safe (BSL-1). A simple but powerful example of the use of AAVs for circuit mapping comes from the Allen Brain Institute's Mouse Connectivity Database (http://connectivity.brain-map.org), a growing collection of projection-mapping experiments (Oh et al., 2014). These experiments are carried out by injecting AAVs expressing yellow fluorescent protein (YFP) as a cell-filling marker, under the control of a partially cell-type-specific promoter or in a recombinase-dependent manner when injected into recombinase-driver mouse lines. Projections of the YFP-expressing population can then be visualized in individual serial sections or with 3D rendering across many sections.

The power of this resource derives from its remarkable breadth; experiments in the Mouse Connectivity Database are not hypothesis-driven, but rather serve as an openly accessible resource for hypothesis generation and testing by other labs. A limitation of the YFP cell-filling approach is that the traced cells, though specified by cell-body location and, in some cases, also by a genetic feature, are not specified by critically important properties of neurons: input and output. For example, it is unclear if the traced cells (which have axonal projections observed in a particular region) actually give rise to axonal terminations in the slice corresponding to that region. To address this issue, other circuit-tracing strategies have been developed that take advantage of the ability of certain viruses (e.g., rabies, herpes simplex virus [HSV], and canine adenovirus [CAV]) to efficiently transduce axon terminals, thus specifying neurons by their outputs. Modern anatomical methods can thus be used to deepen our understanding of communication pathways in the brain by resolving such output-defined elements (ODEs; an abbreviation useful in this context) of the circuit (Figure 2).

Viral definition of ODEs can be further refined by layering onto output definition an additional cell-type characteristic, such as neurotransmitter production defined by a genetic marker. One such early approach to circuit mapping based on both an axonal target and a genetic feature (Fenno et al., 2014) involved delivering an HSV (expressing Flp recombinase in a Cre-recombinase-dependent manner) to the axon target region of interest (in this case, nucleus accumbens [NAc] of tyrosine hydroxylase [TH]-Cre recombinase-driver mice). Only the dopaminergic (TH+) cells arising from the ventral tegmental area (VTA), into which a separate Flp-dependent construct carried by an AAV had been introduced, were able to express the payload from

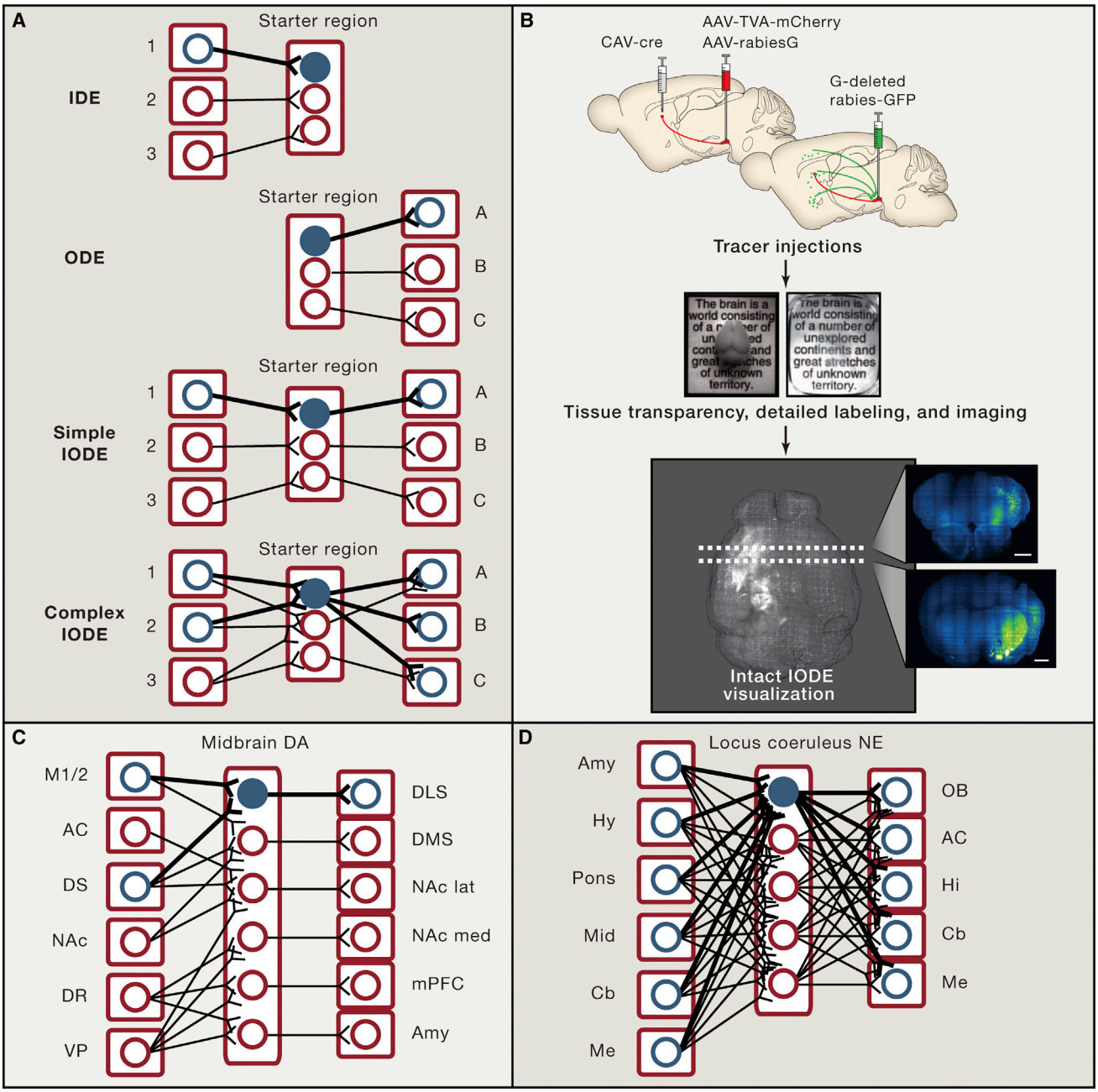

Figure 2. Input/Output-Defined Elements in the Nervous System

(A) Input and output defined elements (highlighted in blue) as nervous system building blocks are schematized. An input-defined element (IDE) is a cell-type defined by location, cell origin, and/or activity of its afferent structures. An output-defined element (ODE) is a cell-type defined by location, cell target, and/or activity of its efferent structures. An I/O-defined element (IODE) is specified by both input and output anatomy and activity as defined above. In a simple case, a cell might serve the purpose of processing and relaying information from input site 1 to output site A. In a more complex case, a cell might integrate and process information from input sites 1 and 2, then relay its output to multiple brain regions (output sites A, B, and C).

(B) Intact-system methods for visualizing IODEs. After IODE tracer injections (e.g., those involved in implementing TRIO; Schwarz et al., 2015), whole brains can be clarified (e.g., using CLARITY; adapted with permission from Chung et al., 2013) and intact IODEs can be visualized in the fully intact organ (Lerner et al., 2015, Menegas et al., 2015). Scale bars on optical coronal sections are 1 mm. The IODE visualized here shows inputs from motor cortex and striatum to DLS-projecting midbrain dopamine neurons, as schematized in C (adapted with permission from Lerner et al., 2015).

(C and D) IODEs observed in recent studies of the midbrain dopamine (DA) system (B) and the locus coeruleus norepinephrine (NE) system (C). Midbrain dopamine neurons form distinct, though sometimes complex, IODEs (Beier et al., 2015; Lerner et al., 2015), whereas locus coeruleus norepinephrine neurons are not readily distinguishable by either input or output (Schwarz et al., 2015). M1/2, primary and secondary motor cortices; AC, anterior cingulate; DS, dorsal striatum; DLS, dorsolateral striatum; DMS, dorsomedial striatum; NAc, nucleus accumbens; NAc lat, NAc lateral shell; NAc med, NAc medial shell; DR, dorsal raphe; VP, ventral pallidum; mPFC, medial prefrontal cortex; Amy, amygdala; Hy, hypothalamus; Mid, midbrain; Cb, cerebellum; Me, medulla; OB, olfactory bulb; Hi, hippocampus.

the AAV, since only these cells had been able to produce Flp from the Cre-dependent construct delivered via HSV. In this way, cells were triply defined and targeted based on cell body location (VTA), a genetic feature (TH+), and an axon termination target (NAc). This example illustrates how multiple features are required to identify cell types, since NAc-projecting VTA cells may be dopaminergic, GABAergic, or even glutamatergic, and VTA dopamine cells may project to the prefrontal cortex, amygdala, and dorsal striatum in addition to the NAc. It is only when the axonal target and genetic criteria are combined that one may specifically isolate NAc-projecting dopamine cells in the VTA for study.

CAV (Soudais et al., 2001) has also been used to direct recombinases (e.g., Cre or Flp) to neurons projecting to a particular output region of interest and can be combined with transgenic mouse driver lines (Beier et al., 2015; Lerner et al., 2015; Schwarz et al., 2015). For example, CAV has been used to examine central monoaminergic cells defined by projection target in order to ask whether noradrenergic or dopaminergic neurons that project to one region of the brain also send collateral projections to other regions (Beier et al., 2015; Lerner et al., 2015; Schwarz et al., 2015). These results revealed disparate circuit properties of different neuromodulator cell populations. While all noradrenergic neurons in the locus coeruleus collateralize broadly, distinct dopaminergic neuron types (especially those intermixed in the substantia nigra pars compacta) form separable output pathways to distinct downstream targets. These examples demonstrate the productive investigation of ODEs using axon-transducing viruses such as HSV and CAV.

Viral tracing is especially versatile because the targeting properties of the virus itself (e.g., axon transduction) can be easily multiplexed with targeting capabilities afforded by the proteins it is engineered to express. For example, AAVs can be used to deliver not just single-marker proteins such as YFP, but also *trans*-synaptic tracer proteins like WGA or the components of even more refined neuronal tracing systems. One important example of the latter is GRASP (GFP-reconstitution across synaptic partners), an elegant split-GFP technique that allows fluorescent marking of close (likely synaptic) contacts between membranes of two cells. Though originally developed in *C. elegans* (Feinberg et al., 2008), GRASP has been adapted for use in mammals (mGRASP; Kim et al., 2012). This system works by tethering split-GFP fragments to synaptic-targeting proteins in two cell populations suspected of forming connections onto one another. In mGRASP, one GFP fragment is fused with the intracellular targeting domain of neurexin-1β to target presynaptic sites, while the other GFP fragment is fused with a sequence from neuroligin-1 to target postsynaptic sites. The pre- and postsynaptic components of the mGRASP system can be directed specifically to two cell populations of interest using AAVs. Connections between these populations are then detected when the GFP fragments come into close contact at synapses and fluoresce as reconstituted GFP. A similar approach, SynView, operates by labeling only those connections where neurexin-1β and neuroligin-1 or neuroligin-2 first bind each other, giving specific information about synapses that naturally use these synaptic adhesion molecules (Tsetsenis et al., 2014). Both mGRASP and SynView can offer information about synaptic locations for enriching information gained by visualizing processes and thus can be used to define with some precision pre- and postsynaptic partners in potential communicative events.

Another neuronal labeling technique that enriches anatomical information beyond single-color labeling is Brainbow (Livet et al., 2007). Brainbow mice have been engineered to express randomized combinations of fluorophores in each cell, a property that allows researchers to trace processes of individual neurons even among other densely packed processes. Brainbow technology is not only available for tracing in mice, but also has been widely adapted for use in *Drosophila* and zebrafish (Hadjieconomou et al., 2011; Hampel et al., 2011; Pan et al., 2011). Recent improvements (e.g., Brainbow 3.0, 3.1, and 3.2; Cai et al., 2013) have enhanced expression and detection; not only can individual fluorophores be stained using specific antibodies to further enhance signals, but also the "default" fluorophore present in nontargeted [i.e., Cre recombinase-negative] cells has been eliminated in favor of a non-fluorescent marker protein (Phi-YFP), which can be visualized by immunostaining if desired. Thus, by crossing Brainbow 3.2 transgenic mice with a Cre-driver line or by injecting a Cre-expressing AAV, one can visualize only a subset of neurons identified by recombinase expression and further distinguish individual cells within that group. The detailed single-axon analyses permitted by Brainbow—and complementary technologies such as MAGIC Marker or CLoNe (García-Moreno et al., 2014; Loulier et al., 2014)—may open the door to applications resolving axon distribution diversity across development and hence accessing different ODEs within circuitry (Figure 2).

The techniques discussed so far primarily involve tracing the outputs of single cells or, in the case of GRASP/mGRASP, identifying the connections of two predefined partner types. In contrast, newer *trans*-synaptic tracing techniques allow the broad labeling of cells across the brain with axons forming connections onto a postsynaptic starter-cell population—the former (labeled afferent) cells are defined by a feature of their output and hence are also ODEs. To the extent that *trans*-synaptic tracing experiments with different starter-cell populations reveal different afferent patterns, the latter (postsynaptic starter-cell) populations can in turn be contrasted and thus considered input-defined elements (IDEs).

Certain of the tracers mentioned above (e.g., WGA and PHA-L) are able to cross synapses; however, with time, these can cross multiple synapses in series and thus the identification of direct connections using these reagents alone is not assured. To limit input tracing to monosynaptic connections, Callaway and colleagues (Wickersham et al., 2007) developed a system in which a modified rabies virus lacks an essential glycoprotein needed for *trans*-synaptic transport. The glycoprotein can then be provided only to the population of starter cells from which input tracing will occur, ensuring rigorous single-synapse definition of the afferent ODEs. Rabies viruses can also be further engineered to refine the starter-cell population (and hence the ODEs synapsing onto the starter-cell population) by pseudotyping with the coat protein EnvA, which causes infection to depend on TVA (avian tumor virus receptor), an avian receptor not found in the mammalian brain. Exogenous TVA in turn can be

selectively expressed in the desired starter-cell subpopulation, such as midbrain dopamine neurons (Ogawa et al., 2014; Watabe-Uchida et al., 2012), serotonin neurons (Ogawa et al., 2014; Pollak Dorocic et al., 2014; Weissbourd et al., 2014), direct- or indirect-pathway striatal projection neurons (Wall et al., 2013), and striatal cholinergic interneurons (Guo et al., 2015).

Recently, several groups have extended this circuit-building toolbox with different approaches to make starter-cell populations not only potentially input defined, but also output defined by integrating the concept of projection-targeted recombinase delivery (Fenno et al., 2014). In one such approach (Beier et al., 2015; Lerner et al., 2015; Schwarz et al., 2015) termed TRIO (tracing the relation between input and output; Schwarz et al., 2015), it is possible to examine monosynaptic inputs to starter-cell neural populations under conditions in which the latter are also defined by their output targets (e.g., using retrograde transport of Cre or Flp recombinase packaged in a CAV). Differing-input starter cells resolved in this way can then be considered IODEs (Figure 2). Using this approach Lerner et al. (2015) showed that the inputs to midbrain dopamine neuron starter cells are biased depending on the projection target of these starter cells, thus resolving IODEs (this result contrasted with noradrenergic neurons in the locus coeruleus, which receive relatively homogeneous inputs regardless of output; Schwarz et al., 2015). In combination with studies of neural collateralization (in the same papers and described above), it was possible to conclude that dopamine neurons are equipped to communicate specific input signals tailored to distinct output brain structures, whereas noradrenergic neurons may broadcast communications more generally across the brain. As in Fenno et al. (2014), TRIO also can be engineered for yet further refinement of the starter cell population by a genetic feature in addition to axonal target (conditional TRIO or cTRIO; e.g., in DAT::cre driver mice; Beier et al., 2015).

Another form of IODE tracing has also recently been published (Menegas et al., 2015). Here, instead of using retrograde transport of a recombinase to specify starter cells, the authors used retrograde transport of TVA, packaged into an AAV (some AAVs can transduce CNS axons, though more variably and weakly than CAV or HSV). It is important to note when using this strategy that the rabies glycoprotein should ideally also be delivered according to a retrograde strategy to prevent tracing from cells not belonging to the correct ODE; otherwise, disynaptic tracing can occur due to connectivity properties among glycoprotein-expressing cells within the local microcircuitry. Many other strategies for IODE definition are now possible (see Table S1 for a summary of available IODE building blocks), which should all involve careful consideration of the scientific question at hand and the relevant circuit anatomy.

Though the above brainwide methods are heavily dependent on fluorescent markers and thus may be generally constrained by the limits of light microscopy, where indicated, these may be followed up with higher-resolution local studies (e.g., leveraging super-resolution light microscopy, electron microscopy, and/or array tomography for detailed synaptic analysis; Atasoy et al., 2014; Kim et al., 2014b; Maglione and Sigrist, 2013; Micheva and Smith, 2007; Ragan et al., 2012) to further resolve the fine structure of IODEs. While engineered-virus, tracer protein, and dye-based methods are generally limited to animal models, the results arising may be compared as needed with corresponding (though less precisely defined) structural features in human brains inferred from diffusion-weighted imaging (DWI), fMRI, or histology (Table S1). Such comparisons may be useful for translating basic findings on circuit structure in animal models into clinical diagnostic tools or interventions.

High-resolution anatomical studies enabled by the technologies described above are substantially advancing the understanding of neural circuit connectivity, yet the throughput of such experiments becomes an issue when dealing with brainwide investigations and in light of the need to be confident that (for example) the source or origin of a population of axons has not been missed or misidentified. Here, intact-brain analyses (as enabled by CLARITY and other whole-organ tissue transparency methods; Chung et al., 2013; Ertürk et al., 2012; Hama et al., 2011; Renier et al., 2014; Susaki et al., 2014; Richardson and Lichtman, 2015), especially when compatible with rich molecular information as may be obtained with multiplexed protein and RNA analysis, have provided key leverage, along with high-speed and high-resolution light-sheet microscopy methods such as COLM (CLARITY-optimized light-sheet microscopy; Tomer et al., 2014; Lerner et al., 2015; Tomer et al., 2015) and emerging methods for automated counting and quantitative analysis of circuit components (Kim et al., 2015b; Menegas et al., 2015). Integration of circuit-labeling techniques (Table S1) with analyses enabled by whole-brain transparency, labeling, imaging, and analysis methods (Table S2) promises to accelerate progress in dissecting IODEs, setting the stage for functional circuit investigation.

Functional Definition of Circuit Elements: Wiring-Dependent Optogenetic Control

The anatomical methods outlined above have greatly enhanced investigation of communication pathways in the brain by allowing highly refined definition of potential sources and targets. However, these methods alone lack access to the actual content and functional significance of the communication. Thus, neuroscientists increasingly seek to combine anatomical observations with both activity perturbations and activity readouts to form a complete picture of circuit information processing, which may include computations performed at the cellular level on incoming information, as well as modulation of the global dynamics of information flow among neural circuit components in vivo. Integration of anatomical maps of communication pathways, with complementary functional approaches to observe and control activity events themselves, has proceeded along several dimensions, all enabled by recent technology development.

The method of optogenetics (Yizhar et al., 2011; Deisseroth 2014) has allowed functional characterization of connectivity motifs by enabling temporally precise manipulation of defined neural circuit elements in living systems, both in slice preparations and in vivo. Optogenetics involves the expression of single microbial proteins, which permit light-activated regulation of ion flow in genetically targeted neurons, resulting in cell-type-specific neuronal control during behavior. Optogenetics thus dovetails well with the structural methods described above that involve expression of single fluorescent marker proteins in

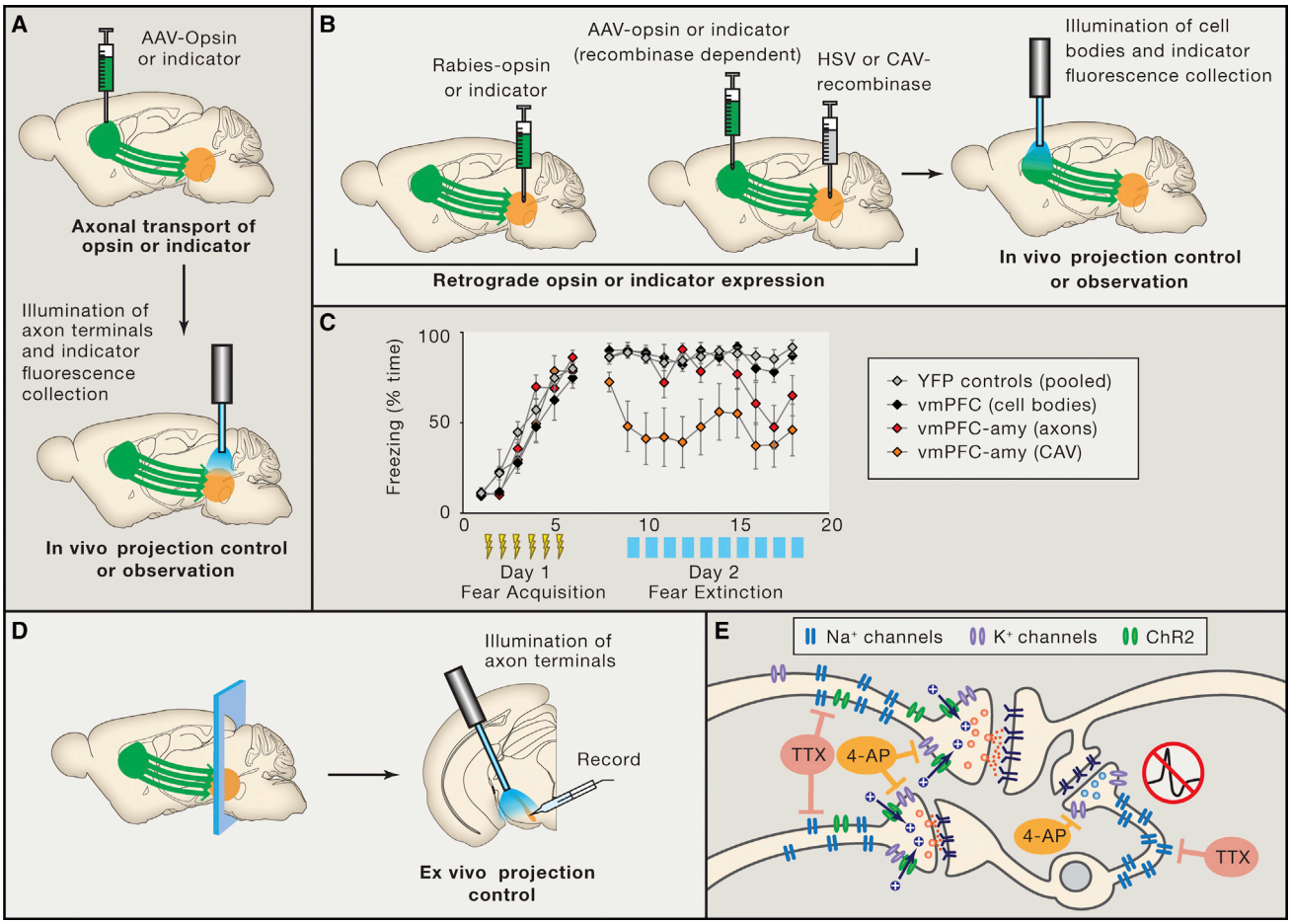

Figure 3. Functional Methods for Circuit Element Mapping In Vivo and Ex Vivo

(A) The expression of opsins or activity indicators in axon terminals allows for functional and anatomical circuit element definition in vivo, achieved by specific fiberoptic-based illumination of axon terminals.

(B) Specific stimulation or observation of a projection can also be achieved by using a retrograde virus such as rabies, HSV, or CAV to express an opsin or activity indicator only in cells that have a specific efferent target. In this case, cell bodies may be illuminated directly.

(C) An example of free mouse behavior during optical control of output-defined elements (ODEs). In this case, the ventromedial prefrontal cortex (vmPFC) to amygdala (amy) projection was manipulated during fear conditioning and extinction using three different strategies. First, non-specific stimulation of vmPFC cell bodies (black symbols); second, stimulation of vmPFC axon terminals in the amygdala (red symbols); third, stimulation of vmPFC cell bodies which send projections to the amygdala (orange symbols; identified by CAV-cre injections in the basomedial amygdala; Adhikari et al., 2015). Increasingly specific output definition of the stimulation circuit element elicits increasingly potent effects on the cued freezing (fear memory) behavior. Yellow bolts indicate six shock-tone pairings given on the training day. Blue bars indicate the time of blue light stimulation of the target circuit element on the extinction day (when tones are played, but no shocks delivered). Gray symbols, YFP (no-opsin) control cohort. Adapted with permission.

(D) Expression of opsins in axons' terminals also allows for optical control of defined circuit elements in the ex vivo slice electrophysiology preparation.

(E) Acute slice preparations allow for fine circuit dissection in controlled conditions, for example using TTX/4-AP to definitively isolate monosynaptic connections (Petreanu et al., 2009; Lerner et al., 2015; Adhikari et al., 2015). First, TTX blocks action-potential-dependent release, preventing disynaptic stimulation through non-opsin-expressing neurons. Second, 4-AP increases terminal excitability by blocking K^+ channels. Channelrhodopsin (ChR) optical drive then induces action-potential-independent depolarization at ChR-expressing terminals only, while patch clamping of different target cells thus allows definition of afferent fibers as output-defined elements (ODEs).

cell-type-specific neuronal populations; other capabilities, limitations, and technical considerations of optogenetics have been recently reviewed (Grosenick et al., 2015; Deisseroth, 2015). Crucial to the success of optogenetics for functional circuit mapping, microbial opsins such as channelrhodopsins and halorhodopsins are trafficked into axons (a process typically enhanced with molecular engineering) and can also be delivered by recombinase-activated labeling strategies traveling retrograde from synapses; hence, the functional communication of ODEs can be modulated using the leverage of their defined anatomical outputs (Figure 3).

Not only can the axons of genetically specified groups of neurons then be controlled (stimulated or inhibited) by light delivered directly to projections during behavior, but subsequent living acute slice preparations can be taken from the target regions and used to study the functional connectivity of these projections in isolation with single-cell resolution (Figure 3D). For example, in cases where it is of interest to define the

monosynaptic (direct) target of ODEs, drugs may be applied to the extracellular recording solution in slice preparations to prevent the occurrence of polysynaptic events (Petreanu et al., 2009). In one version of this method, sodium channels are blocked using tetrodotoxin (TTX), which prevents action-potential-driven release events, while at the same time, voltage-dependent potassium channels are blocked using 4-aminopyridine (4-AP) to facilitate direct depolarization of only channelrhodopsin-expressing axon terminals by blue light, detected by whole-cell patch clamp in putative, directly postsynaptic cells (Figure 3E). This approach is useful for isolating monosynaptic connections between cell types and brain regions as well as for subcellular mapping of functional synapses (Little and Carter, 2012; 2013; MacAskill et al., 2012; Petreanu et al., 2009; Sun et al., 2014), but inasmuch as this approach involves direct actuation in nerve terminals of channelrhodopsin (which fluxes Ca^{2+} ions as well as Na^{+}, K^{+}, and H^{+}), it is important to not draw detailed conclusions about natural synaptic release dynamics (which are highly Ca^{2+}-sensitive) from this sort of work. An as yet unidentified Ca^{2+}-impermeable channelrhodopsin, which would still depolarize the presynaptic terminal strongly enough to drive natural Ca^{2+} influx via voltage-gated Ca^{2+} channels, might be of value in some cases where the focus is not on simple presence or absence of direct synaptic connections.

Nevertheless, this approach to defining direct communication partners has been useful, for example, in facilitating direct IODE definition in the study discussed above (Lerner et al., 2015) that isolated direct striatal inputs to midbrain dopamine neurons and functionally contrasted the inputs arriving from distinct subregions of the striatum to output-defined subpopulations of dopamine cells. Adhikari et al. (2015) also employed this optogenetic monosynaptic connectivity method together with CLARITY and viral tracing to discover a direct connection between ventromedial prefrontal cortical (mPFC) and basomedial amygdala, which turned out to be behaviorally important for top-down regulation of fear and anxiety responses. Also studying communication between the mPFC and amygdala, Little and Carter (2013) used two-photon optogenetic methods to determine the density and distribution of amygdalar inputs onto mPFC cells, demonstrating how optogenetics can be employed for detailed functional mapping of synaptic locations. Demonstrating the potential health relevance of these functional approaches to circuit mapping, optogenetic recruitment of cells and synapses defined by a specific connectivity feature (distinct long-range afferent projections to the NAc) has been applied to probe detailed hypotheses on the synaptic basis of cocaine addiction (Britt et al., 2012; Creed et al., 2015; Pascoli et al., 2014).

Most of the above patch-clamp studies focused on defining direct monosynaptic neuronal connections, but an integrative view of circuit-element output might further consider effects of diverse interacting downstream cell populations, since both monosynaptic and polysynaptic connectivity associated with the output brain region will be important in sculpting elicited activity. Reduced slice preparations may be selected if a specific hypothesis is to be tested regarding local circuit modulation of activity (e.g., the role of sparse interneuron populations recruited by feedback inhibition to modulate local dynamics in slice preparations; Sohal et al., 2009). Conversely, more exploratory brainwide analyses of functional connectivity may be conducted, in which global outputs (resulting from activity in an optogenetically defined cell population) can be measured in a regionally unbiased fashion throughout the brain. The latter approach has been taken using electrophysiological postsynaptic readouts (Chuhma et al., 2011; Mingote et al., 2015) or fMRI (Ferenczi et al., 2016; Lee et al., 2010); in the fMRI case, focal regions in mPFC were found to exert specific and behaviorally relevant influence over the manner in which distant brain regions communicated with each other (Ferenczi et al., 2016). A brainwide database of these second-order interactions was provided (Ferenczi et al., 2016), illustrating how precise modulation of intended direct targets in a brain region (as will occur in natural or experimental settings) exerts influence by accessing the intact brain as a dynamical system, with relevance in this case to top-down control of physiological behavioral state transitions.

Optogenetic analysis of communication in brain circuitry has been employed not only with the physiology readouts noted above, but also in freely moving animals to provide information on the causal relationships between neural circuit activity patterns and behavior (reviewed in Deisseroth, 2014 and Steinberg et al., 2015). Using (for example) fiberoptic neural interfaces, optogenetics can be used in the behavioral setting to stimulate, inhibit, or modulate a population of cell bodies within a brain region or to address a specific ODE in vivo (using either selective illumination of opsin-expressing projection terminals or the retrograde-opsin-expression strategy to recruit cells by a feature of their connectivity; Figures 3A and 3B; Deisseroth, 2014; Steinberg et al., 2015). Behavioral optogenetics experiments have demonstrated the utility of these approaches for resolving the effects of defined circuit elements; indeed, more specific behavioral effects are often observed when resolving cells by projection target instead of generally illuminating cell bodies without regard to projection target (Adhikari et al., 2015; Kim et al., 2013; Warden et al., 2012). For example, Warden et al. (2012) found that optogenetic activation of prefrontal cortical projections to the dorsal raphe nucleus selectively modulated behavioral state (favoring active coping defined by motivated escape behavior in the forced swim test, while not generally increasing locomotor activity) in a manner that depended on specific activation of that pathway (in contrast, nonspecific stimulation of the prefrontal cortex or dorsal raphe, or stimulation of other projections from prefrontal cortex, did not cause the same specific effect profile). In a separate study, Kim et al. (2013) examined several projections arising from a single brain area (the bed nucleus of the stria terminalis [BNST]) that each selectively recruited distinct features (risk avoidance, respiratory rate, or conditioning value) of anxiety-related behavioral state transitions. Upstream of the BNST, Adhikari et al. (2015) studied fear and anxiety modulated by recruitment of distinct cortico-amygdalar projections, observing that fear extinction was enhanced by specific top-down pathway recruitment, but not by non-specific stimulation of cortical cell bodies (Figure 3C).

These studies, and many others like them, indicate that defining circuit elements by structural I/O features (alone or in combination with other features such as genetic markers) is a tractable experimental approach that maps onto nervous system structure-function relationships more precisely than simple

regional stimulation. Notably, optical stimuli (as delivered to these structural elements of interest) can be readily mapped in parametric fashion by varying light intensity and timing. Of course, without incorporation of pre-existing knowledge of native activity patterns, experimenter-defined activity traffic along a given neural communication pathway is unlikely to precisely match the natural dynamics of the pathway. Nevertheless, specific modulation of relevant complex behaviors is still typically observed, revealing that defining these elements by their detailed I/O structure alone may describe meaningful communication in the circuit. When data are available on endogenous activity patterns relating to encoding or transformation of information, optogenetic methods can additionally take into account these data, as discussed next.

Activity Readouts for Delineating Input- and Output-Defined Circuit Elements

A complete picture of I/O properties for specific circuit elements would include not just anatomy, but also activity in the form of naturally occurring neuronal signals along the anatomical I/O pathways of interest. A diverse array of compatible tools for reading out activity and examining the information processing as executed by the circuit (Table S3) indeed now allows layering of this crucial dimension onto anatomical circuit maps. Such an integrated approach allows certain questions across a range of scales to be addressed that would be difficult to answer from anatomy or from optogenetics alone.

Traditional electrophysiological approaches to examining activity in vivo bring the highest temporal resolution but are fundamentally limited in terms of accessibility of cell type and wiring information; moreover, these are not readily able to monitor activity in axons, which would be important for providing pathway specificity just as modulation of axons has provided pathway specificity in optogenetic studies. Crucially, then, it is difficult to cast electrophysiological data in the same framework as the structural (physical and molecular) and optogenetic-control datastreams discussed above. Although temporal resolution with fluorescence Ca^{2+} signals (for example, as recorded with genetically encoded Ca^{2+} indicators) is not as high as with electrophysiology, the cell-type and pathway specificity provided is invaluable when interfacing activity data with anatomical information. This field is rapidly advancing (e.g., Chen et al., 2013) and now extends to faster genetically encoded voltage sensors as well (e.g., Gong et al., 2015); for the purpose of the primer, we focus here on developments that are most immediately and directly linked to the anatomical and optogenetic methods described above for delineating brainwide I/O defined elements.

A fluorescence recording approach, termed fiber photometry (Gunaydin et al., 2014), has been developed for genetically encoded activity sensors and is particularly well suited for monitoring specific ODEs in cell bodies (Cui et al., 2013) and even in deep-brain genetically defined fiber tracts during behavior (Gunaydin et al., 2014; Kim et al., 2016). Fiber photometry is a photon-counting (photometric) strategy built on a fiberoptic interface targeted to the brain area or axonal tract of interest, with optics designed to detect even small-activity fluorescence signals arising from genetically encoded Ca^{2+}-indicator proteins in axons deep in the brain of behaving mammals (Gunaydin et al., 2014). Fiber photometry permits real-time observations of the activity along specific axonal projections defined by origin and target and complements methods for imaging superficially exposed axons with conventional objectives in behaving animals (De Paola et al., 2006; Grutzendler et al., 2002; Lovett-Barron et al., 2014). In the initial demonstration of fiber photometry in deep projections (Gunaydin et al., 2014), Ca^{2+}-indicator expression was targeted to mouse VTA dopamine neurons and activity in the projections of these neurons to the NAc was monitored; thus, the circuit element was defined by outgoing projection anatomy as well as by neurotransmitter phenotype. It was observed that endogenous activity of this output stream was robustly modulated during social interaction but much less so during novel-object interaction in the same mice; optogenetic control over the same projection then revealed that this was a causally significant signal in the behavior. A next-generation version of this method, frame-projected independent-fiber photometry (FIP), has now been developed for recording fluorescence activity signals from many brain regions or deep-brain fiber tracts simultaneously in behaving mice and for tuning optogenetic perturbation to elicit dynamics matching patterns occurring naturally in behavior (Kim et al., 2016). Together, these fiber photometry examples illustrate the utility of optical readouts for resolving activity magnitude and timing in projection-defined elements during free behavior.

In vivo photometry has been applied to many target elements throughout the brain (including cell bodies as well; Chen et al., 2015; Cui et al., 2013; Lerner et al., 2015; Zalocusky et al., 2016; Lütcke et al., 2010; Schulz et al., 2012) and has also recently been used to delineate IDEs, in the sense of differing activity observed in defined circuit elements during behavior. For example, if the somata of two genetically similar cell populations exhibit opposite-direction activity changes in response to the same behavioral stimulus, these two populations are likely to at some level receive different input stimuli and therefore represent IDEs. A recent study (Lerner et al., 2015) demonstrated such IDEs alongside anatomical analysis of inputs and outputs, which also differed for the same populations; using fiber photometry in mice, substantia nigra pars compacta neurons that project to dorsolateral striatum were observed to exhibit activity elevations in response to both appetitive and aversive stimuli, while those projecting to dorsomedial striatum exhibited activity elevations in response to appetitive stimuli but decreased activity in response to aversive stimuli. These two cell populations, though neither genetically nor spatially separable, were in fact thus shown to be communicating separable streams of information (Lerner et al., 2015). This finding opens up avenues for further exploration and illustrates the informative directions that can be taken as IODE characterization reframes models of circuit organization.

Although fiber photometry was designed for ease of use in freely moving behavior as well as direct compatibility with typical anatomical tracing and optogenetic control datastreams, cellular-resolution imaging can be applied as desired for more detailed and complementary information (just as anatomy and optogenetics readily also allow cellular-resolution work in more restricted fields of view). In an example linking behavior,

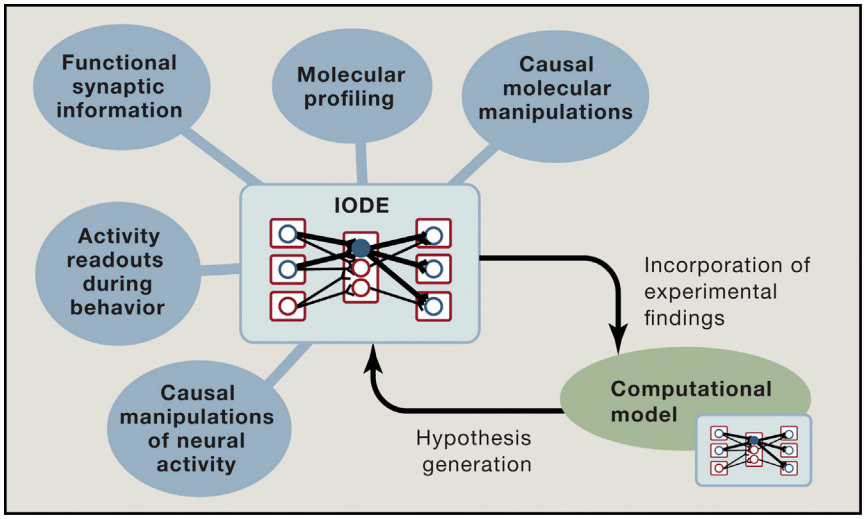

Figure 4. Organizing Principles for Cross-Modal Investigation of Neural Circuits

IODEs may help provide a behaviorally relevant and experimentally tractable framework for guiding and integrating information about neural circuits across many levels of investigation. Once the basic physical structure of an IODE is understood from the anatomical tools described here (including the most relevant convergence and divergence of information through collaterals when considering a specific behavior), activity information during behavior from physiology, imaging, and molecular datastreams can be collected using targeting tools aligned with the IODE structure and then layered onto the diagram to form a more complete understanding of I/O relationships. Computational analyses may facilitate registration and joint interpretation of information gathered by these disparate techniques, as well as generation of higher-order hypotheses to guide further data collection (e.g., through system-identification strategies; Grosenick et al., 2015). Iterations of this data-collection and hypothesis-generation cycle, and crucially the linking of distinct IODEs into loops and more complex topological structures, may continue until experimental and theoretical concepts converge.

anatomy, optogenetics, and cellular-resolution imaging, Rajasethupathy et al. (2015) found that an ODE from anterior cingulate cortex selectively influences a sparse population of "hub" neurons in the hippocampus that are highly correlated with other cells in the local network during memory retrieval. The discovery of this rare cell type and the observation of local circuit dynamics required the use of single-cell-resolution two-photon imaging to effectively link the anatomical and functional lines of evidence pursued.

Such high-resolution and highly local optical readout of activity elicited by control of defined circuit elements is currently complemented by more global (even brainwide) readouts that are also well suited to reporting on effects of ODE activity. Brainwide activity readout (sacrificing spatial and temporal resolution) can be achieved via optogenetic fMRI (ofMRI) BOLD (blood oxygen level dependent) signals (Lee et al., 2010) or (achieving single-cell resolution while still further sacrificing temporal resolution) via immediate early gene (IEG)-based readouts (e.g., IEG-immunohistochemical labeling or IEG-promoter-driven expression of cell-filling fluorescent proteins, as in the TRAP (targeted recombination in active populations) method; Guenthner et al., 2013). Together, these examples illustrate how activity readouts can complement I/O mapping of neural circuit elements and set the stage for diverse research directions combining activity readouts with functional manipulations (e.g., optogenetics) alongside structural anatomical studies (including via whole-brain tissue clearing, pathway tracing, and molecular labeling).

Mesoscale Elements of Communication: the Input/Output-Defined Cell Type

Certain anatomical and functional approaches to circuit mapping rely to some extent on a simplifying assumption: that the group of cells being labeled and traced belongs to a discrete "type." How these cell types are defined can profoundly influence the interpretation of experiments, yet our definitions of cell types are rapidly evolving. A survey of cell types in the brain is among the early goals of the US BRAIN Initiative (Jorgenson et al., 2015), yet consensus is still lacking as to how to best define and organize such categories. Many of the viral/genetic strategies for circuit mapping described above rely, as a matter of practicality, on single-feature recombinase-driver lines to define cell type. Other studies have focused on careful quantifications of morphology to create categories. Nevertheless, each neuron, like each snowflake, is unique. Were fully detailed criteria to be applied, each cell would form its own new class, but such excessively detailed categorization would not provide useful overarching principles describing circuit function (for example, each cell in each mammal's hippocampus is slightly different, but in general, hippocampi appear to solve the problem of spatial navigation similarly). Therefore, neuroscientists must make educated judgments about which elements of cell-type definition are likely to be most meaningful for developing workable theories of brain communication.

We suggest here that the genetic and morphology markers used most commonly thus far are proxies for the neural elements that really matter for circuit function: inputs and outputs defined by wiring and activity. Other markers are not unsuitable by any means, but the field should move where possible toward defining cell types directly in terms of their circuit function. Among other useful aspects, thinking about cell types in IODE terms will create organizing links between molecular/cellular neuroscientists and systems neuroscientists and draw attention to molecular and cellular elements that give neurons particular input- and output-defined circuit properties. Working from such shared concepts may facilitate synthesis of findings and productive interchanges and even promote engagement of computational and theoretical neuroscientists since the resulting datasets will be well-suited to closed-loop and system-identification approaches (Grosenick et al., 2015; Figure 4).

To bring these ideas to a concrete example, we note that dopamine neurons are currently undergoing a revolution in their classification that illustrates how and why shifts in cell-type

definition take place. Until recently, dopamine neurons (as suggested by the name) had been defined primarily by their production and release of the neurotransmitter dopamine; TH, the rate-limiting enzyme in catecholamine production, is often used as one of several molecular markers. Yet, to be useful as a conceptual building block toward understanding brain function, this definition should imply that all dopamine neurons have at least somewhat similar roles in their brain circuits, an assumption that is widely understood in neuroscience to be false, for at least three critical reasons.

First and most simply, dopamine neurons can be subdivided based on their outputs to distinct brain regions, which include the prefrontal cortex, amygdala, NAc core, NAc medial shell, NAc lateral shell, dorsomedial striatum, and dorsolateral striatum, the pituitary gland, the chemoreceptor trigger zone, and many other targets. The dopaminergic projections to these different output regions are largely parallel, meaning that information communicated by a dopamine neuron will be received largely by just a single target output region. It is only when dopamine neurons are viewed from this structural-output-defined perspective that observations of opposite-valence responses to stimuli by different subsets of dopamine neurons become interpretable (Kim et al., 2014a; Lerner et al., 2015; Matsumoto and Hikosaka, 2009).

Second, though the neurotransmitter released might be considered (and is) important, different dopamine neurons also release diverse other neurotransmitters, including GABA and glutamate, which profoundly influence how these neurons participate in a circuit. For example, cholinergic interneurons in the striatum inhibit striatal projection neurons by stimulating release of GABA from dopamine neuron terminals (Nelson et al., 2014). As above, connectivity matters: it appears that dopamine neurons projecting to the NAc, but not to the dorsal striatum, co-release glutamate (Chuhma et al., 2014; Mingote et al., 2015; Stuber et al., 2010). Furthermore, this glutamate release causes burst firing behavior in ventral, but not dorsal, striatal cholinergic interneurons in response to dopamine neuron stimulation (Chuhma et al., 2014), with profound significance for circuit function. This example illustrates the importance of the structural-output- and activity-output-defined perspective for discriminating cell types.

Third and finally, input definitions also turn out to be critical for understanding dopamine neurons. Projection-defined populations of dopamine neurons receive a different balance of inputs from other brain regions, in terms of the numbers of afferents, functional strength, and functional consequences of representing completely different appetitive or aversive (rewarding or punishing) environmental stimuli (Beier et al., 2015; Lerner et al., 2015; Watabe-Uchida et al., 2012). The example of dopamine neurons clearly delineates the shortcomings of defining a cell type by a molecular feature only, as with transgenic mouse lines. However, it is not meant to suggest that other aspects of a cell's phenotype are irrelevant, and I/O must be considered functionally as well as anatomically. For example, VTA GABA neurons may have similar input and output anatomy at some level of inspection compared to VTA dopamine neurons (Beier et al., 2015) but are clearly a different cell type as would be readily distinguished by the effect of their output on downstream structures.

The true power of the IODE-centered definition of different neuronal cell types derives in large part from its ability to interface with other modes of cell-type investigation and will not replace but rather build upon molecular labels. Again turning to the midbrain dopamine system as a model for use of this framework, it has been found that defining dopamine neurons by projection target immediately leads to appreciation of diversity in subtype-specific expression of the dopamine transporter (DAT), dopamine D2 autoreceptors, GIRK channels, and HCN channels (Lammel et al., 2011; Lerner et al., 2015; Margolis et al., 2008). As a result of this diversity, subtypes of dopamine neurons may also differ in their pacemaking mechanisms, an observation that may help explain the progressive pattern of degeneration in Parkinson's disease (Chan et al., 2007; Khaliq and Bean, 2010; Puopolo et al., 2007). Further molecular investigations of dopaminergic IODEs, e.g., via molecular-profiling techniques (Ekstrand et al., 2014; Namburi et al., 2015), are likely to yield even more detailed insights into the overall organization of the system, which is not possible when dopamine neuron subpopulations are grouped as one. Though some unbiased automated discovery of cell types may be possible from single-cell sequencing data (Grün and van Oudenaarden, 2015), it may also be fruitful, perhaps even more so, to pursue unbiased cell-type discovery from connectivity datasets (Jonas and Kording, 2015). At the very least, the use of single genetic or anatomical features in isolation may cause neuroscientists to ignore meaningful sources of variability in data and thus hamper progress toward deeper understanding of the fundamental and versatile building blocks of communication and computation in the brain.

Summary: Confronting Realities of Communication Complexity and Scale in the Brain

Transitioning to I/O definition of cell types is no longer fully technologically limited, but formidable barriers remain. Among these barriers is that conceptual and analytical models have lagged behind experimental and technological advances. The resulting limitation manifests at many levels, ranging from data handling to guiding and interpreting experiments.

Regarding data handling, fast progress in circuit analysis is already dramatically accelerating the rate of dataset acquisition, as well as the size of individual discrete and compressed datasets. Even from a single laboratory, modern structural and activity datasets can reach the petabyte scale each year, creating challenges for both storage and processing. Clearly, utility will be limited by the ability of individual labs as well as the broader community to access and work with these datasets. How can neuroscientists best coordinate data handling to allow for the emergence of transformative new theories of brain function? Central open-access organization may be crucial, and some larger efforts at brain mapping, such as the Allen Institute's efforts, have developed platforms for scientists to search, view, and manipulate the data generated. The Human Connectome Project (http://www.humanconnectomeproject.org/) also maintains an accessible database of human imaging data for download. Nevertheless, there is no single database where reliable data from all levels of analysis (e.g., anatomy, control, and activity) is incorporated together in a common language, nor is

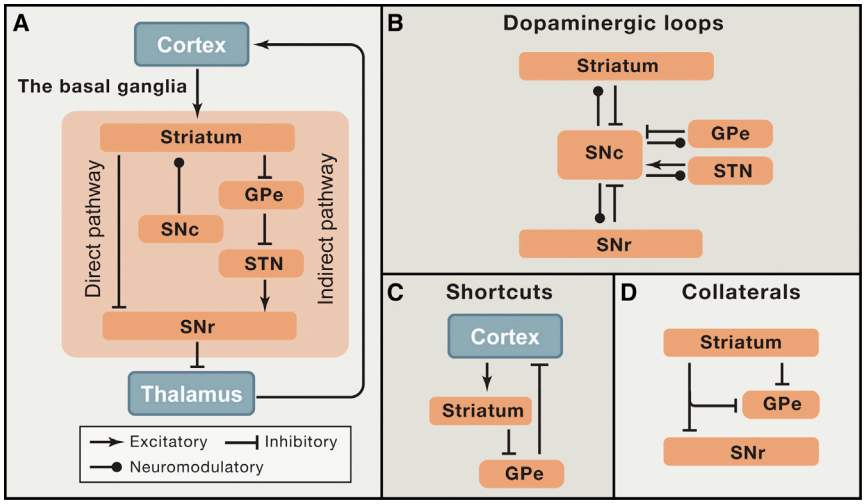

Figure 5. Example: Different Levels of Inspection for Basal Ganglia Circuitry

(A) Simplified diagram of basal ganglia (BG) circuitry depicts the "direct" and "indirect" pathways, which have opposing influences on BG output. While the concepts of the direct and indirect pathways have yielded important insights, the reality of BG circuitry is much more complex.

(B–D) Examples of additional circuit complexity in the BG. (B) Dopamine neurons in the substantia nigra pars compacta (SNc) project not only to the striatum but to other BG nuclei. These dopamine neurons also receive direct projections back from these nuclei. (C) Information need not loop all the way through the cortico-BG-thalamic circuitry. Several shortcuts are available, including the one pictured in which the globus pallidus external segment (GPe) sends projections back to the cortex (Saunders et al., 2015). (D) The "direct" and "indirect" pathways are not absolute. For example, some "direct pathway" striatal neurons also send collaterals to the GPe (Cazorla et al., 2014). A more sophisticated understanding of BG circuit dynamics may emerge as we build testable hypotheses based on a more realistic picture of the circuitry as shown in (B–D). Such an approach will be facilitated by defining, controlling, and observing cells based on their input and output properties in the intact functioning system (see Figure 4).

there an interface that allows easy back-and-forth communication between experimental and theoretical approaches to circuit function, as new information becomes available. We suggest that a unifying database employing the IDE/ODE/IODE framework, in which data from these different modalities can be expressed in the same terms, may help advance the type of rapid-cycle communication between experimentalists and theorists that will soon become indispensible as the complexity of our circuit diagrams increases.

As a current pressing example of circuit complexity, we turn to the basal ganglia (BG), a highly interconnected group of subcortical nuclei that may play a role in (among other behaviors) action selection and motor learning. What circuit-level organizing concepts currently exist for BG? Many discussions of the function of the BG to date have relied on the simple concept of a "direct" and an "indirect" pathway offering two alternative, opposing streams of information flow from the input nucleus of the BG (striatum) to the output nucleus (SNr; Figure 5A). Yet reality is well known to be substantially more complex, involving multiple feedback loops and spirals among nuclei (Figures 5B–D; Alexander et al., 1986; Cazorla et al., 2014; Haber, 2003; Kupchik et al., 2015; Mallet et al., 2012; Nambu et al., 2002; Saunders et al., 2015). The richness of this structure is both enticing and intimidating; there are clearly many more discoveries to be made about BG circuit function, yet already the complexity of our simplified diagram makes forming an intuitive understanding of circuit dynamics (and hence experimental design and data interpretation) very difficult. Of course, due to rich collateralization, individual IODES in the BG and beyond may play separable roles in different brainwide structures, with topologies that become increasingly challenging to visualize and to represent.

To break the standoff between accessibility and realism, computational approaches are a key part of the solution (Figure 4). Such approaches can be used to generate models of neural circuit communication that can be tested further by experimentalists. Results generated by experimentalists can then support further refinement of the circuit model, iterating until the experimental and theoretical concepts converge (see Phillips, 2015 for discussion relevant to biology more generally). As this iteration proceeds, it will motivate and incorporate new technological innovations as well, such as the advent of single-cell control with two-photon and spatial-light modulator-based play-in of optogenetic control over cell ensembles in vivo (Prakash et al., 2012; Packer et al., 2012; Rickgauer et al., 2014; Packer et al., 2015; Reutsky-Gefen et al., 2013; Szabo et al., 2014) or controlling ensembles based on their past involvement in behavior in the same animal (e.g., Liu et al., 2012). Such progress may also eventually bring insight into the development and plasticity of neural circuitry, as IDEs, ODEs, and IODEs may be well suited to serve as primitive building blocks that self-assembling circuitry can employ and adapt, to evolve loops and more intricate (perhaps as yet undiscovered) topologies as well as complex behaviors from individual cells and cell pairs.

In summary, although the challenges involved in understanding intact brain function remain formidable, there are considerable opportunities on the horizon for breakthroughs that may have a substantial impact on basic research as well as on the understanding of disease. Technological developments across many modalities, including progress in anatomical tracing and molecular-profiling techniques, innovations in optogenetic control, and advances in diverse activity readouts, are driving fundamental changes in the way that neuroscientists work. Organized thinking about communication in neural circuits may in itself help in organizing ties among researchers operating within these different modalities and from other biology and engineering disciplines.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.02.027.

AUTHOR CONTRIBUTIONS

T.N.L. and K.D. wrote the paper. L.Y. contributed to the summary of tissue transparency methods.

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Supplemental Information

Communication in Neural Circuits: Tools, Opportunities, and Challenges

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Table S1. Tools for Neural Circuit Mapping

Selected techniques currently available for neural circuit mapping and covering a broad range of capabilities are summarized, with attention given both to major applications/advantages (particularly in terms of characterizing IODEs) and to major caveats. The terms IDE and ODE are defined only relative to the cell population of interest; hence transsynaptic markers are particularly useful in this regard for identifying the inputs to a genetically and anatomically specified starter cell population.

Method		Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
				Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Golgi staining		Detailed local cell morphology	Silver precipitate					✔		widely compatible	• Complete neuronal morphology and fine structure (e.g. spines) are visible • Sparse labeling allows single neurons to be distinguished	• Only sparse labeling is useful • Staining can only be applied to post-fixed samples • Difficult to establish connectivity patterns (esp. long-range)	Ranjan and Mallick, 2010 (modern updates)
Dyes	**DiX Lipophilic Tracers**	Non-specific membrane tracing	Fluorescent dye (variety of wavelengths available) incorporated into cell membranes								• Compatible with tracing in post-fixed brains as well as with live tissue imaging • Efficient transport via membrane diffusion	• No cell type specificity • No directional specificity for tracing	Honig and Hume, 1989
	Dextran Amines	Some anterograde vs retrograde specificity using different mW dextrans leading to preferential uptake by cell bodies vs axons	Fluorophore, biotin or other marker conjugated to the dextran amine.								• Wide variety of marker conjugates • Variety of MWs available to help achieve directional specificity • Can be used to identify ODEs	• No cell type specificity	Reiner et al., 2000
	FluoroGold	Retrograde (axonal uptake)	UV excitation gives gold or blue emission depending on pH	✔				✔ (sparse labeling can allow single cell tracing)		widely compatible	• Efficient uptake by axons • Can be used to identify ODEs • Visualization can be enhanced by immunostraining • Compatible with EM for ultrastructural studies	• No cell type specificity • One color option • High diffusibility can make local injections difficult	Naumann et al., 2000
	Retrobeads	Retrograde (axonal uptake)	Latex microbeads used to deliver red or green fluorescent dyes								• Limited local spread of beads allows local connectivity mapping or very precise ODE tracing • Beads are trafficked quickly, yet are non-toxic, allowing a very wide range of survival times post-injection	• No cell type specificity • Punctate appearance can make cell ID difficult • No labeling of cell morphology • Less efficient axonal uptake than other options (e.g. FluoroGold)	Katz et al., 1984 Katz and Iarovici, 1990

	Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
				Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Tracer Proteins	**HRP**	Retrograde (axonal uptake)	Diaminobenzidine (DAB) reaction								• One component system • Can be used to identify ODEs	• No cell type specificity • One staining option	LaVail and LaVail, 1972
	WGA	Retrograde (transsynaptic) and anterograde (transsynaptic): specific in some contexts	Fluorophore, biotin, HRP, cre or other marker conjugated to tracer protein		✔ (optionally compatible with viral/genetic technqiues for cell specificity)	✔		✔ (sparse expression can allow single cell tracing)		widely compatible	• Transsynaptic labeling • Can be used to identify IDE or ODEs	• Transsynaptic labeling is not highly efficient • Direction of transsynaptic labeling can be mixed, variable, and circuit-dependent	Schwab et al., 1978
	PHA-L	Anterograde (transsynaptic)		✔		✔					• Transsynaptic labeling • Can be used to identify IDEs	• Transsynaptic labeling is not highly efficient • Not strictly anterograde	Gerfen and Sawchenko, 1984
	CtB	Retrograde (axonal uptake)									• Retrograde labeling • Cell type specificity possible • Can be used to identify ODEs	• Not strictly retrograde	Conte et al., 2009
	TTC	Retrograde (transsynaptic)				✔					• Transsynaptic labeling • Can be used to identify IDE or ODEs	• Transsynaptic labeling is not highly efficient • Not strictly retrograde	Kissa et al., 2002
	AAV	Anterograde (axon tracing) Can also be used to express transsynaptic markers	XFP or cre expressed by virus	✔	✔	✔ (if encoded virus is engineered to express a transsynaptic tracer protein)		✔ (sparse expression can allow single cell tracing		mammals	• Versatile, relatively non-toxic package for delivery of numerous tracing components • Allows cell type specificity using specific promoters or when combined with recombinase expression strategies • Can be used to identify IDEs (e.g. when combined with transsynaptic tracer proteins) and/or ODEs (e.g. via axon tracing)	• Packaging size limited to ~5 kB • Inconsistent reports of retrograde transport, may require batch-by-batch characterization	Betley and Sternson, 2011 (review) Wang et al., 2014 (comparison with BDA) Oh et al., 2014 (Allen Mouse Connectivity Atlas)

	Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
				Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
alpha-herpesviruses	**HSV-1**									mammals, some evidence for fish (see References)	• Efficient retrograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs or ODEs (as in Fenno et al., 2014)	• Toxicity • Careful characterization required to assure that spread is restricted to synaptically connected cells • Only particular strains are specifically retrograde	Ugolini et al., 1987 Zemanick et al., 1991 (strain specificity) LaVail et al., 1997 (strain specificity) Fenno et al., 2014 (cell-type specific approaches) Zou et al., 2014 (fish)
	PRV Bartha	Retrograde (transsynaptic)	XFP or cre expressed by virus	✔	✔	✔	✔ (Ba2000 variant; see References)			non-primate mammals	• Efficient retrograde viral tracer • Transsynaptic labeling • Monosynaptic restriction may be possible • Less toxic than other HSV strains • Can be used to identify IDEs or ODEs	• Toxicity	Enquist, 2002 Ekstrand et al., 2008 De Falco et al., 2001 (Ba2001 cell-type specific strain) Callaway, 2008 (see comment on Ba2000 for monosynaptic restriction)
	H129 strain	Anterograde (transsynaptic)								mammals	• Anterograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs	• Toxicity	Sun et al., 1996 Lo and Anderson, 2011 (cre-dependent cell-type specificity)
	VSV	Anterograde (transsynaptic) or retrograde (transsynaptic): Directionality is glycoprotein dependent	XFP label expressed by virus	✔	✔	✔	✔			widely compatible	• Anterograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs • Can be retricted to monosynaptic labeling using G deletion • Cell type specificity using EnvA pseudotyping	• Toxicity • Poorly understood batch variability, requires careful batch-by-batch characterization (see Correction to Beier et al., 2011)	Beier et al., 2011 Mundell et al., 2015
	CAV	Retrograde (axon transducing)	cre or GFP expressed by virus	✔	✔					mammals	• Relatively non-toxic retrograde viral tracer. The lack of toxicity makes this virus particularly appealing for examining functional circuit elements in vivo. • Can be used to identify ODEs	• Not transsynaptic	Soudais et al., 2001 Junyent and Kremer, 2015 also see TRIO references

Method	Connectivity Information/ Tracing Directionality	Mechanism/ Marker	Properties Checklist						Species Compatibility	Major Applications/ Advantages	Major Caveats	References
			Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Rabies	Retrograde (transsynaptic and axon tranducing)	XFP label expressed by virus	✔	✔ (EnvA variants; see References)	✔	✔ (G-deleted variants; see References)			mammals	• Specific, efficient retrograde viral tracer • Transsynaptic labeling • Can be used to identify IDEs or ODEs • Can be retricted to monosynaptic labeling using G deletion • Cell type specificity using EnvA pseudotyping	• Toxicity • Down-regulation of host gene expression	Wickersham et al., 2007 Callaway and Luo, 2015 (review)
TRIO/cTRIO	Retrograde (axon transducing; CAV) and Retrograde (transsynaptic; rabies): Allows three steps of a circuit to be examined	XFP label expressed by virus	✔	✔ (cTRIO variant; see References)	✔	✔			demonstrated in mice, likely compatible with other mammalian systems	• Same advantages of rabies (above) • Specification of inputs based on output target, allowing visualization of the relationship between IDEs and ODEs (IODEs)	• Toxicity • Down-regulation of host gene expression	Schwarz et al., 2015 Beier et al., 2015 Lerner et al., 2015
GRASP/mGRASP	Synaptic partners	Split GFP reconstituted at synapses	✔	✔				✔	currently optimized for worms (GRASP) and mammals (mGRASP)	• Synapse visualization from defined partners • Can be used to further characterize the fine structure of IDEs or ODEs	• Possible bias for false positives in synapse detection	Feinberg et al., 2008 Kim et al., 2011
SynView	Synaptic partners	Split GFP reconstituted at synapses	✔	✔				✔	currently optimized for mammals	• Synapse visualization from defined partners • Can be used to further characterize the fine structure of IDEs or ODEs	• Currently limited to examining synaptic contacts initiated by specific adhesion molecules	Tsetsenis et al., 2014
Brainbow	Anterograde (axon tracing)	Stochastic expression of 3 XFPs	✔	✔			✔		widely compatible - currently adapted for worms, flies, fish, mice	• Combination of single cell resolution and dense labeling is possible (up to 100s of colors) • Can be used to identify ODEs	• Imaging is a major challenge (chromatic aberrations, bleaching, etc can make analysis difficult)	Livet et al., 2007 Pan et al., 2011 (fish) Hampel et al., 2011 (flies) Hadjieconomou et al., 2011 (flies) Cai et al., 2013
Electron Microscopy	Ultrastructural cell morphology	HRP/Diaminobenzidine (DAB) reaction, electron-dense membrane contrast agents, and/or heavy metal-conjugated antibody labeling	✔ (can be combined with long-range techniques e.g. FluoroGold, GESEM)	✔ (limited multifeature immunostaining)			✔	✔ (synapses can be identified by morphology and/or limited immunostaining)	widely compatible	• The most complete picture of neuronal morphology and circuit structure is obtained • Can be used to identify or further characterize the fine structure of IDEs or ODEs	• Extensive time and cost, even for imaging very small tissue volumes	Jurrus et al., 2009 Kleinfeld et al., 2011 Ward et al., 1975 Bock et al., 2011 Briggman et al., 2011 Atasoy et al., 2014 (GESEM)

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			Long-range Connections	Cell-type specificity	Trans-synaptic	Mono-synaptic Restricted	Single Cell Resolution	Synapse Visualization				
Functional Magnetic Resonance Imaging (fMRI)	Functional connectivity	BOLD signal (correlation)	(inference by correlation, need not be direct)						Human, non-human primate, rodent	• Whole brain functional connectivity visible in a live subject • Non-invasive, compatible with human studies	• Indirect (non-anatomical) measure of connectivity precludes IODE identification • No cell type specificity	Friston, 2011 (functional and effective connectivity review) Poldrack and Farah, 2015 (recent review of human imaging methods, with a focus on fMRI)
Diffusion Weighted Imaging (DWI)	White matter tract structure	Visualization of water diffusion preferentially along white matter tracts	(inference by diffusion, need not be direct)						Human, non-human primate, rodent	• Whole brain structural pathways visible in a live subject • Non-invasive, compatible with human studies	• Resolution limited to large white matter tracts • No functional information • No cell type specificity	Le Bihan and Johansen-Berg, 2012