Chapter 11

Genetic Labeling of Synapses

Carlos Lois and Wolfgang Kelsch

Abstract

A major challenge in neuroscience is to unravel how the synaptic contacts between neurons give rise to brain circuits. A number of techniques have been developed to visualize the synaptic organization of neurons. In this chapter, we focus on genetic methods to mark specific types of synapses so that synaptic sites can be visualized throughout the entire dendritic or axonal arbor of single neurons. Genetic synaptic labeling can be achieved by cell-type-specific viral or transgenic delivery of synaptic proteins tagged by fluorescent proteins. Sparse genetic labeling of neurons permits semiautomated quantification of the distribution and densities of selected types of synapses in segregated domains of the axonal and dendritic trees. These approaches can reduce the complexity and ambiguity of attributing synaptic sites to unravel principles of the synaptic organization of identified neuronal types in the circuit.

Key words Synaptic organization, Synaptic markers, Neurons, Genetic synaptic labeling, Synaptophysin, PSD-95, Retroviruses

1 Introduction

A central hurdle toward understanding brain function is the complex organization of synaptic contacts between neurons that form circuits. A plethora of techniques have been developed to solve this challenging task of deciphering the synaptic organization of single neurons and mapping synaptic connectivity in neuronal circuits. In this chapter, we will focus on recently developed genetic markers to identify synaptic contacts and discuss their caveats and limitations.

Traditionally, three main approaches, morphological correlates of synapses, antibody staining, and electron microscopy, had been employed to describe or infer synaptic contacts of neurons:

1. In some cases, synapses can be identified on the basis of their association with neuronal structural specializations. For instance, many excitatory input synapses are located in dendritic spines, in which case spines may be used as a morphological proxy for synapses. One limitation of this method is that a large proportion of synapses, such as excitatory input synapses on cell somata

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and inhibitory synapses, are not associated with spines [1, 2]. In addition, morphological methods are not useful to quantify output synapses, which are mostly located in axon terminals (Ref?). It is easy to identify axon terminals, but it is not possible to accurately quantify the density and measure the size of output synapses by simple morphological analyses. Similarly, the extent of overlap between axons and postsynaptic dendrites has been used to infer the presence of synaptic connectivity based on the fact that synapse formation requires a physical proximity. However, ultrastructural studies reveal that axons and dendrites frequently approach each other without making synapses. For example, in several well-studied systems like C. elegans, the rat hippocampus, or the mouse retina, only 15–25 % of physical contacts are synaptic [3–5]. Thus, close proximity between neurons cannot be used to reliably identify synapses.

2. Antibody labeling against synaptic markers is a powerful method to label synapses in cultured neurons. However, this method is suboptimal in brain sections because the large number of synapses present severely complicates the attribution of synapses to individual new neurons.
3. Electron microscopy (EM) represents the "gold standard" assay for identifying synapses but its main disadvantages are that it is labor intensive, can only be applied to fixed (nonliving) tissue, and cannot be used to trace long-range connections (*see* also below section "Electron Microscopy").

The limitations of these methods have driven the ongoing development of tools to genetically label the synapses of identified neurons.

Recently developed genetic approaches to visualize synapses fall into two broad categories depending on the experimental demand:

- Visualizing specific types of synapses (i.e., glutamatergic input synapses or gabaergic output synapses) in single neurons.
- Visualizing the synaptic contacts between neurons by genetic labeling of both the pre- and postsynaptic neurons.

These approaches reduce the complexity and ambiguity of attributing synaptic sites by genetically targeting a very small subset of neurons in the examined tissue. In this chapter we will discuss these two genetic approaches considering their potential applications and respective limitations.

2 Genetic Labeling of the Synaptic Organization of Single Neurons

Labeling synapses with genetically encoded markers addresses some of the limitations of the abovementioned techniques and significantly simplifies the quantification of synaptic organization and

development in neurons [6–9]. The visualization of pre- and postsynaptic terminals can be achieved via expression of fluorescent proteins fused to proteins specifically located in synapses.

2.1 Molecular Targets for Genetic Labeling of Synapses

Few synaptic proteins have been extensively tested for their use as genetic synaptic markers. We will particularly focus here on two synaptic proteins fused to fluorescent proteins that have been successfully applied in us and others. The first one, PSD-95, is a scaffolding protein restricted to clusters in the postsynaptic density of most glutamatergic synapses [9–13]. The second protein, synaptophysin, can be used to identify release sites on axon terminals as it is selectively localized at presynaptic terminals [14].

Postsynaptic Targets for Excitatory Synapses

To visualize glutamatergic input synapses, we and others have expressed a PSD-95-GFP fusion protein. PSD-95 is a scaffolding protein that localizes to the postsynaptic density of glutamatergic synapses [12] and has been extensively used as a postsynaptic marker of glutamatergic synapses [9–11, 13] both for confocal imaging in fixed tissue and in vivo imaging.

PSD-95-GFP-positive clusters overlap with endogenous PSD-95 expression. PSD-95-GFP-positive clusters are contacted by the presynaptic marker bassoon and are concentrated at asymmetric synapses at the ultrastructural level [6, 7]. Furthermore PSD-95 is already highly expressed at birth [15]. Thus, PSD-95-GFP is useful to follow synaptic development as neurons start to differentiate because it appears early during assembly of the postsynaptic density. For example, expression of PSD-95-GFP fusion protein in progenitor cells with retroviruses was a useful method to investigate the dynamics of synapse formation in newly generated neurons produced in the brain of adult mice [16]. Other postsynaptic proteins like SAP-102 fusion proteins can complement PSD-95-GFP to monitor synapse formation, as they are expressed with a different temporal profile during maturation of glutamatergic synapses.

Finally, retroviral expression of PSD-95-GFP did not change the strength and number of glutamatergic synapses in cultured neurons [6]. The absence of a direct effect of the synaptic marker on synapse number and stability is critical for many experiments, since it has been reported in cultured hippocampal neurons that five- to tenfold overexpression of PSD-95 by transient transfection led to strengthening or increase in the number of AMPA receptor-mediated mEPSCs [17]. Our experiments revealed that the modest level of expression achieved with retroviral expression did not change the strength or number of AMPA receptor-mediated mEPSCs in vitro, further supporting the idea that PSD-95-GFP can be used to genetically label postsynaptic sites. Thus, in all strategies utilizing fusion proteins as genetic markers of synapses, there is a critical balance to achieve, because it is necessary to obtain sufficient expression so that the fusion protein can be reliably detected, but low enough not to interfere with normal cell function.

To the best of our knowledge, other candidate fusion proteins are less established for labeling of glutamatergic synapses. For example, one could imagine visualization of specific subsets of glutamatergic neurons during development. Glutamatergic synapses are characterized by sequential addition of NMDA receptor subunits GluN2B followed by GluN2A. Both NMDA receptor subunits can be engineered as fusion proteins and cluster at the expected sites where glutamatergic synapses are regularly found [18]. However, in contrast to PSD-95-GFP fusion protein, overexpression of these fusion proteins have overt effects on neuronal maturation [18]. Hence, the effects of the expressed fusion protein itself on synapse properties like elimination or strengthening are important to consider. Certain of these effects may be mitigated by directed mutagenesis of the synaptic marker, but there is the possibility that dominant negative effects (such as sequestration of interacting proteins) could occur.

Postsynaptic Targets for Inhibitory Synapses

In contrast to the well-established marker PSD-95-GFP for glutamatergic synapses, less is known about equivalent marker for inhibitory synapses. A scaffolding protein of inhibitory synapses, gephyrin, may be useful [19], but a thorough characterization is necessary to confirm whether gephyrin only clusters at synapses or whether it also clusters at significant levels, i.e., in the cytoplasm. In addition, it has not been determined the fraction of GABAergic synapses that are actually labeled by this marker. Finally, it is not known the extent by which gephyrin labels synapses as neurons mature. Other promising candidates may be $GABA_A$-receptor subunits fused to fluorescent proteins [20]. It is however important to again consider direct effects of these receptor fusion proteins on synapse function or maintenance [21]. Similar gain-of-function problems can arise if other structural synaptic proteins, like neurexins or neuroligins, are used as marker proteins (*see* also next section on GRASP).

Presynaptic Targets for Release Sites

Presynaptic synapses (output synapses) can be labeled by a synaptophysin-GFP fusion protein. Synaptophysin is a 38 kDa synaptic vesicle glycoprotein that is expressed in virtually all neurons in the brain and spinal cord [14]. Despite its well-known interaction with the essential synaptic vesicle protein synaptobrevin, the exact function of synaptophysin remains unclear as it is not an obligatory protein for vesicle formation or fusion [22, 23]. Synaptophysin-GFP has been extensively used to study the distribution and density of presynaptic sites in neurons both in vitro and in vivo [8, 24–26]. There is currently no indication that its overexpression results in gain of additional synapses. Its expression is nearly ubiquitous in all presynaptic sites in contrast to other presynaptic marker proteins like Bassoon. Finally, it appears relatively early during synapse formation [27]. A previous study that examined synapse formation in

zebra fish indicated that transient clusters are formed in the cytosol of developing axons potentially indicating transport of pre-clustered synaptic proteins to the terminals [8]. Such transient clustering should be considered when studying initial synapse formation.

2.2 Genetic Labeling of Contact Between Synaptic Partner Neurons

For certain questions the visualization of synaptic contacts between two types of neurons is critical. For this purpose, complementary genetic labeling techniques have been developed.

Transsynaptic Labeling with Replication-Deficient Rabies Vectors

A technique that has recently gained substantial attention is retrograde transsynaptic labeling using genetically modified rabies viruses, also called "monosynaptic tracing" [28]. Monosynaptic tracing is reviewed in detail in a separate chapter of this book, and here we will focus on its current limitations and opportunities. This method has currently several drawbacks that have to be considered when applying this technique in its current state [29]. Replication of rabies viruses interferes with the protein synthesis machinery of the host cell, thus limiting the time window between its initial expression until the death of the labeled neurons to a few days [30].Thus, it is important to be aware of the effects of rabies replication on the health of the neuron for any imaging and functional studies . Also, once the virus jumps to a presynaptic cell, that cell will remain labeled independently of whether the synaptic contact is lost [29]. Particularly during states of high synapse turnover such as during development, this technique can be used to sample the cumulative history of previous and current synaptic partners. Finally, it appears that rabies virus do not cross over all the synapses. For example, cortical pyramidal cells are estimated to have approx. 10,000 presynaptic partners, but transsynaptic labeling with rabies only reveals hundreds of presynaptic cells [31]. Similarly it is not clear whether rabies viruses will cross different types of synapses with the same efficiency [29]. Again, once these constraints are better understood, they could provide further insight into synaptic properties and their distribution.

GFP Reconstitution Across Synaptic Partners (GRASP)

Another approach that has been initially developed in C. elegans is "GFP Reconstitution Across Synaptic Partners" (GRASP) [32]. GRASP genetically labels synaptic partners utilizing a two-component synaptic labeling system [33]. GRASP takes advantage of a version of GFP divided into two fragments that can emit fluorescence only when the two halves of GFP are combined. To visualize exclusively synaptic contacts, the two GFP fragments need to be appended to the extracellular domains of transmembrane proteins that localize to synapses. GRASP labeling matched ultrastructural synaptic contacts in C. elegans circuits [32, 34]. GFP fluorescence requires no exogenous cofactors and therefore can be monitored in vivo like other fluorescent genetic synaptic marker

proteins. GRASP has now been modified for application in C. elegans, Drosophila, and, most recently, mouse. In transgenic mice [35, 36] the GRASP system currently appears to have low sensitivity. Although signals in especially large photoreceptor synapses are robust, known contacts at smaller synapses in the inner plexiform layer of the retina, the spinal, or the cortex using other Cre transgenic lines were not detected potentially due to insufficient expression of the 2 GRASP partners [36]. In contrast, using high levels of expression via adeno-associated viral vectors, it is possible to detect GRASP signals with higher sensitivity in the mouse hippocampus and cortex [35].

Finally, an important concern regarding GRASP is the potential stabilization of otherwise transient cell-cell contacts, as the binding of the two elements of the split GFP is irreversible. Thus, the two components of the GRASP system act as a strong cell adhesion complex that will keep cell permanently attached to each other. This may become particularly important in dynamic situations such as during brain development where a large fraction of synapses is usually eliminated before achieving the eventual synaptic organization. In summary, GRASP is a promising technique to visualize synaptic connections between neurons that might become widely useful once several key technical limitations are solved.

3 Comparison with Other Techniques

3.1 Histological Approaches to Visualize the Synaptic Organization of Neurons

As already stated above conventional antibody labeling of synaptic proteins in vibratome or frozen tissue sections by light microscopy has two major drawbacks. First, it relies on antibodies against synaptic proteins. However, most antibodies against synaptic proteins that work in cultured neurons do not work well in tissue sections. Second, it is frequently impossible to see the "tree in the forest" due to the extremely high density of labeling in tissue sections, which makes it impossible to attribute or even quantify the number of synaptic sites of single neurons.

Emerging imaging techniques like "array tomography" [37] may be able to overcome some of these problems. Array tomography is a volumetric microscopy method based on physical serial sectioning. Ultrathin sections (50–200 nm) of a plastic-embedded tissue are cut using an ultramicrotome and bonded in an ordered array to a glass coverslip. Due to the ultrathin sectioning, antibody labeling is substantially improved as antibodies penetrate efficiently through the sections. Because these arrays are very effectively stabilized by the glass substrate, they can withstand many repeated cycles of staining, imaging, and elution. This permits using many antibodies serially (20 or more) to each individual section.

The resulting two-dimensional image tiles can then be reconstructed computationally into three-dimensional volume images

for visualization and quantitative analysis. However, "array tomography" relies on the efficiency of antibody staining and preservation of tissue antigenicity and can result in potentially ambiguous and incomplete results.

Electron microscopy is technically more challenging than imaging by light microscopy but allows for high-resolution analysis of pre- and postsynaptic sites by visualizing synaptic vesicles and postsynaptic sites, respectively. Conventional EM using serial sections has been used, for example, to reconstruct the whole set of individual synapses between single adult-born neurons and their synaptic partners [38]. However, this kind of work is extremely labor intensive and can only be used to reconstruct a handful of neurons per experiment. Recent developments within the past few years hint at the possibility of semiautomated sectioning and imaging of large neuropil volumes [39], thus facilitating high-throughput ultrastructural analyses of synapses. However, only relatively small brain volumes can presently be imaged (on the order of $450 \times 350 \times 50$ μm, e.g., [3]), mainly because electron microscopy image acquisition and analysis remain a formidable challenge [39]. In addition, the main limitation for the EM approach is that this technique focuses on the local structure under analysis, because long-range connections from distant parts of the brain cannot be analyzed.

There are two main advantages of electron microscopy and array tomography. First, these techniques provide very high-resolution images. Second, in contrast to genetic methods, they reveal brain circuits in their "native" status, without perturbing their function by addition of extra molecules to their synapses. However, both electron microscopy and array tomography are restricted to fixed (dead) tissue and do not allow functional studies via in vivo imaging or electrophysiological recordings as is possible for genetic labeling of synapses. Due to the extensive tissue processing, e.g., serial electron microscopy, caution has to be applied concerning nonproportional changes in extracellular due to tissue shrinkage and subsequent artifacts that can distort the wiring diagram of circuits during semiautomated analysis [39].

4 Delivering Genetic Synaptic Markers into Neurons

As outlined above delivery of genes to a small number of neurons and obtaining appropriate levels of expression is critical to successfully using genetic synaptic markers. Techniques such as gene gun delivery (in slices), in vivo plasmid electroporation, and adeno-associated viruses are less explored for genetic labeling of synapses as the high copy numbers of transferred genes into single neurons with these methods may result in unpredictable effects on synapse function. In addition, there is a high variation in the copy number

of transgenes delivered per cell using adeno-associated viruses, electroporation, or gene gun. Therefore, analysis of labeled synapses may be complicated by the degree of variation in brightness of clusters among neurons. Finally, above a certain level of expression, a significant level of the marker protein might be located outside of synaptic sites. In contrast, retroviral vectors provide moderate expression levels and a relative consistency in the level of expression between cells. Due to these advantages, retroviruses have been extensively used in many studies of genetic labeling of synapses. An alternative approach to achieve reproducibility of gene expression is provided by generating transgenic animal lines. We will therefore discuss here expression of synaptic markers by retroviruses and tissue-specific transgenesis.

4.1 Viral Delivery of Genetic Synaptic Markers

Retroviral vectors are the most commonly used vehicle to deliver genetic synaptic markers into neurons. Retroviral vectors fall by-and-large into two popular systems: HIV-derived lentiviruses and Moloney oncoretroviruses. Both viral systems are replication deficient, meaning that after the initial infection of a cell, they are not able to replicate and infect other target cells. A major difference between the two systems resides in the cells they can infect [40]. Whereas oncoretroviruses can only infect cells that are in the progress of dividing, lentiviruses can infect both quiescent and dividing cells. Oncoretroviruses have therefore been widely used in developmental biology as they allow birthdating of newly generated neurons and tracking their maturation. The packaging size of the inserted genes is comparable in both systems and sufficient to harbor most genetic synaptic marker and an additional gene of interest, such as those coding for ion channels, growth factors, or cell adhesion molecules. Thus, by introducing both a synaptic marker and a gene of interest, it is possible to assess the effects of various manipulations on synapse formation and dynamics. Retroviral expression under the control of different promoter fragments derived from the human synapsin, CMV, or RSV promoter provides appropriate levels of expression in mammals. Cell-type-specific promoters are however, despite intense search, still not available for most neuron types. For rodents, the CamKII promoter is a good option to label excitatory neurons, but currently there is no good candidate promoter to label inhibitory neurons. Using the abovementioned promoters and retroviral systems, we have observed a limited variability in the brightness of the fusion proteins PSD-95-GFP and synaptophysin-GFP when comparing cells in the same tissue section. Moreover, these differences of expression between cells had little effect on the analysis by confocal microscopy as the brightness of the synaptic clusters was generally saturating and the differences occurred largely in the mostly dim and diffusely distributed fusion protein fluorescence throughout neuronal processes. These low levels of diffusely distributed synaptic fusion

protein do not interfere with detection of synaptic clusters, but can be very helpful as, in the case of in vivo imaging studies, it helps to visualize the neuronal tree along which synaptic clusters form [11, 41]. In case of confocal analysis in fixed tissue, the low levels of diffusely distributed GFP in the neuronal processes can be rendered detectable by amplifying its signal with immunofluorescent staining in a different color (for instance, Texas red antibodies to amplify the PSD-95-GFP diffuse signal) that substantially facilitates the reconstruction of finer neuronal processes.

4.2 Generation of Transgenic Animals Expressing Genetic Synaptic Markers

Another potentially attractive approach is the generation of transgenic lines expressing genetic synaptic markers in selected subsets of neurons. This approach generally can have two advantages. First, transgenic animals provide reproducible levels of gene expression for a given cell type, eliminating the cell-to-cell variability discussed with viral delivery methods. Second, and more importantly, cell-type specificity can be obtained by using knock-in-techniques or large promoter region fragments (>100 kb) for conventional transgenesis. The downside of most cell-type-specific mouse line is that the high density of labeled cells may make it difficult to reconstruct and attribute synaptic clusters to single neurons. This limitation could be overcome using transgenic strategies that result in stochastic and sparse expression of genetic synaptic markers, such as incomplete activation of inducible Cre (Nathans, Luo paper in PLOS ONE). For some of these model systems, the use of genetic synaptic markers, PSD-95-GFP and synaptophysin-GFP, has been successfully applied in combination with in vivo imaging to study synaptic development of single neurons [8].

4.3 Different Ways of Attributing Synapses to Specific Neurites

For most experimental question, genetically labeled synapses need to be attributed to neuronal processes of single neurons. Here, different approaches exist:

1. Immunohistochemical methods.
   (a) For imaging the synaptic organization of single neuron's visualization of the dendritic or axonal tree that contain the genetically labeled synapses in fixed tissue, a simple method based on immunofluorescence antibody labeling can be used. To attribute PSD-GFP-positive clusters to a particular neuron, we took advantage of the presence of low levels of diffuse PSD-95-GFP protein in the cytoplasm, which were not detectable by its endogenous fluorescence. However, this diffuse PSD-95-GFP protein can be easily visualized by amplifying its signal with antibodies raised against GFP (followed by staining with a secondary antibody coupled to a red or blue fluorophore to distinguish it from the intrinsic green fluorescence of PSD positive clusters) and allowed attribution of PSD-GFP clusters to a dendritic arbor belonging to a particular neuron.

2. Co-expression with membrane-tagged XFPs.
   (a) An alternative approach is the expression of both the genetic synaptic marker and a membrane-tagged fluorescent protein of another color. Different approaches exist to co-express two genes with a single retrovirus.

   IRES: internal ribosomal entry sites can be engineered to obtain two different proteins from single mRNA (bicistronic expression). However, this strategy is problematic in many cases as it is usually found that the second protein is expressed at much lower levels than the first protein. In some cases it has been reported that the protein located in behind the IRES may be expressed at levels tenfold lower than the first protein.

   2A linkers: The use of T2A-linker sequences derived from picornaviruses is a relatively recent strategy to obtain bicistronic expression. In this strategy the first gene is engineered without its stop codon, followed by a 2A sequence (about 18 amino acids), and finally followed by the complete coding sequence of the second gene. When the ribosome reaches the 2A sequence, it will release the first protein plus the 2A sequence and will start translating the second protein. In contrast to IRES, the two genes separated by 2A sequences are expressed at similar levels. However, there are a few caveats for bicistronic cassettes with 2A sequences. First, in some situations instead of two independent proteins, a large fusion protein will be produced that includes the open reading frames of the first and second proteins plus the intervening amino acids of the 2A sequence. Second, the first protein will have an extra "tail" consisting of the 2A sequence at its C-terminus, which in some cases can affect its function. We recently observed that the T2A linker provides sufficient levels of expression of synaptophysin-GFP and membrane-tagged dimeric tomato protein in a retroviral vector (unpublished observations, Fig. ???). This approach may be particularly interesting for in vivo imaging that aims at attributing synaptic clusters to processes of specific neurons.

   (b) Bicistronic expression of a synaptic genetic marker plus the recombinase Cre can be used to combine genetic synaptic labeling with conditional mouse or viral transgenic tools. XFP reporter lines are available that displayed high levels of fluorescent protein expression upon Cre recombination. Cre is a highly efficient recombinase, and having a low expression of it (e.g., following an IRES) is not problematic because it is sufficient to induce recombination of the loxP sites. Thus, in this strategy, the XFP would be driven by a strong promoter in a loxP-dependent reporter transgenic mouse or virus.

This approach can be further expanded combining multiple conditional mouse lines and/or viral conditional gene delivery that is Cre dependent.

4.4 Technical Considerations of Genetic Labeling

Two main points have to be considered when designing genetic synaptic markers:

1. *Expression level: not too low and not too high (influence of synapse strength).*
   When introducing these genetically encoded markers, it is critical to ensure that only modest levels of overexpression are achieved because excessive levels of these proteins may interfere with synaptic development or function [17]. Fortunately, retroviral vectors, which deliver single copies of transgenes into their target cells, produce modest levels of expression that are sufficient to detect fluorescent synaptic marker proteins, while at the same time leave synapse number and strength unperturbed [6].
2. *Influence on synapse stability*
   There are concerns to consider in this respect. First, expression of these genetic synaptic markers may influence the number or strength of synapses. For example, neuroligins are known to directly induce synapse formation even with nonneuronal targets [42] and thus have to be carefully tested before using them as fusion proteins to study synaptic development or reorganization as discussed above. Second, labeling approaches based on the transneuronal interaction of transmembrane proteins may interfere with synapse turnover. For example, an important drawback of GRASP is that the reconstitution of the split GFP creates a strong cell-cell adhesion that can stabilize otherwise transient synapses. Thus, it is critical to keep in mind that using proteins to label synapses could corrupt the normal synaptic development and distribution.

5 Imaging and Quantification of Genetic Synaptic Markers

5.1 Two-Photon Microscopy

Two-photon microscope technology has taken off considerably in recent years and is still the only technique that allows for synapse imaging in vivo. This technique is extremely useful for observing real-time changes to experimental manipulations and allows investigators to visualize synapse dynamics.

Neurons up to 800 μm below the brain's surface can be imaged [43]. In some cases it becomes even possible to image even deeper brain structures at high resolution such as superficial dendrites of neurons in CA1 of the hippocampus by removing part of the neocortex and white matter above the hippocampus [44]. Finally, there has been great interest in using two-photon microscopy associated

with endoscope lenses to image deep within the brain, but several technical obstacles to obtain a sufficient spatial resolution need to be solved before this method can be routinely used [45].

5.2 Confocal Microscopy

As deep structures of the brain are beyond the depth limitation of two-photon microscopy in vivo, in vitro time-lapse confocal imaging of brain slices is sometimes carried out to study the dynamics of synapse formation [38, 46]. The main concern about this technique is that the integrity of cultured adult brain slices is dramatically perturbed over long time periods with currently available culture techniques [47], as well as the possibility of abnormal synapse rearrangement due to fluctuations in culture conditions [48]. Confocal imaging of fixed slices is much more commonly used to study synaptic organization, especially because this method of observation is technically straightforward and enables investigators to analyze many neurons simultaneously. Time course experiments can be performed to observe spine formation over days and months, but because only a snapshot of the synapses can be obtained in fixed slices, this technique cannot be used to analyze the short-term dynamics of synapse formation in real time.

5.3 Data Analysis and Quantification

Genetic synaptic markers provide substantial advantages when it comes to the quantification of densities of synapses in dendritic or axonal domains. With genetically encoded markers, one can, in principle, analyze the complete set of a neuron's excitatory input synapses and output synapses, including those not associated with structural specializations such as spines or axon terminals.

Genetically labeled synapses appear as discrete bright clusters that can be semiautomatically detected and measured upon thresholding of images using freely available software packages (e.g., ImageJ-based MacBiophotonics). The obtained data about synapse organization can be combined with reconstruction of the morphology of individual neurons to obtain the spatial distribution of synapses along neuronal trees and measures of local cluster density. Most of the current analysis is limited to two-dimensional projections of 3D image stack. With the advancement of existing software tools, analysis in 3D may soon be amenable.

The main challenge of these approaches is the still highly labor intense reconstruction of neuronal processes, because it is essential to attribute genetically labeled synapses to specific neuronal domains such as axons or dendrites. One may imagine that reconstruction of dendritic and axonal trees of sparsely labeled neurons is an easy task to be automated. This however turned out to be a formidable challenge [49]. As a first step in this process, accurate semiautomated reconstruction software in 3D (e.g., [50]) could facilitate neuronal reconstruction to then attribute synaptic organization to neuronal trees in 3D.

6 Future Directions: Combination of Synaptic Labeling and Transneuronal Tracing

Genetic synaptic markers provide powerful tools that can help answer many open questions on the development and synaptic wiring of brain circuits when considering their currently existing limitations and caveats. With the continuous efforts to improve the existing tools, validation of novel marker proteins for specific types of synapses, these tools become increasingly more flexible for specific research questions. They may thus help to find the "tree in the forest" among the complex meshwork of neuronal processes and synapses in brain circuits. We believe that for many circuits understanding the typical wiring diagram of neurons may help to build realistic, unifying models of their function without necessarily having to know each detail of the entity of each individual's circuit under study.

Finally the strength of genetic tools might come into full action when genetic synaptic markers and transneuronal tracing will be combined. One example for such a dual approach shall be outlined here. Once a presynaptic neuron has been labeled at a given time point via transneuronal labeling with rabies, it will remain so independently of whether the synaptic contact is lost. In contrast to this permanent labeling (a trace of the neuron's connectivity history), labeling with genetic synaptic markers, e.g., with PSD-95-GFP, will be lost immediately when the synapse is lost. Hence, a combination of both techniques can serve to identify the history of cells that had been presynaptic to the target neuron at one point and the current state of synaptic contacts between the neurons.

7 Appendix: Gene Delivery of Genetic Synaptic Markers with Retroviruses and Quantification of Synapses

This appendix describes some specific procedures to deliver retroviruses carrying genetically encoded synaptic markers into the brain of rodents. General details about production and titration of lentiviral and retroviral vectors can be found elsewhere.

8 Injection of Viruses into the Brain

Viral prep: It is critical that the viral suspension is very clean. During the preparation of the viruses, there will be some cellular debris that can be strongly autofluorescent. To eliminate this debris it is useful to centrifuge the viral prep with a 20 % sucrose cushion.

Stereotaxic injection: It is critical to minimize the damage to the brain during injection. In particular, bleeding associated with the

injection will cause very high levels of autofluorescence that will make quantification of synapses very difficult. To minimize damage it is useful to use thin borosilicate pipettes pulled to an outer diameter of approx. 15–20 μm. It is not advisable to inject through metallic needles as this will cause severe tissue damage on the injection site. Similarly, it is advisable to inject the viral prep slowly, at a rate of approx. 1 μl over 5 min. Rapid injection can severely damage and distort the tissue. Regarding the timing of imaging after injection, for lentiviral vectors, the expression of the transgene peaks as early as 3 days, but there will likely be some acute damage in the injection area at this early time. Thus, it is advisable to wait at least a week so that the autofluorescence due to damage is resolved before perfusion of the animal.

9 Acquisition and Analysis of Genetically Labeled Synapses

This section describes the procedure that we optimized to visualize the synaptic organization of single genetically labeled neurons, which can be easily modified for individual experimental needs. The procedure is divided into three main steps, and technical issues are highlighted that are critical in our experience: preparation of the tissue ("Preparation of Tissue"), image acquisition by confocal microscopy ("Image Acquisition"), and semiautomated image analysis ("Quantification of Synaptic Clusters").

9.1 Preparation of Tissue

1. The protocol is described for small rodents, but can be easily adapted to other species. Animals are transcardially perfused initially with phosphate buffered saline (PBS, 1×) for 10–15 s, followed by 4 % paraformaldehyde (PFA) for 3–5 min. The animal should become rigid within the first 30 s to 1 min of perfusion with PFA. It is optimal to use an overdose of an injectable anesthetic drug (such as Ketamin/Xylazin) and to start perfusion when the heart is still beating. PBS should be infused at a pressure such that the liver becomes pale within 10–15 s and clear PBS flows out of the right atrium after this period. It is equally important to perfuse with relatively low pressure, because at high perfusion pressure, the small capillaries in the brain will break and perfusion will not be homogeneous throughout the brain. PBS should be set to pH 7.0–7.4 and pre-warmed to 32–37 °C to prevent contraction of smaller blood vessels in the brain. Following PBS, perfusion should be immediately switched to room-temperature PFA. Incorrect perfusion leads to delayed fixation with PFA, which results in beaded structures of dendrites and loss of genetically labeled synaptic clusters. After perfusion is complete, the brains are extracted from the skull and post-fixed in 4 % PFA overnight at 4 °C.

2. After preparation of floating sections with a vibratome (e.g., 50 μm sections), tissue can be incubated (overnight at 4 °C) with primary antibody raised against the fluorescent protein tagged to the synaptic marker. The following day sections are rinsed in PBS and stained with a secondary fluorescent antibody for two hours at room temperature. Sections are then washed with PBS and mounted with an aqueous mounting medium that preserved fluorescent molecules. This procedure allows for the visualization of the neuronal tree and to attribute synaptic clusters to a neuron and specific dendritic domains. Blocking solutions (PBS containing 1 % bovine serum albumin or related serum proteins) for antibody incubation usually contain a detergent, i.e., Triton X-100, to permeabilize the tissue. We keep the procedure and times as constant as possible to avoid introducing additional variability, i.e., by differentially affecting the brightness of the fluorescence of the synaptic clusters.

9.2 Image Acquisition

Neurons expressing synaptic marker proteins can be conveniently imagined using confocal laser scanning microscopy. In most experimental conditions, it is advantageous to image sections that are sparsely labeled, where individual neurons are clearly separated from each other. In this case it is easy to analyze the full dendritic arbor of a single neuron without having to deal with neurites that could belong to neighboring cells. Confocal stacks are acquired at high magnification (with a 60–63× oil immersion objective) to efficiently capture emitted light from the clustered fluorescent proteins. The pixel size should be sufficiently small to obtain high intensity of all the pixels that are grouped in individual synaptic clusters, and to easily distinguish them from the occasionally observed noise that may result in random isolated pixels with high intensity. As most synaptic clusters have a size around 1 μm, we found a cluster size between 0.2×0.2 and 0.3×0.3 μm^2 most reliable for subsequent analysis. Laser excitation intensities should be set to levels that result in little or no obvious bleaching of the clusters. This can be easily tested by imaging the same neuron twice in the same day and comparing the clusters among the two images. A reference section containing neurons with good synaptic cluster intensity should be used to guarantee comparable acquisition conditions over time. The sensitivity of the photomultipliers (PMT) should be set to a level that clusters just saturate but low enough that individual clusters do not become confluent due to overexposure. Similarly, the pinhole size should be kept in the recommended range [51] for the chosen magnification. Once the settings are initially defined with a test sample, the conditions should be kept constant throughout the different imaging session. Upon acquisition of confocal stacks, maximum density projections are prepared for further image analysis. Two-dimensional projections are generally used for analysis, as current version of most image processing software cannot handle 3D data for quantification.

9.3 Quantification of Synaptic Clusters

Analysis of densities and distribution of genetically labeled synapses can be semiautomated. Fully automated analysis is currently limited by the still challenging task for computers to reconstruct neuronal trees due to overlap of labeled neurons and discontinuities in the processes deriving from histological processing and incomplete filling with fluorescent proteins. Thus, reconstruction of processes has to be performed individually or at least be supervised.

Contrary to reconstruction of dendritic trees, analysis of clusters can be fully automatized provided the original image quality has a good signal-to-noise ratio. Signal-to-noise ratio for these experiments means high intensity of fluorescence in the synaptic cluster and low levels of autofluorescent background outside of synaptic sites. Similarly, it is important that there is a low level of fluorescence originating from diffusely distributed XFP in the cells' processes outside the synapses. Another potential source of "contamination" with artifactual autofluorescent clusters can be due to lipofuscin granules observed in some brain structures and species. The appearance of these autofluorescent granules is difficult to predict. For example, we have found them in mouse olfactory bulb and dentate gyrus, but not in the rat olfactory bulb or mouse neocortex. These autofluorescent artifacts can be easily diagnosed as they are excited by all wavelengths. In contrast, real genetic synaptic markers containing XFPs can only be detected at a specific wavelength (e.g., 550 nm for GFP). In addition, these autofluorescent granules can usually be excluded from analysis as they are mostly present in cell bodies.

Given these considerations the analysis is relatively straightforward using different analysis software packages. We will describe the different steps of analysis and particularly refer to the ImageJ-based MacBiophotonics software (www.macbiophotonics.ca/). Similar procedures can be performed in Metamorph software from Molecular Probes.

Steps:

1. Open maximum density projection (*File>Open*).
2. Define pixel size for subsequent distance measurements (*Analyze>Set scale*).
3. Split color channels (*Image>Color>Split channels*).
4. Choose the channel that displays the fluorescent synaptic clusters.
5. Set inclusive threshold so that only clusters are included (*Image>Adjust>Threshold*). The threshold value should be kept constant throughout the analysis. Therefore, it proves useful to use a reference as described in the acquisition part to set the threshold.
6. Draw a contour using the freehand selection tool to define a region of interest to measure a specific dendritic domain and exclude neighboring neurons.

7. Perform region measurement (*Analyze>Analyze particles*). Desired parameter data can be set in the results window (*Analyze>Set Measurements*) and copied to a data sheet of a given statistics program.

Measure the length of the neuronal processes in the region of interest using the freehand line tool and *Analyze>Measure*.

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