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Molecular Neuroanatomy: A Generation of Progress

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Abstract

The neuroscience research landscape has changed dramatically over the past decade. An impressive array of neuroscience tools and technologies have been generated, including brain gene expression atlases, genetically encoded proteins to monitor and manipulate neuronal activity and function, cost effective genome sequencing, new technologies enabling genome manipulation, new imaging methods and new tools for mapping neuronal circuits. However, despite these technological advances, several significant scientific challenges must be overcome in the coming decade to enable a better understanding of brain function and to develop next generation cell type-targeted therapeutics to treat brain disorders. For example, we do not have an inventory of the different types of cells that exist in the brain, nor do we know how to molecularly phenotype them. We also lack robust technologies to map connections between cells. This review will provide an overview of some of the tools and technologies neuroscientists are currently using to move the field of molecular neuroanatomy forward and also discuss emerging technologies that may enable neuroscientists to address these critical scientific challenges over the coming decade.

Introduction

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 many cell types and tissues in the nervous system. While 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 connectivity among various cell types. Improved cell-centric strategies, such as the ability to functionally manipulate specific neuronal cell types and circuits, are critical for understanding the nervous system and may be essential for a full mechanistic understanding of important brain disorders and the eventual development of next generation cell type-targeted therapeutics to treat important brain disorders. Furthermore the development of nanoparticles that are targetable to specific cell types (as defined by molecular phenotype and neuronal circuit) could enable the non-invasive mapping, monitoring and manipulation of the activity of millions of neurons at the single cell and millisecond resolution as conceived of by projects such as the Brain Activity Map (BAM). [1-3]

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This review will describe several of the most important gene-centric technologies and resources that have been developed and 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, and genetically-encoded biosensors and modulators, and molecular phenotype datasets[4]. Cell-specific genetic manipulation has been inhibited by: 1) The limited number of cell type-specific promoters; 2) the very few genes that are selectively expressed in a given cell type and; 3) 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, provides a means to overcome this barrier.

Brain Atlases

Eighty percent of 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 the 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 2-D or 3-D would identify cell type-specific molecular markers. The identification of cell type-specific molecular markers would provide targets that would facilitate the delivery of genes and gene products to these cell types for the analysis of cellular development, connectivity, and function as well as 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. Targeted mutation in the rat can now be achieved using Transcription Activator-Like Effector Nucleases (TALEN), Zinc Finger Nucleases (ZFN), and CRISPR. Fig 1.

The Allen Mouse Brain Atlas

The Allen Mouse Brain Atlas, is a high resolution 2-D and 3-D 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 doing 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 expression of multiple genes to infer the number of cells 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 just 170 neuron-specific genes (Fig. 2). Work by Pascal et al[11] also suggests that anatomical boundaries for cortex, hippocampus, striatum, ventral midbrain, medulla, and cerebellum can be delineated based on the density of cell types and gene expression profiling of 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 needed to provide combinatorial gene expression data that can be used to better characterize brain anatomical boundaries and cell-type specific organization.

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, the eGFP reporter or cre-recombinase is placed in frame in the first exon with an inframe stop codon to prevent overexpression of the protein. Since BACs are usually of large size, they typically include control elements that recapitulate 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 positional effects are still possible. GENSAT BAC mice are widely used by the scientific community since 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 genes[15-17]. GENSAT has characterized over 10,000 BAC transgenic founder lines and 1347 eGFP and Cre founder lines (Fig. 3) have been made available to the scientific community through Mutant Mouse Regional Resource Centers (MMRRC) (see Table I and Table II 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 systematically map the connections of the nervous system at the macroscopic, mesoscopic, and microscopic levels. Maps at all three of these levels provide a critical 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 level. Cell-based connectivity maps based on molecular phenotypes are just 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 creates structural constraints on the functional properties of the nervous system by limiting and directing the flow of information[23]. In support of this hypothesis, Varshney, et al[24], Sohn et al [25], Jerrell et al [26], have been able to make testable predictions about functional connectivity underlying behavior in C. elegans based on EM reconstruction of the wiring diagram of the C. elegans and computational methods that weight synaptic strength instead of synaptic number. Their analysis is limited by the inability to discern from electron micrographs 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 five year project designed to map the brain connectivity of 1200 healthy adults, including 300 twin pairs and their non-twin siblings, to correlate connectivity with genetic and behavioral data (Fig. 4). The four methods that will be used for mapping structural and functional connections in the human brain include diffusion MRI (d-MRI), task functional MRI (T-fMRI), resting-state fMRI, T1- and T2-weighted MRI. To compensate for the limited temporal resolution of the MRI, EEG and magneto-electroencephalography will also be used. The first phase of the project (2010 to mid-2012) has performed optimization of hardware and imaging protocols, while the second phase (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 the Cold Spring Harbor Laboratory from 2006 to 2008. The published recommendations [31] advocated the brain-wide mapping of mesoscopic level neuronal connectivity across species. The decision to initially map 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. (Fig 5.)

In the Brain Architecture and the iConnectome projects, anterograde and retrograde tracers are systematically injected together at 200 to 250 different sites in a grid dependent fashion and micrographs are obtained using automated wide field fluorescent microscopy. The inputs and outputs of each brain region are mapped to a grid based on the Allen Reference Atlas. Both projects 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 the 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 AAV (rAAV) virus expressing a fluorescent protein only labels the anterograde projections of neurons expressing CRE in a region. Fluorescent 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 being 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.

The Cre-dependent recombinant AAV (rAAV) virus used by the Allen Brain Mouse Connectivity Atlas is not transynaptically transported and thus cellular connectivity cannot be established. This problem can be surmounted by exploiting other viruses. Rabies and Pseudo Rabies Virus (PRV) are used as retrograde trans-synaptic tracers, while the herpes simplex virus type 1 strain H129 virus has been used as an anterograde trans-synaptic tracer[32, 33, 34,35]. The direction of transport appears to be determined by the viral glycoprotein [36]. This directionality is an advantage over classic transynaptic tracers such as wheat germ agglutinin (WGA), which are transported in both anterograde and retrograde directions[35]. Since neurotropic viruses replicate within neurons, the problem of serial dilution faced by conventional transynaptic tracers is minimized.

Wickersham, et al[37, 38] used a complementation strategy to restrict the transsynaptic spread of rabies virus to monosynaptic connections in genetically defined populations of neurons. This method uses a PRV in which the G glycoprotein needed for replication is replaced with EGFP linked to the glycoprotein, EnvA from ASLV-A. This PRV 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] (TEXT 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] that allows translation across the different coordinate systems used by different atlases such that the datasets are interoperable. The Whole Brain Catalog is an open source 3-D atlas of the mouse brain that allows linkage and integration of information and data from a number of 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 electron microscopy 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; super-resolution array tomography uses protein expression based on antibody detection and morphology. The development of a genetically encoded tag based on the Arabidopsis phototropic2 protein, a fluorescent protein, called miniSOG (mini Singlet Oxygen Generator) permits 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 electron microscopic visualization. Improved correlation of images obtained with fluorescent microscopy and EM can be obtained because the same tag is used 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 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 comprised 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 and 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 uniquely tag a cell type. Selective expression of GEBs/GEAs using viral vectors and plasmids is critically dependent upon tissue specific promoters.

GEBs 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, while the bioluminescent proteins (e.g. aequorin or luciferase) are typically enzymes that emits light upon oxidizing substrates such as

coelenterazines[50]. Fluorescent protein GEBs 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 GEB 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 simultaneous use of blue-activated channel rhodhopsin 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 with the activity 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. A number of versions of genetically encoded voltage sensors have been developed[59-61] . These include Flash, SPARC, FLARE, VSFP, and Arch3 (Arch) [62,63-65]. These voltage sensors, except for Arch, have time constant greater than 1ms, making it difficult to temporal resolve action potentials. However 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 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%, ability to target to the soma or dendrites, and are non-toxic as well as being genetically encodable.[60, 61]

Genetically Encoded Sensors of Neurotransmitter Release

pHflourins are pH sensitive fluorescent proteins used as genetically encoded reporters to measure synaptic release. pHfluorins can be targeted to synaptic vesicles by fusing pHflourin to synaptic vesicle proteins such as SNARE, synaptophysin, and the vesicular glutamate transporter. The acidic environments inside synaptic vesicles quench fluorescence while 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 with calcium concentration at the same 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 ten 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 bluelight, neuronally-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 produces hyperpolarization. (Fig, 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 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 laser confocal microscopy[71]. This depth can be extended by penetrating tissue with a fluorescent microendoscope with gradient refractive index (GRIN) microlenses[72] that 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 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 promises to help disentangle neuronal circuitry involved in Deep Brain Stimulation 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 opto-epigenetic 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 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 designer receptors exclusively activated by designer drugs (DREADDs) [84, 85]. The discovery of Lynx, a family of endogenous peptides with homology to nicotinic acetylcholine receptor blocking snake toxins, has led to 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 expression of a transgene to any specific cell type of interest would greatly enhance our ability to investigate nervous system circuitry and mechanisms. Yet 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 NHGRI-funded Encyclopedia of DNA elements (ENCODE) project have been generating molecular phenotype data sets for a variety of Drosophila, C. elegans, mouse and human cell and tissue types [87-91]. These data sets can be used to identify molecular signatures and enhancer elements specific for particular cell types. In particular, identification of unique cell-specific enhancer elements may facilitate our ability to spatially and temporally express transgenes 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[92]. These maps typically include DNA methylation information, chromatin immunoprecipitation data for several informative activating or silencing histone modifications, chromatin accessibility information using the DNAse1 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[93].

While the transcriptional profile of a number of cell types or tissue types have been characterized and published[94-103], no systematic analysis of the transcription profiles of nervous system cell types has yet been reported. Several different approaches have been used to transcriptionally profile 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 cell (manual) and Translating Ribosome Affinity Purification, and Ribo-tag. LCM and manual sorting of fluorescently labeled cells are limited by throughput [104, 105]. FACS for isolating cells[106, 107] and PAN can introduce artifactual gene expression changes due to cellular stress[108]. These problems quantitating translated mRNAs are overcome with genetic methods of tagging of ribosomes using BACs/ TRAP [109] or the induction of the floxed ribosomal tag (Ribo-Tag) with a “cell specific Cre”[110].

The BAC-TRAP (Bacterial Artificial Chromosome- Translating Ribosome Affinity Purification) method dynamically measures translated mRNAs in genetically identified cells, providing molecular phenotype information for cell types in the nervous system[109, 111] . In this method, the L10 ribosomal subunit is inserted into the first exon of a gene tagged with eGFP into 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 measurement made with eGFP BACs or in situ data, which is a static representation of gene expression. Already this method has identified and characterized a number of specific cell types on the basis of molecular phenotype[112-115].

Ribo-tag uses a gene targeting approach in which the ribosomal gene, Rpl22, is floxed with 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. The epitope-tagged Rpl22 incorporated ribosomes is used to immunoprecipitate ribosome-specific transcripts from specific tissues.[110]

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[116]. Tissue-specific CRE drives expression of the Ago2 trangene 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 knowledge 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[117, 118]. However, dissociation of nervous 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 on 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. Recently cellular barcodes based on DNA sequence have been used to track hematopoietic cell lineages[119-124]. 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 [125, 126]. In Brainbow, different genes encoding different fluorescent proteins are placed in between loxp sites (Fig. 7). A palette of different color neurons are generated when CRE acting on the loxp sites causes random rearrangements and deletions of the fluorophores. However, the number fluorophores and their dynamic range given the number of cells and synapses limit the utility of using Brainbow.

As suggested by Zador et al[127] 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 transynaptic virus with a unique sequence generated by a recombinase (Fig. 8). Invader viruses would be ligated together with host neuronal viral sequence by phi31 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 identification of synaptic connections.

The use of DNA barcodes overcomes the lack of dynamic range of fluorophores used in Brainbow since there are far more barcodes available than there are fluorophores[128]. 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[129, 130] or the 8.6×10^{10} neurons and 8.5×10^{10} non neuronal cell in the human brain, and sufficient to label 8.8×10^{11} synapse in the mouse brain [131]. 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. Secondly, 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 zinc finger DNA or RNA binding proteins[132] may provide a method to correlate cell type with connectivity and unambiguously label specific cells (Fig 9). Isolation of synaptic adhesion molecules from a pre-and post-synaptic neurons that bind one another tagged with a cell-specific barcode would show two different barcodes of 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 to 30 DNA nucleotides inserted between LoxP sites in a transgene that is expressed throughout the nervous system. By crossing transgene with a CRE mouse a unique barcode identifier is created.

Wouldn't sequencing barcodes to establish connectivity destroy useful 3-D 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 RNA seq 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 that include in situ sequencing[133-138].

The use of cellular barcodes will not lead directly to the ultimate goal of unambiguously identified 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[139] that 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 shRNAs, viral vectors, and nanoparticles to specific cells with a unique set of surface markers. Even if such a cell surface marker code is not discovered, DNA barcoded Zinc finger proteins expressed on cell surface may facilitate the targeted delivery of viruses and nanoparticles and may provide unique identifiers[140, 141].

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 doing transgenic delivery of gene through the germline is limited.

Conclusion

While significant progress has been made in the gene-based analysis of the nervous system, improved cell-centric strategies based on molecular phenotype being developed are necessary for a full understanding of brain function. The organizational principle of nervous system is the cell; 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, connectivity, improved voltage sensors, and new methods that non-invasively image the activity of thousands of single neurons at the depth of centimeters opens new horizons for a cell-centric understanding of brain function. The identification of cell types and their connectivity based on molecular phenotype provide not only a means to observe nervous system function, but to experimentally manipulate specific cell types and connectivity. The ability to manipulate specific cell type is fundamental to the development of the next generation of cell-type targeted therapeutics to treat brain disorders.

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Text Box 1. High Resolution Microscopic Methods for Mapping Neuronal Circuits

Super Resolution Microscopy

Advances in light microscopy and high throughput electron microscopy 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 (150-300nm) is now overcome with super-resolution fluorescent microscopy that provides 2-10 times the resolution of conventional light microscopes[142, 143]. 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[144], using STORM to image proteins at the synapse, were able to show the 3-D 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, an automated serial section/volume reconstruction imaging with super-resolution fluorescent microscopy like 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 of 50 to 200nm in width. These sections can be repeatedly stained with, and stripped of, by 20 or more immunofluorescent antibodies for repeated imaging of 20 or more antigens before the final staining with heavy metals for imaging with electron microscopy is performed. The stack of images is placed in register to produce high resolution large volumetric 3D images. When super-resolution imaging is used in array tomography throughput is decreased. Thus, decisions in experimental design require evaluations of the tradeoffs between resolution and throughput needed to resolve the cellular and molecular structures of synapses, neurons, and circuits.

Automated Sectioning, Image Acquisition, and 3-D Reconstruction

Despite automated methods to collect images[145, 146] months to years are now required to reconstruct the synaptic connections at the microscopic level in a cubic millimeter of nervous tissue[147]. Development of improved algorithms for segmentation of images and new computational methods will automate image analysis and increase throughput[148, 149]. Toward this goal Helmstaedter[150] has developed KNOSSOS software for visualization and annotation of imaged sections that is 50 times 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. Redundant-skeleton consensus procedure (RESCOUP), a statistical method, is used to resolve discrepancies among annotators.

Text Box 2: Unanswered Questions

How many cell types are defined by their molecular profile and connectivity in the Mammalian Brain?

Dot invariant gene expression profiles define a cell type? What are they?

Are there species-specific brain cell types?

How do cell types 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 the fidelity of microelectrodes with temporal resolution less than 500 microseconds and are highly sensitive to changes in voltage?

Do any laws of physics prevent the non-invasive recording of single neurons at millisecond resolution through centimeters of tissue?

Highlights

- Compelling technologies and resources for neuroscience are highlighted.
- Atlases, the connectome, genetically encoded sensors and activators are reviewed.
- Methods for molecular phenotyping of brain cell and tissue types are described.
- The use DNA barcoding to catalog neuronal cell types and connections is presented.
- The argument for new technologies to define cell types and connectivity is given.

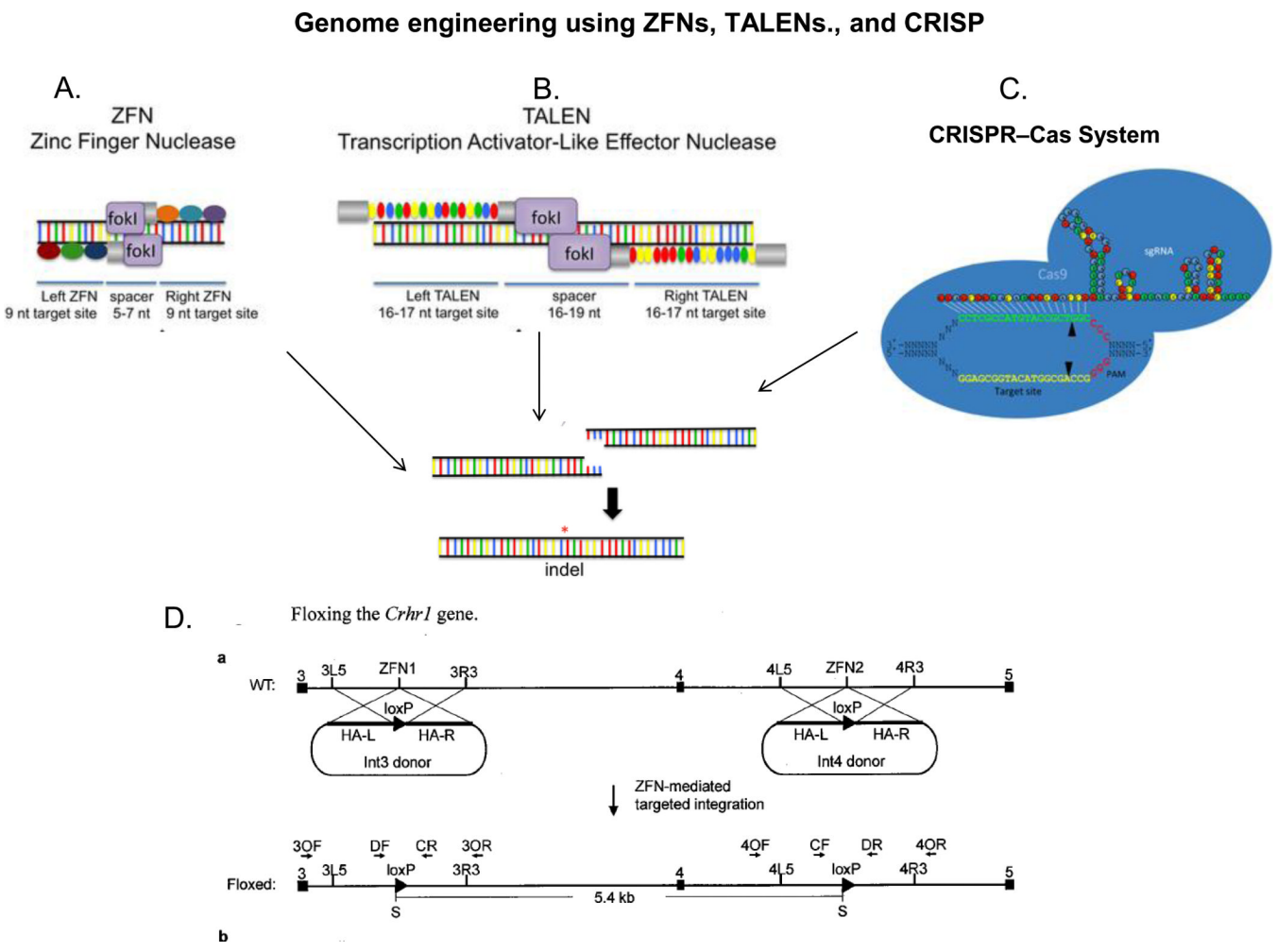


Fig 1. Creating CRE/LoxP rat using Zinc Finger Nuclease, TALEN, and CRISPR technologies for inducible gene knock-in or knock-out

A. Zinc Finger Nuclease (ZFN) links engineered DNA binding zinc finger (ZF) domain with a DNA-cutting nuclease domain, which contains a Fok1 restriction enzyme site. Each zinc finger 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 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. ZFNs induced chromosomal breaks are then randomly reconnected by endogenous cellular DNA repair mechanisms called non-homologous end joining, leading to gene knockout. Nonetheless, if there are manipulated DNA strands ending with nucleotides on each side that can form homologous bindings to the DNA break at the time of ZFN incision, a homology dependent repair can occur, resulting in gene knock-in. Adapted with permission[151]

B. TALENs bind DNA using TAL effector 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[151]

C. CRISPRs (clusters of regularly interspaced short palindromic repeats) were first found in the Escherichia coli genome likely involved in its 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 RNA (crRNAs) serve as sequence-specific guides for the Cas proteins/nucleases to target and generating double strand break (DSB) in the DNAs. In CRISPR genome editing, the crRNA and the trans-activating crRNA (tracrRNA) forms a double strand RNA structure that directs the Cas9 to generate DSBs in the target DNA. At the genomic site where is complementary to the crRNA, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the non-complementary strand. Reprinted with permission from[152].

D. Floxing the Crhr1 gene. In conditional knockin rats, a plasmid DNA that encodes the CRE or LoxP proteins 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 called homologous end joining during DNA repair. This allows CRE-LoxP to be inserted at desired genomic sites to created inducible mutations and generate CRE/LoxP rat for specific neural circuitries in the brain. Figure Courtesy of Dr. Xiaoxia Cui, Sage Labs, St. Louis MO..

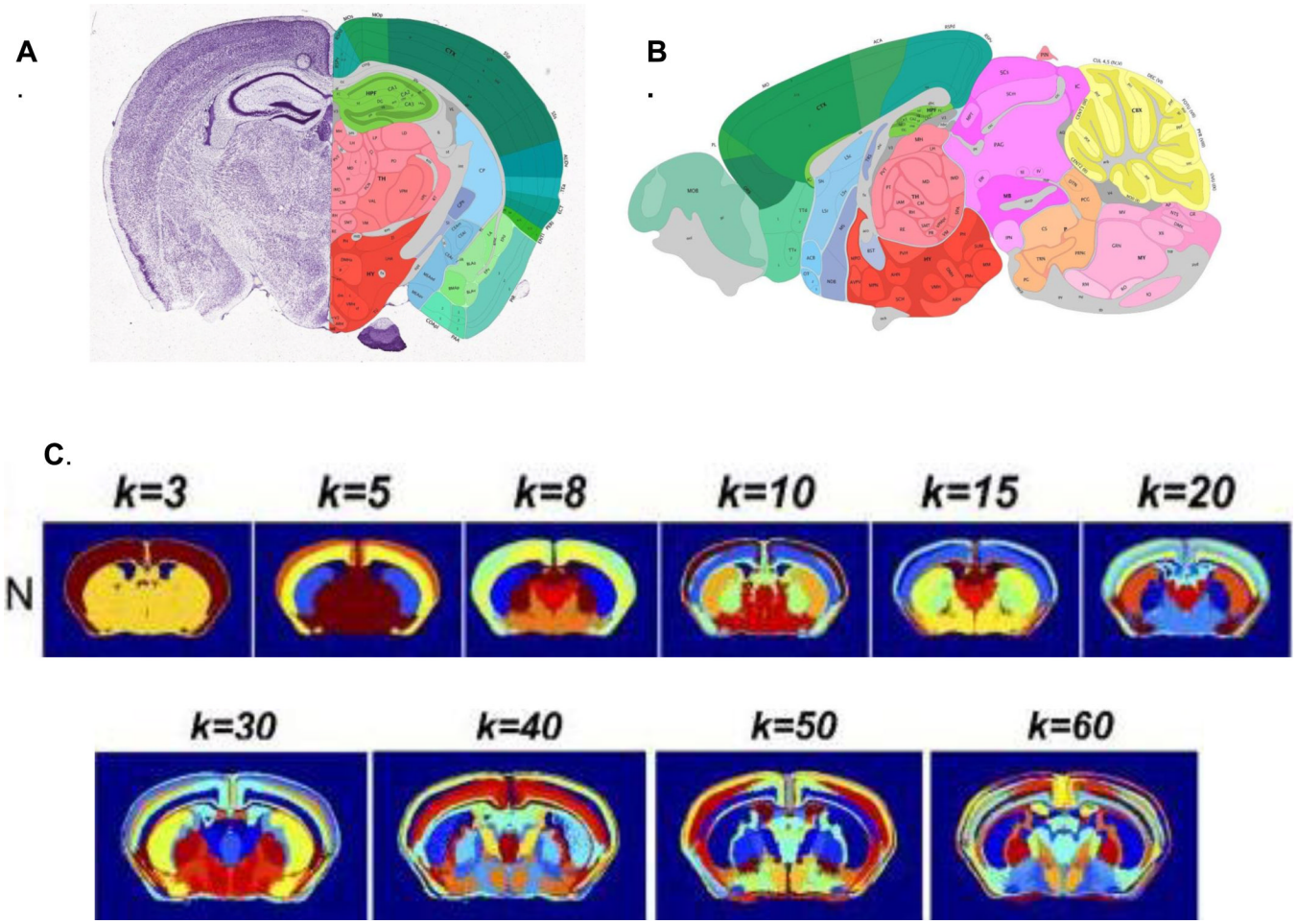


Fig 2.

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 then number of k-mean clusters of neuron-specific transcripts used in the analysis increases. Further refinement of c ell types and their projection may increase anatomical resolution. A. Coronal section from Allen Brain Reference Atlas. B. Sagital section from Allen Brain Atlas. C. Spatial cluster analysis of neuron-specific transcripts. Adapted with permission[9].

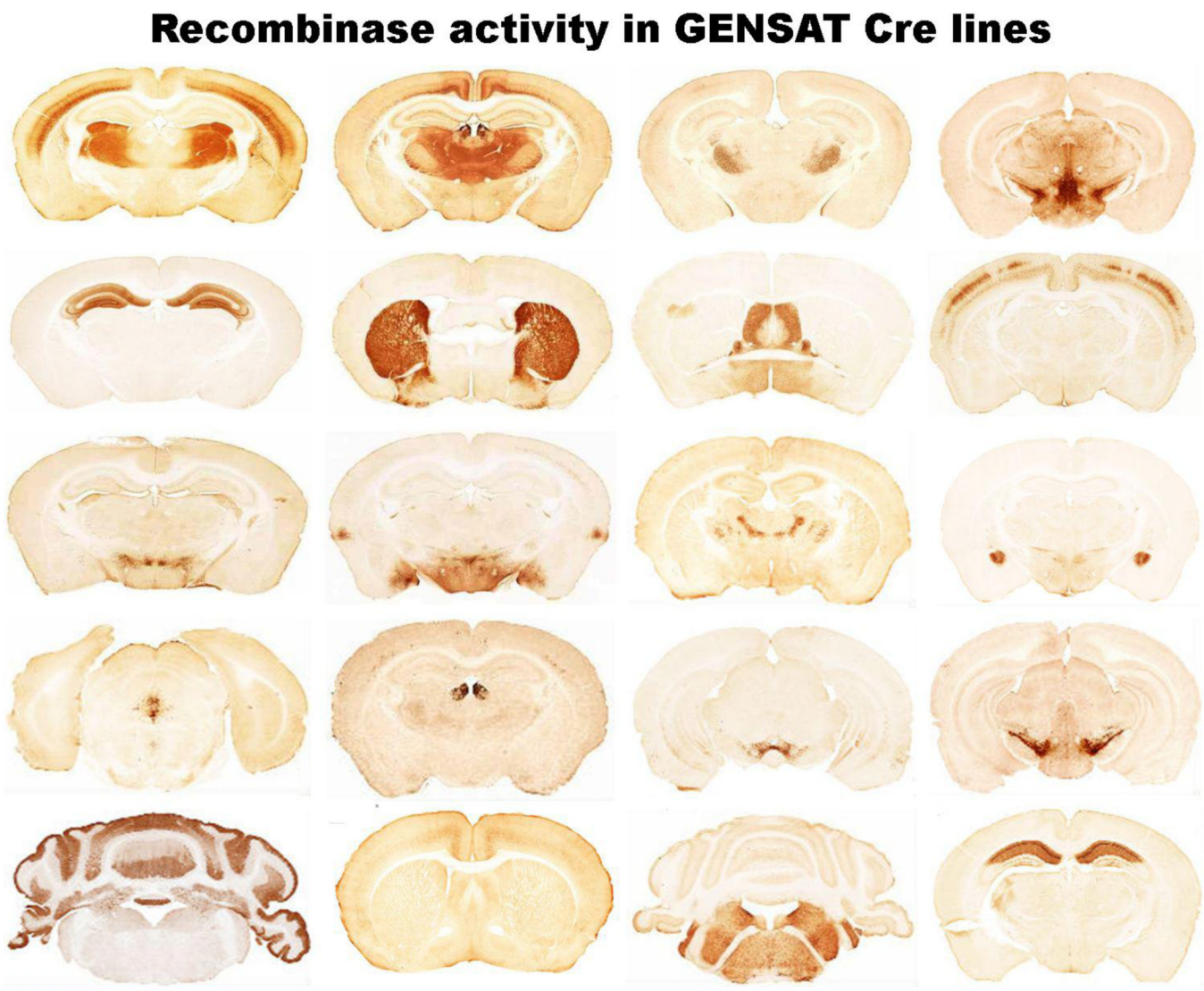


Fig 3.

Micrograph of anti-EGFP stained coronal section through the brains of a subset of Cre recombinase BAC transgenic mouse line generated by the GENSAT project. All Cre lines are tested for recombinase activity by breeding with a standard Rosa26-loxP-Stop-loxP-EGFP reporter. Examples of over two hundred 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, NIMH.

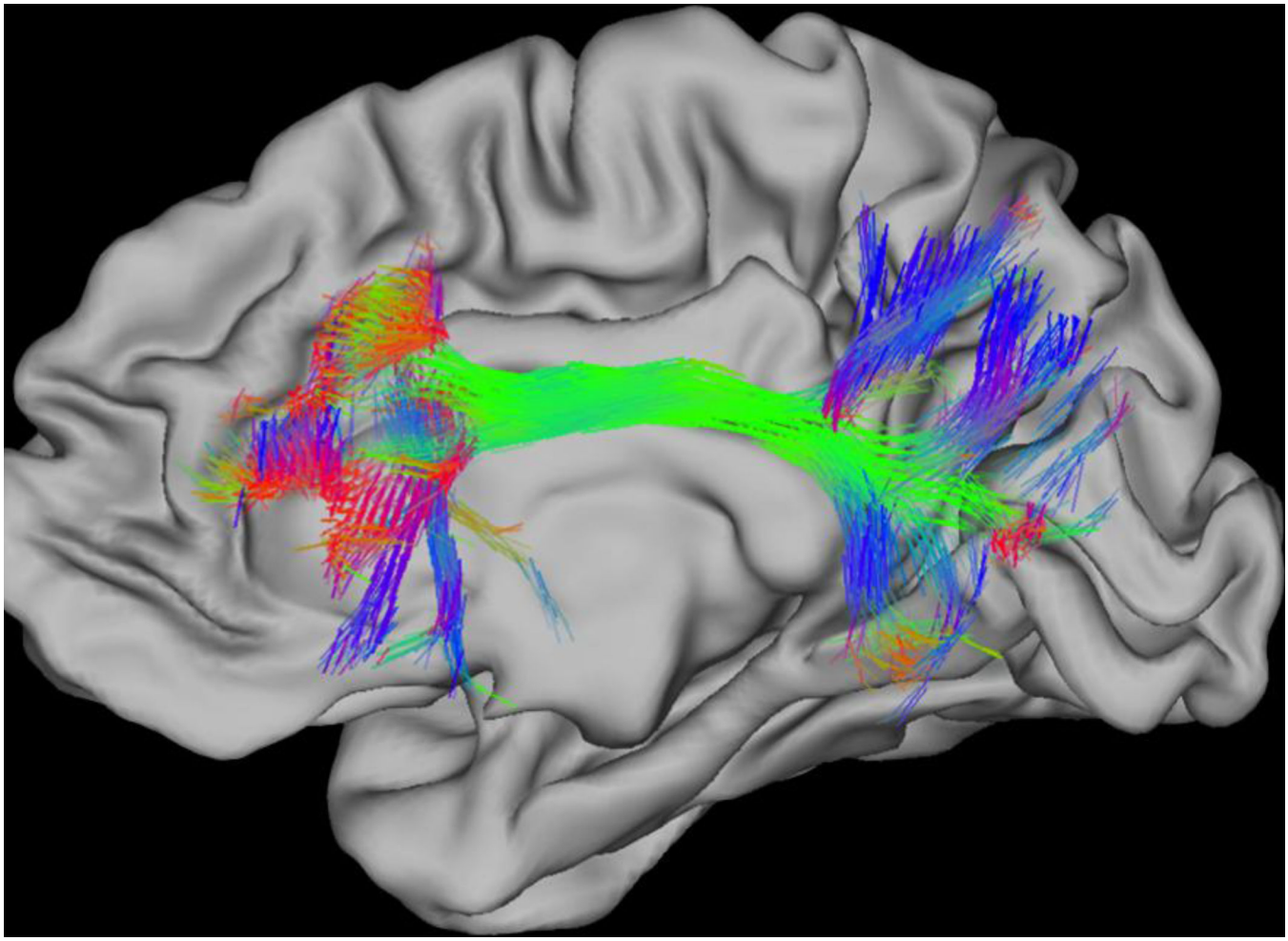


Fig 4. Human Connectome

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 1.25mm cubic volume of brain (voxel), the resolution of the diffusion MRI data. Intersection of color tracts suggests connectivity. Adapted with permission[153]

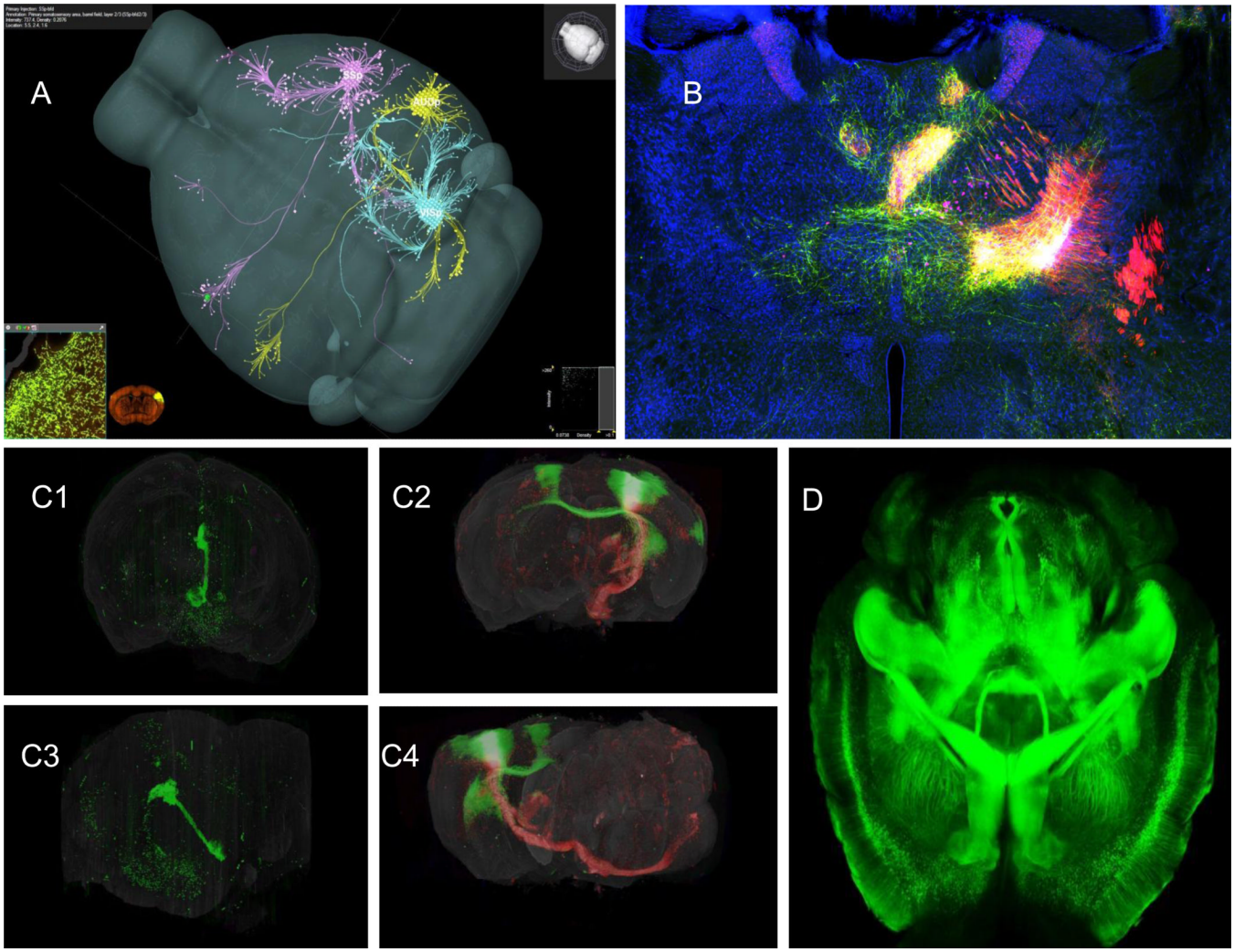


Fig 5. Mouse connectome

A. Three 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 B. A representative digital image of the iConnectome project (www.MouseConnectome.org) showing multiple fluorescent labeled neuronal pathways through one coronal thalamic level of the mouse brain, after multiple fluorescent tracers were injected into the infralimbic (PHAL and CTb) 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 associated with two anterograde/retrograde tracer coinjection sites within the same brain and simultaneously exposes reciprocal connections . Courtesy of Hongwei Dong, Univ. Southern California. C. The 3-D 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) Left, GFP labeled modified ‘retrograde’ rabies virus injected in 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) . (2) Right, double injection of primary motor cortex (MOp) with modified ‘anterograde’ adeno-associated virus (AAV2/1 CAG) 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. D. CLARITY 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[154] .

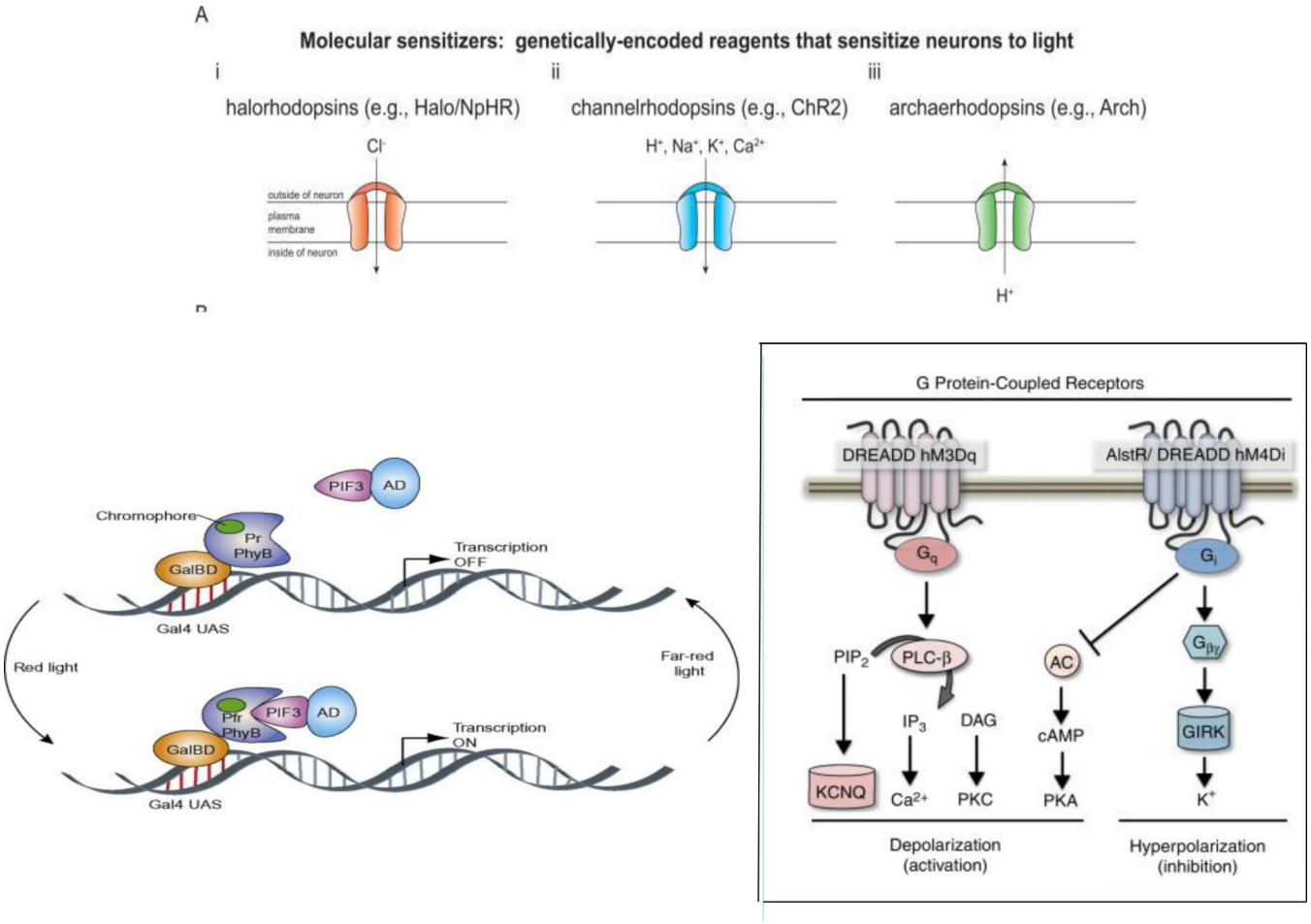


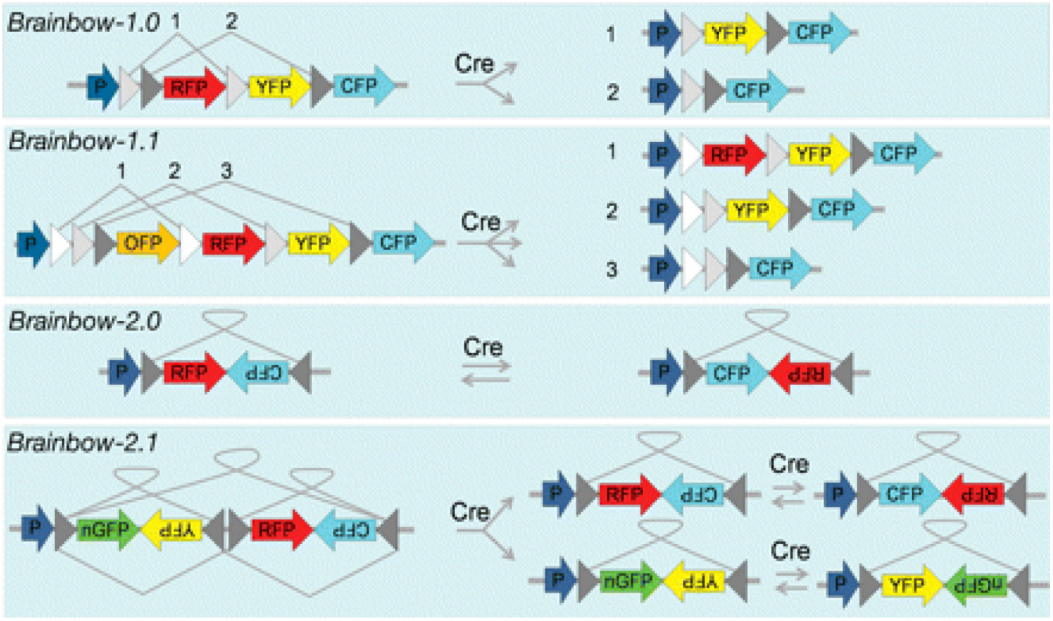
Fig. 6.

Genetically encoded biosensors A. Some genetically encoded biosensors have been used as molecular sensitizers to manipulate neuronal activities. Neurons express them can be activated or silenced by light. a. Halorhodopsins, such as N. pharaonis halorhodopsin, pump chloride ions inward and hyperpolarize the neuron upon yellow light. b. 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 neuron. c. Stimulation of Archaerhodopsins (H. sodomense opsin) with yellow or green lights shuts down neuronal activity by hyperpolarizing neurons. Adapted with permission[70].

B. Biosensors can be designed to control protein-protein interactions. Here red light triggers PhyB binding of PIF3. Since PhyB is fused to a Gal4-binding domain (GalBD) and PIF3 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 upon far-red light PhyB reverts to the non-binding conformation and releases PIF3, shutting down transcription. In this way, light can be used to either induce or shut down transcription. Adapted with permission from[155].

C. Biosensors are effective tools to rapidly manipulate neuronal activity. DREADDs are engineered from muscarinic acetylcholine receptors (mAChRs). It is potently activated by the pharmacologically inert molecule clozapine-N-oxide (CNO). When it is coupled to Gq, Gi or Gs the GPCR signaling pathways can be manipulated without obvious interference with endogenous GPCR signaling. In this figure the presence of CNO selectively activates (Gq-coupled hM3Dq) or inhibits (Gi-coupled hM4Di) G-protein signaling. The response is

robust and reversible for the study of neuronal activity. Activation of Gq signaling leads to neuronal depolarization and increased activity. In contrast, CNO activation of Gi signaling triggers inward rectifying potassium channels (GIRKs), resulting in hyperpolarization and inhibition of the neuron.



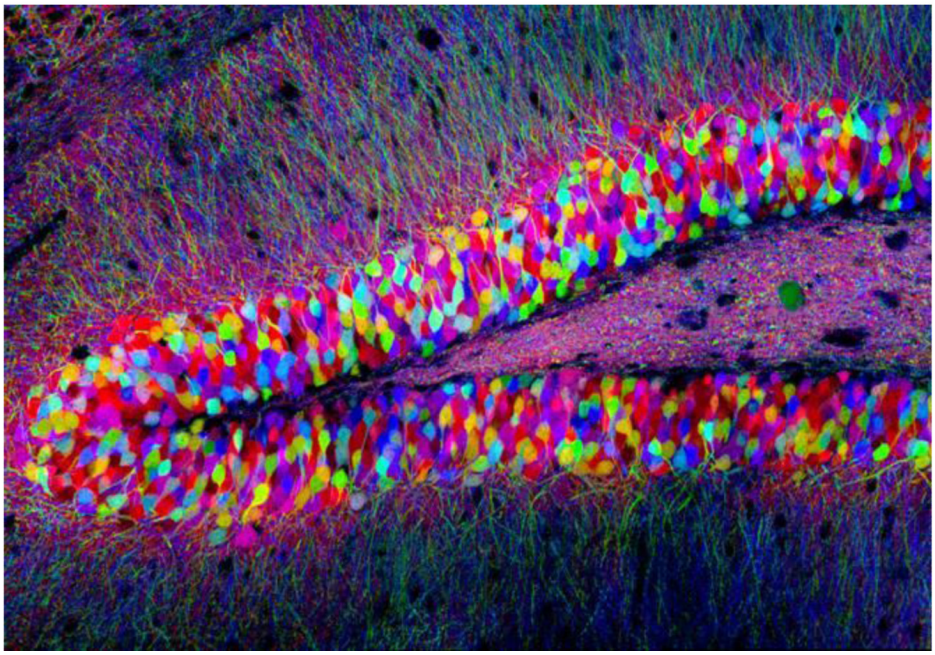


Fig 7.

Brainbow. A. Strategy for creating Brainbow mice. In Brainbow 1.0 and Brainbow 1.1 excision of different fluorophores from incompatible lox variants generates 2 to 3 different color combinations of XFP. In 2.0 the XFP genes are placed head to head between two asymmetric loxP sites. Activation of recombination by CRE causes inversion and change in the expression of the fluorophore from red to cyan. Place the inverted fluorophore genes between asymmetric loxP sites in tandom increases the number of combinations to four. B. Fluorescent fluorophore expression in mouse hippocampus. Adapted with permission from [128].

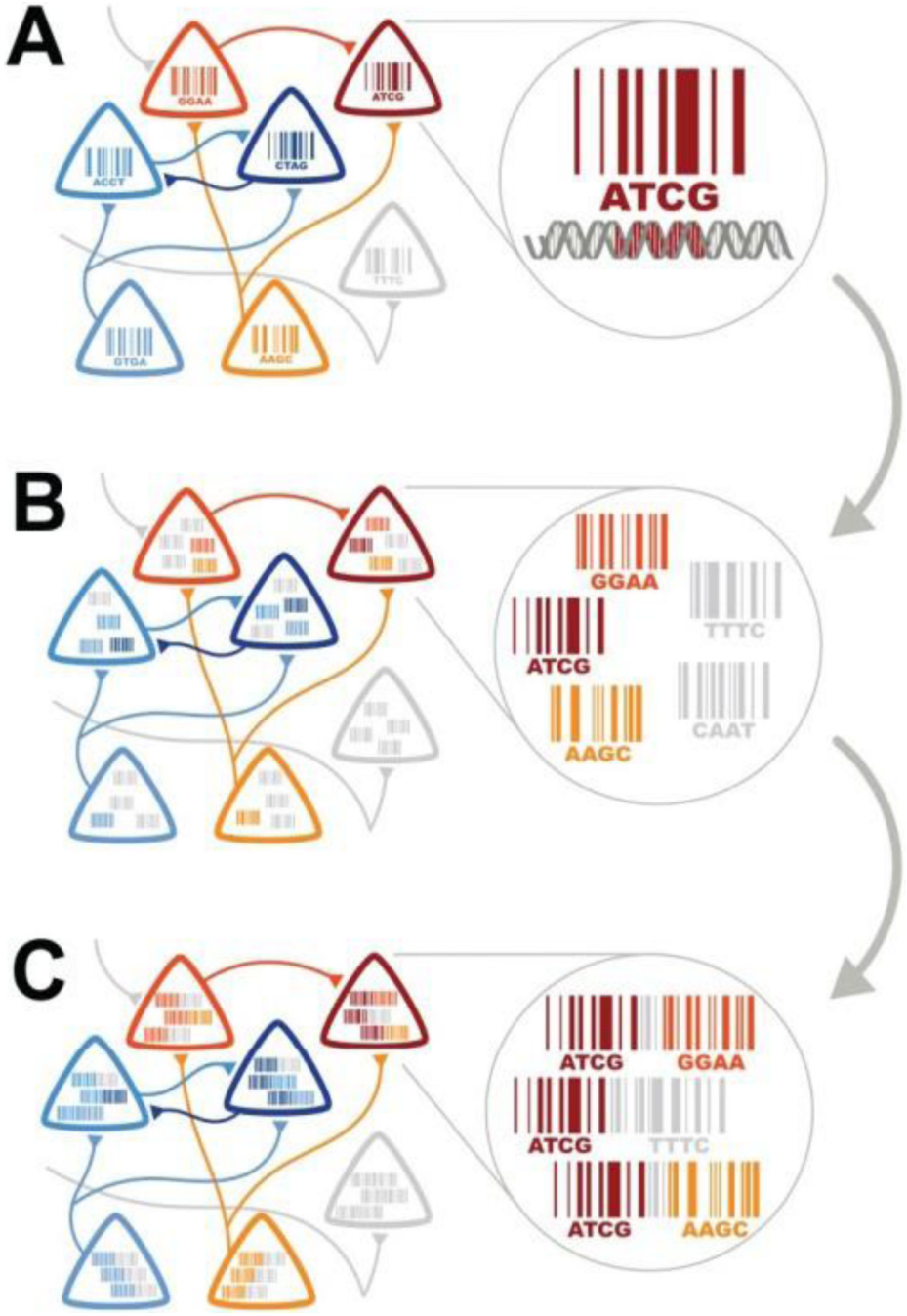


Fig 8.

DNA Barcoding of Individual cells and neuronal connectivity converts connectivity problem into a sequencing problem. A. Each neuron is assigned a unique identity by labeling the cell with PRV virus that expresses a unique DNA barcode. B. PRV virus expressing a unique DNA Barcode is transynaptically transported (the invader virus) across a synapse. C. The DNA Barcodes from host PRV virus and the Invader PRV virus are joined by PhiC31 integrase and high throughput sequencing is performed. Reprinted from Zador[119].

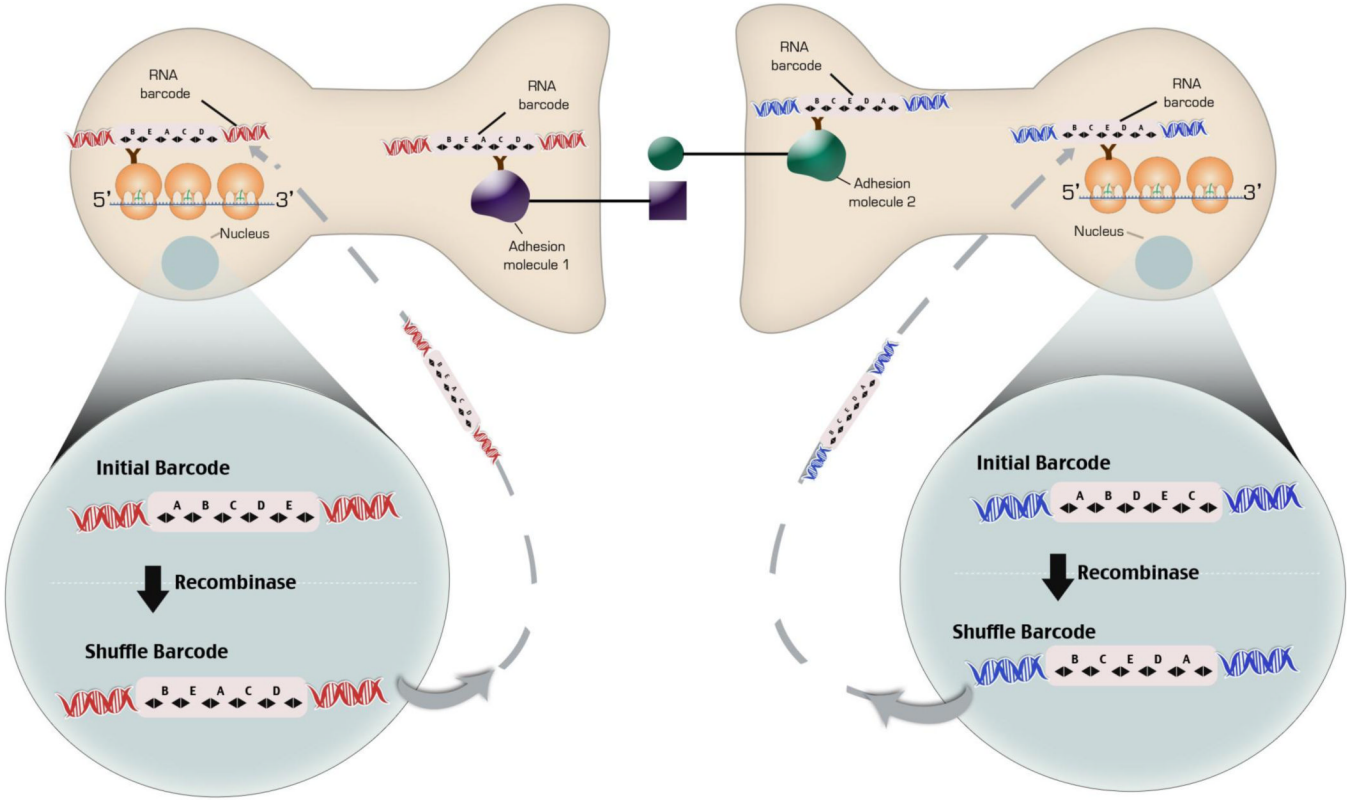


Fig 9.

Schema for correlating cell-type gene expression with connectivity. A Lentiviral vector carrying a unique DNA barcode infects neurons so that each neuron expresses a unique cellular barcode. The DNA element in the viral 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 another site specific recombinase. Recombination of the barcode sequence is induced by the activation of the recombinase and RNA barcode is generated. The RNA barcode binds to an RNA binding protein inserted in into a ribosomal subunit with EGFP of the cells ribosomes. A RNA binding protein is also inserted into pre- and post-synaptic adhesion molecules that bind to each other. The ribosomes are purified. Complementary DNA sequences attached to a longer DNA sequence are hybridized to the RNA Barcode on the ribosome. PCR primers to the 5' sequence of the complementary DNA sequences attached to a longer DNA and to polyA tail are made such that they join upon the PCR reaction. PCR and sequencing performed. Only mRNAs from that cell will contain the unique barcode sequence. At the same time synaptic adhesion complexes are isolated from synaptomsomes with immunoprecipitation. The DNA barcodes are hybridized to beads containing the complementary sequence and sequenced in a microfluidic chamber.

Table I

Resource	Summary	References or Website
General Resource to 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, multi-scale, 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 11.5, e15.5, postnatal (p) day 7 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	*In situ* hybridization of developing mouse brain at E11.5, E13.5, E15.5, E18.5, P4, P14, P56	http://developingmouse.brain-map.org/

Resource	Summary	References or Website
Molecular Anatomy of Mouse Embryo Project (MAMEP)	A comprehensive information resource for the functional analysis of pattern formation, tissue development, and organogenesis in mice. Whole-mount *in situ* 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 whole-mount mouse embryo (ICR strain) of mid-gestational stages (Embryonic Day 9.5, 10.5, 11.5), in which the most striking dynamics in pattern formation and organogenesis is 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)	A 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 alignment of individual scans to improve localization and statistical power	http://brainmap.wisc.edu/monkey.html

Resource	Summary	References or Website
	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.	
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 multi-modal, multi-resolution 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 of human brain development. Such information is referenced to MRI and diffusion weighted imaging (DWI) data.	http://www.brainspan.org
Multiple Species Atlases		
Brain Maps	Interactive multi-resolution 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	http://www.loni.ucla.edu/Atlases/

Resource	Summary	References or Website
	and adult human, Alzheimer's, monkey, vervet, mouse, and rat	
Adult Rat Brain Atlas		
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 *Drosophila* Nervous System Armstrong HD et al 1995	Atlas of the adult and developing *Drosophila* 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 *C. elegans*	http://www.wormatlas.org/hermaphrodite/nervous/Neuroframeset.html
Zebra Finch Expression Brain Atlas	A NIH-funded online public repository of *in situ* 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 five-year project sponsored by NIH including tween two consortia of research institutions to build a “network map” in order 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	http://www.mouseconnectome.org/ http://brainarchitecture.org/ http://connectivity.brain-map.org/

Resource	Summary	References or Website
	connectivity in the mouse brain.	
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

Table II

Enabling Technologies and Resources

General Resource for Data and Resources		
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
Geneweaver	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	A 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.rrrc.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	http://www.genetrap.org/

	for nominal handling fees.	
International Knockout Mouse Consortium	Knockout Mouse Project (KMP, funded by NIH) European Conditional Mouse Mutagenesis Project (EUCOMM, funded by European Union) North American Conditional Mouse Mutagenesis Project (NorCOMM, funded by Genome Canada) Texas A&M Institute for Genomic Medicine (TIGM)	www.knockoutmouse.org
Mutant Mouse Regional Resource Centers (MMRRCs)	Distributes and cryopreserves scientifically valuable, genetically engineered mouse strains and mouse ES cell lines with potential value for the genetics and biomedical research community.	http://www.mmrrc.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 profile of Cre driver lines supported by the NIH Blueprint	www.credrivermice.org
The Jackson Laboratory Cre Repository	Provides the scientific community 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 Mt 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 *Xenopus* Gene Collection	Full length cDNAs for *Xenopus*	http://xgc.nci.nih.gov/
The *Zebrafish* Gene Collection	Full length cDNAs for Zebrafish	http://zgc.nci.nih.gov/
Viral Vectors		
National Gene Vector Biorepository (NGVB)	A 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	http://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/
Ensembl Genome Browser	Produces genome database for the eukaryotic and	http://www.ensembl.org/index.html

	vertebrate organisms.	
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	A consortium leverages experimental pipelines built around next-generation sequencing technologies to map DNA methylation, histone modifications, chromatin accessibility, and small RNA transcripts in stem cells and primary *ex vivo* tissues selected to represent the normal counterparts of tissues and organ systems frequently involved in human disease.	http://www.roadmapepigenomics.org/
The ENCODE Project	The ENCyclopedia Of DNA Elements provides information about the functional elements in the genome	http://www.genome.gov/10005107
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 & 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	http://www.neuinfo.org/products/antibodyregistry/
Protein Database	Databases of protein sequences and 3dimensional	http://www.ncbi.nlm.nih.gov/protein

	structures of proteins	
Other Important Research Resources		
Drosophila Genomics Resource Center (DGRC)	A NIH-funded Center which serves the *Drosophila* 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	A NIH-funded Resource Center to provide a central repository for wild-type and mutant strains of zebrafish *(Danio rerio)* 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/