

Feature Review

Molecular neuroanatomy: a generation of progress

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The neuroscience research landscape has changed dramatically over the past decade. Specifically, an impressive array of new tools and technologies have been generated, including but not limited to: brain gene expression atlases, genetically encoded proteins to monitor and manipulate neuronal activity, and new methods for imaging and mapping circuits. However, despite these technological advances, several significant challenges must be overcome to enable a better understanding of brain function and to develop cell type-targeted therapeutics to treat brain disorders. This review provides an overview of some of the tools and technologies currently being used to advance the field of molecular neuroanatomy, and also discusses emerging technologies that may enable neuroscientists to address these crucial scientific challenges over the coming decade.

From gene-centric to cell-centric strategies

Progress in neuroscience over the past decade has relied heavily on gene-centric strategies, such as the genetic or pharmacological manipulation of gene function, affecting multiple cell types and tissues in the nervous system. Although progress in the gene-centric realm has been substantial, the fundamental organizing principle of the nervous system is the cell and not the gene. Transmission of information and the generation of behavior are directly determined by cell type and by the connectivity among various cell types. Improved cell-centric strategies, such as those permitting functional manipulation of specific neuronal cell types and circuits, are crucial for understanding the nervous system, and may be essential for both a full mechanistic understanding of important brain disorders and the eventual development of next-generation cell type-targeted therapeutics for these disorders. Furthermore, the development of nanoparticles that are targetable to specific cell types (as defined by molecular phenotype and

neuronal circuit) could enable non-invasive mapping, monitoring, and manipulation of the activity of millions of neurons at the single cell and millisecond resolution, as conceived by projects such as the Brain Activity Map (BAM) [1–3].

This review will describe several of the most important gene-centric technologies and resources that have been developed, and will describe the ways in which they provide a firm foundation for the further development of new and improved cell-centric strategies for analysis of the nervous system in the coming decade. These technologies and resources include gene expression atlases of the brain, gene expression profiling, knockouts and transgenic animals, Cre driver lines, viral vectors, connectivity maps, genetically encoded biosensors and modulators, and molecular phenotype datasets [4]. Cell-specific genetic manipulation has been inhibited by: (i) the limited number of cell type-specific promoters, (ii) the very few genes that are selectively expressed in a given cell type, and (iii) our still limited knowledge of the mechanisms that specify cell type. Emerging single-cell technologies used to profile cell types and synapses in heterogeneous tissues, such as cell-specific barcoding strategies, provide a means to overcome this barrier.

Brain atlases

Eighty percent of the 20 000 genes in the mammalian genome are expressed in the central nervous system [5]. These distinct patterns of gene expression underlie neuronal identity, anatomical boundaries, and the specification of neuronal circuits. Characterization of changes in neuronal gene expression has provided key insights into neural development and the response of the nervous system to the environment and drugs of abuse. The Allen Brain Gene Expression Atlas and the GENSAT atlas were developed with the expectation that gene expression arrayed in either 2D or 3D would identify cell type-specific molecular markers. This would then provide targets to facilitate the delivery of genes and gene products to these various cell types for the analysis of cellular development, connectivity, and function, as well as casting light on the principles by which genes organize the nervous system. Early advances in mouse genome sequencing and in manipulating the mouse genome through transgenesis and homologous recombination led to a strong preference for the mouse over other organisms such as the rat. However, targeted mutation in the rat can now be

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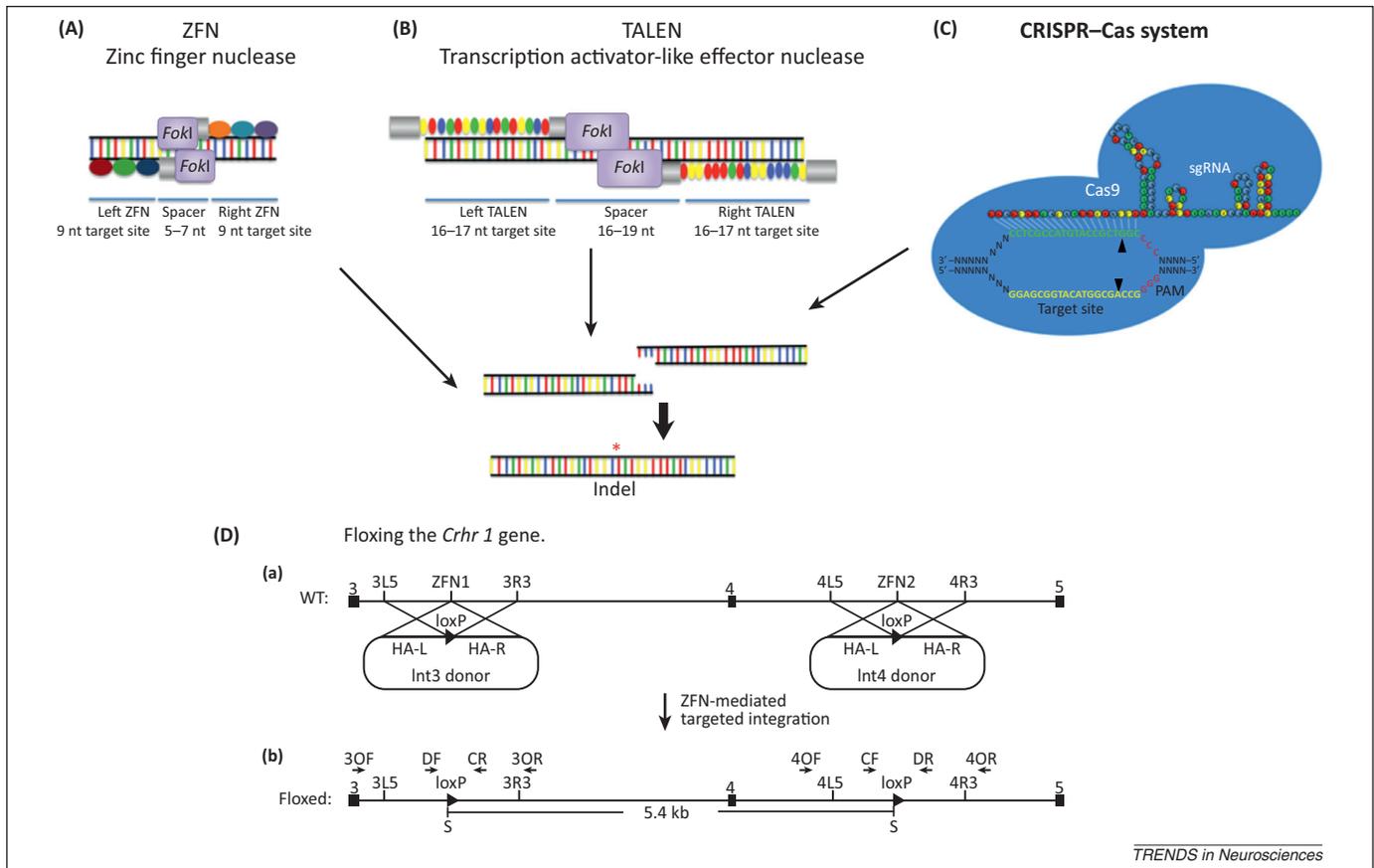


Figure 1. Genome engineering: creating a *Cre/loxP* rat using ZFN (zinc-finger nuclease), TALEN (transcription activator-like effector nuclease), and CRISPR (clusters of regularly interspaced short palindromic repeats) technologies for inducible gene knock-in or knockout. **(A)** ZFN links an engineered DNA-binding zinc-finger (ZF) domain with a DNA-cutting nuclease domain, which contains a *FokI* restriction enzyme site. Each ZF recognizes and binds to three targeted nucleotides. The pairing of left ZFN and right ZFN acts like a pair of genomic scissors to produce a double-strand (ds) DNA incision in the spacer region. The ZF domains are often extended, doubled, or tripled for longer sequence recognition and increased specificity on each side. ZFN-induced chromosomal breaks are then randomly reconnected by endogenous cellular DNA repair mechanisms ('non-homologous end-joining'), leading to gene knockout. Nonetheless, if manipulated DNA strands ending with nucleotides on each side can bind via homologous pairing to the DNA break during ZFN incision, homology-dependent repair will take place, resulting in gene knock-in. Adapted, with permission, from [140]. **(B)** TALENs bind to DNA using transcription activator-like effector (TALE) repeat domains derived from *Xanthomonas* that recognize individual nucleotides. These TALE repeats are ligated together to create binding arrays that recognize extended DNA sequences. The coupled nuclease domain of TALEN cleaves the DNA, in the same fashion as ZFN, within the intervening spacer region. Adapted, with permission, from [140]. **(C)** CRISPRs were first found in the *Escherichia coli* genome and are probably involved in immune defense against foreign DNA. CRISPR loci are surrounded by a cohort of conserved CRISPR-associated genes (*Cas* genes) adjacent to the cluster repeats. The processed CRISPR RNAs (crRNAs) serve as sequence-specific guides, bringing the Cas proteins/nucleases to the target and generating a ds break (DSB) in the DNA. In CRISPR genome editing, the crRNA and the transactivating crRNA (tracrRNA) form a dsRNA structure that directs Cas9 to generate DSBs in the target DNA. At the genomic site complementary to the crRNA, the Cas9 HNH nuclease domain cleaves the complementary strand, and the Cas9 RuvC-like domain cleaves the non-complementary strand. Reprinted, with permission, from [141]. **(D)** Floxing the *Crhr1* gene. In conditional knock-in rats, a plasmid DNA containing *Cre* or *loxP* is used together with the genomic editing methods. The plasmid DNA contains homologous binding sequences to bind to the DSB. Insertion of the plasmid DNA is achieved through a mechanism termed homologous end-joining during DNA repair. This allows *Cre/loxP* to be inserted at desired genomic sites to create inducible mutations and to generate *Cre/loxP* rats for specific neural circuitries in the brain. Figure courtesy of Dr Xiaoxia Cui, Sage Labs, St. Louis, MO, USA.

achieved using transcription activator-like effector nuclease (TALEN), zinc-finger nuclease (ZFN), and clusters of regularly interspaced short palindromic repeat (CRISPR) technologies (Figure 1).

The Allen Mouse Brain Atlas

The Allen Mouse Brain Atlas is a high-resolution 2D and 3D digital atlas of the C57BL/J mouse brain populated by 20 000 transcripts [5–7]. This effort led to the development of high-throughput methods for performing *in situ* hybridization, a standardized coordinate system for displaying mouse brain gene expression, and an informatics framework for data integration and analysis. The standardized coordinate system is particularly important for correlating the expression of multiple genes to infer the number of cell types in a given brain region, delineate anatomical boundaries, and ultimately correlate gene expression patterns

with neuronal connectivity [8]. Using data from the Allen Mouse Brain Atlas, Wolf *et al.* [9] suggest that regional gene expression predicts neuronal connectivity. Recent work by Ko *et al.* [10] suggests that the anatomical boundaries within a mouse brain can be defined by the clustering of only 170 neuron-specific genes (Figure 2). Work by Grange *et al.* [11] also suggests that anatomical boundaries for cortex, hippocampus, striatum, ventral midbrain, medulla, and cerebellum can be delineated based on the cell type density and gene expression profiling for 64 cell types from these different regions. Experiments in which these gene expression patterns are subtracted from each voxel suggest that many more mouse brain cell types may exist. Expression profiling of many additional neuronal cell types is necessary to provide combinatorial gene expression data that can be used to characterize better the anatomical boundaries and cell type-specific organization.

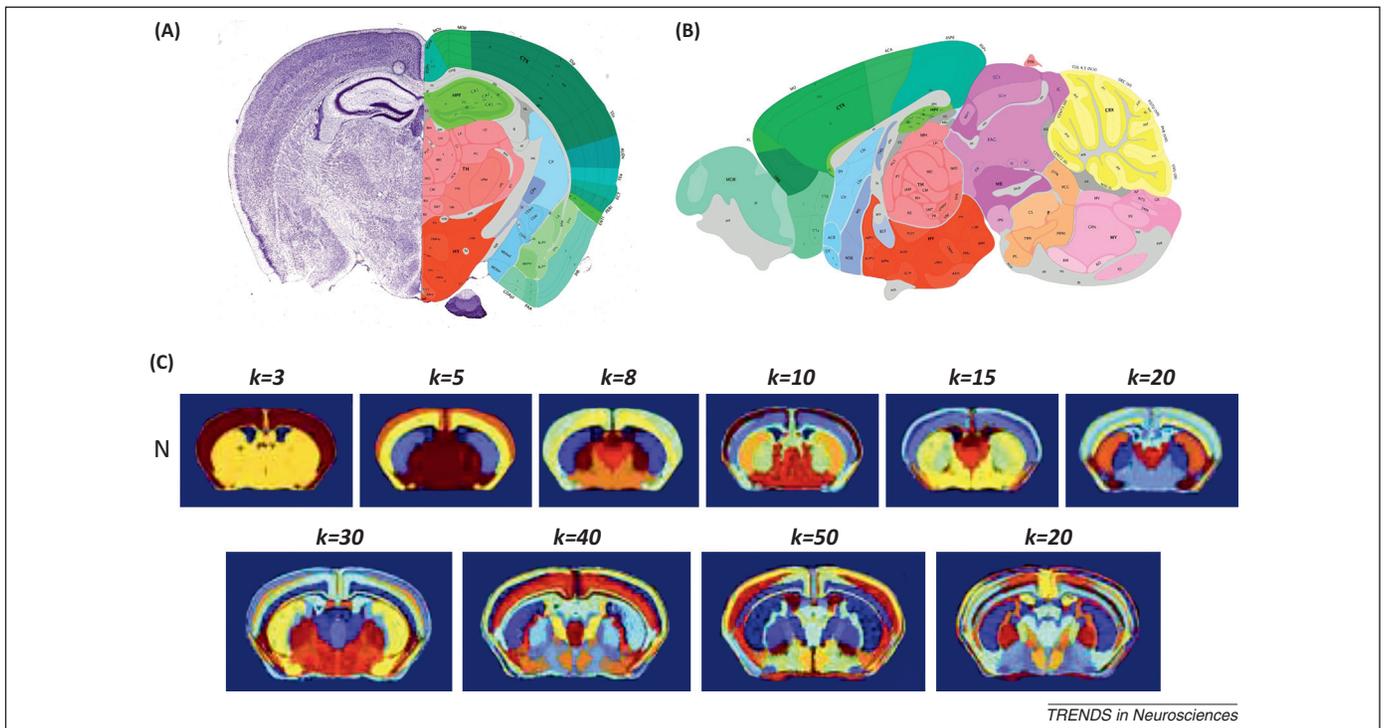


Figure 2. Spatial clustering of neuron-specific transcripts delineates anatomical boundaries in the brain, revealing more than 50 brain regions. The spatial resolution and delineation of brain structures increases as the number of k -mean clusters of neuron-specific transcripts used in the analysis increases. Further refinement of the cell types and their projections may increase anatomical resolution. **(A)** Coronal section from the Allen Brain Reference Atlas. **(B)** Saggital section from the Allen Brain Atlas. **(C)** Spatial cluster analysis of neuron-specific transcripts. Abbreviation: N, neurons. Adapted, with permission, from [10].

GENSAT

GENSAT provides images of gene expression in the adult and developing nervous system of the FVB/N mouse, as demonstrated by transgenic bacterial artificial chromosome (BAC)-expressing green fluorescent protein (GFP) or Cre recombinases [12–14]. In BAC recombineering, EGFP reporter or Cre recombinase coding sequences are placed in frame in the first exon with an in-frame stop codon to prevent overexpression of the protein. Because BACs are usually of large size, they typically include control elements that recapitulate the *in vivo* temporal and spatial expression of that gene. This increases the likelihood that the BAC transgene expression pattern will be congruent with the wild type expression pattern, but position effects are still possible. GENSAT BAC mice are widely used by the scientific community because they enable biochemical and electrophysiological analysis of individual EGFP-expressing neurons and glia. The Cre driver lines provide a method for temporal and spatial control of gene activity or reporter gene expression [15–17]. GENSAT has characterized over 10 000 BAC transgenic founder lines, and 1347 EGFP and Cre founder lines (Figure 3) have been made available to the scientific community through Mutant Mouse Regional Resource Centers (MMRRC) (see Tables 1 and 2 for links to digital atlases and resources for molecular neuroanatomy).

Connectome

The detailed description of neuronal circuitry of the mammalian brain remains highly incomplete, a knowledge gap that was emphasized in an article by Francis Crick and Ted Jones in 1993 [18]. This knowledge gap has led to the

development of projects to map systematically the connections of the nervous system at the macroscopic, mesoscopic, and microscopic levels. Maps at all three levels provide a crucial foundation for understanding brain function and dysfunction [19–22]. To meet this need, the human connectome and mouse projectome projects have begun to characterize neuronal connectivity at the macroscopic and mesoscopic levels. Cell-based connectivity maps based on molecular phenotypes are now beginning to be developed.

Cell-based connectivity is important because the location of cell types and the connectivity between types of cell in the nervous system imposes structural constraints on the functional properties of the nervous system by limiting and directing the flow of information [23]. In support, Varshney *et al.* [24], Sohn *et al.* [25], and Jarell *et al.* [26] have been able to make testable predictions about functional connectivity underlying behavior in *Caenorhabditis elegans* based on electron microscope (EM) reconstruction of the wiring diagram of the *C. elegans* nervous system and computational methods that weight synaptic strength instead of synaptic number. Their analysis is limited by the inability to discern from EM whether a synapse is excitatory or inhibitory and the effect of neuromodulators on these synapses when released from distant sites [27].

The Human Connectome Project

The NIH Neuroscience Blueprint Human Connectome Project is a 5 year project designed to map the brain connectivity of 1200 healthy adults, including 300 twin pairs and their non-twin siblings, to correlate connectivity

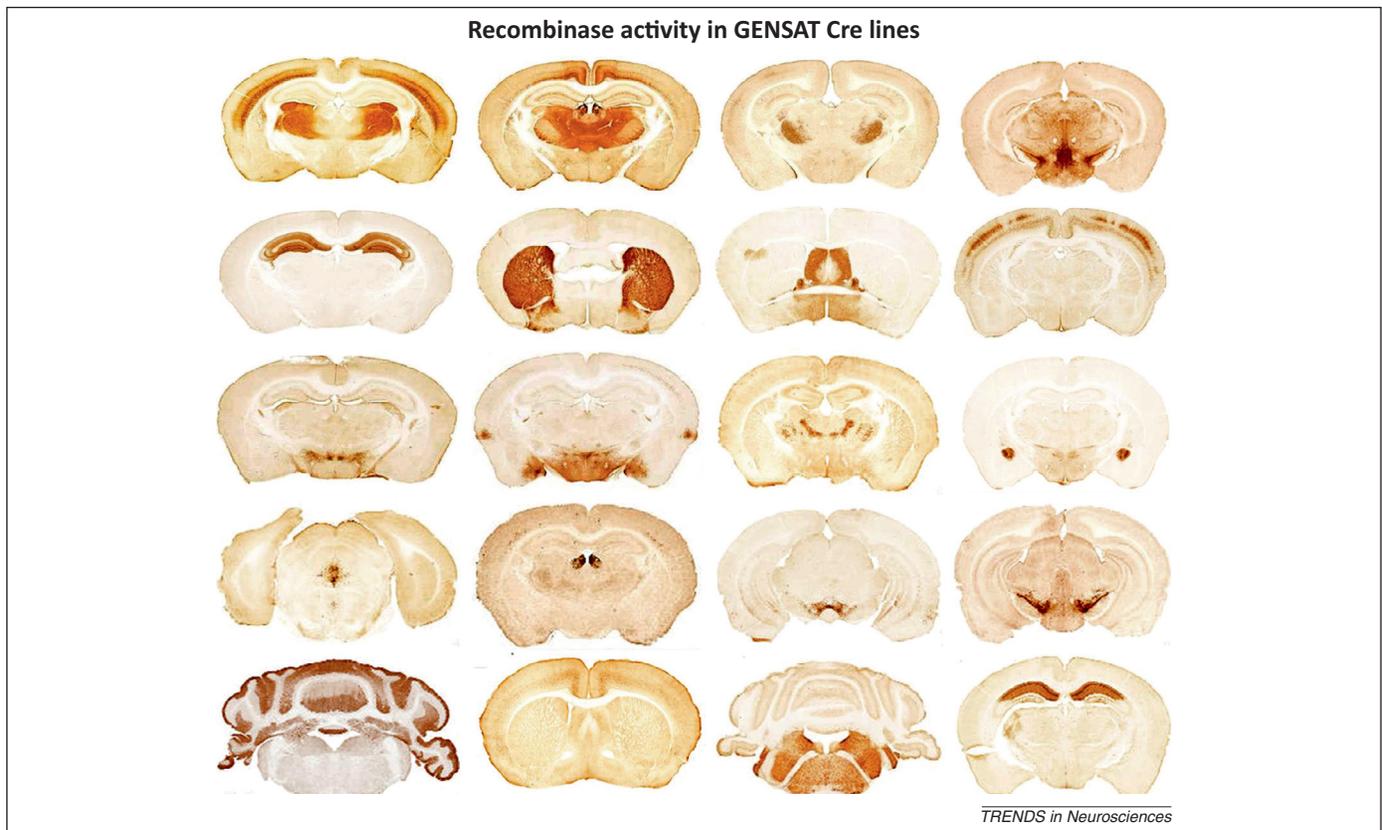


Figure 3. Micrograph of anti-EGFP stained coronal section through the brains of a Cre recombinase BAC transgenic mouse line generated by the GENSAT project. All Cre lines are tested for recombinase activity by breeding with mice harboring a standard *Rosa26-loxP-Stop-loxP-EGFP* reporter. Examples of over 200 other Cre recombinase lines can be viewed on the Gensat website. Mice are available to investigators via the Mutant Mouse Regional Resource Center (MMRRC). Image courtesy of Nathaniel Heintz, Rockefeller University and Charles Gerfen, National Institute of Mental Health (NIMH), USA.

with genetic and behavioral data (Figure 4). The four methods that will be used for mapping structural and functional connections in the human brain include diffusion magnetic resonance imaging (d-MRI), task functional

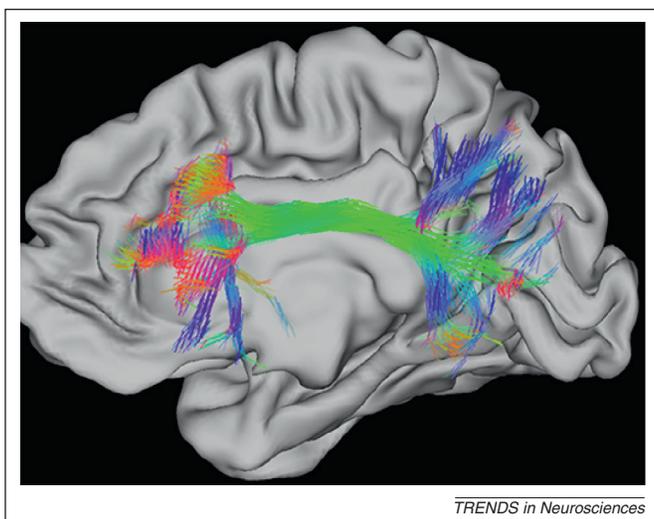


Figure 4. Human connectome. The diffusion of water in the brain is greater along the lengths of axons than it is across axons. Diffusion MRI (dMRI) reveals the directions along which water diffusion is greatest. The probabilistic trajectories from a frontal cortex starting-point that end at other locations on the cortical surface are shown. 3D orientation vectors shown as RGB colored sticks (red, left-right; green, anterior-posterior; blue, inferior-superior) show the estimated directions of white-matter fibers through each 1.25 mm cubic volume of brain (voxel), the resolution of the diffusion MRI data. Intersection of color tracts suggests connectivity. Adapted, with permission, from [142].

MRI (T-fMRI), resting-state fMRI, and T1- and T2-weighted MRI. To compensate for the limited temporal resolution of MRI, electroencephalography (EEG) and magneto-EEG will also be used. The first phase of the project (from 2010 to mid-2012) has performed optimization of hardware and imaging protocols, whereas the second phase (from mid-2012 to 2015) will acquire data from 400 research participants each year. The ultimate goal of the project is to make the data freely available to the scientific community [28,29].

The mouse connectome

Three complementary projects, the Brain Architecture Project, the iConnectome, and the Allen Mouse Brain Connectivity Atlas [30], are aligned with the scientific recommendations of meetings held at the Banbury Conference Center at Cold Spring Harbor Laboratory, USA, from 2006 to 2008. The published recommendations [31] advocated the brain-wide mapping of mesoscopic-level neuronal connectivity across species. The decision to map initially the whole male C57BL/6J mouse connectome at the mesoscopic level will enable the connectome data to be integrated with the Allen Reference Brain Atlas and exploit the genetic resources that exist for the mouse (Figure 5).

In the Brain Architecture and the iConnectome projects, anterograde and retrograde tracers are systematically injected together at 200–250 different sites in a grid-dependent fashion, and micrographs are obtained using

Table 1. Atlases and online resources for neuroanatomical research

Resource	Summary	Website
General resource for brain atlases		
Neuroscience Information Framework	Provides links to many brain atlases	https://www.neuinfo.org/mynif/search.php?q=atlas&t=registry
Mouse brain/CNS atlases		
Allen Mouse Brain Atlas	Reference Atlas of C57BL/6J adult mouse brain and high resolution gene expression atlas	http://mouse.brain-map.org/
Gene Expression Nervous System Atlas (GENSAT)	An NIH-funded, publicly available, gene expression atlas of the developing and adult CNS in mice	http://www.gensat.org/index.html
Whole Brain Catalog	An open source, downloadable, multiscale, virtual catalog of the mouse brain and its cellular constituents	http://wholebraincatalog.org/
Rodent Brain Workbench	A consortium website and portal to atlases, databases, and tools	http://www.rbwb.org/
Waxholm Standard Space	A coordinate-based reference space for the mapping and registration of neuroanatomical data	http://incf.org/programs/atlasing/projects/waxholm-space
GenePaint	A digital atlas of gene expression patterns in the mouse	http://www.genepaint.org/Frameset.html
Mousebrain Gene Expression Map	A database containing gene expression patterns assembled from mouse nervous tissues at 4 time points throughout brain development, including embryonic (E) day E11.5, E15.5, postnatal (P) day P7 and adult P42	http://www.stjudebgem.org/web/mainPage/mainPage.ph
Mouse Spinal Cord Atlas	A gene expression atlas of mouse spinal cord at P4 and P56	http://mousespinal.brain-map.org/
The Mouse Brain Library	High-resolution images and databases of brains from many genetically characterized strains of mice	http://www.mbl.org/
Atlases of the developing mouse		
Allen Brain Developing Mouse Brain Atlas	<i>In situ</i> hybridization of developing mouse brain at E11.5, E13.5, E15.5, E18.5, P4, P14, P56	http://developingmouse.brain-map.org/
Molecular Anatomy of Mouse Embryo Project (MAMEP)	A comprehensive information resource for the functional analysis of pattern formation, tissue development, and organogenesis in mice. Wholemount <i>in situ</i> hybridizations on mid-gestation mouse embryos are used to assign genes to cell types and organs	http://mamep.molgen.mpg.de/
EMBRYS	A database of gene expression patterns mapped in the wholemount mouse embryo (ICR strain) of mid-gestational stages (embryonic days E9.5, E10.5, E11.5), in which the most striking dynamics in pattern formation and organogenesis are observed	http://embrys.jp/embrys/html/MainMenu.html
Eurexpress	A transcriptome Atlas database for mouse embryo	http://www.eurexpress.org/ee/
e-mouse Atlas (EMA and EMAGE)	EMA is a 3D anatomical atlas of mouse embryo development with histology. EMAGE is a database of mouse gene expression which can be mapped into EMA 3D space	http://www.emouseatlas.org/emap/home.html
Gene Expression Database	A collection of gene expression information during mouse development	http://www.informatics.jax.org/expression.shtml
Mouse Phenome Database (MPD)	An NIH-funded database with characterizations of hundreds of strains of laboratory mice to facilitate translational discoveries and to assist in selection of strains for experimental studies	http://phenome.jax.org/db/q?rtn=docs/home
Non-human primate brain atlases		
NIH Blueprint NHP Atlas	A suite of gene expression data, neuroanatomical data, and informatics tools for exploring the cellular and molecular architecture of the developing postnatal rhesus macaque brain.	http://www.blueprintnhpatlas.org/
Rhesus Macaque Brain Atlases	These atlases enable the alignment of individual scans to improve localization and the statistical power of the results, and allow comparison of results between studies and institutions. A set of multi-subject atlas templates is constructed specifically for functional and structural imaging studies of rhesus macaque.	http://brainmap.wisc.edu/monkey.html
Human brain atlases		
Big Brain: An Ultrahigh Resolution 3D Human Brain Model	An ultrahigh resolution 3D human brain model	https://bigbrain.loris.ca/main.php
Allen Human Brain Atlas	A multimodal, multiresolution atlas detailing gene expression across the adult human brain	http://human.brain-map.org/
BrainSpan (Atlas of the Developing Brain)	An atlas describing the transcriptional mechanism of human brain development, which includes RNA sequencing and exon microarray data profiling up to 16 cortical and subcortical structures across the full course	http://www.brainspan.org

Table 1 (Continued)

Resource	Summary	Website
	of human brain development. Such information is referenced to MRI and diffusion weighted imaging (DWI) data	
Multiple species atlases		
Brain Maps	Interactive multiresolution brain atlases based on over 20 million megapixels of sub-micron resolution, annotated, scanned images of serial sections of human, non-human primate, cat, rat, mouse, barn owl, opossum, red jungle fowl, spiny anteaters, platypus and goldfish brains. Brain structures and functions can be quickly queried and retrieved over the internet	http://brainmaps.org/
The Scalable Brain Atlas	A web-based display engine for different atlases created for mouse, macaque, and human	http://scalablebrainatlas.incf.org/main/index.php
Laboratory of Neuroimaging Atlas	Website contains brain atlases for fetal and adult human, Alzheimer's, monkey, vervet, mouse, and rat	http://www.loni.ucla.edu/Atlases/
Adult rat brain atlases		
Adult Wistar Rat Brain	Atlas combines the Paxino Watson atlas with magnetic resonance histology and diffusion tensor imaging	http://www.civm.duhs.duke.edu/neuro2012ratatlas/
Rodent Brain Workbench	A consortium website and portal to atlases, databases, and tools	http://www.rbwb.org/
Other species		
Atlas of the <i>Drosophila</i> Nervous System Armstrong <i>et al.</i> (1995)	Atlas of the adult and developing <i>Drosophila</i> central nervous system	http://flybrain.neurobio.arizona.edu/
Zebrafish Brain Atlas	Curated information and images of neuroanatomical structures in the developing zebrafish	http://zebrafishbrain.org/
Wormatlas	A description of anatomical location and function of 302 neurons in the adult <i>Caenorhabditis elegans</i>	http://www.wormatlas.org/hermaphrodite/nervous/Neuroframeset.html
Zebra Finch Expression Brain Atlas	An NIH-funded online public repository of <i>in situ</i> hybridization images from a large collection of genes expressed in the brain of adult male zebra finches	http://www.zebrafinchatlas.org/
Human connectome		
Human Connectome	A 5 year project sponsored by NIH including two consortia of research institutions to build a 'network map' to better understand the anatomical and functional connectivity in healthy subjects	http://www.humanconnectome.org/ http://www.humanconnectomeproject.org/
Human Brain Connectivity Database	Curated by the Brain Architecture Project, this is a preliminary database of neuroanatomical connectivity reports specifically for the human brain	http://mitraweb1.cshl.edu:8080/BrainArchitecture/pages/publications.faces
Mouse connectome		
Mouse Connectome	An NIH-funded online database of neuroanatomical connectivity in the mouse brain	http://www.mouseconnectome.org/ http://brainarchitecture.org/ http://connectivity.brain-map.org/
Allen Mouse Brain Connectivity Atlas	A cell-based connectivity map of the C57BL/6J mouse brain	http://connectivity.brain-map.org/
Mouse Brain Architecture Project	A connectivity map of the C57BL/6J mouse brain	http://brainarchitecture.org/mouse
Imaging resources		
NITRC	The source for neuroimaging tools and resources	http://www.nitrc.org/

automated wide-field fluorescence microscopy. The inputs and outputs of each brain region are then mapped to a grid based on the Allen Reference Atlas. Both projects will provide a detailed map of axonal projections and are likely to reveal a distributed network map with overlapping circuitry instead of the classic labeled lines. Convergent and divergent projections will be visualized with this approach. This method does not distinguish cell types within a region from which a projection arises. These regions are often composed of heterogeneous cell types, with each cell type possibly having its own unique projection. This method also does not permit connections to be defined directly as excitatory or inhibitory.

To address this issue of cell heterogeneity giving rise to different projections within a given region, the Allen

Mouse Brain Connectivity Atlas further characterizes projections from 300 anatomically defined structures by using genetically defined populations of neurons within these regions that express Cre recombinases. Injection of Cre-dependent recombinant adeno-associated virus (rAAV) expressing a fluorescent protein only labels the anterograde projections of neurons expressing Cre in a given region. Fluorescence images of the rAAV tracer are acquired with serial two-photon (STP) tomography. The limitation of this gene-based method is that cell type is defined by a single gene and its projection. Furthermore, only a limited number of Cre transgenic mice can be generated with cell-specific or region-specific expression. Thus, many cell type-specific projections not defined by a single gene are missed.

Table 2. Enabling technologies and resources

General resource		
Neuroscience Information Framework	A web portal and search engine that links to data and research resources and has the capability of searching data and resources in the hidden web	http://neuinfo.org
Genetic resources for rat and mouse		
Mouse Genome Informatics Database	International database of biological resources for the mouse	www.informatics.jax.org
GeneNetwork	GeneNetwork is a database and open source bioinformatics software resource for systems genetics	www.genenetwork.org
Gene Weaver	Gene Weaver allows users to integrate phenotype centered gene sets across species, tissue and experimental platform	http://geneweaver.org/
Mouse Phenome Database	Collaborative collection of baseline phenotypic data on inbred mouse strains	http://phenome.jax.org
International Mouse Phenotyping Consortium (IMPC)	Phenotype and characterize the function of 20 000 known and predicted mouse genes	http://www.mousephenotype.org/
The Rat Genome Database	Genetic resources for rat	http://rgd.mcw.edu/
Rat Resource and Research Center	An NIH-funded Resource Center to supply biomedical investigators with the rat models, embryonic stem cells, related reagents, and protocols they require for their research	http://www.rrcc.us/
Resources for knockout mice		
International Gene Trap Consortium (IGTC)	IGTC represents all publicly available gene-trap cell lines, which are available on a non-collaborative basis for nominal handling fees	http://www.genetrap.org/
IMPC	A world-wide consortium of institutions dedicated to the production and phenotyping of germ line transmission of targeted knockout mutations in embryonic stem cells for 20 000 known and predicted mouse genes	https://www.mousephenotype.org/
Mutant Mouse Regional Resource Centers (MMRRCs)	Distributes and cryopreserves genetically engineered mouse strains and mouse ES cell lines with potential value for the genetics and biomedical research community	http://www.mmrc.org/about/generalInfo.php
International Mouse Strain Resource (IMSR)	A searchable database of mouse strains, stocks, and mutant ES cells that are available in public repositories	www.findmice.org
Cre-driver and FLP-driver lines		
Cre-driver Network	Database and expression profiles of Cre driver lines, supported by the NIH Blueprint	www.credrivermice.org
The Jackson Laboratory Cre Repository	Provides information about a comprehensive set of well-characterized Cre driver lines and their availability	http://cre.jax.org/index
The Jackson Laboratory FLPe repository	Provides information about available FLP-FRT recombinases in mice and constructs	http://www.jax.org/search/Main.jsp?qt=Research+tools+FLP-FRT+System&sg=0
Cre-X-Mice: A Database of Cre Transgenic Lines	A database of Cre transgenic lines maintained by Andras Nagy at Mount Sinai Hospital, Toronto, Canada	http://nagy.mshri.on.ca/cre_new/index.php
Allen Brain Atlas expression map of Cre and other drivers	Expression maps of transgenic Cre and other driver lines in mice	http://connectivity.brain-map.org/transgenic/search/basic/
Gensat Cre-Mice	Provides tissue-specific expression of Cre recombinases expressed in bacterial artificial chromosomes	http://www.gensat.org/cre.jsp
Full-length cDNAs		
Mammalian Gene Collection (MGC)	Full-length cDNAs for human, mouse, rat, and cow	http://mgc.nci.nih.gov/
The <i>Xenopus</i> Gene Collection	Full-length cDNAs for <i>Xenopus</i>	http://xgc.nci.nih.gov/
The <i>Zebrafish</i> Gene Collection	Full-length cDNAs for Zebrafish	http://zgc.nci.nih.gov/
Viral vectors		
National Gene Vector Biorepository (NGVB)	An NIH-funded Center to address the increasing needs of the research community for access to the use of viral transneuronal tracing tracers. This approach is the most widely used method to gain a circuit perspective on the functional architecture of the nervous system at a cellular level	https://www.ngvbcc.org/Home.action
Tissue-specific enhancers and promoters		
Pleiades Promoter Project:	This resource provides mini-promoters to drive tissue-specific expression	http://www.pleiades.org/
The ENCODE Project	Then ENCyclopedia Of DNA Elements provides information about the functional elements in the genome	www.genome.gov/10005107
Genome browsers		
University of California at Santa Cruz Genome Browser	Provides reference sequences and working draft assemblies for large collection of genomes and access to the ENCODE and Neanderthal projects	http://genome.cse.ucsc.edu/

Table 2 (Continued)

Ensembl Genome Browser	Produces genome database for the eukaryotic and vertebrate organisms	http://www.ensembl.org/index.html
NCBI Genome	This resource organizes information on genomes including sequences, maps, chromosomes, assemblies, and annotations	http://www.ncbi.nlm.nih.gov/genome
Epigenomes		
NIH Roadmap Epigenomics Mapping Consortium	Reference epigenome maps (e.g., DNA methylation, histone modifications, chromatin accessibility, and RNA transcripts) from 'normal' human cells and tissues	http://www.roadmapepigenomics.org/
Epigenomics	Genome-wide maps of DNA and chromatin modifications	http://www.ncbi.nlm.nih.gov/epigenomics/
MicroRNA		
microRNA.org	A website service to predict microRNA targets and target downregulation scores; experimentally observed expression patterns	http://www.microrna.org/microrna/home.do http://www.rnainterference.org/ (for background)
Proteins		
NIF Antibody Registry	Provides a stable, traceable, permanent identifier to all antibody products created by large commercial vendors and individual laboratories	http://www.neuinfo.org/products/antibodyregistry/
Protein Database	Databases of protein sequences and 3D structures of proteins	http://www.ncbi.nlm.nih.gov/protein
Other important research resources		
<i>Drosophila</i> Genomics Resource Center (DGRC)	An NIH-funded center which serves the <i>Drosophila</i> research community to provide services on DNA clones, cell lines, microarrays and the emerging genomics technologies	https://dgrc.cgb.indiana.edu/
Zebrafish International Resource Center	An NIH-funded Resource Center to provide a central repository for wild-type and mutant strains of zebrafish (<i>Danio rerio</i>) and for materials and information about zebrafish research	http://zebrafish.org/zirc/home/guide.php
Model Organisms for Biomedical Research	Provides information about resources for model organisms	http://www.nih.gov/science/models/

The Cre-dependent rAAV virus used by the Allen Brain Mouse Connectivity Atlas is not trans-synaptically transported and thus cellular connectivity cannot be established. This problem can be surmounted by exploiting other viruses. Rabies virus and pseudorabies virus (PRV) are used as retrograde trans-synaptic tracers, whereas the herpes simplex virus type 1 strain H129 virus has been used as an anterograde trans-synaptic tracer [32–35]. The direction of transport appears to be determined by the viral glycoprotein [36]. This directionality is an advantage over classic trans-synaptic tracers such as wheatgerm agglutinin (WGA) which are transported in both anterograde and retrograde directions [35]. Because neurotropic viruses replicate within neurons, the problem of serial dilution faced by conventional trans-synaptic tracers is minimized.

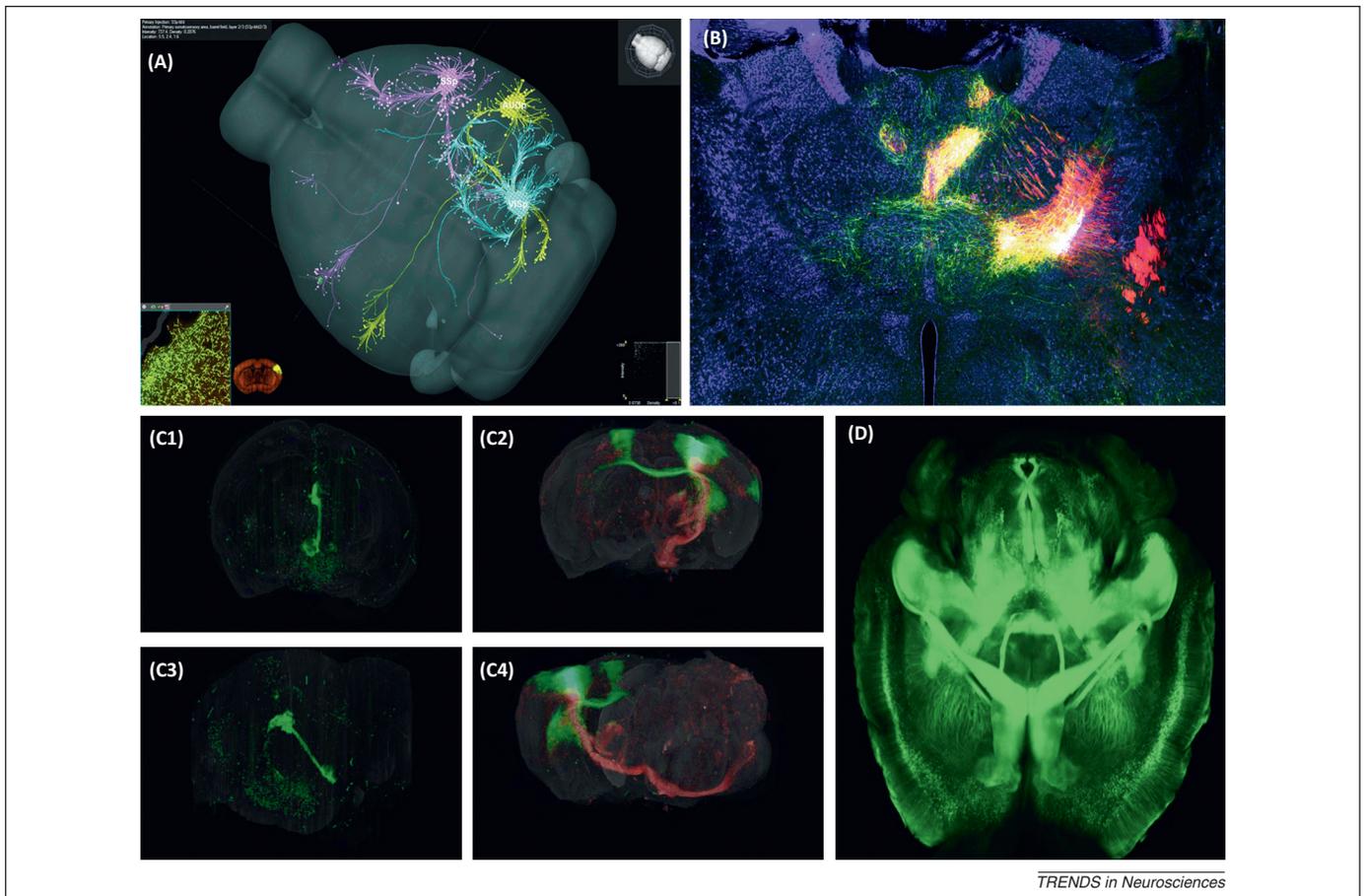
Wickersham *et al.* [37,38] used a complementation strategy to restrict the trans-synaptic spread of rabies virus to monosynaptic connections in genetically defined populations of neurons. This method uses a rabies virus in which the G glycoprotein needed for replication is replaced with EGFP linked to the glycoprotein, EnvA from avian sarcoma and leukosis virus, subgroup A (ASLV-A). This rabies virus can only infect mammalian neurons if its receptor TVA, a protein found in birds but not mammals, is expressed on the cell surface. Expression of the TVA receptor can be restricted to a subpopulation of neurons by placing TVA under the control of an inducible promoter. Thus, cells expressing TVA replicate the virus and presynaptically infect a neighboring cell. The presynaptic cell

not expressing TVA renders the virus replication-incompetent, preventing further spread to adjacent neurons, thereby labeling only monosynaptic connections.

Integration of the connectivity datasets described above with super-resolution array tomography [39,40] (Box 1) and other data in the Whole Brain Catalog will enhance the mesoscopic connectivity data in these atlases by combining cellular connectivity data and receptor types, thereby enabling the determination of whether major projections are excitatory or inhibitory. The Whole Brain Catalog utilizes Waxholm space [41], and this allows translation across the different coordinate systems used by different atlases, thus rendering the datasets interoperable. The Whole Brain Catalog is an open-source 3D atlas of the mouse brain that allows linkage and integration of information and data from several sources, including the Cell Centered Database (CCDB) [42] and other sources cataloged in the Neuroscience Information framework [43].

Reconstruction of microcircuits

Reconstruction of microcircuits has been based on automated serial block-face EM with semi-automated image reconstruction [44], serial section electron tomography [45], and super-resolution array tomography [39,46]. EM techniques recognize cell types based on morphology, whereas super-resolution array tomography uses protein expression based on antibody detection and morphology. The development of a genetically encoded tag based on the *Arabidopsis* fluorescent protein phototropic2, termed miniSOG (mini singlet oxygen generator), now permits



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Figure 5. Mouse connectome. **(A)** Three adeno-associated virus (AAV)–GFP tracer injections into the mouse primary somatosensory (SSp), the primary auditory (AUDp), or the primary visual (VISp) cortical area, respectively, are co-displayed in a common 3D mouse brain model in the Brain Explorer program. Courtesy of Hongkui Zeng, Allen Brain Institute, USA. **(B)** A representative digital image of the iConnectome project (www.MouseConnectome.org) showing multiple fluorescently labeled neuronal pathways through one coronal thalamic level of the mouse brain, after multiple fluorescent tracers were injected into the infralimbic (*Phaseolous vulgaris leucoagglutinin*, PHAL; and CTb, cholera toxin B subunit) and prelimbic (AAV and CTb) cortical areas. Green axons are labeled with PHAL; red axons are labeled with AAV; magenta neurons are labeled with CTb. The yellow regions also contain fluorogold-labeled neurons, which are mostly intermixed with AAV axons. This approach effectively reveals two sets of afferents and two sets of efferent pathways that are associated with two anterograde/retrograde tracer coinjection sites within the same brain, and simultaneously exposes reciprocal connections. Courtesy of Hongwei Dong, University of Southern California, USA. **(C)** The 3D reconstruction of two whole mouse brains injected in the left hemisphere with fluorescent virus tracers to visualize specific sets of connectivity. Top, frontal view; bottom, left lateral view. (C1/3) GFP-labeled modified ‘retrograde’ rabies virus injected into the medial nucleus of the habenula (mH) showing retrogradely labeled cell bodies in the hypothalamus and frontal cortex, as well as the passively filled output projection from the mH (fasciculus retroflexus). (C2/4) Double injection of primary motor cortex (MOp) with modified ‘anterograde’ AAV2/1 harboring a CAG promoter (hybrid CMV enhancer coupled to a modified chicken β -actin promoter) injection to highlight axons of efferent projections by supragranular neurons (injection with GFP–AAV), and infragranular neurons (injection with RFP–AAV). Courtesy of Partha Mitra, Cold Spring Harbor Laboratory, USA. **(D)** CLARITY (clear, lipid-exchanged, anatomically rigid, imaging/immunostaining compatible, tissue hydrogel) is a newly developed technology to transform intact and opaque biological tissue into a hybrid form of formaldehyde-fixed tissue and hydrogel monomers which is physically stable and permeable to exogenous macromolecules. Because the hybrid is permeable to exogenous macromolecules, the whole tissue can be uniformly stained with antibodies to reveal specific cell types and allows multi-round molecular phenotyping of an intact brain. Adapted, with permission, from Chung and Deisseroth [143]. Abbreviations: A, the promoter, the first exon and the first intron of chicken beta actin gene; C, cytomegalovirus (CMV) early enhancer element; G: the splice acceptor of the rabbit beta-globin gene.

long-distance serial EM reconstruction of neural connections of cells expressing this genetic tag [47]. Exposure of miniSOG to blue light generates singlet oxygen that polymerizes diaminobenzidine, permitting tissues to be stained with osmium for EM visualization. Improved correlation of images obtained with fluorescent microscopy and EM can be obtained because the same tag is used, thereby enabling direct association of structure with cellular physiology using genetically encoded sensors in the same cell.

Genetically encoded biosensors and activators

Electrophysiological recording from identified neurons in a simple model organism such as *Aplysia* has contributed greatly to the functional analysis of neural circuits and to the understanding of the cellular basis of behavior.

Systems with identified neurons can permit assignment of function to a given cell type within a neuronal circuit and can enable analysis of structure–function relationships. Tissues tagged with genetically encoded biosensors (GEBs) and genetically encoded activators (GEAs) are beginning to allow functional dissection of specific tissues and brain regions composed of highly heterogeneous cell types. Neuronal tissues can be tagged via transgenes, through infection with viral vectors, and/or by electroporation of embryos. In the case of transgenes, cellular specificity is dependent on the *cis* regulatory elements driving expression of the GEB, GEA, or the recombinase used to activate the GEB/GEA. Attempts to use split recombinases (e.g., split Cre) [48,49] may increase cellular selectivity, but the expression of two genes may not be sufficient to tag a cell type uniquely. Selective expression of GEBs/

Box 1. High-resolution microscopic methods for mapping neuronal circuits

Super-resolution microscopy

Advances in light microscopy and high-throughput EM have significantly improved our ability to analyze the structure of the nervous system at the microscopic level. The limit of light-microscopic resolution for subcellular structures and organelles when diffraction reaches half the wavelength of light (150–300 nm) is now overcome with super-resolution fluorescent microscopy that provides 2–10-fold the resolution of conventional light microscopes [144,145]. These methods include stochastic optical reconstruction microscopy (STORM), stimulated emission depletion microscopy (STED), saturated-illumination microscopy (SSIM), photoactivated localization microscopy (PALM), and fluorescent photoactivation localization microscopy (FPALM).

Array tomography combined with super-resolution microscopy

Dani and colleagues [146], using STORM to image proteins at the synapse, were able to show the 3D relationship among many synaptic proteins using immunolabeling with three spectrally-distinct activated dye pairs. STORM only produces high-resolution 3D images at tissue surfaces because antibody penetration and optical restrictions in thick tissue limit the resolution at deeper depths. By integrating array tomography and automated serial section/volume reconstruction imaging with super-resolution fluorescent microscopy such as STORM, the problems with variable antibody penetration and optical aberrations can be resolved (because ultrathin sections can be used). In the technique of array tomography [39,40], tissue is imbedded in acrylic resin and serially sliced into a stack of ultrathin sections 50–200 nm in width. These sections can be repeatedly stained with, and stripped of, 20 or more immunofluorescent antibodies for repeated imaging of 20 or more antigens, before final staining with heavy metals for EM imaging. The stack of images is placed in register to produce high-resolution large volumetric 3D images. Throughput is decreased when super-resolution imaging is used in array tomography, and therefore experimental design requires evaluations of the trade-offs between resolution and throughput needed to resolve the cellular and molecular structures of synapses, neurons, and circuits.

Automated sectioning, image acquisition, and 3D reconstruction Despite automated methods to collect images [147,148], months to years are now required to reconstruct the synaptic connections at the microscopic level in a cubic millimeter of nervous tissue [149]. Development of improved algorithms for segmentation of images and new computational methods will automate image analysis and increase throughput [150,151]. Toward this goal Helmstaedter [152] has developed KNOSSOS software for visualization and annotation of imaged sections that is 50-fold faster than volume labeling. Instead of standard volume labeling, point-to-point lines along the length of a neurite or skeletons are created. Multiple human annotators create skeletons for the same neurite. The redundant-skeleton consensus procedure (RESCOUP), a statistical method, is used to resolve discrepancies among annotators.

GEAs using viral vectors and plasmids is crucially dependent upon tissue-specific promoters.

GEs encode a fluorescent or bioluminescent protein linked with a functional sensing or target-binding sensor. The fluorescent proteins used are often modified GFP derivatives or analogous proteins from marine organisms, whereas the bioluminescent proteins (e.g., aequorin or luciferase) are typically enzymes that emit light upon oxidizing substrates such as coelenterazines [50]. Fluorescent protein GEs depend either on a single fluorescent protein, on a biomolecular fluorescent complementation strategy (BiFC), on a double dimer fluorescent strategy (ddFP) [51], or on fluorescent energy transfer (FRET). The functional or target-detecting component of the GE is a linked protein that may bind to ions (e.g., Ca^{2+} , Zn^{2+} , Cl^- ,

and H^+), small molecules (e.g., cAMP or hydrogen peroxide), or enzymes (e.g., myosin light chain kinase) [52]. Thus, biochemical, metabolic, and physiological changes occurring within cells can now be visualized.

Genetically encoded calcium indicators (GECIs)

The most widely used sensors of neuronal activity are genetically encoded calcium indicators based on GFP. GCaMP5 and GCaMP6, which can sense calcium influx, are currently the most sensitive of these detectors [53,54]. New genetically encoded calcium indicators with different emission wavelengths have been created, enabling multi-color imaging of different cell types and organelles. Overcoming the problem of spectral overlap between genetically encoded calcium indicators (GECI) and optogenetic activators allows the simultaneous use of blue-activated channel rhodopsin, to stimulate neuronal activity, with a red-shifted GECI to record neuronal activity [55]. GECIs have been used to map the sequential activation of neurons in the parietal cortex during a memory task [56]. GECIs, combined with high-speed light-sheet microscopy, have permitted whole-brain functional imaging in larval zebrafish; activity was determined for 80% of neurons at single-cell resolution [57,58].

Genetically encoded voltage sensors

Imaging of calcium transients is an indirect measure of neuronal activity, and does not provide information about hyperpolarization and sub-threshold events. Several versions of genetically encoded voltage sensors have been developed [59–61]. These include Flash, SPARC, FLARE, VSFP, and Arch3 (Arch) [62–65]. These voltage sensors, except for Arch, have time constants greater than 1 ms, making temporal resolution of action potentials difficult. Moreover, Arch suffers from low brightness. A major advance for the field will be the development of voltage sensors that have time constants of less than 1 millisecond, a large change in fluorescence in relationship to baseline ($\Delta F/F$) with a signal-to-noise ratio (SNR) greater than 2, some detectable fluorescence at the resting potential, a peak molecular brightness equivalent to EGFP, limited bleaching, a capacitance change of <1%, the ability to be targeted to the soma or dendrites, and that are non-toxic as well as being genetically encodable [60,61].

Genetically encoded sensors of neurotransmitter release

pHluorins are pH-sensitive fluorescent proteins used as genetically encoded reporters to measure synaptic release. pHluorins can be targeted to synaptic vesicles by fusing pHluorin to synaptic vesicle proteins such as SNARE, synaptophysin, or the vesicular glutamate transporter. The acidic environments inside synaptic vesicles quench fluorescence, whereas exocytosis exposes the lumen of the vesicle to neutral pH, which reduces quenching and increasing fluorescence [66]. The development of mOrange2-pHluorins tagged to VGLUT1 together with a green calcium reporter GCaMP3 fused with synaptophysin-GaMP3 (SyGCaMP3) permits analysis of vesicle release as a function of calcium concentration at a single synapse [67]. In addition, glutamate-sensitive fluorescent reporters

have also been developed to visualize the release and diffusion of glutamate at the synapse [68].

Genetically encoded activators and inhibitors manipulate neuronal activity

Optogenetic methods

Over the past 10 years a large number of genetically encoded proteins that affect the electrophysiological characteristics of neurons and that are activated by light or controlled by small molecules have been developed to manipulate the activity of neurons. Lasers, mercury arc lamps, and light-emitting diodes are used to activate optogenetic probes [69]. Three different optogenetic methods have been used to control membrane potential [70]. When exposed to blue light, neuron-expressed channelrhodopsin depolarizes neurons by activating a non-selective cation ion-channel. In response to green-yellow light, neurons expressing halorhodopsin hyperpolarize by eliciting an inward chloride current. Suppression of neuronal activity

can also be achieved with archaerhodopsin which, when stimulated with green-yellow light, generates an outward proton current that produces hyperpolarization (Figure 6).

The utility of optogenetic methods and genetically encoded reporters to monitor and control brain activity *in vivo* is limited by the need to use invasive methods to stimulate the optogenetic tool or visualize light being emitted by a genetically encoded reporter. This is because the opaque nature of nervous tissue scatters light. The depth of fluorescent imaging by two-photon microscopy, which images to a maximum depth of 1000 μM , exceeds that of laser confocal microscopy [71]. This depth can be extended by penetrating tissue with a fluorescent micro-endoscope with gradient refractive index (GRIN) microlenses [72]; these also permit recording in alive and awake animals. Activation of neurons in awake behaving animal via light-stimulation of genetically encoded proteins in deep brain structures requires fiber optic probes with light-emitting diode arrays to be inserted deeply in the

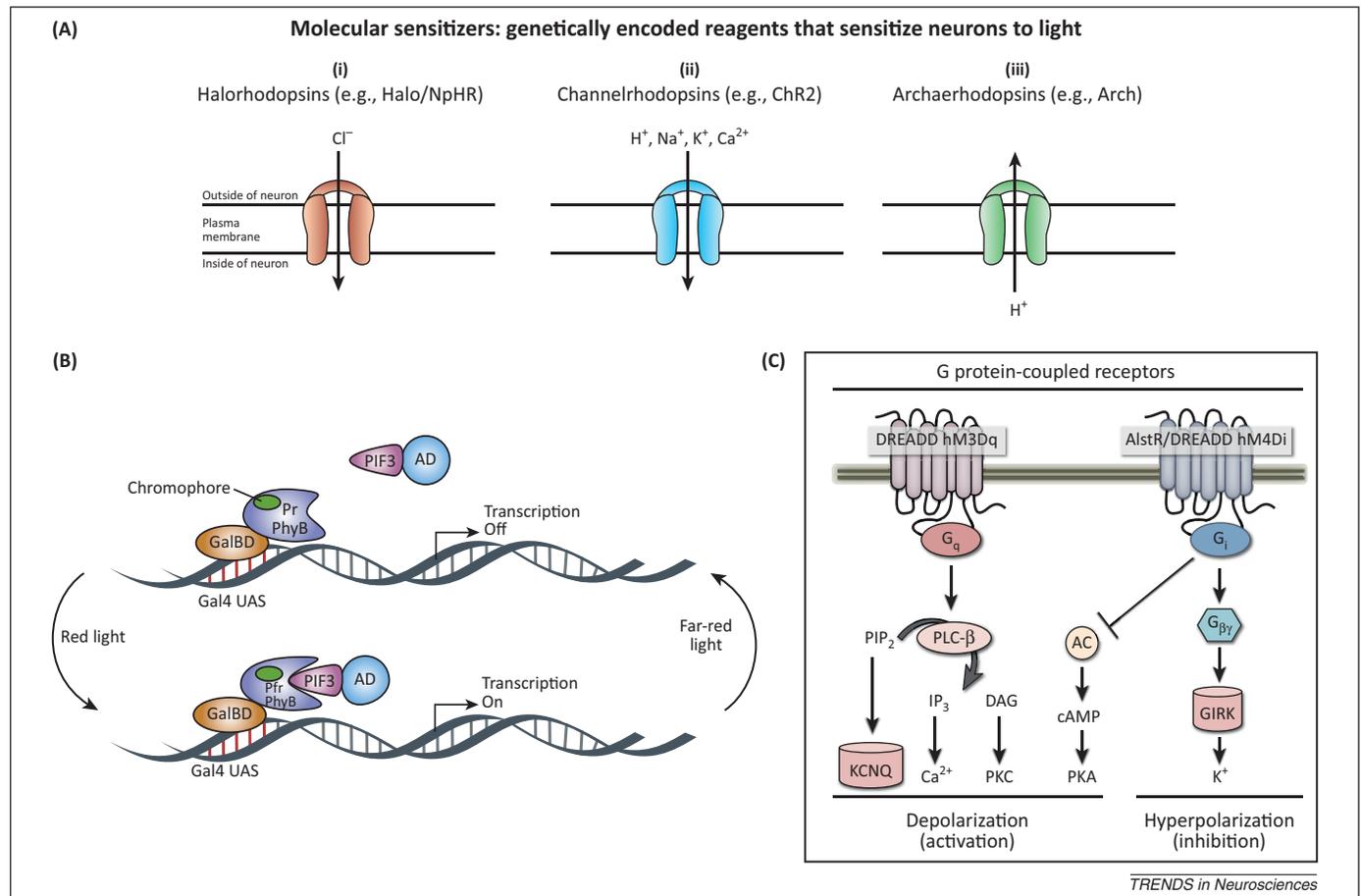


Figure 6. Genetically encoded biosensors. **(A)** Some genetically encoded biosensors have been used as molecular sensitizers to manipulate neuronal activities. Neurons expressing them can be activated or silenced by light. (i) Halorhodopsins, such as *N. pharaonis* halorhodopsin, pump chloride ions inward and hyperpolarize the neuron on yellow light illumination. (ii) Channel rhodopsins are inward nonspecific cation channels for H^+ , Na^+ , K^+ , and Ca^{2+} . They respond to brief, millisecond-timescale pulses of blue light, transporting cations inward and depolarizing the neuron. (iii) Stimulation of Archaerhodopsins (*H. sodomense* opsin) with yellow or green light shuts down neuronal activity by hyperpolarizing neurons. Adapted, with permission, from [70]. **(B)** Biosensors can be designed to control protein-protein interactions. Here red light triggers PhyB binding to PIF3. Because PhyB is fused to a Gal4-binding domain (GalBD), and PIF3 is fused to a Gal4 activation domain (Gal4AD), binding of PhyB to PIF3 in turn dimerizes GalBD with Gal4AD, and initiates gene expression. Remarkably, in the dark or under far-red illumination, PhyB reverts to the non-binding conformation and releases PIF3, shutting down transcription. In this way, light can either be used to induce or shut down transcription. Adapted, with permission, from [153]. **(C)** Biosensors are effective tools to manipulate neuronal activity rapidly. DREADDs (designer receptors exclusively activated by designer drugs) engineered from muscarinic acetylcholine receptors (mAChRs) can be potentially activated by the pharmacologically inert molecule clozapine-*N*-oxide (CNO). When coupled to G_q , G_i , or G_s the G protein-coupled receptor (GPCR) signaling pathways can be manipulated without obvious interference with endogenous GPCR signaling. In this figure the presence of CNO selectively activates (G_q -coupled hm3Dq) or inhibits (G_i -coupled hm4Di) G-protein signaling. The response is robust and reversible for the study of neuronal activity. Activation of G_q signaling leads to neuronal depolarization and increased activity. By contrast, CNO activation of G_i signaling triggers inwardly rectifying potassium channels (GIRKs), resulting in hyperpolarization and inhibition of the neuron.

brain to reduce light-scattering [73,74]. An advantage of the optogenetic method is that it permits the brain-wide mapping of activity generated by stimulating neurons (potentially of identified type) in the region near the stimulating optical fiber. This has permitted the development of optogenetic fMRI [75,76] which, for example, promises to help disentangle neuronal circuitry involved in deep brain stimulation (DBS) therapy of neurological and neuropsychiatric disease. Furthermore, recent advances may enable locus specific and/or temporal manipulation of gene expression levels in neurons through targeted alteration of transcription factor binding or chromatin state via optogenetic or chemo-epigenetic methods [77–79].

Chemogenetic methods

An alternative to the optogenetic approach is to express genetically engineered receptors that respond to a ligand without activating endogenous receptors. These approaches include the expression of TRPV1 and P2X activated by photocaged ligands [80], activation of a TRP (transient receptor potential) channel coupled to transferrin by a pulsing magnetic field [81], *C. elegans* glutamate-gated chloride channel activated by ivermectin [82], the *Drosophila* allatostatin receptor [83], and DREADDs (designer receptors exclusively activated by designer drugs) [84,85]. The discovery of Lynx, a family of endogenous peptides with homology to nicotinic acetylcholine receptor-blocking snake toxins, has led to the development of tethered toxins to permit long-term activation or inactivation of a neural circuit [86].

Molecular phenotyping of brain cell and tissue types

The transgenic technologies described have significant therapeutic potential but their translational utility will remain limited unless transgenes can be systemically delivered and expressed in specific cell types and circuits to correct circuit abnormalities that underlie brain diseases. Tools and technologies enabling researchers to restrict the expression of a transgene to any specific cell type of interest would greatly enhance our ability to investigate nervous system circuitry and mechanisms. However, it has become apparent that no single gene expressed in the nervous system defines a cellular phenotype. For example, work by the Rubin lab suggests that multiple enhancers are required to drive cell specific expression of a transcriptional activator such as GAL4 in *Drosophila* [80,81].

To begin to understand the genome-wide molecular characteristics and enhancer elements for specific cell types, scientific consortia such as the NIH Roadmap Epigenomics Program and the National Human Genome Research Institute (NHGRI)-funded Encyclopedia of DNA elements (ENCODE) project have been generating molecular phenotype datasets for a variety of *Drosophila*, *C. elegans*, mouse, and human cell and tissue types [87–90]. These datasets can be used to identify molecular signatures and enhancer elements selective for specific cell types. In particular, the identification of unique cell-specific enhancer elements may facilitate our ability to express transgenes spatially and temporally with much greater specificity.

The NIH Roadmap Epigenomics Program has been generating comprehensive maps of chromatin from a wide variety of ‘normal’ human cell and tissue types, including components of the nervous system from post-mortem adult and fetal tissue [91]. These maps typically include DNA methylation information, chromatin immunoprecipitation data for several informative activating or silencing histone modifications, chromatin accessibility information using the DNase I hypersensitivity assay, and gene expression information. A major discovery made using data generated by the NIH Roadmap Epigenomics Program is the finding that GWAS variants are highly enriched in regulatory DNA regions and often have activity during fetal development [92].

Although the transcriptional profiles of several cell or tissue types have been characterized and published [93–102], no systematic analysis of the transcription profiles of nervous system cell types has yet been reported. Several different approaches have been used to profile transcriptionally individual cell types in the nervous system. These methods include immunopanning (PAN), laser capture microscopy (LCM), fluorescent activated cell sorting (FACS), manual sorting of fluorescently labeled cells (manual) and translating ribosome affinity purification (TRAP), and Ribo-tag. LCM and manual sorting of fluorescently labeled cells are limited by throughput [103,97]. FACS for isolating cells [104,105] and PAN can introduce artifactual gene expression changes due to cellular stress [106]. These problems quantitating translated mRNAs are overcome with genetic methods of tagging of ribosomes using BACs/TRAP [107] or the induction of the floxed ribosomal tag (Ribo-Tag) with a ‘cell-specific Cre’ [108].

The BAC-TRAP bacterial artificial chromosome translating ribosome affinity purification method dynamically measures translated mRNAs in genetically identified cells, thereby providing molecular phenotypic information for cell types in the nervous system [107,109]. In this method the L10 ribosomal subunit is inserted into the first exon of a gene tagged with EGFP within a BAC. Polysomes are affinity purified using an antibody against EGFP, and the mRNAs are profiled using microarrays or RNAseq. Background transcriptional contamination from other cell types is removed by subtracting the unbound RNA isolated from the tissue from the RNA isolated from the bound polysomes or from a BAC-TRAP that is ubiquitously expressed. This method provides a quantitative measure of gene expression, unlike the qualitative measurements made with EGFP BACs or *in situ* data which provide a static representation of gene expression. Already this method has identified and characterized several specific cell types on the basis of molecular phenotype [110–113].

Ribo-tag uses a gene targeting approach in which the ribosomal gene, *Rpl22*, is floxed with a hemagglutinin (HA) epitope tag inserted before the stop codon. Crossing the Ribo-Tag mouse with a Cre tissue-specific driver induces the expression of the epitope-tagged *Rpl22*. Epitope-tagged Rpl22 incorporated into ribosomes is used to immunoprecipitate ribosome-specific transcripts from specific tissues [108].

For profiling miRNAs, floxed Ago2 tagged with GFP and Myc (miRAP) has also been used to identify miRNAs in glutamatergic and GABAergic cells in the cortex and cerebellum [114]. Tissue-specific Cre drives expression of the *Ago2* transgene in a specific neuronal region, and the Myc tag permits immunoprecipitation of the miRNA–Ago2 complex, followed by deep sequencing.

The determination of cell-specific expression by TRAP, Ribo-Tag, and miRAP is limited by either BACs that tag specific cell types or the availability of cell-specific promoters. Microfluidic and lab-on-chip technology may offer a solution to an unbiased analysis of gene expression, epigenetic marks, protein expression, and metabolomics of single cells in a heterogeneous population of cell types [115,116]. However, dissociation of neural tissue disrupts the ability to correlate cell type and cellular physiology with cell type connectivity, as well as introducing artifacts. The ability to analyze changes in cellular connectivity with cellular epigenetic changes in individual neurons at a network scale is essential for understanding the mechanisms of synaptic plasticity and learning, a

major goal of neuroscience, as well as for determining the importance of structural connectivity in influencing function.

Using barcodes to inventory neuronal cell types and connections

Correlation of cell type with cell type-specific connectivity in the nervous system is ultimately an inventory problem. In commerce, optical barcodes have been used for decades to handle inventory problems. Cellular barcodes based on DNA sequences have recently been used to track hematopoietic cell lineages [117–122]. The development of robust cell-specific DNA barcode technology that uniquely labels ribosomes could provide a means to compare the gene expression profiles of a specific cell with other cells. This unique tag could also label proteins at the synapse, making possible the identification of connections between cells. Brainbow provides a partial solution to this inventory problem [123,124]. In Brainbow, different genes encoding different fluorescent proteins are placed between *loxP* sites (Figure 7). A palette of differently colored neurons are

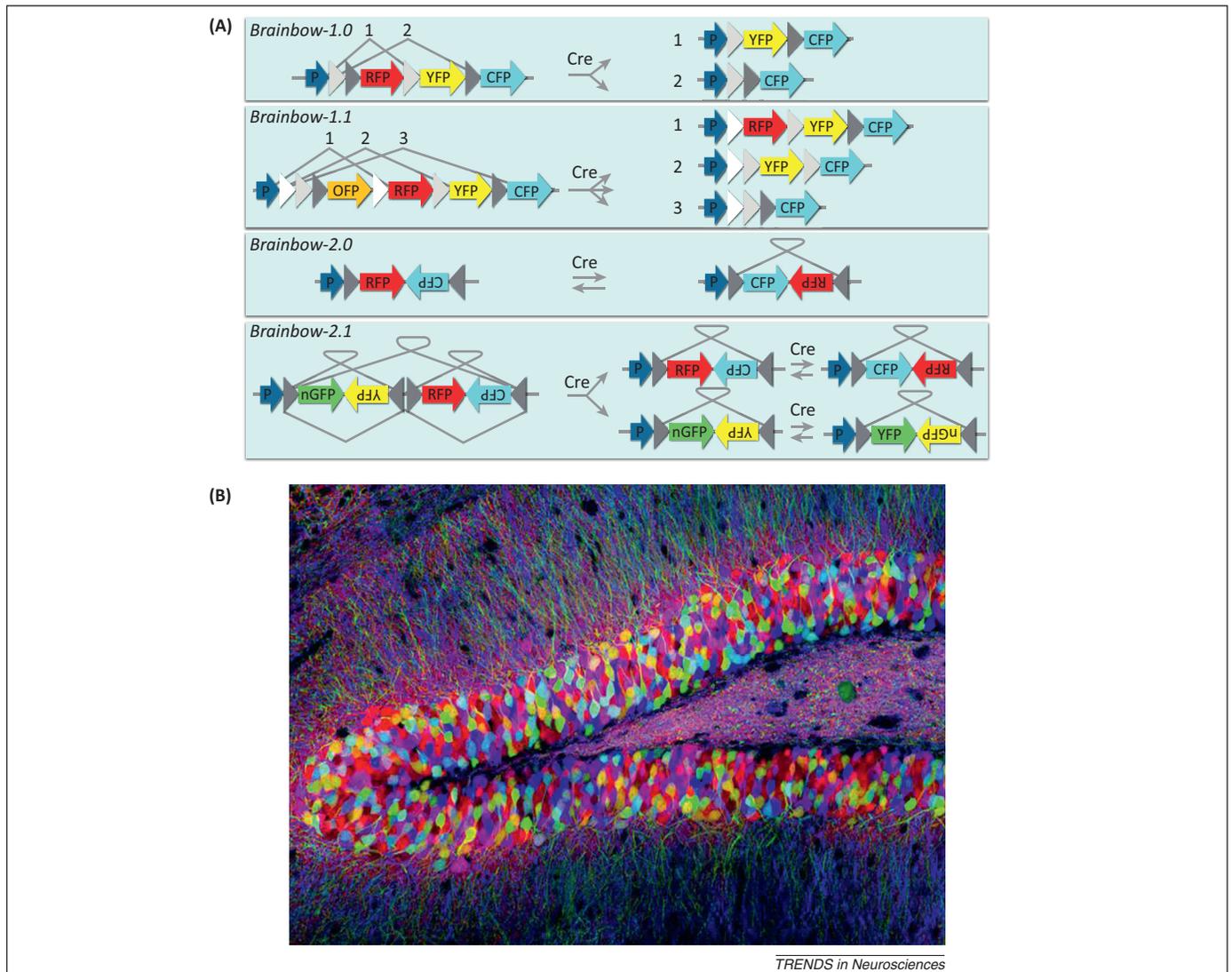


Figure 7. Brainbow. (A) Strategy for creating Brainbow mice. In Brainbow 1.0 and Brainbow 1.1 excision of different fluorophores from incompatible *loxP* variants generates 2–3 different color combinations of XFP. In version 2.0 the XFP genes are placed head-to-head between two asymmetric *loxP* sites. Activation of recombination by Cre causes inversion and a change in the expression of the fluorophore from red to cyan. Placement of the inverted fluorophore genes between tandem asymmetric *loxP* sites increases the number of combinations to four. (B) Fluorescent fluorophore expression in mouse hippocampus. Adapted, with permission, from [126].

generated when Cre acting on the *loxP* sites causes random rearrangements and deletions of the fluorophores. However, given the number of cells and synapses, the number of fluorophores and their dynamic range limit the utility of using Brainbow.

As suggested by Zador *et al.* [125], a solution to the connectivity problem could be to tag cells with viruses expressing DNA barcodes and profile the DNA barcodes with NextGen sequencing. In this scheme each neuron is infected with a trans-synaptic virus with a unique sequence generated by a recombinase (Figure 8). Invader viruses would be ligated together with host neuronal viral sequence by phiC31 integrase and sequenced to identify synaptically connected cells. In the future, neurons tagged with DNA barcodes may enable molecular profiling of single cells with RNA-seq as well as the identification of synaptic connections.

The use of DNA barcodes overcomes the lack of dynamic range of fluorophores used in Brainbow because there are far more barcodes available than there are fluorophores [126]. For example, 10^{15} barcode combinations can be generated with 25 nucleotides (4^{25}), more than enough to label either 6.3×10^7 neurons and 5×10^7 non-neuronal

cells in the mouse brain [127,128], or the 8.6×10^{10} neurons and 8.5×10^{10} non-neuronal cells in the human brain, and sufficient to label 8.8×10^{11} synapses in the mouse brain [129]. The problem of inventorying neuronal identity and connectivity is then essentially transformed into a more tractable high-throughput DNA sequencing problem. Our current ability to generate and analyze large amounts of DNA sequence at low cost is extremely robust.

The barcode invader method described by Zador may be limited by the problem of infection of host neuron with multiple viruses, although statistical methods may help overcome this problem. In addition, this method does not provide a way to correlate cell type (as defined by transcription profile) with connectivity. Nor does it allow quantification of changes in gene expression or epigenetic changes with changes in synaptic strength.

The development of new methods to tag ribosomal subunits and synaptic proteins with cell-specific nucleic acid barcodes using ZF DNA- or RNA-binding proteins [130] may provide a method to correlate cell type with connectivity, and label specific cells unambiguously (Figure 9). Isolation of synaptic adhesion molecules from pre- and postsynaptic neurons that bind to one another, each tagged with a cell-specific barcode, would show two different barcodes for two mono-synaptically connected neurons. Any increase in the number of sequenced correlated barcodes would indicate a change in synaptic strength. Transcriptional profiles obtained from ribosomes tagged with the same cell-specific barcode can then be associated with the barcode connectivity data and changes in synaptic plasticity. Cell-specific barcodes are generated by combining a unique nucleic acid sequence with 20–30 DNA nucleotides inserted between *loxP* sites in a transgene that is expressed throughout the nervous system. By crossing mice harboring the transgene with a Cre-expressing mouse a unique barcode identifier is created.

Would sequencing barcodes to establish connectivity destroy useful 3D information needed for reconstruction of circuits? The problem of loss of spatial resolution might be partly overcome by voxelating the brain into small cubes followed by RNAseq profiling of the DNA-barcoded neurons. Alternatively, strategies to image cellular DNA barcodes in serial sections of nervous tissue may help to overcome this obstacle, including *in situ* sequencing [131–136].

The use of cellular barcodes will not lead directly to the ultimate goal of unambiguously identifying neurons, as in *Aplysia*, because the barcodes labeling each cell occur randomly in each animal and are not specified by a developmental program. The use of barcodes to identify cell types and cellular connectivity may help to identify a molecular combinatorial code of cell-surface molecules that defines cell types and connectivity, as has been suggested for cadherins [137], and which could ultimately lead to the discovery of a complete catalog of cell types in the nervous system. The generalization of this code across species is testable.

Association of a unique set of specific cell-surface makers with a particular neuronal cell type would enable the targeting of nanovesicles containing short hairpin RNAs (shRNAs), viral vectors, or nanoparticles to specific

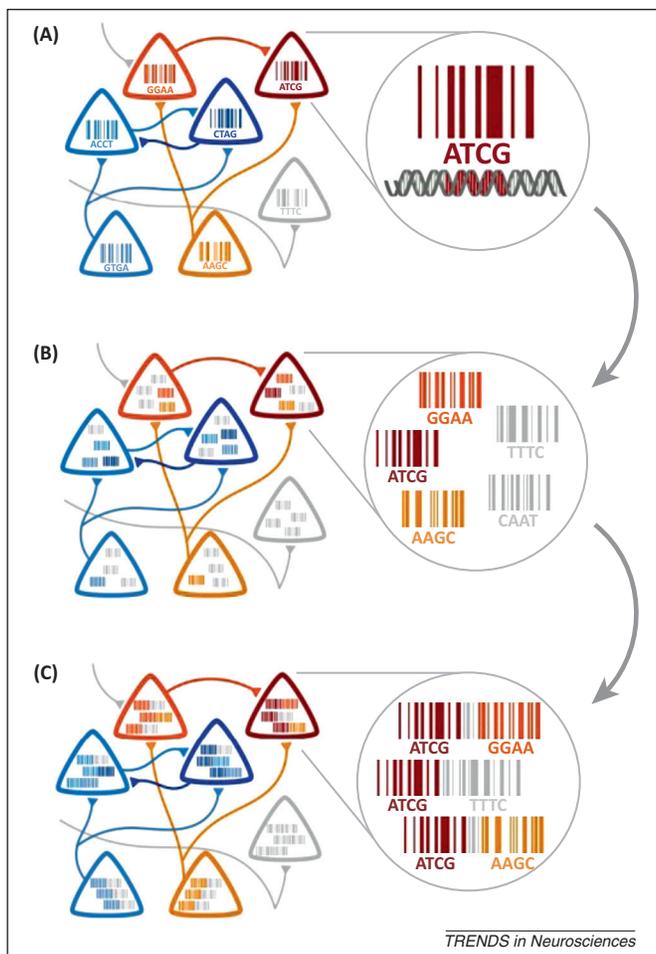
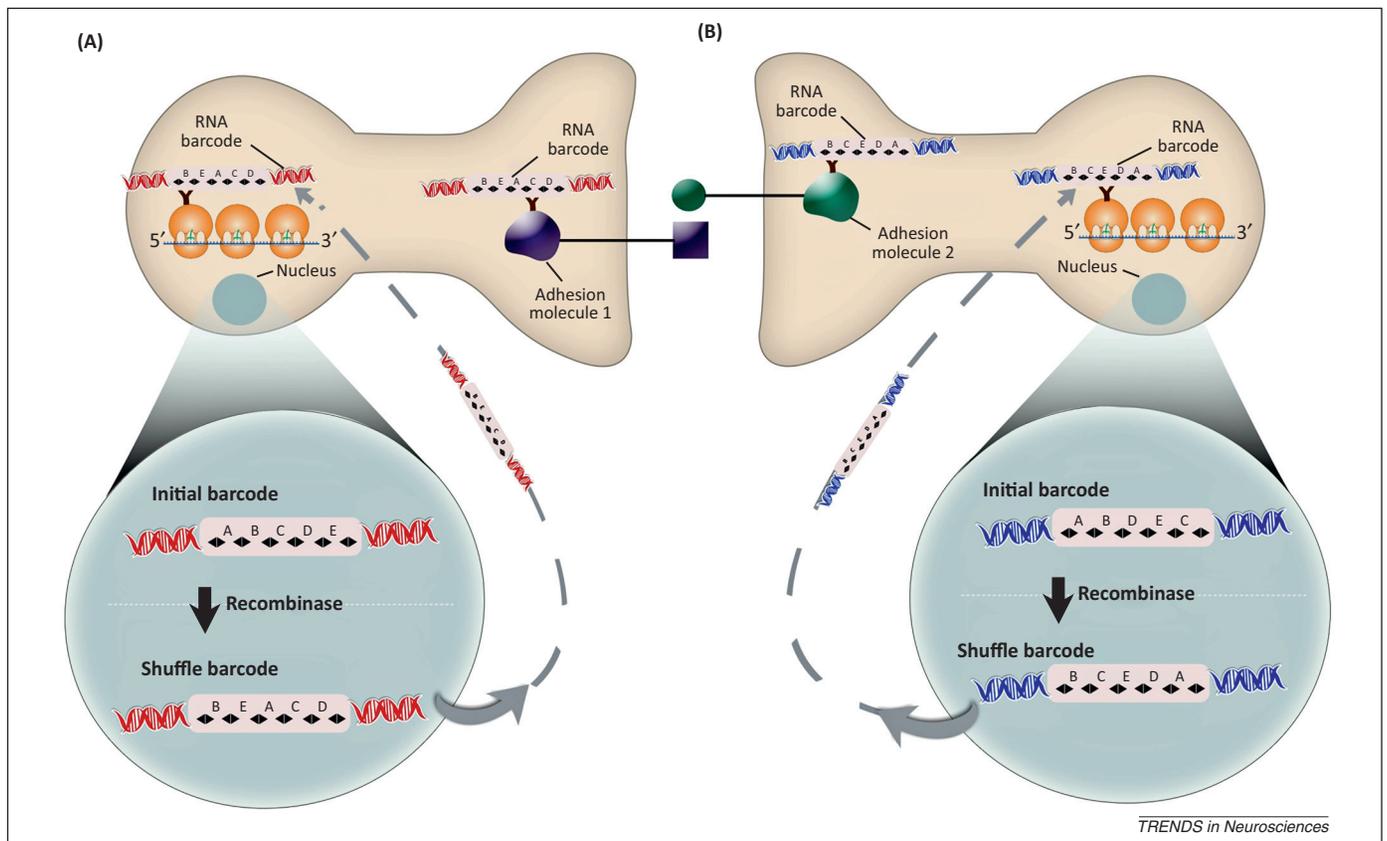


Figure 8. DNA barcoding of individual cells and neuronal connectivity converts a connectivity problem into a sequencing problem. (A) Each neuron is assigned a unique identity by labeling the cell with pseudorabies virus (PRV) that expresses a unique DNA barcode. (B) PRV expressing a unique DNA barcode is trans-synaptically transported (the invader virus) across a synapse. (C) The DNA barcodes from host PRV and the invader PRV are joined by Φ C31 integrase, and high-throughput sequencing is performed. Reprinted, with permission, from Zador [125].



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Figure 9. Schema for correlating cell type gene expression with connectivity. **(A)** A lentiviral vector carrying a unique DNA barcode infects neurons such that each neuron expresses a unique cellular barcode. The DNA elements in the virus genome are transcribed and translated such that an RNA barcode is generated. **(B)** In the second schema DNA barcodes inserted into the *Rosa26* locus are placed between *loxP* sites or targets for another specific recombinase. Recombination of the barcode sequence is induced by the activation of the recombinase, and an RNA barcode is generated. The RNA barcode binds to an RNA-binding protein tagged with EGFP inserted into a ribosomal subunit of the cell ribosomes. An RNA-binding protein is also inserted into pre- and postsynaptic adhesion molecules that bind to each other. Ribosomes are purified, and complementary DNA sequences attached to a longer DNA sequence are hybridized to the RNA barcode on the ribosome. PCR primers (i) to the 5' sequence of the complementary DNA sequences attached to a longer DNA, and (ii) to the polyA tail, are designed such that they join upon the PCR amplification. PCR and sequencing are then performed. Only mRNAs from that cell will contain the unique barcode sequence. At the same time, immunoprecipitation is used to isolate synaptic adhesion complexes from synaptosomes. The DNA barcodes are hybridized to beads containing the complementary sequence and sequenced in a microfluidic chamber.

cells with a unique set of surface markers. Even if such a cell-surface marker code is not discovered, DNA-barcoded ZF proteins expressed on the cell surface may facilitate the targeted delivery of viruses and nanoparticles, and may provide unique identifiers [138,139].

The transcriptional profiling of cell types using barcodes and the tagging of nuclei with cell-specific barcodes could also assist in the identification of enhancer combinations that specify neuronal cell types. The discovery of an enhancer combinatorial code that specifies gene expression in specific cell types would aid the development of viral vectors that are uniquely expressed in specific cells. The development of such vectors would be not only useful for gene therapy approaches but could greatly aid in mapping functional connectivity of the primate nervous system – where the opportunities and capability for delivery of transgene constructs through the germline are limited.

Concluding remarks

Although significant progress has been made in the gene-based analysis of the nervous system, the improved cell-centric strategies based on molecular phenotype currently being developed are necessary for a full understanding of brain function. The organizational principle of nervous system is the cell; that is, the cell types and the connectivity

pattern among cell types control the organization of behavior and the processing of information. The development of new technologies to define cell types and their connectivity, together with improved voltage sensors, and new methods to image non-invasively the activity of thousands of single neurons at the depth of centimeters will open new horizons for a cell-centric understanding of brain function. Outstanding questions are listed in Box 2. The identification of cell types and their connectivity based on molecular phenotype

Box 2. Outstanding questions

- How many cell types are defined by their molecular profile and connectivity in the mammalian brain?
- Do invariant gene expression profiles define a cell type? What are they?
- Are there species-specific brain cell types?
- How does cell type impact the functioning of specific genes and their variants?
- Can barcode technologies be exploited to provide high-throughput methods to identify cell types and connectivity in the brain?
- Can improved voltage sensors be developed with a temporal resolution less than 500 microseconds (the fidelity of microelectrodes), and that are also highly sensitive to changes in voltage?
- Are there absolute physical barriers that prevent the non-invasive recording of single neurons at millisecond resolution through centimeters of tissue?

provides not only a means to observe nervous system function but also to manipulate experimentally specific neural circuits. Thus, the ability to manipulate specific cell types is fundamental to the development of the next generation of cell type-targeted therapeutics to treat brain disorders.

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