From Cajal to Connectome and Beyond

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Abstract

One goal of systems neuroscience is a structure-function model of nervous system organization that would allow mechanistic linking of mind, brain, and behavior. A necessary but not sufficient foundation is a connectome, a complete matrix of structural connections between the nodes of a nervous system. Connections between two nodes can be described at four nested levels of analysis: macroconnections between gray matter regions, mesoconnections between neuron types, microconnections between individual neurons, and nanoconnections at synapses. A long history of attempts to understand how the brain operates as a system began at the macrolevel in the fifth century, was revolutionized at the meso- and microlevels by Cajal and others in the late nineteenth century, and reached the nanolevel in the mid-twentieth century with the advent of electron microscopy. The greatest challenge today is extracting knowledge and understanding of nervous system structure-function architecture from vast amounts of data.

Contents

INTRODUCTION

The complementarity between form and function is a fundamental principle of biology, just as in other areas such as electrical engineering, architectural renderings, machine schematics, and genomes. Exploitation of this relationship in the twentieth century gave deep insight into how neurons function at the cellular, subcellular, biophysical, and molecular levels. It is now clear that the general structure-function organization of neurons is the same in all animals from hydra to humans—the neuron is the basic logic unit of neural circuitry (McCulloch & Pitts 1943). What differs between species is the architecture of nervous systems constructed from these circuit elements. As the twenty-first century progresses, it is becoming increasingly obvious that tools needed to understand the design features of entire nervous systems are either available or within our grasp (Pechura & Martin 1991, Bota et al. 2003, Sporns et al. 2005, Lichtman & Sanes 2008, Bohland et al. 2009, Lichtman et al. 2014).

The nervous system is the only part of the vertebrate body where the general relationship between structure and function remains unclear—because of the system's unique complexity as a biological computer controlling the internal bodily state of an animal and coordinating underlying homeostatic mechanisms with behavioral interactions directed toward surviving in the external environment. But researchers can now envision the creation of a biologically based dynamic or functional wiring diagram model of the nervous system for clinical, experimental, and theoretical systems neuroscience, which currently lacks a powerful conceptual framework analogous to the periodic table of the elements for chemistry, the double-helix model of DNA for molecular biology, or Harvey's model of the circulatory system for physiology.

This review focuses on conceptual underpinnings and strategies for constructing the structural half of a dynamic model of global nervous system organization, a necessary but certainly not sufficient model of physical reality that on one hand constrains the range of functionality and hypotheses of underlying mechanisms and on the other hand provides the neuroinformatics skeleton for organizing related molecular, genetic, physiological, behavioral, and cognitive data.

A fundamental principle of systems or network analysis is that to understand function and mechanism in a particular system or network, one must start with a parts list, understand how each part works, and know how the parts are connected to work as a whole (Meadows 2008). After defining more carefully what we mean by nervous system architecture (including parts and connections), we argue below that a great deal of knowledge already exists in this domain—although it has been difficult to access and digest—and that considerable technical and scientific expertise now exists to formulate realistic strategies for understanding global nervous system organizing principles at successively deeper levels of analysis.

Successful application of these strategies is not straightforward for two interesting reasons. First, reductionist approaches tend to view individual genes or microscopic features as explaining particular behaviors, whereas in reality, networks of molecules, cells, and larger structures are involved in all function. This is where systems biology applies to the nervous system. And second, collecting vast amounts of digital data is now relatively easy compared with learning how to mine those data for knowledge and understanding. This is where neuroscience will benefit greatly by collaboration with mathematicians, computer scientists, and others interested in taking on the challenge of understanding how the nervous system works through the interaction of structure and function.

CAJAL'S CENTRAL DOGMA: NEURON DOCTRINE WITH FUNCTIONAL POLARITY

Santiago Ramón y Cajal (1852–1934) spurred a revolutionary paradigm shift in neuroscience by introducing the basic conceptual framework of neural circuit organization used today, updated with all the refinements emerging from 125 years of research. Before Cajal's first groundbreaking paper in 1888 (Cajal 1888), scientists generally thought the cellular organization of neural circuits was reticular, with the cytoplasmic extensions (dendrites and axons) of different neurons in physical continuity, without the intervention of plasma membranes, allowing neural activity to spread in any direction through the network, biased by sensory inputs (Shepherd 1991).

Based first on observations in bird cerebellum with the Golgi method (Cajal 1888), and eventually on a systematic analysis of the developing and adult vertebrate nervous system (Cajal 1909–1911), Cajal proposed two broad generalizations about the organization of neural circuits that became central dogma: First, individual neurons interact with other neurons and effector cells (such as muscle and gland) via contact or contiguity, not continuity; and second, neural impulse flow directionality through circuitry (not within individual neurons; **Figure 1*a***) is predicted from the structure of a neuron's individual components, with dendrites and the cell body being the input side and the axon being the output side. The first generalization became known as the neuron doctrine and was confirmed unequivocally in the 1950s with electron microscopy (Shepherd 2010). The second generalization became known as the law of functional (dynamic) polarity, and its predictions were largely confirmed by elucidating the physical basis of graded and action potentials (Hodgkin 1964).

Structure and function were inextricably interwoven in Cajal's thinking, with profound functional predictions based on careful, critical observation of structural organization. At the level of the entire nervous system, he used observation and his conceptual framework to explain for the first time (Cajal 1893) the reflex and voluntary control of behavior in cellular terms (**Figure 1*a***). This model involved the entire body with nervous system circuitry containing three major neuronal classes based on structure, function, and location: sensory neurons, interneurons, and motor neurons. And it was a minimal or canonical model in that one prototypical neuron was shown at each circuit node. This modeling convention was replaced in specific brain

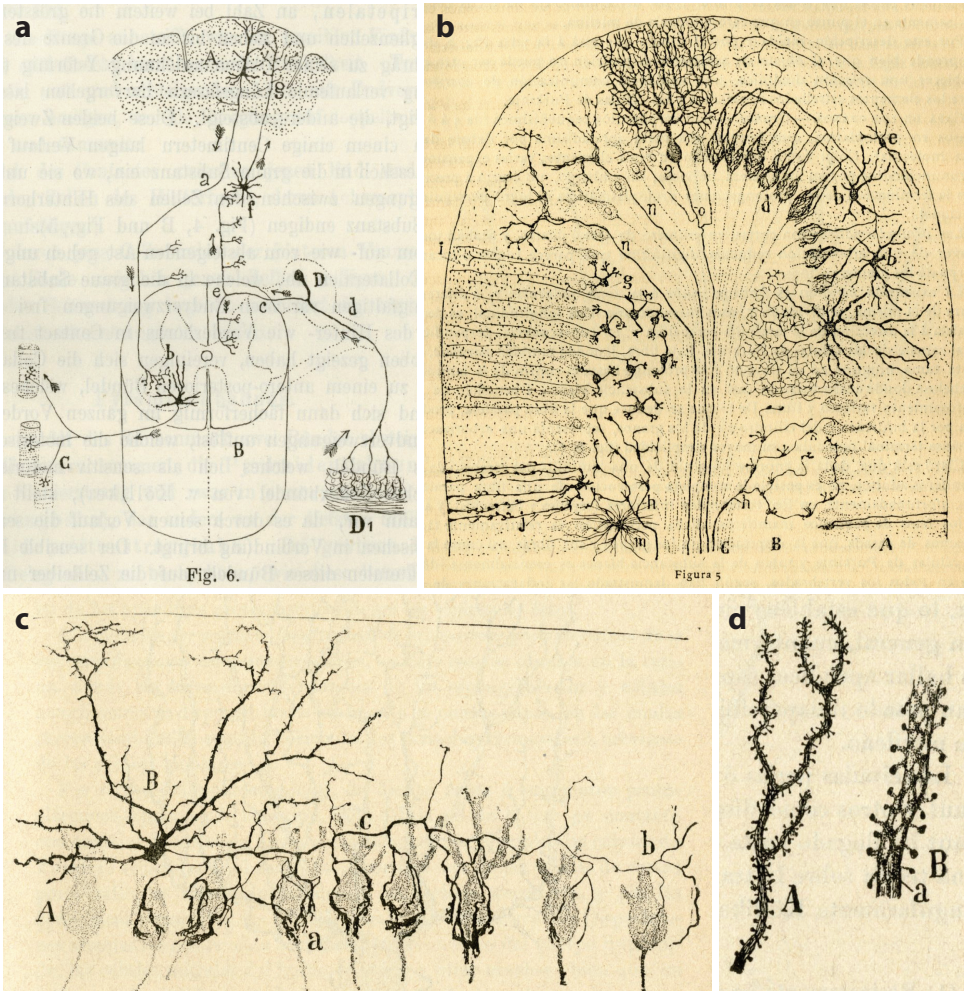


Figure 1

Levels of neural circuit analysis from Cajal's work. (*a*) A nervous system–wide diagram of reflex and voluntary control of behavior based on the Golgi method. Sensory information from the skin (D) is transmitted by dorsal root ganglion cells (d) to spinal cord (B) gray matter and to pyramidal neurons in the cerebral cortex (A), which in turn transmit impulses to motor neurons (b) in the spinal cord. For clarity, an interneuron between the spinal ending of (c) and an ipsilateral motoneuron are not shown. In this diagram, function (*arrows*) is predicted from structure. Panel reproduced from Cajal (1893). (*b*) Neuron types in a gray matter region, the cerebellum, demonstrated with the Golgi method. Panel reproduced from Cajal (1892). (*c*) The morphology and structural connections of an individual neuron, a cerebellar basket cell (B), with axon terminals on lightly outlined Purkinje cells (B), demonstrated with the Golgi method. (*d*) Detailed structure of part of a neuron—in this example, dendritic spines on segments of Purkinje cell dendrites—demonstrated with the Ehrlich methylene blue method. Panels *c* and *d* reproduced from Cajal (1899–1904). As a fundamental generalization, most neurons have a single axon that branches in a more or less complex way to form multiple connections, as illustrated in panels *a*–*c*; this feature is not represented in current connectomes.

regions, where neuron sets constituting different neuron types, with their local connections, were represented (**Figure 1*b***); even more detail was provided for individual neurons, such as the cerebellar basket cell for which he described the neuron doctrine (**Figure 1*c***; Cajal 1888).

Cajal referred to structurally identified axon terminals typified by those for the basket cell (**Figure 1*c***) as *articulaciones* or *terminales* in Spanish, and the functional equivalent was soon called a synapse by Sherrington (1897). The detailed examination of individual synapses and their distribution is a final level of analysis before (and after) electron microscopy (**Figure 1*d***).

NEURAL CONNECTIONS AND CONNECTOMES TODAY

Current network analysis recognizes four basic neural connection types depending on which data set is used: structural, functional, effective, and hypothetical (Friston 1994, Rubinov & Sporns 2010, Brown & Swanson 2013). This review focuses on structural connectivity, and more specifically on neuron-based connectivity, as a tangible, physical starting point. Magnetic resonance diffusion tractography (Birnbaum & Weinberger 2013, Jones et al. 2013, Thomas et al. 2014)—with a practical resolution in humans of about a cubic millimeter, compared to the micron to nanometer resolution of histological analysis—is beyond the review's scope.

Defining Connections

Cajal's foundational view of neural connectivity has been greatly elaborated and extended with the recognition of electrical and chemical synapses (inhibitory and excitatory); axo-axonic, dendrodendritic, reciprocal, and silent synapses; neurotransmitter volume conduction; synaptic plasticity; and so on (Kruger & Otis 2007). Nevertheless, the basic starting point for modern neural circuit analysis remains the spatial distribution of individual neurons and the pattern of axonal connections between them and effector cells. How this scaffolding functions and changes over time poses a complementary set of challenges constrained by structural organization because information flow timing in the circuit depends partly on conduction distances, conductor diameters, and conductor branching patterns.

Contemporary structural neuroscience (and neuroscience generally) suffers from relatively chaotic nomenclature (compared to, say, chemistry) that obscures communication and is disastrous for formal network analysis and knowledge management systems using inference engines (Swanson 2014). To address this problem, a foundational model of nervous system structural connectivity, with a defined set of terms and relationships between them, was created (Swanson & Bota 2010), along with a human- and machine-readable formal modeling language (notation) for neural connectivity (Brown & Swanson 2013).

In this context, a structural neural connection in any animal at any level of analysis is defined simply as "the overall structural link between two nodes in the wiring diagram of the nervous system" (Swanson & Bota 2010, suppl. inf. p. 19). This is where a node can be an individual neuron, a neuron type, or a gray matter region (see the section titled Analysis Levels below).

Connectomes, Connectomics, and Connectopathies

The term connectome was first used to define "a comprehensive structural description of the network of elements and connections forming the human brain" (Sporns et al. 2005, p. 0245). Connectome has since come to mean any systematic account of connections, from local circuits to networks constituting entire nervous systems, which have been called neuromes (Bota et al. 2015). Some researchers have suggested that, for clarity, long-range projection maps should be

differentiated from local connection maps, hence the term projectome (Kasthuri & Lichtman 2007, Shepherd 2014).

Connectomes are connection (adjacency) matrices that can be undirected or directed, and binary or weighted. Since Cajal's time, neuron-based connectivity data have generally been published as directed (from-to) and weighted (most often qualitative, on an ordinal scale, rather than quantitative). In one sense, connectomes are simply compilations of neuroanatomical data. However, connectomes have added a revolutionary dimension to neuroanatomy. Comprehensive matrices encourage systematic entry of all relevant data, exposing known and unresearched connections, and lend themselves to complex network analysis (see the section titled Circuit Architecture Beyond Connectomes below).

The connectome concept soon generated an encompassing research field, connectomics, originally defined as "a branch of biotechnology concerned with applying the techniques of computer-assisted image acquisition and analysis to the structural mapping of sets of neural circuits or to the complete nervous system of selected organisms using high-speed methods, with organizing the results in databases, and with applications of the data (as in neurology or fundamental neuroscience)—compare GENOMICS" (Lichtman & Sanes 2008, p. 346). Applications to neurology were immediately obvious: Perturbations of the normal human connectome—renamed connectopathies—are undoubtedly associated with a wide range of clinical problems affecting the nervous system (Lichtman & Sanes 2008). A definition today would include psychiatry as an obvious application area, and proteomics as an obvious comparison.

Circuit Architecture Beyond Connectomes

Although systematic, directed connectomes alone are the barest possible representation of connectional data because they are just from-to lookup tables—vast amounts of associated structural, molecular, biophysical, and other functional data are unrepresented, although they can be associated in a database (Bota et al. 2003). More importantly, connectomes alone do not display how individual connections are arranged in nervous system circuitry. Instead, the transforming advantage of connectomes is the ability to subject their systematic connectional data to network analysis, currently based largely on graph theory dealing with nodes and the pattern of connections (edges) between them. The result is computational identification of universal network properties such as small-world organization, stereotyped node connection motifs, node modules with heavy local connections, nodes with high centrality measures of connectivity (hubs), and hubs with high centrality measures of connectivity between modules (rich club) (see Bullmore & Sporns 2009, 2012; Rubinov & Sporns 2010; Sporns 2011; van den Heuvel & Sporns 2011). Nodes and connection numbers in any nervous system are too large to understand global organizing principles without the aid of computational and computer graphics methodologies.

Analysis Levels

A successful strategy for solving complex problems is to start with a tractable general solution and work from coarse- to fine-grained levels of analysis and understanding, as suggested for global nervous system wiring diagrams (Bota et al. 2003, Sporns et al. 2005, Lichtman & Sanes 2008). In the foundational model of structural connectivity (Swanson & Bota 2010, Brown & Swanson 2013), researchers defined a simple, systematic, three-level hierarchical (nested) approach based on gray matter regions (such as retina and suprachiasmatic nucleus), the neuron types forming each gray matter region (such as granule and Purkinje cells in the cerebellar cortex gray matter region), and individual neurons forming each neuron type population.

Macroconnections are at the highest, most coarse-grained level, and the two nodes are gray matter regions treated as black boxes (such as from retina to suprachiasmatic nucleus). Mesoconnections are at the next level, and the two nodes are neuron types (such as from granule cells to Purkinje cells). Microconnections are at the third level, and the two nodes are individual neurons, for example, between a particular granule cell and a particular Purkinje cell. Nanoconnections are a logical extension and are at the most fine-grained level, where the two nodes are pre- and postsynaptic elements of a synapse, static or dynamic.

In healthy animals, macro- and mesoconnections are considered hardwired and genetically determined. Micro- and nanoconnections, by contrast, are modified by experience, including by learning, hormones, and drugs (Swanson & Bota 2010).

MACROCONNECTOMES

Unraveling nervous system connectivity has a long, rich history going back to classical antiquity (Clarke & O'Malley 1996, Swanson 2000). The first global structure-function account of nervous system organization—the three-cell theory—lasted from the fifth to the seventeenth century. It postulated that psychic pneuma (our neural information) flows in through sensory nerves to the lateral ventricles (Cell 1), where it is combined and processed before sequential processing in the third (Cell 2) and fourth (Cell 3) ventricles; it is then passed down the spinal cord to motor nerves, producing behavior (Manzoni 1998). Thomas Willis (1664) postulated instead that psychic pneuma is generated in gray matter regions and transmitted by white matter tracts, making the ventricles a waste disposal system—a paradigm shift that remains foundational over 350 years later. Based on gross dissection, Willis also proposed a global structure-function model with 4 regions (nodes) and 12 connections (directed edges) between them. Although none of these connections hold up to modern analysis, Willis's terminology and conceptual framework had a profound and lasting influence. Meynert (1872) provided the only other attempt at a similar global nervous system model, based on the appearance of mammalian central nervous system tissue observed histologically just before introduction of the Golgi method (Golgi 1873).

From today's perspective, what are the boundary conditions of macroconnectome complexity? The mammalian brain has on the order of 500–1,000 gray matter regions (a range easily accommodating spinal cord and peripheral ganglia) and 25,000–100,000 macroconnections between them (Bota et al. 2003). A recent meta-analysis indicated there are about 2,400 association macroconnections between 73 cerebral cortical regions in rat (**Figure 2**; Bota et al. 2015). This study also suggested there are enough published connection data to assemble a qualitatively accurate rat nervous system macroconnectome. Previously, an informal assessment of the connectional literature suggested a global four-systems network model of mammalian nervous system macroarchitecture, with motor, sensory, cognitive, and state subsystems interconnected asymmetrically (Swanson 2012).

All the methodology needed to assemble high-resolution, global, nonhuman mammalian nervous system macroconnectomes is readily available. Researchers have made promising starts on the mouse brain macroconnectome (Zingg et al. 2014, Oh et al. 2014), which can readily be extended to the mesoconnectome level.

MESOCONNECTOMES

Global mesoconnectomes are the sets of axonal connections between specific neuron types within and between gray matter regions of a gender, age, or disease state of a species. One critical problem with mesoconnectomes is disagreement about defining neuron type. Recently developed genetic

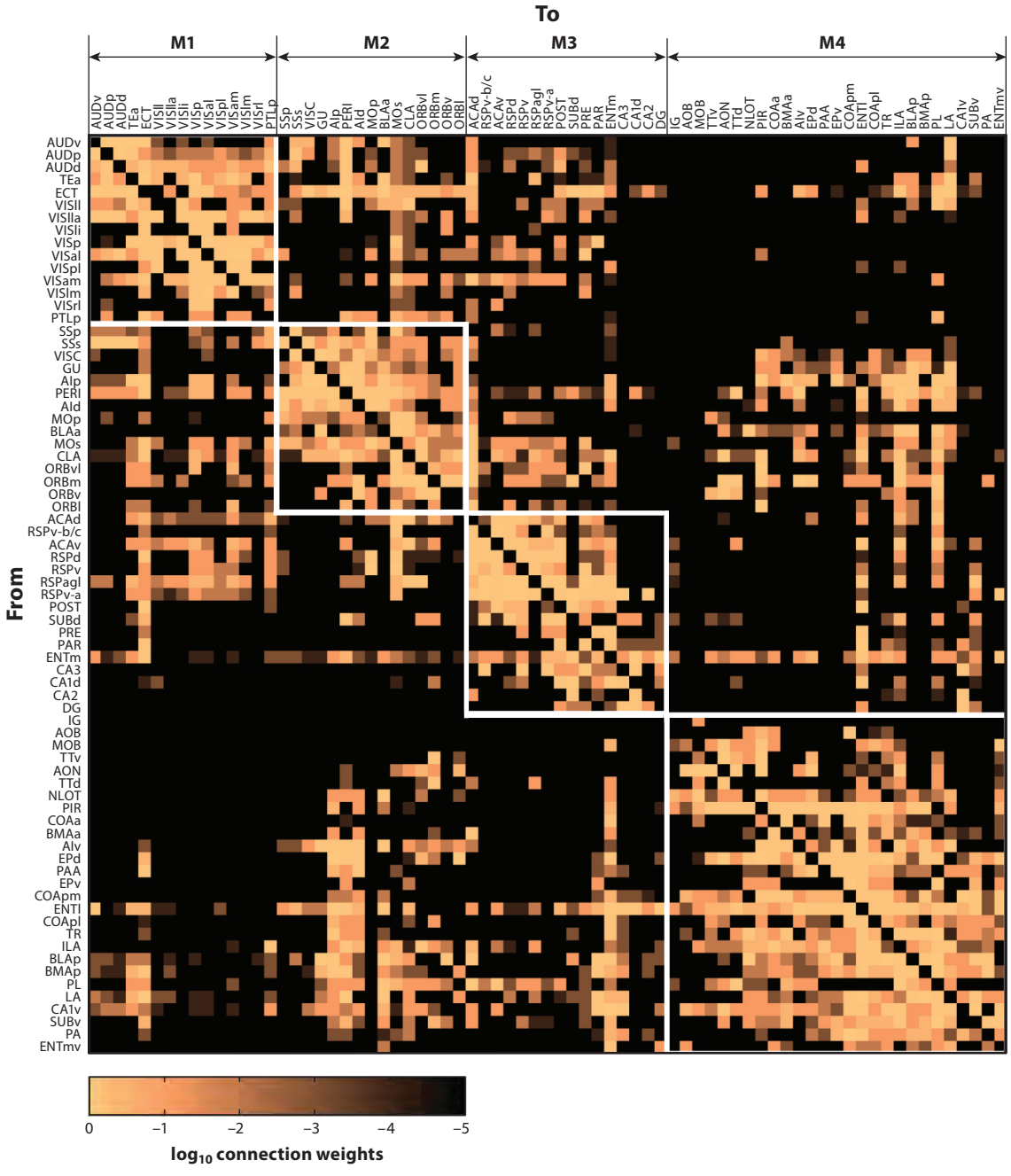


Figure 2

A rat cerebral cortical association macroconnectome, involving 73 gray matter regions or nodes (isocortex, allocortex, cortical plate, and cortical subplate). It is based on directed (from-to relationships) and weighted (scale at the *bottom*) connections established with experimental axonal transport pathway tracing methods, and network analysis shows that the cortical mantle can be divided into four modules (M1–M4) based on association connection weight. Topographically, the modules form a shell-core arrangement, with dorsal and ventral shell modules essentially representing the classic limbic region, and rostral and caudal core modules representing the rest of the cortical mantle. Figure adapted from Bota et al. (2015) with permission.

dissection methods for neural circuitry add new dimensions to connectomics (Luo et al. 2008), but older criteria for classifying neurons—location (spatial distribution), size and shape, connections, and function (**Figure 1**)—are also important (Swanson 2004). Polythetic classification, in which all relevant data are evaluated statistically with multiparametric methods such as principal component and cluster analysis, has been recommended and applied to the discovery of neuron types—distinguishable clusters formed in parametric space, subject to revision based on new data (Bota & Swanson 2007).

Neuron classification is not a mysterious process, just another example of biological taxonomy (Simpson 1961). Both Golgi (1885) and Cajal (1899–1904) recognized that a complete gradation between neuron shapes is not the rule (**Figure 1**). Instead, in the cerebellum, retina, olfactory bulb, and all other gray matter regions, neurons fall into recognizable populations, each with a distinct set of locations, shapes, connections, gene expression patterns, and functions, although many details remain to be clarified. Based on complete literature reviews for seven gray matter regions in rat (including retina, cerebellar cortex, and trochlear nucleus) and a systematic polythetic-monothetic approach, investigators proposed a seven-level hierarchical classification scheme for all neuron types, starting at the top with neuron as a cell type and ending at the bottom with neuron types (analogous to the species level in animal taxonomy) and then neuron varieties (Bota & Swanson 2007). Compared to a common taxonomic hierarchy with 21 levels for classifying mammals (Simpson 1961), this is a reasonable starting point.

None of the leading neuroanatomists of the nineteenth and early twentieth century attempted a synthetic, global wiring diagram model of the vertebrate nervous system at the mesoconnection level. The only example remains C. Judson Herrick's description of the tiger salamander based on over 50 years of personal research (Herrick 1948). The magnitude of the problem is indicated by estimates that the mammalian brain has on the order of 2,500–5,000 neuron types, assuming its 500–1,000 gray matter regions have an average of 5 neuron types each (Bota et al. 2003). Rapid progress is currently being made on the fly mesoconnectome using a combination of genetic, imaging, and neuroinformatics technologies (Chiang et al. 2011, Shih et al. 2015, Wolff et al. 2015).

MICROCONNECTOMES

Researchers widely believe a canonical synaptic connectivity matrix interconnects neurons of different types. Any introductory neurobiology textbook will show such connectivity diagrams for retina, cerebellum, and even cerebral cortex. Cajal's great legacy was to not only bring to light the vast number of differently appearing neurons but also show how they might be functionally interconnected by noting the proximity of axons of one cell type impinging on the dendrites and somata of other cell types. Nowadays, upgrades to these diagrams depend less on Golgi staining and more on methods that verify synaptic connections between individual nerve cells. Detecting these synaptic connections requires either electrophysiological or high-resolution anatomical methods. Recordings (optical or electronic) of neural activity, coupled with a means to selectively stimulate particular types of input, have provided a wealth of information about who talks to whom (Nikolenko et al. 2007, Oberlaender et al. 2012, Packer et al. 2015).

The great strength of the Golgi stain was the sparsity of labeling that allowed unambiguous identification of putative pre- and postsynaptic partners even though they are labeled the same color. This monochromatic approach is, however, also a drawback if the goal is to identify all the kinds of inputs to particular neurons because dense staining is impossible to untangle. Thus, using sparse labeling to get all the connectivity information can only occur by looking at a great number of cells of a particular type and pooling all those results. However, the wrong inference can be drawn by pooling if some neurons differ not so much by their appearance but by their

connectivity per se. Alternatively, if one could see all the connections at once, then individual cells could be defined by the particular cohort of neurons that innervate them. One potential way to accomplish this is to use a dense, multicolor, Golgi-like approach to disambiguate each input. Researchers developed Brainbow transgenic strategies with this idea in mind (Livet et al. 2007, Cai et al. 2013, Loulier et al. 2014, Richier & Salecker 2015, Weissman & Pan 2015, Zhang et al. 2015). Diverse colors in different neurons come about by a stochastic combinatorial expression of three or four different fluorescent proteins by Cre-lox recombination. This method has been useful in determining the projections of neurons in insects (Hampel et al. 2011, Boulina et al. 2013, Shimosako et al. 2014), fish (Pan et al. 2013, Robles et al. 2013, Xiong et al. 2015), and glia in the mammalian central nervous system (Dumas et al. 2015), but to date, the extraordinary density and numbers of converging inputs to cortical neurons have made this technique less than ideal. Another approach is to specifically label only the subset of neurons that are presumably presynaptic to a particular cell. Transneuronal viral labeling is such an approach (Wickersham et al. 2007, Feinberg et al. 2008, Kim et al. 2012, Pivetta et al. 2014, Callaway & Luo 2015). Even retrograde labeling techniques can be multiplexed to allow identification of many individual axonal arbors in the same sample (Tsuriel et al. 2015).

These approaches are, however, piecemeal and hence inadequate to generate a complete microconnectome that identifies all cell types that innervate each neuron in the brain. Therefore, researchers have considered some more industrial methods. One idea is to translate the connectivity problem into a format that can take advantage of the breakthroughs in DNA sequencing, which at present can be done at rates of hundreds of millions or even billions of nucleotides in a single day. Zador and colleagues (2012) are developing a means of barcoding individual neuronal connections that could achieve this goal. This approach does away with the need for an anatomical connectome and replaces it with a connectivity matrix.

NANOCONNECTOMES

The high resolving power of electron microscopy made it the first way to unambiguously identify synapses in the brain (Robertson 1955, 1956; De Robertis 1959). In the ensuing decades, electron microscopy imaging of brain sections led to many hundreds of papers and the identification of synapses between different classes of neurons. The main disadvantage of electron microscopy is the requirement of imaging ultrathin ($\ll$1 μm) histological sections, which means that a single section is never adequate to generate connectomics information except in situations in which the pre- and postsynaptic cells are identifiable by some other means, such as immunohistochemistry. To overcome the problem of thin sections, scientists have used serial sectioning, in which each brain section is part of a sequence that transects a volume into hundreds or even tens of thousands of sections. Tracing objects from one section to the next reconstructs the geometry of the neurons and can also be used to identify the sites and cellular participants of each synapse.

The first attempt to do serial sectioning to understand nervous system structure occurred at the beginning of the twentieth century, when Richard Goldschmidt, as a young man working at the University of Munich, explored the nervous system of the intestinal roundworm *Ascaris*. Of course, electron microscopy was not yet invented, so he reconstructed its nervous system from 5-μm serial sections using Nissl-stained material in high-resolution (1,030×) light microscopy (Goldschmidt 1903, 1908, 1909). Among the many anatomical drawings he produced were wiring diagrams that seem to have been barely interpreted in more modern literature (Chitwood & Chitwood 1974). He produced a color wiring diagram of nerve fibers outside the worm's nerve ring and three extraordinarily detailed diagrams of the neuronal processes that entered and traversed the nerve ring (**Figure 3*a***).

With hindsight of the past century of neuroscience, the inadequacies of these drawings are clear. They show no synaptic connections, and so they are more like a high-resolution version of a mesoconnectome in which connections between individual cells are plotted but the direction of those connections is entirely absent. Goldschmidt could deduce that some of the pathways were motor (outgoing) and others sensory (ingoing), but for the many association connections between, the lines drawn do not convey directionality. Indeed, he admitted that even the straightforward idea of whether this is a syncytial (à la Golgi) or contact-based (à la Cajal) nervous system is not knowable from the data he had.

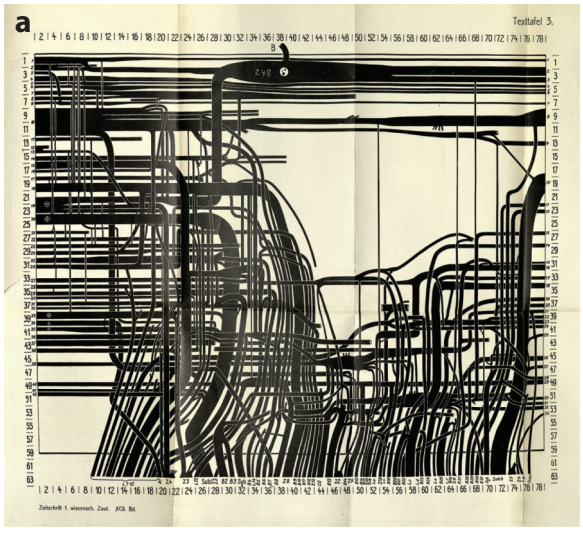
Owing to the large size and inadequacy of the light microscopy reconstruction to document synaptic connections, Brenner and his colleagues decided to reconstruct the nervous system of a smaller soil roundworm, *Caenorhabditis elegans*, in which the complete structure of a nervous system might be accomplished by electron microscopy (Ward et al. 1975). Their goal was to ascertain how the nematode's behavior was generated by the structure of the nervous system. They also noted that *C. elegans* is a good species for study of the genetics of complex processes, both because behavioral mutants exist and because its life cycle is brief.

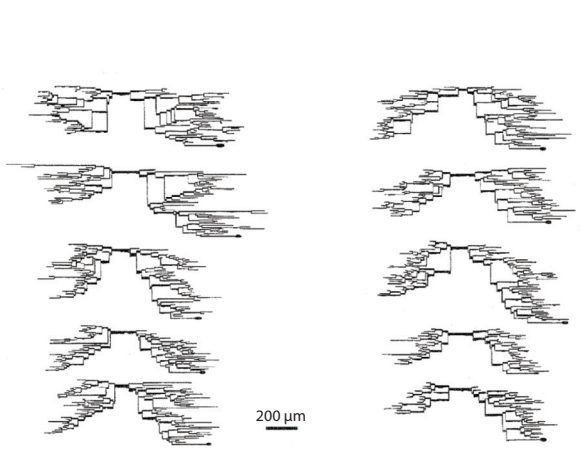
However, even this small animal with about 300 neurons required a heroic, decade-long effort to complete its connectome: a project dubbed "the mind of a worm" (White et al. 1986, Emmons 2015). The effort was precomputer and required manual image acquisition, darkroom work to print out pictures from glass negatives, and manual tracing. S. Brenner (personal communication) said that they tried to use computers to automate the process, but it was too difficult at the time. They were helped by the assumption of a largely stereotypic wiring diagram, allowing them to splice the results of two hermaphroditic worms because of lost material. *C. elegans* wiring diagrams have been further improved by Emmons and colleagues for both hermaphrodites and males using software to trace and analyze the connections (Jarrell et al. 2012; S.W. Emmons, personal communication). In addition to added data, the connectivity has been reanalyzed into connectivity maps showing 280 nonpharyngeal neurons, with 6,393 chemical and 890 electrical synapses, plus 1,410 neuromuscular junctions (**Figure 3*b***; Varshney et al. 2011).

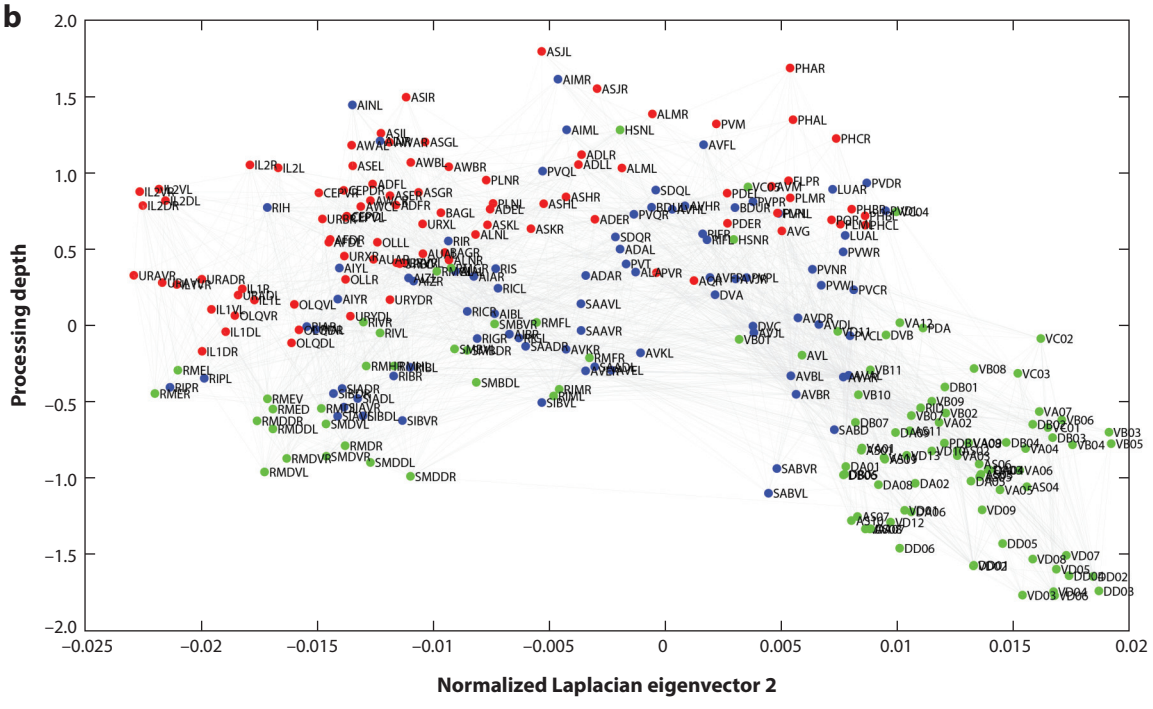
Many interesting features are brought to light with a bird's-eye view of this connectome. First, although the connectivity is perhaps far too complicated to get a gestalt by just looking at it, the network does have properties that are akin to a wide range of other networks (including the Internet). In particular, it shows so-called scale-free properties in which a subset of neurons is far more interconnected in the network than would occur if the network were random (Barabási & Bonabeau 2003, Varshney et al. 2011). It remains unknown if and where single neurons in mammalian neural circuits serve as hubs. It is possible that responsibilities analogous to hub neurons in worms are served instead by multicellular neural groups, such as ventral horn motoneuron pools. Indeed, one interesting difference between vertebrate nervous systems and those of many invertebrates is the tendency of vertebrates to use groups of similar neurons (neuron types) to serve functions carried out by individual identified neurons in invertebrates (Lichtman & Colman 2000). This difference may mean that functional motifs will be represented in different ways in different subphyla of animals.

This distinction between the nervous systems of terrestrial vertebrates on one hand and invertebrates (such as nematodes and insects) on the other may have important ramifications for nanoconnectomics. In the 1960s, numerous molecular biologists began to consider the new frontier of neuroscience by looking at invertebrate nervous systems. Sydney Brenner, as already mentioned, chose worms; Seymour Benzer flies; Gunther Stent the leech; and Cyrus Levinthal the small, freshwater planktonic crustacean *Daphnia magna* (Macagno et al. 2014). Levinthal was interested in learning the extent to which genetic information specifies the connections and geometry of neurons and chose *Daphnia* animals because they can be genetically identical clones by

parthenogenetic replication. Levinthal and his coworkers developed a serial electron-microscopy approach in which prints from electron microscopy glass negatives were initially overlain by clear acetate for tracing, and the resulting traces were subsequently aligned and used to build three-dimensional models. Technical difficulties limited the number of neurons that could be traced, and Levinthal and his colleagues realized that automation was essential. This led them to develop a digital drawing tablet and computer-aided reconstruction tracing of serial sections. This







computer-aided approach was ahead of its time and is still the mainstay of many modern attempts to generate wiring from serial electron-microscopy images (see, for example, Kasthuri et al. 2015). His group also tried to get automated image segmentation working but was not really successful. This challenge is still a central stumbling block (see below).

The invertebrate work made it clear that the Cajal notion of cell type could be extended to the single-cell level in these animals. Thus, investigators could inquire about the neural connections of the same individual neuron from one animal to the next. In *Daphnia* and, more recently, crayfish, the results suggested that arbors are recognizably similar but not identical between animals (**Figure 3*c***). This idea has been strongly supported by more recent studies in flies and comparisons of neurons in different worms and other animals that have stereotyped structure and connectivity (see, for example, Hikosaka et al. 1996, Chen et al. 2006, Chiang et al. 2011, Randel et al. 2015, Wolff et al. 2015).

Although few neuroscientists doubt the monumental contribution of Cajal to the notion of neural circuit, nanoconnectomics may push circuitology into a realm with a different and, to some degree, opposing emphasis. The strength (and potentially also a limitation) of neural circuits developed prior to the advent of nanoconnectomics is that conclusions are based on approaches in which small numbers of neurons are used to infer the organization of a population. Such approaches most easily highlight the ways different (often different-looking) kinds of neurons are connected to each other. This is a different emphasis than what is gained from nanoconnectomics, in which differences in the synaptic connectivity among many individual neurons of one type can be revealed. Indeed, a central conclusion based on Cajal-style circuits is that neurons of the same type, by definition, connect the same way. Nanoconnectomics, on the other hand, can easily label the connections of many or all pre- and postsynaptic cells and is therefore not constrained to focus only on which different types of neurons are connected to each other.

Although nanoconnectomics is too nascent to provide a definitive answer, it seems likely that when an animal has many neurons of the same type, no two are doing exactly the same thing. For example, in a pool of motor neurons, each has a unique wiring pattern, and even the functionally homologous neuron innervating the identical contralateral muscle has a different innervation pattern than its ipsilateral homolog (Lu et al. 2009). Thus, what seems like a stereotyped pattern at a lower level of analysis may hide an immense diversity at another, higher-resolution level. The differences in the wiring diagrams between multiple neurons of the same type (such as the precise Purkinje cell connectivity of each of thousands of parallel fibers originating from different

Figure 3

The beginnings of microconnectomics. (*a*) Probably the first attempt to generate a wiring diagram comes from the serial light microscopy of the intestinal roundworm *Ascaris* by Goldschmidt (1903, 1908, 1909). He managed to trace out many hundreds of nerve fibers, albeit without any synaptic connections. Shown is a detailed drawing he made of the layout of nerve fibers entering and exiting the nerve ring. Panel reproduced from Goldschmidt (1909). (*b*) The decade-long effort of White, Brenner, and colleagues (1986) gave rise to a description of the connectivity of the small nervous system of the nematode *Caenorhabditis elegans*. That connectivity has been reanalyzed with computational tools and rendered in this panel into a connectivity map in which neurons with similar connectivity are closer to each other than less-connected neurons. This diagram shows all 280 nonpharyngeal neurons, and their interconnections are based on descriptions of 6,393 chemical, 890 electrical, and 1,410 neuromuscular synaptic connections. Panel reproduced from Varshney et al. (2011) with permission. (*c*) Confocal reconstructions of ten local directionally selective interneurons from the terminal abdominal ganglion of ten separate crayfish (*Procambarus clarkii*) that were intracellularly injected with the fluorescent dye Lucifer yellow CH. This mechanosensory neuron is nonspiking, so the precise organization and length of its processes are critical to its function. Superficially, they all look quite similar; however, dendrograms that plot the dendritic structure reveal these neurons differ from each other quantitatively severalfold in total membrane area, total dendritic length, length of the longest dendrite, number of dendrite branches, and electrotonic length. Panel reproduced from Hikosaka et al. (1996) with permission.

individual granule cells in the cerebellum) may provide a wealth of information about how these neurons are actually used.

CURRENT STATUS AND FUTURE DIRECTIONS

Connectome Versus Genome

The most successful biological omics is genomics, and it may provide some lessons for the more nascent connectomics field. The first is that what began as a monumentally difficult, expensive, and slow-going process has with time become easy, cheap, and fast. Although researchers are in the process of completing connectomes of small animals such as worms and flies, it is still hard to imagine a complete map of a rodent or human brain at the nanoscale. At the resolution required to see every synaptic vesicle, a cubic millimeter of gray matter would require in the range to 2 million gigabytes (2 petabytes). At that scale, a human brain might require nearly 2 million petabytes, an absurdly large amount of data, whose storage would likely require a large fraction if not all of the digital storage available on the planet today.

But one of the lessons of genomics is that what seems like big data in one decade is not so intimidating in the next. At the time genomes were being considered, some noticed with alarm that this effort would require thousands of megabytes of data (gigabytes) (Lichtman & Sanes 2008). Data acquisition speed is another bottleneck that is already showing signs of becoming unstoppered with the advent of machines that use parallel processing for image capture (Eberle et al. 2015, Hayworth et al. 2015). Image data segmentation has also been sped up by crowdsourcing approaches (Kim et al. 2014). There can be little doubt that, with time, connectomics will also become less difficult, quicker, and cheaper.

Critics of connectomics often forget another important lesson from genomics. This relates to the profound limitations of genomic information as an explanation for the phenotype. It is well understood that although almost all the features of an animal's structure and function can be traced back to its genes, the timing and locale of gene expression provide the diversity and complexity of cellular, organ, and animal form. Thus, the dynamics of the genome hold the key to relating the structure of the genome to the structure of an animal. However, a dynamic map of when and where genes are turned on and off, which is critical for making sense of the genome's role, has not yet been generated. Despite this limitation of genomic data per se, no one has argued that structural genomics (mapping the nucleotide sequences) is not worth doing. In connectomics, however, we hear the standard critique that structure without function is valueless. But just as transcriptomics could hardly get going until there was a genome, the same argument could apply to the nervous system's wiring diagram role in making sense of the function of neural circuits.

Systems Neurobiology in the Twenty-First Century

The resurgent interest in systems biology is a sign of the changing views of all manner of biologists who seek to understand complex phenomena. Success with systems approaches "will certainly require modeling and simulation tools from engineering, where experience shows that brute-force computational approaches are hopeless for complex systems. . . . Experiments, modeling and simulation, and theory all have fragilities, but they are complementary" (Csete & Doyle 2002, p. 1668). In systems neuroscience, success can be measured partly by the formulation of frameworks that have predictive power. The frameworks, however, need not be easy to understand; they must only give the right answer consistently. One hopes that researchers will eventually develop animal-wide structure-function algorithms of the nervous system. Efforts like this are already under way

in a variety of model organisms such as hydra, *Platynereis* (Randel et al. 2015), worm, fly, zebrafish, rodent, monkey, and human. Importantly, these rely on minimal a priori assumptions.

Interestingly, there may be a deep link between systems biology at the level of individual cells and modern systems neuroscience. In cellular systems biology, the focus is on the molecular pathways or circuits that underlie cellular behavior. These circuits are based on the upstream and downstream regulators of a particular cellular process. These networks have both excitatory and inhibitory parts, converging and diverging signals, and sensory (receptors) and motor (cell motility) aspects. In many ways, then, the molecular machinery of a cell seems analogous to the role in a cell's behavior that neural processes play in the behavior of multicellular animals. This link may be more than a coincidence because behaving single-celled organisms (think protozoa) must have all their behavior encoded in molecular mechanisms as opposed to neural connections.

Multicellular organisms may have needed to replace pure molecular paths, given the severe limitations on the diffusion speed of chemicals. In this sense, the wires that emerge from neurons are surrogates for the molecular mechanisms that work adequately in small (single-celled) animals. For neuroscience, the good news is that axons and dendrites tell us where the signals from one cell are coming from and going to. In molecular systems biology, tracing the network is much harder because there are no explicit physical connectors between chemical signals and their responders.

Rapid Technology Advances Generating Really Big Data

It is not particularly gratifying for scientists to attempt to predict the future, especially given how the pace of technological advance seems to outpace the expectations of even highly optimistic researchers. Nonetheless, we think it safe to assume that increases in computational power through use of computer clusters and ever larger and less expensive data storage modalities will be positive forces in the future of brain mapping. In addition, computer-driven automation will certainly help industrialize data collection, giving rise to extraordinary data sets: complete connectomes at all size scales.

The fact that our visual system is our strongest sense is likely the main reason why visualization tools are so useful as we attempt to fathom brains. Interactive online computer graphics visualization tools will be critical for wide community use of data from connectomics and from structural neuroscience concepts in general. Like the Earth, the brain and nervous system as a whole are three-dimensional objects (embedded themselves in the body), and like the Earth, both two- and three-dimensional maps of the brain and nervous system, and their connectivity, are useful.

A topologically representative flat map of the rat central nervous system, based on embryological fate mapping from the neural plate stage (Swanson 1992, Alvarez-Bolado & Swanson 1996), is available and has been used to display over 5,000 experimentally determined macroconnections in adult rat (see Canteras et al. 1992, Hahn & Swanson 2015). Combined with connectional data in the online Brain Architecture Knowledge Management System (Bota et al. 2012), the flat map is now the template for version 1.0 of an interactive Google Maps for the brain (Brown & Swanson 2015).

Researchers are also beginning to develop interactive flat maps for nanoconnectomics. So far, these take their inspiration from subway maps (Al-Awami et al. 2014). A much larger challenge is to make three-dimensional maps. Although rendering three-dimensional data is becoming the norm in connectomics by using a combination of semitransparency and movement and taking advantage of sophisticated shading models (see, for example, **Figure 4** and Kasthuri et al. 2015), these are not maps. Even in the most three-dimensional contexts, humans work mostly in a flat world despite excellent stereopsis. For example, so-called upper-area air traffic controllers mentally create three-dimensional pictures of airplane traffic on the basis of the two-dimensional radar

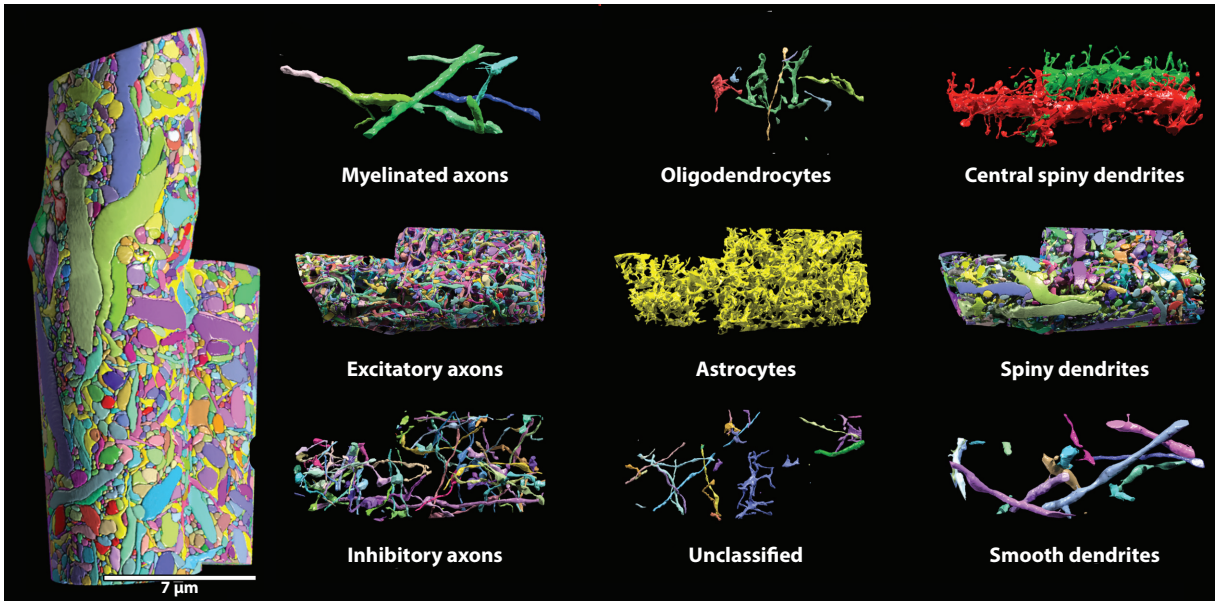


Figure 4

Reconstructions using modern, serial electron-microscopy connectomics approaches. On the far left is a fully reconstructed volume surrounding two apical dendrite segments of layer 5 pyramidal neurons in somatosensory cortex. Each colored object is a separate neuronal or glial cell process or extension. These processes are categorized in the images to the right. Adapted from Kasthuri et al. (2015) with permission.

display information, supported by other textural information concerning altitude (Eurocontrol 2015). Thus, the development of a robust three-dimensional platform remains an unsolved problem in brain mapping.

However, no matter how well neuroscientists render these data, a big crisis is looming, one that may be a direct byproduct of the success of generating connectomics data. The fact is that accumulating data is not equivalent to accumulating understanding (see, for example, the discussion in Kasthuri et al. 2015). Indeed, the biggest casualty of big data may be those big ideas that offer an explanation of how brains work. The problem stems from the intrinsic lack of intelligibility of connectomics data. The inherent complexity of data that simultaneously show thousands of streams of information flowing into and out of each of many nodes (individual neurons, neuron types, or regions) is likely beyond any individual human's comprehension. Making sense of such data may therefore be beyond the limits of the explanatory power of the human mind. The long-debated challenge of bridging the divide between mind and brain is unfortunately not likely to be overcome by a detailed accounting of each and every synaptic connection in the brain.

The so-called explanatory gap (Horgan 1999) between what we can know and what we want to understand is not necessarily made smaller by more information. Rather, such omics information pushes neuroscience into a different realm where information rather than ideas is the currency. In this realm, a detailed, bottom-up description of a biological system is mined for whatever conceptual insights might be revealed rather than top-down concepts of what we care about being used as a setup for experiments. In this sense, the data give us a more accurate view of the way things are and, at the same time, push us away from intelligible, albeit facile, answers to the enduring puzzles of the brain.

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