Deep Molecular Diversity of Mammalian Synapses: Why It Matters and How to Measure It

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SUMMARY

- Synapses of the mammalian central nervous system are highly complex structures composed of thousands of distinct proteins. Synaptic proteins are often expressed in variable patterns across different brain regions and neuronal populations, suggesting a high degree of molecular diversity in synapses.
- Synapses types have long been discriminated on the basis of neurotransmitter molecules, but it is now necessary to recognize much deeper diversity of both synaptic protein composition and function within each traditional neurotransmitter type.
- Intra-type synapse diversity reflects many factors, including diversity of presynaptic and postsynaptic parent neurons, activity histories, and influences of third-party neurons and glial cells.
- Intra-type functional diversity is reflected in strength, kinetics, voltage-dependence, and plasticity of synaptic transmission. Improving knowledge of the relationships between molecular and functional aspects of diversity is sure to illuminate principals of synaptic circuit function.
- Proteins identifiable at mature synapses include adhesion molecules that guide selective synaptogenesis during development. Such molecules may encode circuit context and connectivity in the mature brain and aid circuit reconstruction efforts.
- Many mental and neurological disorders reflect mutations in synaptic proteins expressed in subsets of synapses. A better grasp of synapse molecular diversity is certain to contribute to our understanding of specific brain disorders.
- New techniques that probe the combinatorial expression of proteins at the level of individual synapses within the brain are bolstering our efforts to link intra-type diversity to distinct physiological and structural properties of synaptic connections and brain circuits.
- The potentially vast diversity of mammalian synapses poses major challenges to efforts to reconstruct brain circuit structure and function. With improved means for the analysis of synapse populations at the single-synapse level, such challenges seem likely to yield to the development of “synaptomic” sub-type classification schemes based on cell-biological principles.

DEEP MOLECULAR DIVERSITY OF MAMMALIAN SYNAPSES: WHY IT MATTERS AND HOW TO MEASURE IT

SUMMARY

Pioneering studies during the middle of the twentieth century revealed substantial diversity amongst mammalian chemical synapses and led to a widely accepted synapse type classification based on neurotransmitter molecule identity. Subsequently, powerful new physiological, genetic and structural methods have enabled the discovery of much deeper functional and molecular diversity within each traditional neurotransmitter type. Today, this deep diversity continues to pose both daunting challenges and exciting new opportunities for neuroscience. Our growing understanding of deep synapse diversity may transform how we think about and study neural circuit development, structure and function.

"Unfortunately, nature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity."

Ramón y Cajal, 1906[1]

The synapse was envisioned originally as a simple gap across which nerve impulses must leap to travel from one neuron to the next. This early view suggested immediately that synapses must play a key role in neural information processing, but appreciation of the remarkable richness and variety of synapse functions has grown only gradually over the last century. Synapses are recognized today as being highly plastic in structure and function, influenced strongly by their own histories of impulse traffic and by signals from nearby cells. The synaptic contact is also fundamental to the development, homeostasis, and remodeling of complex neural circuitry. This functional and developmental diversity dictates that synapses must also be highly diverse in molecular composition, a conclusion ratified directly by increasingly sophisticated molecular biological investigations. Nonetheless, many important dimensions of synapse molecular diversity remain unexplored.

Early investigations of synaptic transmission resulted in a classification of synapses into "electrical" types, where ions can flow directly from one neuron into the next, and "chemical" types, where transmission is mediated by a neurotransmitter. Chemical synapses were also classified as excitatory, inhibitory, or modulatory in their actions. The discovery of numerous small molecule neurotransmitters led eventually to a widely used classification of chemical synapse types on the basis of neurotransmitter identity (as adrenergic, cholinergic, GABAergic, dopaminergic, serotonergic or glutamatergic, for example). Today, however, evidence for the molecular diversity of chemical synapses in the mammalian central nervous system (CNS) extends well beyond traditional neurotransmitter type classification. We now recognize that within these synapse classes there exists deep intra-type molecular diversity that includes both absolute differences in the complement of distinct proteins expressed and differences in their relative expression

levels. Rapidly growing lists of disparately expressed proteins include receptor subunits, vesicular transporters, scaffold proteins, and adhesion molecules, as well as proteins regarded as neurodevelopmental cues, "generic" signaling molecules, or "housekeeping" proteins. Importantly, diversity in function, such as the strength, kinetics or plasticity of synaptic transmission, often appears to mirror such protein expression diversity.

Exploration of this synapse diversity stands as a major challenge to neuroscience. Left unfathomed, synapse diversity threatens to confuse efforts to better understand synaptic physiology and neural function. Conversely, sounding the depths of synapse intra-type diversity is likely to enable more productive approaches to the study of both individual synapses and the circuits they define. Although the experimental obstacles are formidable, emerging methods offer exciting new opportunities for both functional and molecular insights.

This review will focus first on the status and implications of our present knowledge of synapse molecular diversity. Although it seems almost certain that deep molecular diversity will be a feature of synapses of every neurotransmitter type, most studies to date have focused on glutamatergic or GABAergic synapse types. Therefore, the present treatment will be limited to these two predominant synapse types and will focus on a few illustrative examples, selected to frame subsequent discussion, though regrettably this will leave important examples untouched. The review will conclude by describing the evolution of methods for visualizing single synapse molecular diversity and highlighting exciting opportunities for future explorations of synapse diversity that may open new windows on neural circuit development and organization, synaptic function and plasticity, memory, and disease.

Proteomic Complexity and Deep Diversity

The synapse is an extraordinarily complex organelle, spanning two distinct cells and comprising large numbers of distinct protein species contributed by both the presynaptic and postsynaptic parent neurons. An evolutionary study of synapse proteomes suggested that there has been a great expansion in the number of proteins present at the mammalian postsynaptic density (PSD) relative to those of *Drosophila* and other invertebrates, highlighting the potential for tremendous complexity [2]. Box 1 illustrates the vast number of synaptic proteins distributed across synaptic compartments at a canonical glutamatergic synapse of the mammalian CNS. Although technical limitations preclude certainty as to the total number of protein species composing any single synapse, **proteomic** studies in rodent brain using subcellular fractionation and large-scale mass spectrometry have provided extensive lists of protein species found in various synaptic fractions. Synaptic vesicle fractions contain over 400 protein species [3], clathrin-coated vesicles about 200 [4], postsynaptic densities from 200 to over 1000 species [5-7], and **synaptosomes** more than 3000 [8, 9]. Given that neurons are highly differentiated and diverse cells, the large number of proteins found in mammalian CNS synapses implies that there is an enormous potential for intra-type molecular diversity. Interestingly, it appears that during evolution the most recent additions to the synapse proteome in mammals are the ones that contribute most to synapse diversity [2], thus suggesting a logical link between complexity and diversity.

Compared to the rapidly growing understanding of synapse complexity, much less is known about synapse diversity. Mass spectrometry-based proteomic studies typically require the pooling of heterogeneous synaptic populations, and thus do not inform us about differences in the molecular composition of single synapses. Thus far, only a few proteomic studies have analyzed different synapse types or synapses from different brain regions. For example, a study of PSDs from rat forebrain and cerebellum revealed marked molecular heterogeneities between the two regions [10]. On the other hand, a recent comparison of synaptic vesicle pools between glutamatergic and GABAergic synapses reached the conclusion that the two pools have very similar molecular compositions, with the major difference being the vesicular transporters [11]. In another study, the postsynaptic proteome of parallel fiber to Purkinje cell synapses was isolated based on the specific expression of a glutamate receptor subtype in this population. The authors identified 60 highly enriched proteins, some of which were not seen in heterogenous synapse purifications and may be specific to this synapse type [12]. Future studies such as this will greatly contribute to a better understanding of synaptic diversity.

Multiple lines of evidence from genomic, immunohistochemical and physiological studies further point to the potential for vast diversity and its significant implications. It is now well known that within the brain, multiple **isoforms** of synaptic proteins are expressed differentially between different brain regions and neural cell types [13, 14]. For example, the Allen Brain project used in situ hybridization to localize 20,000 different genes at the level of single neurons in the mouse brain [14]. The analysis revealed that 70% of the genes were expressed in less than 20% of the cells, implying a high degree of molecular variability between cell types. These large-scale surveys along with in situ hybridization studies of individual proteins or protein families highlight the regionally restricted expression of many synaptic protein genes (for example [15], Fig. 1a). Protein translation, as revealed by immunohistochemistry, is also highly variable between brain regions (for example [16], Fig. 1b) and at the subcellular level (for example [17], Fig. 1c). In addition, physiological studies describe highly varied synaptic properties that must also be based on distinct patterns of protein expression (for example [18], Fig. 1d). Taken together, these data imply that a tremendous depth of synaptic diversity remains to be unearthed.

WHY DOES DEEP DIVERSITY MATTER?

Clearly, the potential for molecular combinatorial diversity is vast. Moreover, growing evidence suggests that synapse molecular diversity contributes in fundamental ways to the function of neural circuits and in particular to distinct properties of synaptic transmission and plasticity, cell type and projection-specific differences, development of specific connectivity patterns, and differential susceptibility to neurological disease

Molecular Diversity and Synaptic Transmission. The physiological properties of synapses within a given neurotransmitter type vary extensively. These functional phenotypes must necessarily be tied to molecular-level mechanistic differences. Such connections are complex and difficult to pinpoint experimentally, but have the potential to reveal the crucial role of intra-type molecular diversity in cellular and network function.

Most synaptic connections exhibit either an increase or decrease in transmission efficacy over short trains of repeated stimuli – **short-term facilitation and depression**, respectively. These activity-dependent shifts in **response probability** are related to molecules regulating presynaptic calcium concentration and vesicle availability [19] and are usually a stable characteristic of a given synapse type [20, 21]. For instance, calcium binding proteins such as parvalbumin (PV) and calbindin (CB) are expressed in different GABAergic interneurons, where they act as calcium buffers and produce distinct frequency-dependent firing dynamics [22-24] (Fig. 2A, B). Such simple molecular differences have important functional consequences. In neocortex, PV+ basket cell synapses respond robustly to low-frequency activity, but depress at higher frequencies, whereas the initially weak synapses of PV– bitufted cells facilitate at these higher input regimes [25], enabling the network to maintain effective inhibition over a broad range of input frequencies.

Diversity within the complex vesicle-trafficking and recycling pathway also affects frequency-dependent dynamics. For example, of the three distinct vesicular glutamate transporters (vGluTs), VGluT1, but not VGluT2 or 3, binds endophilin, an interaction that is necessary for efficient endocytosis of vesicles during prolonged high-frequency stimulation[26, 27]. This may enable VGluT1+ synapses to recover faster from stimulation, and thereby reduce frequency-dependent depression. A number of other vesicle proteins, including synaptophysin, VAMP/synaptobrevin, and synaptogyrin, also exhibit significant intra-type isoform diversity [28]. Indeed, immunofluorescence studies have shown that particular isoforms of these proteins are co-expressed at individual synapses with the different vGluT isoforms[29, 30], which are expressed in complementary patterns across the brain (Fig. 1A)[15]. Such modules of molecular diversity deserve further study to elucidate their potential for synergistic effects on the functional differences between synapse populations.

In contrast to the variations in release probability described above, differences in synaptic response amplitude and kinetics are primarily associated with the expression and membrane trafficking of different neurotransmitter receptor subtypes. AMPA-type glutamate receptors (AMPARs) differ in GluA subunit composition between brain regions and cell types [31]. For example, most mature excitatory synapses express GluA2-containing (GluA2+) AMPARs [32, 33], whereas many hippocampal and neocortical GABAergic interneurons express GluA2-lacking (GluA2–) AMPARs. At least a dozen functional subtypes of NMDA-type glutamate receptors (NMDARs) have been observed at different synapses, but 80 distinct combinations of the multiple isoforms and splice variants of their GluN1 and GluN2 subunits are possible [34-37]. Finally, $GABA_A$ receptors, the principal ionotropic receptors for fast inhibitory transmission, are encoded by an incredible 19 distinct genes, which are known to produce at least 26 receptor subtypes with distinct functional properties [38-42].

Differential receptor subtype expression can produce functional diversity related to both pre- and post-synaptic identity. For instance, thalamocortical axons make significantly stronger synapses onto fast-spiking (FS) inhibitory interneurons than onto neighboring

regular-spiking excitatory cells as a result of specific expression of GluA2– AMPARs at the FS-cell synapses (Fig. 2C) [43]. AMPARs lacking the GluA2 subunit are calcium permeable and produce faster, higher amplitude excitatory responses [44-46], a variation which in this case proves critical for effective thalamocortical feedforward inhibition. Differential receptor subtype expression can also enable post-synaptic cells to respond differently to distinct presynaptic inputs. For example, neocortical layer 5 pyramidal neurons frequently express GluN2B-containing (GluN2B+) NMDARs when contacted by local intracortical axons, but express GluN2A+ NMDARs at long-distance callosal inputs. GluN2B+ NMDARs have slower activation and decay kinetics and pass more current, which may allow more time for integration across the local inputs [47]. In contrast, the more precise kinetics of GluN2A+ NMDARs could improve coincidence detection at the callosal inputs (Figure 2D) [34, 48]. Similarly, it is thought that differential expression of GABA(A)R α1 and α2 subunits at inhibitory inputs from two interneuron networks onto hippocampal pyramidal neurons underlies distinct functional roles for these populations in modulating hippocampal network activity [39, 42, 49].

Extensive work is needed to fully understand the effect of receptor subtype diversity on synaptic physiology. Indeed, postsynaptic response transduction involves complex molecular cascades beyond the receptors themselves, with chaperones, kinases, and receptor modulators that also impart significant functional diversity [50-52]. Better comprehension of the relationship between molecular and functional synaptic diversity would greatly enhance our understanding of the differences between synapse populations that enable appropriate circuit and network-level function.

Molecular Diversity and Plasticity. Synaptic function and molecular composition are not stable over time. Indeed, a fundamental property of neural circuits is plasticity, which provides a mechanistic link between learning and memory and specific changes in the molecular composition of synapses [53, 54]. For instance, a recent genomic study demonstrated that environmental enrichment produces distinct changes in the expression levels of many synaptic proteins in different layers of rodent somatosensory cortex, presumably due to the widespread induction of plasticity [55]. However, this relationship goes beyond a simple one-way causality: not only do plastic modifications of synapses induce corresponding molecular changes, but also pre-existing molecular differences underlie distinct modes of plasticity available to different synapse populations.

Immediate-early genes are a well-known example of activity-driven expression. For example, Arc mRNA is enriched at active synapses, where the protein is involved in homeostatic AMPAR endocytosis and the stabilization of many forms of plasticity. However, although Arc protein is reliably observed within minutes of synaptic activity, it is just as rapidly degraded (reviewed in ref [56]). More persistent molecular modifications of individual synapses include plasticity-induced changes in postsynaptic receptor trafficking. For example, prototypical Hebbian **long-term potentiation (LTP)** and **long-term depression (LTD)** at glutamatergic synapses [57-59] involve insertion and removal, respectively, of AMPARs at the synaptic membrane. Because NMDAR concentrations remain relatively constant, these forms of plasticity produce long-lasting alterations in the AMPAR-NMDAR ratio at these synapses [60, 61].

Circuit activity can also induce synaptic plasticity through more subtle alteration of receptor subunit ratios. In particular, activity-dependent trafficking of AMPARS with and without the GluA2 subunit alters response amplitude, kinetics and calcium permeability at glutamatergic synapses [37, 62, 63]. For instance, at many glutamatergic synapses, LTP involves insertion of GluA1+ AMPARs, while LTD often produces preferential removal of GluA2+ AMPARs [64, 65]. Such modifications are implicated in homeostatic responses of neural circuits to changes in net input level. For example, silencing inputs to cultured neurons drives increases of synaptic strength by heightened use of calcium-permeable GluA2– AMPARs [66]. Conversely, increased activity has been shown to drive replacement of GluA2– AMPARs with GluA2+ AMPARs at parallel fiber to purkinje cell synapses in cerebellum [63]. A different activity-dependent change in subunit ratios is seen in hippocampal pyramidal neurons, however, where activity shifts AMPAR insertion from GluA2/3 to GluA1/2 heteromers [67]. Analogous activity-dependent alterations of GABA receptor subunit ratios have recently been demonstrated at inhibitory synapses [38]. For instance, sensory deprivation in the rodent whisker system up-regulates expression of α1 subunit-containing GABA(A)Rs in certain cortical interneurons, which produces faster and stronger responses at affected synapses [68].

As the above examples suggest, plasticity is not a unitary phenomenon, but involves distinct molecular mechanisms in different synaptic connections (reviewed in refs [69-71]). For instance, although the classic form of LTD originally demonstrated in the CA1 region of hippocampus depends on postsynaptic NMDAR activation [58], several other LTD mechanisms have been found at different synapse populations. Many synapses rely instead on activation of postsynaptic metabotropic glutamate receptors (mGluRs) [72, 73], while others require activation of presynaptic type-1 cannabinoid receptors (CB1Rs) by retrograde transmission of endocannabinoids to induce LTD [74]. Differential expression of mGluR subtypes and CB1Rs at these synapse populations produces the diverse forms of plasticity that underlie learning and memory in complex neural circuits [75-77].

Thus, although some molecular features may be persistent throughout the life of a mature synapse, including those related to parent neuron identity and certain physiological properties, the specific history of synaptic and network activity for a given synapse is also reflected in its molecular architecture. Activity-dependent plasticity can produce absolute differences in protein expression and ratiometric differences between proteins or their isoforms, but also more subtle molecular modifications such as protein phosphorylation [78]. In addition, intra-type molecular diversity among synapses is a key basis for the many varieties of functional modification particular circuit connections undergo in response to activity.

Molecular Diversity and Connectivity. In addition to supplying clues about the physiological properties of synapses, knowledge of their molecular composition can provide meaningful structural information as well. The proteins expressed in a synapse are primarily dependent upon the identity of its pre- and postsynaptic parent neurons. Thus, the knowledge of the complement of proteins expressed at a synapse, its "molecular signature", has the potential to provide a code for connectivity linking that synapse to the identity of its parent neurons.

The remarkable information processing capabilities of the brain depend on the formation of precisely wired circuitry during development. The establishment of brain circuits requires the targeting of axons and dendrites to specific brain areas, neuronal targets, and even specific locations on the surface of a neuron (reviewed in refs [79, 80]) . One class of proteins that is well positioned to provide molecular markers for the formation of brain circuits and synaptic connections is the adhesion proteins. Large numbers of distinct adhesion molecules are present in the developing brain and help regulate the formation of specific neuronal connections. Many of these proteins are retained at synapses in the mature brain [81-85]. For example, cerebellar basket cells form large **pinceau synapses** on the axon initial segment (AIS) of Purkinje cells. Neurofascin 186, a member of the L1 adhesion protein family, helps guide their processes to the AIS through interactions with the post-synaptic scaffold protein Ankyrin G (Fig. 3A) [86]. In the mature synapse, both Neurosfascin 186 and Ankryin G expression are maintained [87] and can act as a markers for pinceau synapses. Thus, the proteins crucial to the initial development of the synapse are retained and their presence in the adult reveals the identity of the parent neurons and position of the synapse within the circuitry of the mature brain. As recent interest focuses on mapping of circuits in the mature brain [88-94], these adhesion proteins have the potential to provide clues about the parent neurons forming an individual synapse and their pattern of connectivity within the circuitry of the brain.

Clearly, a vast number of proteins would be needed to encode all the circuits in the brain. Two classes of synaptic adhesion proteins in particular, the neurexins and protocadherins (Pcdhs), have large numbers of isoforms and are present both during the initial formation of synaptic connections and in mature synapses. Vertebrates have 3 neurexin genes, each with two promoters that can drive transcription of a large α-neurexin and smaller β-neurexin[95, 96] (reviewed in ref [83]). Alternative splicing can generate over 1000 neurexin isoforms, which are expressed in regionally distinct but overlapping brain regions [97]. Neuroligins, the ligands for neurexins, are encoded by four genes which each contain either one (Neuroligin 1,3, and 4) or two (Neuroligin 2) alternative splicing sites. Neuroligin 1 is expressed in excitatory synapses [98], 2 and 4 in inhibitory synapses [99, 100] , and Neuroligin 3 appears to be in both [101]. Similarly, the Pcdhs are a group of 50 or more ([102, 103], see review [80]) members of the cadherin family encoded by a large gene cluster that form homophilic bonds and are also expressed in disparate but partially overlapping regions of the brain [104, 105]. Additional adhesion protein families with fewer members also contribute to synaptic diversity, including the ephrins and members of the immunoglobulin superfamily [81]. With the potential for combinatorial expression of these large numbers of adhesion proteins, a vast number of unique codes would be available to provide molecular signatures for specific brain circuits in the mature brain.

In addition to adhesion proteins, other types of synaptic proteins can also serve as part of the molecular signature for a specific synaptic subtype. For instance, some identifiable classes of GABAergic interneurons express cell-type specific proteins that are expressed at the synapse. Although there is still no fully comprehensive classification [106], GABAergic interneurons in rat cortex have been divided into several classes based on their morphology, connectivity, and expression of molecular markers such as calcium binding proteins and neuropeptides [106, 107] – chandelier cells, large basket cells, small basket cells, nest basket cells, double bouquet cells, bipolar cells, bitufted cells and

Martinotti cells. The chandelier and large basket cells in the cerebral cortex both express the calcium binding protein PV. The large basket cells innervate the cell bodies and proximal dendrites of cortical pyramidal neurons and interneurons, whereas the terminals of the chandelier cells are restricted to the AIS of the pyramidal neurons and have a characteristic candle-like shape. Thus, a GABAergic synapse on the soma of a pyramidal neuron in the cerebral cortex (Fig. 3B) that expresses PV can be identified as a large basket cell synapse. In the cerebellum, the basket cells that form pinceau synapses also express PV (Fig. 3A).

Vesicular transport proteins also supply useful information about the synaptic connections formed by specific sets of afferents. Neurons in the hippocampus, cerebral and cerebellar cortex express predominantly VGluT1 and those in the thalamus, brainstem and deep cerebellar nuclei express primarily VGluT2 (Fig. 1A)[15]. In the adult cerebellum, Purkinje cells receive two types of excitatory inputs that differ in their VGluT content. The parallel fibers, axons of granule cells, synapse onto the distal dendrites and express VGluT1, while the climbing fibers, which originate in the inferior olive and innervate the proximal dendrites, express VGluT2 (Fig. 3A) [15, 108]. Similarly, the majority of synapses made by the thalamocortical axons that project to layer 4 in the cerebral cortex express VGluT2 and those made by corticocortical axons express VGlut1 (Fig. 3B). Thus, the expression of the VGluTs provides one clue to the origin of synaptic inputs. The identification of these and other molecular synaptic markers that can be linked to specific parent neurons of individual synapses have the potential to provide valuable clues in identifying specific synapse subtypes within the complex circuitry of the brain.

Molecular diversity and susceptibilty to disease. Synaptic proteins are thought to play a primary role in many brain diseases[109]. A number of synaptic proteins linked to disease have regionally distinct expression patterns, raising the possibility that some subsets of synapses or brain circuits could be more vulnerable to a particular brain malady than others. Recently, great headway has been made in uncovering the links between synaptic proteins and brain disease in screens for genetic mutations linked to neurodevelopmental and psychiatric disorders in humans.

In one example, mutations in genes for neurexin 1, neuroligin 3 and 4, and Shank 3, a synaptic scaffold protein, have all been implicated in familial **autism spectrum disorders** (ASDs)[110] (reviewed in refs [83, 111]). Shank 3 interacts indirectly with neuroligins through their common binding partner PSD 95. More recently, two more proteins from the same complex, SAPAP2 (synapse-associated protein 90/postsynaptic density-95-associated protein 2) and Shank 2, have also been linked to human ASDs [112]. To examine the role of these proteins in ASDs, mutant mice were produced with a point mutation in neuroligin 3[113, 114], a loss of function mutation in neuroligin 4 [115], and deletions in the Shank3 genes [116]. Analysis of the mutant mice consistently shows deficits in social interactions that mirror some of the symptoms seen in human ASD patients [114, 116]. Although the mechanisms that underlie the aberrant behaviors in the transgenic mice are still being determined, defects in synaptic transmission appear to play a significant role in ASD pathology. Because neurexins and neuroligins are expressed in subsets of

neurons and in regionally specific patterns, this suggests that ASDs may affect specific synapse types and neuronal circuits in the brain [83].

In a related study, the gene disrupted in schizophrenia 1 (DISC1) was discovered in a genetic study of a Scottish family with a translocation between chromosomes 1 and 11 that correlates with schizophrenia, bipolar disorder and depression [117, 118]. Subsequent studies further supported a role for DISC1 in psychiatric disease [119]. Although some studies indicate that DISC1 mutations disrupt early neurogenesis and neuronal migration, DISC1 also affects synaptogenesis and could be acting at the level of brain circuits. DISC1 is a scaffold protein and is expressed postsynaptically in the mature brain [120]. In autopsy studies of schizophrenic patients, the number of dendritic spines on cortical pyramidal neurons was found to be reduced compared to normal patients [121]. Similarly, a reduction in dendritic spines was found in a transgenic mouse expressing a truncated version of DISC1 [122] and in rat cortical cultures and whole brains after DISC1 RNAi treatment [123]. In the adult mouse brain, DISC1 is expressed in only some subsets of neurons [124] suggesting that the DISC1+ synapses and their parent neurons could be more vulnerable to disease related changes. Although the exact mechanism for the role of DISC1 in psychiatric disorders is yet to be revealed, it is provides another example of specific synapse proteins implicated in brain disorders. The search for synaptic proteins associated with nervous system disorders promises to be a fruitful strategy for exploring the molecular mechanisms underlying brain disease.

How to Meaure Deep Molecular Diversity

Ideally, investigations of synapse molecular diversity require methods capable of probing large numbers of individual synapses in specific brain tissues. Unfortunately, such methods are still few in number, and are often limited in capacity or slow to apply. The first method to distinguish individual synapses was the **Golgi stain** (developed in 1873, Fig. 4), which allows visualization of dendritic spines, the postsynaptic part of most excitatory synapses in cerebral cortex. Although this method was indispensible for Cajal's early studies and is still widely used, it does not provide information about the molecular composition of synapses. The first intimate view of the whole synapse was provided by electron microscopy (EM) [125]. With its sub-nanometer resolution, this method revealed the ultrastructural organization of synapses and their morphological diversity. In combination with immunostaining, EM has enabled the study of the ultrastructural distribution of synaptic molecules. ImmunoEM, however, is limited for the study of synaptic diversity, because in general it can detect only a couple of antigens at the same synapse. Recent advances, such as **EM tomography**, are beginning to improve on that limit by identifying different molecular species by their structure instead of with antibody labeling [126].

Another recently developed EM method, automated transmission electron microscopy (ATEM) [88, 89], is particularly well suited for synaptic diversity studies. ATEM is based on automated EM imaging of large series of ultrathin sections, periodically intercalated with ultrathin sections immunolabeled for small molecules (such as glutamate or GABA) and proteins and imaged with light microscopy. Alignment of the EM sections with the immunostained sections allows mapping of the antibody labels onto the tissue

ultrastructure. In contrast to traditional EM, large numbers of molecular markers (at least 10) can be accommodated [127]. ATEM has now been used for the full reconstruction of a circular segment of retina, 0.25 mm in diameter and spanning three retinal layers, with 2 nm resolution and 6 molecular labels, including a marker for neuronal activity [88].

Until recently, light microscopy, even **confocal** and **two-photon microscopy**, did not have sufficient resolution to identify individual synapses *in situ*. This is now rapidly changing with the advent of new high- and super-resolution methods. Array tomography [128, 129] is a new high-resolution proteomic imaging method that allows the imaging of dozens of different antibodies at individual synapses within large volumes of brain tissue (Fig. 5A). The high spatial resolution of this wide-field fluorescence microscopy based method is enabled by the use of **ultrathin** serial tissue **sections** (70 nm) and its multiplexing capabilities are enabled by multiple rounds of immunofluorescent labeling, imaging and antibody elution. Large volumes, containing millions of synapses, can be acquired with array tomography, because both immunological labeling and imaging of the ultrathin sections are not limited by depth within the tissue. Array tomography is, however, currently limited to a lateral resolution of ~200 nm, the diffraction limit of conventional light microscopy.

A number of recent super-resolution fluorescent microscopy methods have broken the diffraction limit and two approaches in particular, STED and STORM/PALM, show significant potential for the analysis of synaptic structures. STED (stimulated emission depletion microscopy) is a confocal scanning method that achieves diffraction-unlimited lateral resolution by spatially confining the fluorophores emitted from the sample with a second overlapped “depletion” beam that forces excited molecules back to the ground state [130]. STORM (stochastic optical reconstruction microscopy) [131] and PALM (photoactivation localization microscopy) [132, 133] on the other hand are based on single-molecule imaging of photoswitchable fluorescent probes. As an illustration of the power of super-resolution methods for single synapse analysis, STORM has enabled the mapping of 13 proteins onto a common synaptic coordinate system using immunofluorescence [134], albeit across multiple distinct sections. However, these super-resolution approaches have not yet been applied to large tissue volumes and still have limited multiplexing capabilities. Up to three differently coloured labels in the same sample have been imaged with STED [135], and only recently 6 different colours were attained with STORM in a model sample of differentially labeled streptavidin molecules [136].

None of these imaging methods, in their present state, is sufficient for a thorough characterization of the deep molecular diversity of mammalian synapses. These methods, however, are potentially compatible with each other, which could result in a synergistic approach building on the strengths of each technique. For example, a possible integration of ATEM and array tomography would combine ultrastructural and connectivity information with detailed molecular characterization. A combination of STED or STORM with array tomography would allow for super-resolution imaging and high multiplexing capabilities. Furthermore, integration of the proteomic imaging tools described above with other methods, such as axon tracing or single synapse live imaging and physiology is also possible and crucial for enhancing our capabilities to grasp

synaptic diversity (Fig. 5B). Thus, the complement of tools is finally beginning to emerge to enable a future systematic exploration of deep synapse diversity.

FATHOMING DEEP DIVERSITY: WHAT'S NEXT?

Synapses of the mammalian central nervous system are complex and deeply diverse structures that mediate complex, diverse and highly plastic signaling functions. Mounting evidence indicates that a careful accounting of synapse molecular and functional diversity will be necessary to understand neural circuit structure and function. The construction of "synaptomes", bodies of knowledge resulting from quantitative reckoning of synapse diversity, will not be easy. This will entail extensive and very challenging exploration of both molecular composition and function at the level of individual synapses. Fortunately, new technologies, as discussed above, are now poised to begin the necessary single-synapse surveys of the brain's vast and diverse synapse populations. We conclude with some thoughts as to how a quantitative appraisal of deep synapse diversity might unfold and impact neuroscience.

Synapse Classification. A synapse classification framework, with strong empirical and biologically principled foundations, is likely to offer the most practical approach to grappling with molecular and functional synapse diversity and provide the basis for useful aggregation of synaptomic information. At present, however, because complete and thorough single-synapse data have not been available, we can only begin to envision what a robust synapse classification might look like. The cell biology of protein biosynthesis affords one obvious principle that may aid development of synapse classification frameworks. Because a synapse receives its component proteins exclusively from its two parent neurons, parent neuron identity seems likely to predict synapse similarity: synapses sharing the same two parents are likely to share common proteins and thus common functional properties, as schematized in Figure 6A. The potential power of this principle to guide synapse classification expands enormously if it refers to two parent neuron cell types, not just two particular parent neurons, as in Figure 6B. If all neurons of a given cell-type are assumed to express identical sets of synaptic proteins, biologically principled synapse subtype classes might be derived directly from parent neuron cell-type classes. The caveat here, however, is that neuron cell-type classification remains a work in progress [21, 88, 106, 107, 137], and still there is no guarantee that neuron cell-types, as presently defined, express entirely common sets of synaptic proteins. As additional characteristics of neuronal differentiation and gene regulation are identified, they are likely to contribute insights useful for biologically principled synapse classification.

Classification and Synapse Mutability . Synaptic mutability, including use-dependent plasticity, synapse homeostatis, and modulatory effects of nearby neurons and glial cells, poses additional challenges to synapse classification. It is clear that both synaptic function and protein composition are mutable over both short and long periods of time. Changes in synaptic proteins can involve changes in transcription and translation as well as post-translational protein modifications. Such changes might be framed as driving a synapse to change from one subtype class to another, or, alternatively, viewed as driving a synapse to change status, perhaps reversibly, and perhaps in graded fashion, while remaining within a given subtype, as schematized in by Figure 6C. At present, this

distinction may seem purely semantic. It might become more meaningful, however, with advances in our broader understandings of developmental, homeostatic and plasticity-related gene expression and our abilities to survey single-synapse characteristics.

Memory Synaptomes. Most present models postulate that memory is encoded and accessed primarily as change in synaptic connections. Many different forms of memory are now recognized, and may be encoded by various forms of dynamic synapse change, including formation of new synapses and strengthening, weakening, or elimination of existing synapses. Combinations of behavioral and physiological methods with single-synapse proteomic imaging (Figure 5) may offer revolutionary new opportunities to discover molecular markers of memory. Synaptomic studies that establish molecular markers reflecting both circuit connectivity and plasticity state could prove to be powerful tools for exploration of the encoding of memories in synaptic circuit architectures. The discovery of such markers could lead eventually to the development of memory biomarkers useful for human molecular neuroimaging research and clinical applications.

Disease and Disorder Synaptomes. Synapse abnormalities are now recognized as fundamental to numerous mental and neurological disorders, including those associated with aberrant development, neurodegeneration, trauma, and addiction. In addition, there is now evidence suggesting that pathological conditions can impact distinct synapse subsets and perhaps specific brain circuits. This raises the intriguing prospect that we may someday recognize particular mental and neurological disorders by distinctive molecular "signatures" of abnormal synaptic protein distributions [109] that is, by "disease synaptomes". Thus, the emergence of new methods for surveying and systematizing synapse molecular diversity may lead to powerful new approaches to the diagnosis and treatment of mental and neurological disorders.

The Synaptome Meets the Connectome. Quantitative understanding of neural circuit function in the light of neural circuit structure stands as one of the ultimate challenges to neuroscience. Given the obvious complexities of neural circuit structure and function, however, achieving such an understanding seems certain to require computational reconstruction and modeling. Two kinds of information necessary to reconstruct a neural circuit quantitatively are the complete pattern of the neural circuit's synaptic connectivity (that is, its "connectome") and the functional properties of the synapse. One of the many challenges to obtaining such information is the fact that in living brains, these connections and their properties are constantly changing in response to ongoing circuit activity. Although it should be possible, in principle, to obtain a complete circuit diagram as a 'snapshot in time' (from fixed tissue, for example), it is much harder to imagine a method for obtaining a snapshot of the physiological properties of all of a circuit's myriad individual connections. With the deepening of synaptomic information that relates diverse synaptic functional properties to synapse molecular composition, however, it may be possible to skirt this apparent obstacle by obtaining a molecular snapshot of all of a circuit's synapses from a fixed specimen and inferring functional properties from molecular composition [94].

Synaptomic information also may provide an important technological complement to connectomic efforts to reconstruct complete circuit wiring diagrams. While new high-throughput electron microscopy (EM) and image analysis methods are showing great

promise for the extraction of circuit connectivity information [88-93, 138], the associated rates of sporadic image segmentation errors threaten to thwart reliable automated completion of circuit diagrams for the foreseeable future [139]. A molecular connectivity code reflected in naturally occurring synapse molecular diversity, as reviewed above, could provide ancillary information to help detect and correct the infrequent but devastating errors that may be inevitable from straight EM segmentation approaches to complete connectivity mapping.

Conclusions. Present evidence establishes forcibly that the depth of molecular diversity of mammalian synapses is far in excess of that envisioned by any traditional synapse classification scheme. Indeed, it is clearly in excess of any classification that could be proposed with any cogency today. We must recognize that uncharted synapse diversity is a scientific liability capable of severely restricting our ability to understand neural circuit function and even basic mechanisms of synapse function. On the other hand, a more complete understanding of synapse diversity is certain to be a strong asset to both synapse and circuit science. Newly emerging tools now offer great promise to convert currently unexplored dimensions of synapse diversity from scientific liabilities to assets.

GLOSSARY TERMS

Proteomic - The study of the proteome, or the entire set of proteins expressed by an organism, tissue, cell or subcellular organelle. A variety of large-scale techniques are employed such as mass spectrometry, immunolabeling or tagging of proteins, and yeast two-hybrid screens.

Synaptosome -An artificially formed membranous structure that is generated by subcellular fractionation of brain tissue homogenates and contains most of the presynaptic terminal, including synaptic vesicles and mitochondria, as well as the postsynaptic density and adjacent postsynaptic membrane.

Isoforms – Alternate forms of the same protein generated from either related genes or from alternate splicing of the same gene.

short-term facilitation (STF) – Increase in the amplitude of synaptic transmission over multiple stimuli on the scale of milliseconds, thought to result from frequency-dependent buildup of presynaptic calcium, which increases the release probability for upcoming spikes.

short-term depression (STD) – Decrease in the amplitude of synaptic transmission with repeated stimulation on the scale of milliseconds, thought to result from frequency-dependent depletion of fusion-ready vesicles, which decreases the release probability for upcoming spikes.

release probability – The probability that a single presynaptic spike will result in the release of a vesicle of neurotransmitter into the synaptic cleft, determined by multiple presynaptic factors.

long-term potentiation (LTP) – Long-lasting increase in synaptic strength between neurons, usually resulting from synchronous or temporally coordinated pre- and post-synaptic activity.

long-term depression (LTD) – Long-lasting weakening of synaptic strength between neurons, often resulting from asynchronous pre- and post-synaptic activity.

Pinceau synapse – a synapse shaped like a paintbrush ("pinceau" in French) that is formed at the base of the Purkinje cell axon by a cerebellar basket cell.

Autism spectrum diseases – a group of complex neurodevelopmental disorders characterized by difficulties in social interaction, poor verbal and nonverbal communication and abnormal repetitive behaviors.

Golgi stain A technique based on precipitations of metallic salts within cells and used for visualization of sparse subsets of neurons and glial cells in their entirety.

EM tomography A method for the three-dimensional reconstruction of objects from a series of projection images that are recorded with a transmission electron microscope. It offers the opportunity to obtain spatial information on structural arrangements of cellular components.

Confocal microscopy- A fluorescence imaging technique that increases resolution through 'optical sectioning'. To attain optical sectioning, excitation light is scanned across an object, illuminating a single point at a time, and the emitted fluorescence light is detected through a pinhole aperture, limiting the light originating from outside the focal plane.

Two-photon microscopy A form of microscopy in which a fluorochrome that would normally be excited by a single photon is stimulated quasi-simultaneously by two photons of lower energy. Under these conditions, fluorescence increases as a function of the square of the light intensity, and decreases as the fourth power of the distance from the focus. Because of this behaviour, only fluorochrome molecules near the plane of focus are excited, greatly reducing light scattering and photodamage of the sample.

Ultrathin sections – Histological sections of resin-embedded or frozen tissue with a thickness of 30 – 200 nanometers, typically used for electron microscopy.

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BOX AND FIGURES

Box 1. The molecular complexity of the CNS glutamatergic synapse. A transmission electron micrograph (courtesy of T. S. Reese, bottom left) illustrates the ultrastructural features of four main synaptic compartments: synaptic vesicles, the presynaptic active zone, synaptic cleft, and postsynaptic density (PSD). Each compartment is composed of large numbers of distinct proteins. Thus, the chemical synapse emerges as an extremely complex molecular machine.

Synaptic vesicles are the transport packets for neurotransmitters. The vesicle membranes contain neurotransmitter transporters and ion pumps to load the vesicles, trafficking and docking proteins to help deliver the vesicles to the membrane via the cytoskeleton, priming proteins to expedite exocytosis, and clathrin-related proteins to aid in endocytosis. At least 410 different proteins have been identified in synaptic vesicles[3].

The active zone is specialized to expedite synaptic vesicle release in response to calcium influx. An electron dense region adjacent to the membrane, the cytomatrix, contains the molecular machinery necessary for exocytosis, including voltage-gated calcium channels, scaffold and signaling proteins, and actin and other cytoskeletal proteins, as well as proteins involved in endocytosis. Because of difficulties isolating this compartment, reliable estimates of the number of proteins present are not available.

The synaptic cleft is a specialized site of cell contact that resembles a tight junction with precisely spaced and rigidly parallel membranes about 25 nanometers apart in glutamatergic synapses. Over 1000 different protein isoforms from several major families of adhesion proteins are thought to span the synaptic cleft [80-85]. Therefore, the adhesion proteins contribute greatly to synaptic complexity.

The PSD contains the machinery to generate the response to neurotransmitter release. Neurotransmitter receptors and ion channels are stabilized in the PSD membrane by scaffold proteins. The PSD contains signaling proteins, such as protein kinases and phosphatases, which modulate the synaptic response as well as proteins associated with the cytoskeleton that extends into the dendritic spines, short branches that protrude from dendrites at the site of many glutamatergic synapses. The results of proteomic studies suggest that the PSD contains anything from 200 to more than 1000 different proteins [5-7].

Figure 1. Evidence for distinct patterns of synaptic protein expression. A) In situ hybridization studies using mRNA from the synaptic protein isoforms VGluT1 and VGluT2 reveal distinct regional gene expression of VGluT1 at high levels in the hippocampus, cerebral and cerebellar cortex and VGluT2 concentrated in the thalamus, brainstem and deep cerebellar nuclei [15]. **B-C)** Immunohistochemistry studies using antibodies against metabotropic glutamate receptor isoforms reveal distinct expression patterns at both the regional level in the adult rat brain (**B**)[16] and at the subcellular level (**C**) [17]. In **C)**, a CA1 hippocampal pyramidal cell axon forms two different synapses in CA3, one expressing mGluR7 (colloidal gold, ImmunoEM) with an interneuron and the other with a pyramidal cell that does not express mGluR7. **D)** Corticothalamic synapses exhibit paired-pulse facilitation, whereas corticolemniscal synapse show paired-pulse

depression[18]. differences between the two synapse types are likely to underlie the differences in physiological responses.

Figure 2. Synaptic molecules and physiological diversity. (A-B) Expression of different calcium binding proteins by two interneuron populations produces distinct transmission phenotypes. **A)** In mouse cerebellar slices, synapses between parvalbumin (PV) positive interneurons and purkinje cells exhibit paired-pulse depression (PPD) (WT: blue), but in knockout animals this connection exhibits paired-pulse facilitation (PPF) (KO: red, [24]). **B)** In neocortical slices, synapses between calbindin (CB) positive interneurons and pyramidal cells exhibit paired-pulse facilitation (PPF) (WT: blue), but in knockout animals, PPF is abolished (KO: red, [22]). **(C-D)** Selective receptor subtype expression is implicated in the ability of individual afferents to produce distinct responses in different post-synaptic cell-types, and of individual neurons to respond differently to distinct presynaptic inputs. **C)** In thalamocortical slices, the response of regular-spiking (RS) cells to thalamic stimulation is unaffected by pharmacological blockade of GluA2–AMPARS. However, the much more potent response of fast-spiking (FS) interneurons is significantly reduced[43]. **D)** In neocortical slices, the response of layer 5 pyramids to long-distance transcallosal inputs is not affected by pharmacological blockade of GluN2B+ NMDARs. However, the response of these cells to local intracortical inputs is significantly reduced[48].

Figure 3. Synaptic proteins as a connectivity code. A) The axon initial segment (AIS) of a Purkinje neuron (green) in the cerebellum receives input from the PV+ synapses of a basket interneuron (brown). Neurofascin (red) is expressed in a gradient within the AIS during development and in the synaptic cleft of the mature synapses. Thus, PV and Neurofascin are part of the molecular signature of the pinceau synapse. Granule cell neurons (magenta) extend their axons, parallel fibers, to form VGluT1+ synapses on the distal dendrites of Purkinje cells. Climbing fibers extend from the inferior olive to innervate the proximal dendrites of Purkinje cells where they form VGluT2+ synapses. ML–molecular layer; PL-purkinje layer; GL-granule layer. **B)** The dendritic spines of a layer 5 (L5) pyramidal neuron (green) in the cerebral cortex are innervated by VGluT2+ synapses (blue) from thalamocortical axons and VGluT1+ synapses (magenta) from corticocortical axons in layer 4 (L4). The soma of the pyramidal neuron receives input from PV+ synapses (brown) formed by a basket cell from layer 2/3 (L2/3). The presence of these pre-synaptic markers can be used to help identify the pre-synaptic parent neurons that form individual synapses.

Figure 4. Methods probing ultrastructure and molecular composition of single synapses. A) Golgi stain of cortical dendrites (drawing from Cajal [140]). Entire neurons and their processes can be visualized with this method, which is based on precipitation of metallic salts within random sparse subsets of cells within brain tissue. **B)** GABA immunogold staining of a synapse in rat cortex [141]. Immunoelectron microscopy reveals the ultrastructural distribution of proteins using antibodies conjugated to gold particles or other electron-dense reagents. **C)** ATEM, Ca, a reconstructed fragment of the mammalian AII amacrine cell network with Cb, electron micrographs of synaptic connections[88]. ATEM is based on automated EM imaging of large series of ultrathin sections. Molecular information is obtained from periodically intercalated sections that are immunolabeled

and imaged with light microscopy. **D)** Array tomography (reconstruction of a segment of cortical dendrite with synaptic markers associated with dendritic spines) [129]. This method is based on ultrathin serial sectioning, and multiple rounds of immunofluorescent labeling, imaging and antibody elution providing three-dimensional high-resolution molecular information. **E)** STORM (mapping of synaptic proteins onto a common coordinate system defined by Homer 1 and Bassoon [134]). This super resolution method is based on single-molecule imaging of photoswitchable fluorescent probes.

Figure 5. Multidimensional views of single synapses. A) High dimensional proteomic data of individual synapses can be currently generated using array tomography. Such data are viewed as "synaptograms" with columns representing individual serial sections through a synapse and rows representing each marker. The two synaptograms show examples of a glutamatergic and GABAergic synapse with glutamatergic markers boxed in red and GABAergic markers boxed in blue, respectively. **B)** Array tomography is compatible with super-resolution imaging methods [130-136] that increase the resolution of array tomography data, as well as with intracellular dye fills [142], two-photon in vivo imaging [143-146], two-photon glutamate uncaging [147] and high throughput EM [88-93, 138]. In combination with these other methods, array tomography can be utilized to explore multiple facets of synaptic diversity.

Figure 6. A mechanistic classification of synapses based on protein biosynthesis principles. A) Such a classification scheme would be based on the fact that the molecular composition of an individual synapse depends on the molecular composition of its parent neurons. **B)** The cell types of the parent neurons M and N would then provide the basic definition of a generic synapse sub-type MN. **C)** Specific local factors may more precisely define synapse type MN1a and also affect temporally varying status X within the subtype.

Box 1 Figure

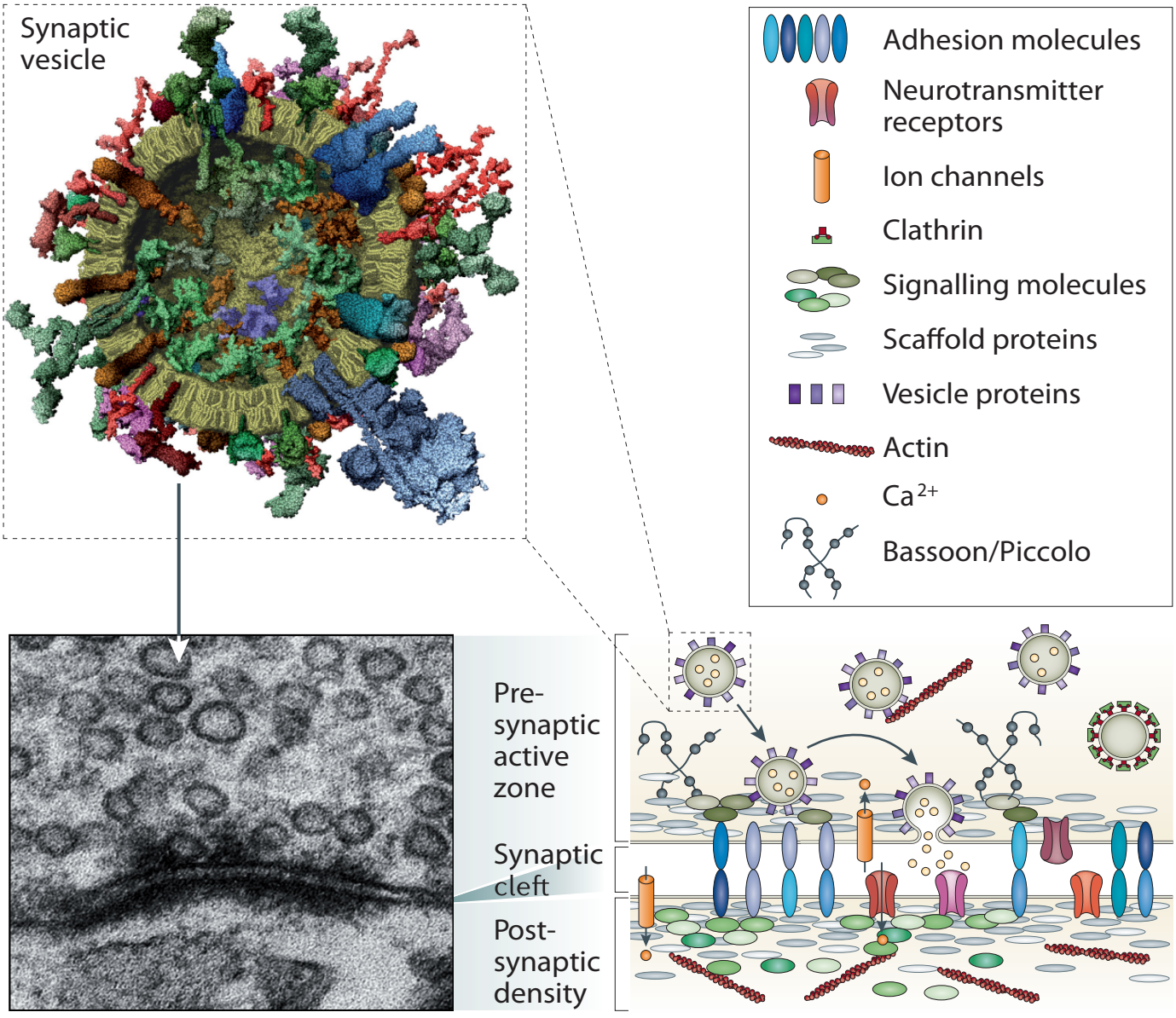

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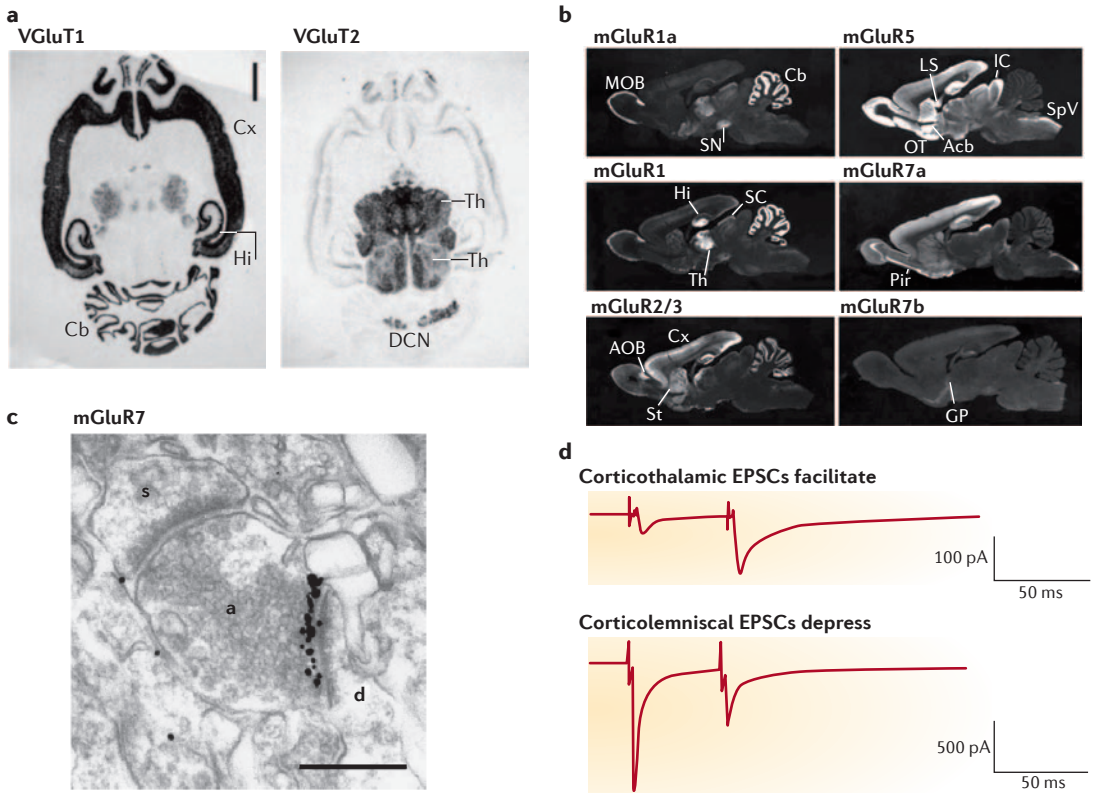
Figure 1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Corticolemniscal EPSCs depress
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Figure 2

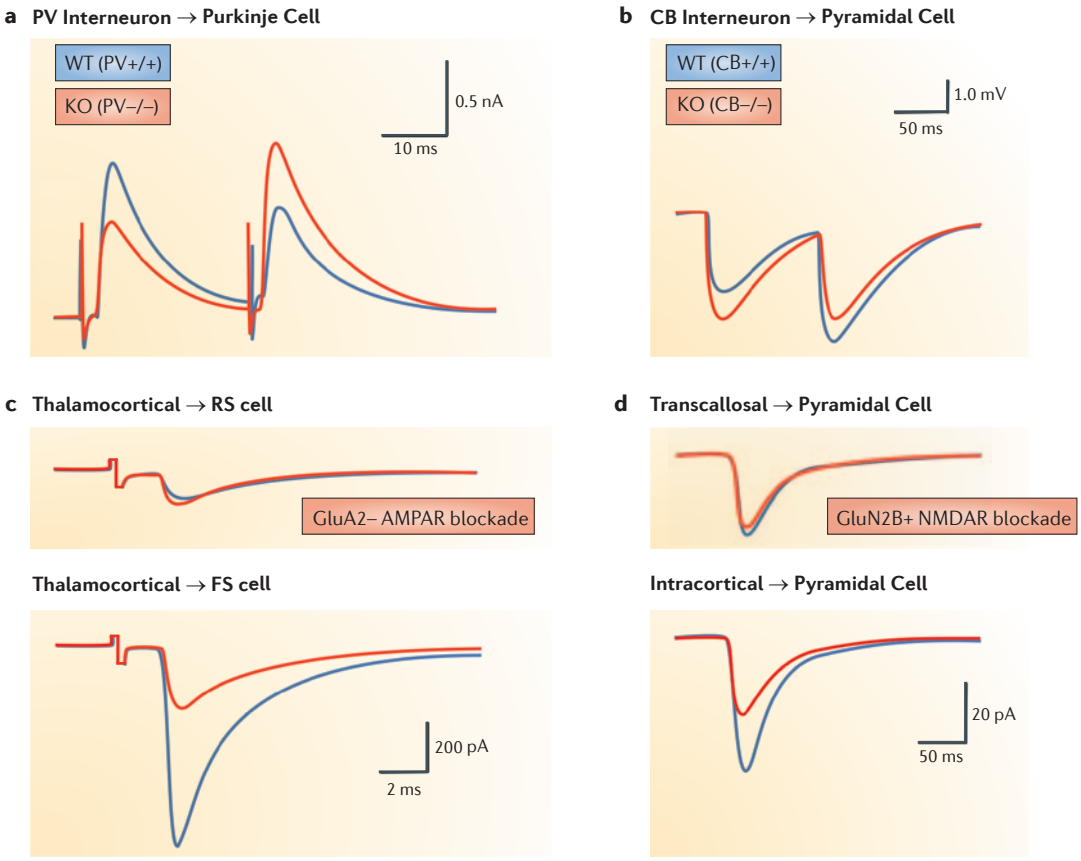

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Figure 3

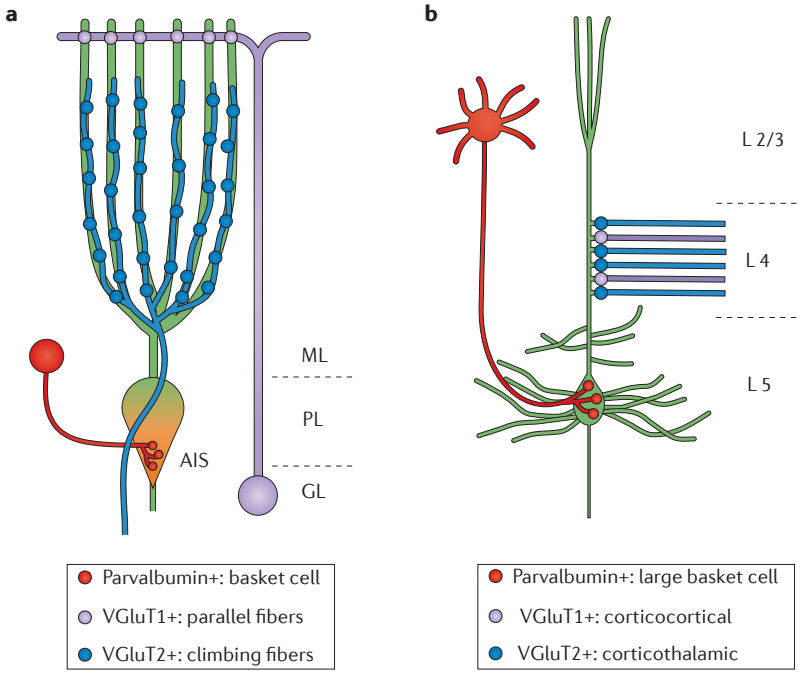

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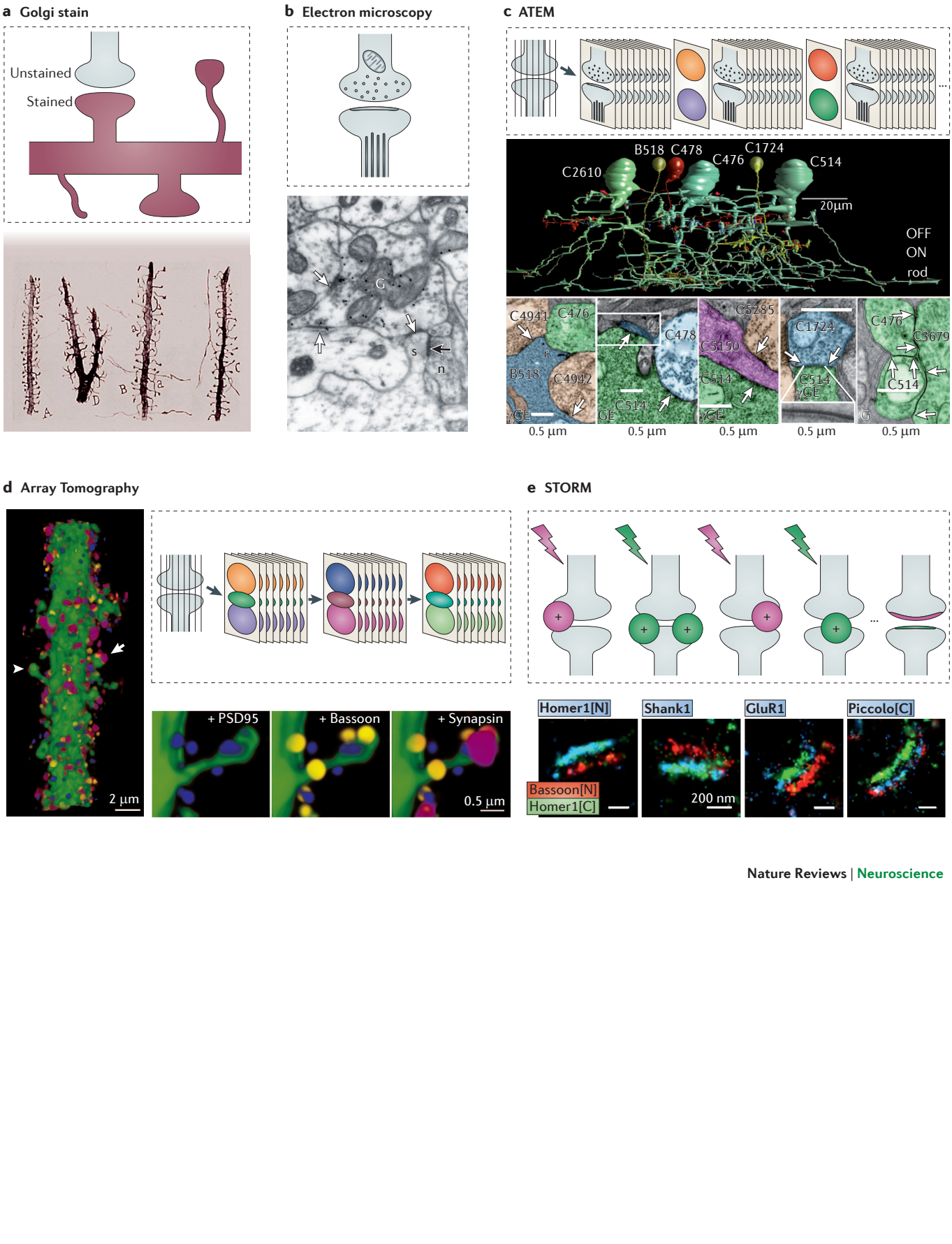
Figure 4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Homer1[C]
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Figure 5

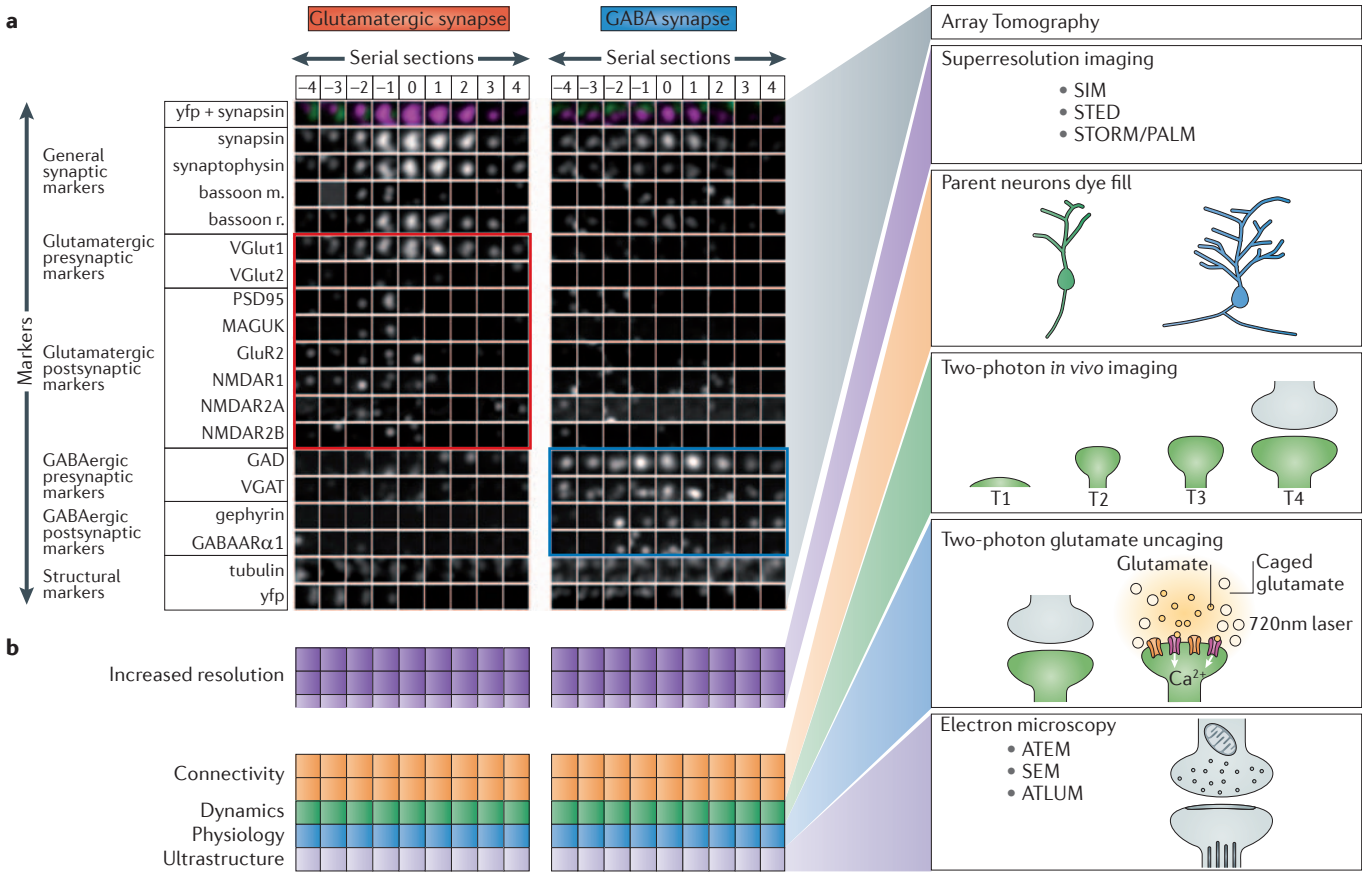

Figure 6