Imaging of synaptic proteins dynamics in vivo

CANE, Michele

Abstract

In the adult somatosensory cortex the majority of the spines are stable. However some spines appear and disappear over time. EM analysis showed that many newly formed spines do not bear a morphologically defined synapse. In this thesis I describe studies that we have conducted to directly probe the dynamics of excitatory and inhibitory synapses in vivo. We used in utero electroporation of GFP-tagged PSD-95 to investigate the relationship between spine and PSD stability in neocortical L2/3 pyramidal cells in vivo. We then adapted a single cell electroporation method for chronic imaging of synaptic proteins. The technique has a high spatial and cell type selectivity, a low background fluorescence and does not affect developmental processes. As a proof of principle we transfected various synaptic proteins, some of which where thus far only successfully studied in vitro.

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*La Faculté des sciences, sur préavis du jury de thèse formé par :

Prof. Anthony Holtmaat, Dépt. des neurosciences fondamentales, Faculté de Médecine, Université de Genève, directeur de la thèse
Prof. Jean-Pierre Hornung, Dépt. des neurosciences fondamentales, Université de Lausanne
Dr. Jean-Marc Matter, Département de biochimie, Section de chimie et biochimie, Faculté des sciences, Université de Genève
Prof. Dominique Muller, Dépt. des neurosciences fondamentales, Faculté de Médecine, Université de Genève

autorise l’impression de la présente thèse, sans prétendre par là émettre d’opinion sur les propositions qui y sont énoncées.

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Le Doyen, Jean-Marc TRISCONE

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Abstract

One of the most extraordinary properties of the brain is its plasticity. The main substrate for plasticity in the brain are the synapses. The synapse is a physical entity connecting two neurons. It is composed of two different structures: a presynaptic component (an axonal bouton) and a postsynaptic component (a spine). Evidence for structural remodeling of synaptic circuits has mostly been based on static assessments of synapses using electron microscopy (EM) or on time-lapse imaging of dendritic spines and axonal boutons as proxies for synapses. It has been shown that, in the adult somatosensory cortex, the majority of the spines are stable. However some spines appear and disappear over time. Moreover some of the new spines stabilize and the number of new spines undergoing stabilization can be increased by certain plasticity paradigms. Retrospective EM analysis of imaged spines showed that many newly formed spines do not bear a morphologically defined synapse, and those new spines that contain synapses mature slowly. These data suggest that the outgrowth of spines and the stabilization of spines with synapses are regulated by different processes, but the temporal relationship remains unknown. In this thesis I describe studies that we have conducted to directly probe the dynamics of excitatory and inhibitory synapses in vivo. We used in utero electroporation of DsRedExpress and GFP-tagged postsynaptic density protein 95 (PSD-95) to investigate the relationship between spine and PSD stability in neocortical L2/3 pyramidal cells in vivo. Spine head size and PSD-95 levels co-varied over time suggesting a continuous change in synapse strength. The majority of newly formed
spines did not acquire PSD-95 puncta and rarely survived more than 1 day. Although PSD-95 incorporation increased spines’ lifetimes, the majority of the spines with puncta did not stabilize for long periods of time. This indicates that transient spines incorporating PSD-95 may serve as short-lived synaptic contacts. Although the majority of persistent spines that were meant to be pruned lost their PSD concomitantly, they showed reduced PSD-95 levels well before the actual disappearance.

We then adapted a single cell electroporation method for chronic imaging of synaptic proteins. This technique was developed to overcome some problems that we experienced in pilot experiment in which we aimed at studying the relationship between structural synapse dynamics and synaptic plasticity. The technique has a high spatial and cell type selectivity, a low background fluorescence and does not affect developmental processes. As a proof of principle we transfected various synaptic proteins, some of which where thus far only successfully studied in vitro.
Résumé

Une des qualités les plus extraordinaires du cerveau est sa plasticité. La cible principale de la plasticité sont les synapses. La synapse est l'entité physique qui relie deux neurones et elle est composée de deux structures: une composante présynaptique (le bouton axonal) et une composante postsynaptique (l'épine dendritique). Les preuves de remodelage structurel des circuits synaptiques sont basées principalement sur des observation statiques des synapses par microscopie électronique (EM) ou par imagerie d'épines dendritiques considérées comme formant une synapse. Il a été montré que, dans le cortex somatosensoriel adulte, la plupart des épines sont stables mais certaines d'entre elles disparaissent au cours du temps. De plus, certaines nouvelles épines se stabilisent et le nombre de ces dernières peut être augmenté par des protocole de plasticité. L'analyse d'images acquises par EM montre que la plupart des nouvelles épines ne constituent pas une synapse morphologiquement définie et que les nouvelles épines qui forment une synapse se développent lentement. Ces données laissent penser que la croissance d'épines et la stabilisation des épines formant une synapse sont des mécanismes régulés par des processus différents mais leur relation temporelle reste inconnue. Dans cette thèse, je décris des études que nous avons menées pour comprendre la dynamique des synapses excitatrices et inhibitrices in vivo. Nous avons utilisé l'électroporation in utero de DsRedExpress et de la protéine postsynaptique 95 (PSD-95) fusionnée a de la GFP pour étudier la relation entre la stabilité de la PSD et les épines dans les cellules pyramidales de la couche corticale 2/3 in vivo. La taille de la tête des épines et les niveaux
de PSD-95 co-varient au cours du temps pouvant indiquer un changement dans la force des synapses. La plupart des nouvelles épines n’incorporent jamais de la PSD-95 et survivent rarement plus d’un jour. Même si l’incorporation de PSD-95 prolonge la durée de vie des épines, la plupart des épines ayant incorporé la protéine ne sont pas stabilisées pour des longues périodes de temps. Cela pourrait indiquer que les épines temporaires qui incorporent la PSD-95 puissent servir comme des contacts synaptiques de courte durée. Même si la plupart des épines persistantes qui sont destinées à être éliminées perdent leur PSD en même temps, elles ont des niveaux réduits de PSD-95 avant l’élimination.

Ensuite, nous avons adapté le protocole d’électroporation d’une seule cellule (single cell electroporation) à l’imagerie chronique in vivo. Cette technique a été développée pour résoudre les problèmes que nous avons rencontré lors des expériences pilotes dans lesquelles nous avons cherché à étudier la relation entre les dynamiques structurelles de l’épime et la plasticité synaptique. Cette technique a une très haute sélectivité spatiale et cellulaire, une fluorescence basale faible, et n’affecte pas les processus liés au développement. Comme démonstration de principe, nous avons transfécté plusieurs protéines synaptiques, dont une partie qui a seulement été testée in vitro jusqu’à présent.
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Chapter 1

Introduction

1.1 The synapse

The pioneering work of Ramón y Cajal can be considered as the beginning of modern neuroscience. In his work he formulated the concept that neurons are the fundamental functional signalling units of the nervous system and are interconnected in very specific ways (Cajal Ramón y, 1894). Using a silver staining method developed by Camillo Golgi he was able to observe that neurons are connected by specialized points of apposition which would later be named 'synapses' by Charles Sherrington (Sherrington, C.S., 1924).

1.1.1 Composition and role

Synapses can be grouped in three different ways according to their mode of transmission, their localization or their polarity. Synapses transmit signals in
two different ways: electrically or chemically. For the former the continuity is provided by gap junctions, which are clusters of ions channels and connexins, allowing potassium ion (current) to pass freely between two neurons. Their main function is to provide neuronal synchronization. Recent studies suggest a secondary role in the mammalian adult brain providing lateral spread and forward transmission (Bennett, Zukin, 2004). In the latter the neurons are separated by a synaptic cleft. Neurotransmitter release from synaptic vesicles, triggered by an axon potential, in the synaptic cleft is followed by the binding of these molecules to receptors localized on the other neuron, which induces a change in the receiving neuron’s polarization. This complex mechanism can be modulated presynaptically, by changing the type of neurotransmitter or its quantity, or postsynaptically by changing the receptor composition (Lisman et al., 2007).

Axo-dendritic synapses (formed between axons and dendrites) are the most abundant types. Axo-axonic (formed between two axons) and axo-somatic (formed between axons and cell bodies) also exist but account for a smaller number of contacts (Toni et al., 2007). Dendro-dendritic spines (formed between two dendrites) are found in the case of electrical synapses. All future references in the text to “synapse” refer to the chemical synapse.

1.1.2 Excitatory and inhibitory

Synapses can also be grouped based on their polarity. If receptor binding of the neurotransmitter induces an EPSP (excitatory post-synaptic potential)
by causing an influx of cations, the synapse is termed excitatory. If the neurotransmitter induces an IPSP (inhibitory post-synaptic potential) by causing an influx of anions, the synapse is inhibitory. The two main neurotransmitters in the brain are glutamate and \( \gamma \)-aminobutyric acid, acting mainly on excitatory and inhibitory receptors respectively. In the cerebral cortex the proportion of excitatory synapses is 80-90\% versus 10-20\% of inhibitory synapses (DeFelipe et al., 2002).

1.1.3 Structure

The inhibitory and excitatory synapses have different components but the structures are similar. To describe the structure of and basic function the glutamatergic synapse can be used as an example.

1.1.3.1 Axonal boutons

The presynaptic zone is composed, among other constituents, of Ca\(^{2+}\) channels which are opened by the arrival of an action potential (AP). Ca\(^{2+}\) induces budding of synaptic vesicles to the membrane and glutamate is then released in the synaptic cleft (which is approximately 20 nm long) (Lisman et al., 2007). The synaptic cleft contains a plethora of heterogeneously located proteins, mainly adhesion molecules.
1.1.3.2 Dentritic spines

On the exterior of the dendritic spines glutamate binds to AMPA receptors inducing an EPSC (excitatory post-synaptic current). On the membrane other receptors such as NMDA receptors can be found. The zone approximately 35-50 nm beneath the membrane is called post synaptic density (PSD) and contains a large diversity of proteins with scaffolding and signaling roles (Rochefort, Konnerth, 2012).

1.2 Plasticity

Plasticity is one of the most extraordinary characteristics of the brain. It allows the brain to interact with the sensory world by learning and remembering patterns, predict, obtain reward and recover after damage. In order to understand the cellular and synaptic mechanisms of plasticity many models have been used. One that offers many advantages is the neocortex.

1.2.1 Cortical plasticity

The ability of neocortex to perform sensory, motor and cognitive tasks combined with learning components, makes it an exquisite model for studying plasticity. Another reason for using the cortex to study plasticity is the fact that primary cortical areas are somatotopically organized according to their
function. For example, in rodents, the barrel cortex contains a somatotopic map of the whiskers on the snout. Thus, each whisker is represented in a small but separate region of the cortex, known as a barrel. Each barrel column is mainly activated by one principal whisker (Fox, 2002). Most cortical areas, such as the auditory, visual cortex and the rest of the somatosensory cortex are topographically organized (Fox, Wong, 2005; Nelken et al., 2004).

Two major phenomena have been observed in the sensory neocortex with regard to plasticity. The first is experience-dependent map plasticity where the representational area can be modified by behavior and other kinds of input manipulation (Blake et al., 2002). The second is sensory perceptual learning, where the associative process of learning induces field and/or map representation changes in the cortical area that is relevant to the learned perception (Weinberger, 2007). Both experience-dependent and learning-dependent plasticity take place with similar underlying functional components in the somatosensory (S1), visual (V1) and auditory (A1) cortices probably induced by shared cellular mechanisms.

1.2.2 Whisker map plasticity

Similarly to other cortical areas, the barrel cortex receives thalamic inputs, which in turn receives information from the whiskers via the trigeminal nucleus in the brain stem. The thalamocortical axons mainly project onto cortical layer 4 neurons, which in turn project to layer 2/3 and finally to layer 5 providing cortical and sub-cortical outputs (Lübke, Feldmeyer, 2007;
Petreanu et al., 2007, 2009). When whiskers are trimmed in a chessboard pattern an increase in spiking responses of spared whiskers and a decrease of spiking responses of deprived whiskers are observed. This process results in a shrinkage of deprived whisker and expansion of spared whisker representations (Fox, 1992; Glazewski, Fox, 1996). The plasticity is more pronounced in young animals targeting mainly L4 neurons in an early stage (less than 6 days old) and switching almost exclusively to L2/3 neurons in juveniles and adult mice (Stern et al., 2001; Drew, Feldman, 2009). The potentiation in spared barrel columns and depression in deprived barrel columns have different mechanisms of action (Glazewski et al., 2000). Both are involved in young adult mice of less than 2 month whereas on older mice only potentiation is observed (Fox, 2002; Fox, Wong, 2005). This mechanism is probably used to optimize information processing by reallocating deprived inputs to spared inputs.

1.2.3 Ocular dominance plasticity

The rodent visual cortex does not have defined ocular dominance columns but has a region where neurons show responses to both eyes. Monocular deprivation during the critical period (19-32 days), which is defined as the time window during early postnatal development showing high or unique aptitude to plasticity, leads, similarly to whisker map plasticity, to a decrease in responses of the deprived eye and an increase in responses to the open one. The similarity to whisker map plasticity is reflected in the different
mechanism of action of potentiation and depression, with L2/3 neurons being
the main early target for plasticity and older mice displaying plasticity mainly
in the form of potentiation (Trachtenberg et al., 2000; Maffei et al., 2004;
Maffei, Turrigiano, 2008; Liu et al., 2008).

1.2.4 Other types of plasticity

The pairing of stimuli with positive or negative reinforcement or neuromod-
ulation and with perceptual learning task increases plasticity in adult mice.
Training on visual discrimination tasks changes visual cortex tuning of the
trained characteristic (Dan, Poo, 2006). Classic conditioning paired with
acoustic stimuli increases auditory cortex responses to presented frequencies
(Weinberger, 2007). Similarly, when paired with whisker stimuli, it induces
an expansion of the whisker representation in the barrel cortex (Siucinska,
Kossut, 2004). Recurrent activation of a distinct sensory input leads also to
potentiation of responses to that input. The repeated exposure of young rats
to auditory stimuli reinforces the representation of the stimuli frequencies
and intensities in the auditory cortex (Keuroghlian, Knudsen, 2007). In a
similar way display of high contrast oriented grids to young mice induces in-
creased responses in the visual cortex (Frenkel et al., 2006). The same kind of
potentiation is observed both in visual and somatosensory cortex in response
to temporally correlated inputs (Wang et al., 1995; Dan, Poo, 2006). Finally,
extreme use or deprivation induces homeostatic compensatory mechanisms
aimed at restoring, completely or partially, neuronal activity to a base level.
Prolonged visual deprivation increases responses in the monocular region of rodent visual cortex (Mrsic-Flogel et al., 2007) and prolonged and continuous whisker stimulation reduces the representation of the activated whisker in the somatosensory cortex (Knott et al., 2002).

Five types of cortical plasticity can therefore be defined. Rapid depression to deprived inputs and slow potentiation to spared inputs, which are Hebbian and driven by competing active and inactive inputs. Potentiation paired with learning tasks and potentiation driven by active inputs or inputs temporal correlation, both consistent with Hebbian plasticity. The fifth is the homeostatic adjustment to massive increase or decrease of inputs (Feldman, 2009).

1.2.5 Synaptic plasticity

1.2.5.1 Long term potentiation

LTP implements, in young mice, temporal correlation and use-dependent enhancement of sensory responses, in adult mice, learning related dependent enhancement of responses and finally enhancement of spared inputs during deprivation. Several forms of LTP have been identified taking place in several cortical synapses (Feldman, 2000; Froemke, Dan, 2002; Hardingham, Fox, 2006). In the majority of the cases the LTP is post-synaptic and NMDA dependent (Malinow, Malenka, 2002). In this type of LTP the calcium influx through post-synaptic NMDA receptors activates CaMKIIα, which in
turn induces specific phosphorylation of AMPA receptors, and the insertion into the synapse membrane of AMPA receptors containing GluR1. The effect of NMDA dependent LTP is observed in silent synapses, expressing only NMDA receptors, which upon potentiation show AMPA currents (Rumpel et al., 1998). If the potentiation is protracted over time autophosphorylation of CaMKII induces CREB activation which in turn leads to changes in proteins expression levels. Another form of LTP observed in the cortex acts presynaptically by increasing the release probability and affecting short term changes. Both types of LTPs are observed for example in L4 to L2/3 synapses in the barrel cortex by post-synaptic GluR1 enrichment and presynaptic retrograde nitric oxide signaling (Hardingham, Fox, 2006).

Some studies link NMDA dependent LTP to potentiation induced by deprivation in the somatosensory cortex. Single whisker sparing experiments, in which all but one whisker are clipped, induces increased AMPA to NMDA ratios and increased quantal size in L4 to L2/3 synapses of the spared whisker compared to control animals or deprived regions. This paradigm also shows AMPA current rectification of spared whiskers neurons and increased sensitivity to joro spider toxin, a specific antagonist of GluR2 defective AMPA receptors, which is compatible with an increased insertion of GluR1 subunits (Clem, Barth, 2006). Knock out of GluR1 alone or in combination with a neuronal nitric oxide synthase block, partially or completely inhibit response potentiation induced by single whisker sparing suggesting the involvement of both pre and postsynaptic LTP (Dachtler et al., 2011). Earlier studies on knock out mice show a requirement of CREB, CaMKIIα and its phosphorylation for response potentiation (Glazewski et al., 1999, 2000). In the visual
cortex proof that response potentiation acts during monocular deprivation are not very clear. Several manipulations of molecules involved in LTP do not inhibit it (Hofer et al., 2009) or their participation to other pathways influence the visual system development (Rao et al., 2004).

In the developing barrel cortex, over expression of GluR1 in L2/3 neurons induces AMPA current rectification which is lacking in whisker trimmed mice. Preventing insertion of endogenous GluR1 into the membrane by expression of GluR1 cytoplasmic tail inhibits AMPA current rectification. These results are conform with NMDA-LTP identified in vivo and suggest a strong implication of use-dependent enhancement of cortical synapses in young mice (Takahashi et al., 2003). This mechanism is also dependent on the presence of PSD-95 (Ehrlich, Malinow, 2004). In a similar way, NMDA antagonist and GluR1 cytosolic tail expression in the visual cortex prevent response strengthening upon visual stimulation by high contrast grids (Frenkel et al., 2006).

### 1.2.5.2 Long term depression

Three different mechanisms of LTD have been described in different neocortical regions. The NMDA receptor dependent LTD, observed both visual and somatosensory thalamocortical synapses (Feldman et al., 1998; Crozier et al., 2007). In this type of LTD calcium influx through post-synaptic NMDA receptors activates phosphatases inducing Glur1 AMPA receptor subunit dephosphorylation finally leading to AMPA receptors internalization. The same
mechanism is observed on other cortical regions (Feldman, 2009). Another form of LTP is the one mediated by metabotropic glutamate receptors which can act on the synapse by different biochemical pathways (Barbara et al., 2003; Czarnecki et al., 2007). Finally LTD can be presynaptically induced by decreasing the release probability mediated by presynaptic cannabinoid type 1 receptors activation stimulated by post-synaptic type I metabotropic glutamate receptors cannabinoid synthesis (Crozier et al., 2007; Lafourcade et al., 2007).

LTD is strongly correlated with a reduction of deprived sensory inputs. In ex vivo slices of the somatosensory cortex from whisker deprived rats a decrease in the paired pulse ratio at L4 to L2/3 synapses is observed along with a slowed use dependent MK-801 inhibition of NMDA receptor currents. This is consistent with a presynaptic decrease in release probability (Bender et al., 2006a). Moreover whisker deprivation inhibit further cannabinoid receptor 1 induced LTD suggesting that this mechanism is the main drive of deprivation induced weakening of L4 to L2/3 synapses in vivo (Allen et al., 2003; Bender et al., 2006b). Other evidence supporting this model shows that blocking cannabinoid receptor 1 with the antagonist AM251 prevent the depression induced by deprivation (Li et al., 2009). In visual cortex LTD, upon monocular deprivation, is probably expressed by different mechanisms in L4 and L4 to L2/3 synapses. L4 synapses display NMDA dependent LTD whereas L4 to L2/3 synapses exhibit cannabinoid receptor 1 dependent LTD (Crozier et al., 2007). Inhibition of CB1 receptors prevents depression of responses in L2/3 neurons but not in L4 neurons. Moreover monocular deprivation decrease GluR1 fraction and is prevented by knock out of PKA RIIβ subunit
or AKAP150 (Daw et al., 2004). However the manipulation of other protein previously linked to LTD, such as BDNF over-expression and knock out of PKA RIβ, do not inhibit monocular deprivation induced plasticity (Hensch, 2005).

1.2.5.3 Spike-timing-dependent plasticity

One of the rules proposed to mediate experience-dependent plasticity in vivo is spike-timing-dependent plasticity (STDP). The mechanism has been extensively studied in vitro. It drives LTP when the post-synaptic excitatory current occurs before the action potential and the interval is lower than 20 ms. It drives LTD when the post-synaptic excitatory current occurs after the action potential and the interval is typically lower than 100 ms (Sjöström et al., 2008). There are only a few in vivo studies of STDP. In cat visual cortex the presentation of brief flashing stimuli with an interval of 10 ms on two different retinotopic regions induces rapid changes in cortical representation of visual space, which was also observed in similar experiments in the rat visual cortex (Yao, Dan, 2001; Fu et al., 2002; Meliza, Dan, 2006). In a similar way, in the ferret auditory cortex, presentation of asynchronous auditory stimuli (8 to 10 ms delay) induced shifts in neuronal frequency selectivity (Dahmen et al., 2008). This experiment suggests that STDP drives perceptual learning in the visual and auditory cortex. STDP was also shown to drive LTD in the rat barrel cortex on L2/3 cells by whisker stimulation coupled to but lagging spontaneous axon potentials (Jacob et al., 2007). L2/3 barrel cortex neuron
STD-LTP can be induced by simultaneous whisker stimulation and action potential injection (Gambino, Holtmaat, 2012).

1.2.5.4 Homeostatic plasticity

Homeostatic plasticity, formed by the Greek words homeo (similar), stasis (standing still) and plastos (molded), can be interpreted as 'remaining the same through changes'. This form of non-Hebbian plasticity was first observed in cortical cultures, where an induced increase in network activity over relatively long time (hours to days) elicit a multiplicative decrease in the strength of excitatory synapses and an induced decrease in network activity elicit an increase in the strength (Turrigiano et al., 1998). In the neocortex a mechanism regulating AMPA receptor expression at the membrane, similar to what happens during LTP and LTD, mainly expresses homeostatic synaptic scaling (Turrigiano, Nelson, 2004). As already mentioned above, homeostatic plasticity occurs in vivo in response to overuse or massive deprivation. In the monocular region of the visual cortex an increase in network excitability and spontaneous firing, and an increase in responses to the closed eye is observed upon monocular deprivation (Maffei et al., 2004). Different paradigms of deprivation such as eyelid suture and TTX injection, induce homeostatic plasticity by different mechanisms including upscaling of excitatory synapses, changes in inhibitory circuits and intrinsic excitability (Maffei, Turrigiano, 2008). Whereas in auditory cortex homeostatic plasticity is observed upon peripheral hearing loss (Kotak et al., 2005) in somatosensory
cortex lacks proof of existence.

1.2.5.5 Metaplasticity

Metaplasticity is a form of plasticity acting on the rules of plasticity themselves. The ability of a neuron to undergo LTP and LTD driven by a certain stimuli can be altered by changes in inhibitory tone, activity of modulatory afferents, dendritic excitability or NMDA receptor function. In somatosensory cortex a new form of LTP, driven by metabotropic GluR1 receptors, appears only after NMDA dependent LTP on L4 to L2/3 synapses induced by single whisker experience is initiated in order to maintain synapse strengthening (Clem et al., 2008). In visual cortex the expression of different NMDA receptor subunits is used to alter the capacity for LTP and LTD on L4 to L2/3 synapses upon dark rearing (Philpot et al., 2003).

1.2.5.6 Molecular components of synaptic plasticity

Synaptic potentiation and depression ultimately leads to molecular alterations at the synapse that may support long-term structural changes in the connectivity between neurons. Structural changes vary from simple synapse size changes and dendritic spine size dynamics to the complete removal or addition of new synaptic connections (see 1.4).

The post-synaptic molecular cascade that is associated with structural changes is vast. It is beyond the scope of this thesis to review in depth the
molecular mechanisms underlying synaptic plasticity and stability. There are many excellent reviews that discuss the interplay between the various post and presynaptic components and synaptic plasticity (e.g. Sheng, Hoogenraad (2007); Cingolani, Goda (2008); Caroni et al. (2012)). I will briefly introduce a few of the most prominent members of the postsynaptic signaling cascade that have been associated with plasticity.

The structure of a spine/synapse is largely maintained by dynamics of Actin, which constitutes a major component of the spine cytoskeleton. Many synapse signaling pathways interact with the actin polymerization process to control spine size and stability (Cingolani, Goda, 2008). LTP/LTD-like processes activate or inactivate various GTPases, Guanine nucleotide exchange factors (GEF) and kinases, which in turn modulate the restructuring of the cytoskeleton (Tolias et al., 2011). These mechanisms may also control local protein synthesis, and thereby influence the supply of synapse building blocks. Synaptic adhesion is also regulated directly or indirectly through LTP/LTD-associated signaling. For example, N-cadherin mediates the stabilization of potentiated synapses (Cingolani, Goda, 2008; Mendez et al., 2010). Synaptic adhesion proteins have also been shown to regulate LTP. For example, Neuroligin-1 has been shown to regulate the sequestration of NMDARs to postsynaptic sites (Budreck et al., 2013). LTP also critically depends on CaMKII, a calmodulin-dependent kinase that is activated by Ca^{2+}-entry through NMDARs, which subsequently drives AMPARs to the synapse (Hayashi et al., 2000; Coultrap, Bayer, 2012). Postsynaptic scaffold proteins such as PSD-95, Gephyrin and Shank provide the substrate on which postsynaptic signaling components dock and interact, but they may also have more
active and direct roles in regulating plasticity (Sheng, Hoogenraad, 2007). In paragraph 1.4.1 I will discuss studies that have used synaptic signaling components to image synaptic dynamics and function.

1.3 **In vivo imaging**

1.3.1 **Techniques**

The development of 2-photon laser scanning microscopy (2PLSM) (Denk et al., 1990) in conjunction with the creation of transgenic mice lines expressing fluorescent proteins in neuronal subsets (Feng et al., 2000; De Paola et al., 2003), proved to be a pivotal for the studies of structural and functional plasticity. The low wavelength excitation (near infrared) and the low diffraction typical of 2PLSM allow in-vivo imaging at depths up to 500 \( \mu \text{m} \) in the brain with reduced photo-toxicity (Zipfel et al., 2003; Helmchen, Denk, 2005; Svoboda, Yasuda, 2006; Kerr, Denk, 2008). Two different techniques are used to perform optical imaging on the superficial layers of the cortex: skull thinning (Grutzendler et al., 2002; Yang et al., 2010) or cranial window (Trachtenberg et al., 2002; Knott et al., 2009). Each one has different advantages over the other technique. Using a cranial window one can perform repeated long-term imaging of a broader imaging surface. It also provides, during the implantation period, access to brain for labeling methods such as virus injection or single cell electroporation. The blood vessel pattern under the window can be used as landmarks to retrieve the location of previously imaged regions. 2PLSM was not only used to study cortical dynamics
but also to observe structural dynamics in the olfactory bulb (Mizrahi, Katz, 2003) and the cerebellum (Nishiyama et al., 2007). Lately new techniques have been developed in order to overcome the limitations of 2PLSM. Fiber optics and miniaturized portable microscopes allow imaging in behaving animals (Helmchen et al., 2001; Wilt et al., 2009). Super-resolution techniques, such as STED, can be used to study dynamics of microstructures (Berning et al., 2012; Bethge et al., 2013). Finally endoscopes (Wilt et al., 2009), regenerative amplification of laser pulses (Mittmann et al., 2011) and 3-photon excitation (Horton et al., 2013) allow imaging of deeper brain regions (Barretto, Schnitzer, 2012).

1.3.2 Expression of fluorescent proteins

Perfecting and developing new imaging techniques goes along with the development of new methods to express fluorescent proteins in vivo. GFP, or spectral variants, expressed in transgenic mice under the control of Thy-1 promoter was the first fluorescent protein used in combination with 2PLSM (Feng et al., 2000; De Paola et al., 2003) (Fig. 1.1 c). Some of the most notorious lines are GFP-M, YFP-H, YFP-S and YFP-G expressing cytosolic fluorescent protein (Feng et al., 2000) and mGFP-L15 and mGFP-L21 expressing membrane-bound GFP (De Paola et al., 2003). Those transgenic mice show high GFP levels expressed in a relatively small subset of neurons, resulting in a sparse labelling with low background fluorescence. This allows imaging with a resolution close to the point spread function of the microscope and, due to the relatively large 2-photon cross section of GFP
and its resistance to bleaching, slow and repeated scanning over short times reducing potential photo-toxicity effects (Tsien, 1998). Some of the above transgenic lines display a too dense expression patterns for imaging of single cell structures. Others lines have the opposite problem: a very sparse expression pattern makes it difficult to predict a fluorescent cell’s location. The potential labeling of different cell subtypes is another issue to take in account which could lead to a high variability in some parameters such as, for example, spine densities or spine volumes. Finally, they do not allow the imaging of sub-cellular compartments.

To increase the spatial specificity, the expression of fluorescent proteins can be achieved using recombinant viral vectors (Fig. 1.1b). Lenti-viral vectors (LV) and adeno-associated viral vectors (AAV) are among the currently most used vectors. Despite its limited packaging size, which is anyway sufficient to carry the coding sequence of most fluorescent proteins, AAV became the preferred vector for studies requiring a long term expression for longitudinal imaging in vivo (Stettler et al., 2006; Mizrahi, 2007; Nishiyama et al., 2007). The protein expression takes two or three weeks to reach imaging levels but after this period is stable for months (Tenenbaum et al., 2004). Another way to increase spatial specificity is to use in utero electroporation (Fig. 1.1a). By varying the embryo age at which the actual electroporation is performed, from E12 to E18, different layers of the cortex can be specifically targeted. The plasmid(s) encoding the recombinant protein(s) are injected in the embryo’s ventricle. Electrical pulses are applied to force the cells to take up the plasmids. The cells then migrate into the cortex where they differentiate and integrate into their cortical layer (Saito, Nakatsuji, 2001; Saito, 2006). An
even more precise targeting can be achieved by single cell electroporation. A patch glass pipette is inserted through a craniotomy to specifically target a cell in a relatively small area such as a single barrel column (Kitamura et al., 2008; Judkewitz et al., 2009). The power of the techniques described above can be drastically increased when used in combination with knock-in mice. For example the Allen Institute of Brain conceived several knock-in mouse lines expressing fluorescent proteins at levels suitable for \textit{in vivo} imaging. In these mice a Cre-dependent activation STOP-cassette between two loxP sites followed by the recombinant fluorescent protein is inserted in the ROSA locus (ROSA-LSL) (Madisen et al., 2012). These lines can be crossbred with Cre expressing mice lines in specific cells subpopulation providing. An example is the crossing of ROSA-LSL-TdT mice with mice expressing Cre recombinase in parvalbumin-positive interneurons (Fig. 1.1 d). The ROSA-LSL and the crossbred mice can be used in combination with viral injection (Fig. 1.1 e), \textit{in utero} electroporation or single cell electroporation to increase spatial selectivity (intended as region of the brain), cell type specificity, the variety of fluorescent recombinant proteins and temporal control (i.e. express proteins after a critical period).

\section*{1.4 Structural plasticity}

The techniques mentioned above allow longitudinal imaging of spines \textit{in vivo}. In this context the spine is considered to be a proxy for a synapse even if the presence of a synapse cannot be directly confirmed. Electron microscopy
Figure 1.1: a. *In utero* electroporation. b. Viral vectors. c. Simple transgenic animal. d. Cre-loxP system. e. Combination of Cre mice and viral vectors. (Knöpfel, 2012)
studies have shown that all spines aged four or more days do have a synapse but only 30% of spines younger than four days do have a synapse (Knott et al., 2006). *In vivo* studies have shown that, except for small changes in tip length, the dendrite is mostly stable (Mizrahi, Katz, 2003). Under constant sensory experience in the barrel cortex of young mice the majority of spines of L5 neurons are stable but some appear and disappear over time (Fig. 1.2). Moreover, the fraction of stable spines increases with the age of mice, suggesting that synaptic circuits continue to stabilize during adulthood (Holtmaat et al., 2005). Spines showed a higher turnover in young mice (2 month old) (Trachtenberg et al., 2002) and similar results were found in the visual cortex where a higher persistent fraction in older mice (more than 4 month old) was shown (Grutzendler et al., 2002). Spine shapes also appear to be an important indicator of plasticity processes. Some studies consider all dendritic protrusions to be spines that can potentially form synapses (Trachtenberg et al., 2002; Holtmaat et al., 2005; Tropea et al., 2010), whereas other studies tend to classify spines based on their form and particularly tend to differentiate between spines, protrusions with a bulbous head, and filopodia, long and thin protrusions without a defined head (Zuo et al., 2005a; Majewska et al., 2006). They suggest that filopodia act as spine precursors. Their fraction in the total pool of protrusions gradually decreases from more than 50% in two week old mice to less than 10% in 1 month old mice, and less than 3% in adult mice (Zuo et al., 2005b).

Experience dependent plasticity in the barrel cortex induced by chessboard
whisker trimming increases the stabilization of newly formed spines (Holtmaat et al., 2006) on L5 neurons. This happens preferentially at cells located in barrel septa, which are the locations between spared and deprived barrels (Wilbrecht et al., 2010). This increased formation of new stable spine is impaired in mice that carry a mutation in the CaMKIIα gene (Wilbrecht et al., 2010). In these mice the CaMKIIα does not autophosphorylate, which was shown to abolish response potentiation (Glazewski et al., 2000). Enriched environment has similar effects on spine formation as trimming a subset of the whiskers. It increases spine formation and elimination in the barrel cortex. The majority of the stable spines that were present before enrichment of the environment survive the experience dependent pruning and a fraction of the newly formed spines during enriched environment survive during
adulthood (Yang et al., 2009). Similarly, in the visual cortex monocular deprivation induces new spine stabilization on L5 pyramidal cells located in the binocular zone and not on L2/3 neurons. Interestingly, spines formed during this period persisted during the reestablishment of normal vision. A second monocular deprivation did not increase the number of stabilized spines (Hofer et al., 2009), but increased the volume of new spines that were formed during the first deprivation period. Mice undergoing dark rearing show higher spine motility, which is measured as the index of changes in length over seconds to minutes. This motility is restored to normal levels after seven days of light exposure. Dark rearing also increased the number of thin spines and filopodia. This phenotype was also restored upon light exposure (Tropea et al., 2010).

Learning related tasks also have an effect on spine structural plasticity in the cortex. In the motor cortex a forelimb-reaching task rapidly induces new spine formation (one hour) (Xu et al., 2009). A rotarod running task induces new spine formation after two days (Yang et al., 2009). In both cases the new spine formation process is later followed by spine elimination leading to spine reorganization. Interestingly a fraction of the newly formed spines during task learning tend to appear in clusters (Fu et al., 2012). On L5 pyramidal neurons of the mouse frontal associative cortex fear conditioning induced by and auditory cue paired with a foot shock increases the spine elimination. The effect is reversed by fear extinction, which increases spine formation. Subsequent conditioning preferentially induces elimination of the spines that were formed after fear extinction (Lai et al., 2012). In the HVC forebrain nucleus of zebra finches a high spine turnover is highly correlated
with the ability to learn a song from a tutor. Hearing the song from a tutor induces a rapid stabilization, accumulation and enlargement of dendritic spines (Roberts et al., 2010).

In order to correlate functional and structural changes different approaches can be followed.

1.4.1 Imaging of synaptic proteins

Spine and synaptic structures contain a high density of proteins. Many of them are associated with synaptic plasticity. In vivo imaging of these proteins will deepen our understanding of plasticity. The majority of studies on synaptic protein imaging have been conducted in vitro but the adoption of the latest imaging techniques will allow to partially or fully translate them in vivo.

Hereafter I will describe relevant studies that have imaged synaptic proteins in vitro or in vivo.

1.4.1.1 AMPA and NMDA receptors

As discussed above, AMPA and NMDA receptors are tightly related with plasticity phenomena. Therefore, tracking their expression and translocation in spines is a potential method to visualize synaptic plasticity. In hippocampal neuronal cultures, FRAP experiment have shown that a chemical LTP protocol results in an increased mobility of GluR1 AMPA receptor subunit with slower kinetics (Sharma et al., 2006). AMPA and NMDA receptor are found in two different states, internalized and expressed at the membrane. Surface receptors are recruited from the pool of internal receptors and are
the most relevant for plasticity. Unfortunately the use of GFP-tagged AMPA or NMDA receptor subunits does not allow to clearly discriminate between internalized and externalized receptors (Kolec et al., 2006). To solve this problem a pH sensitive version of GFP, called super ecliptic pHluorin (SEP), has been developed. SEP is fluorescent when exposed to neutral pH like the one found in extracellular environment and quenched when exposed to an acidic environment like the one found in the intracellular compartment (Miesenböck et al., 1998) (Fig. 1.3). In hippocampal slices induction of chemical LTP increases GluR2 and even more GluR1 expression at the spine membrane. About six minutes later, this is followed by an enlargement of the spine. Furthermore, NR2B NMDA subunit levels remain relatively stable after chemical LTP, whereas NR2A slightly decreases (Kolec et al., 2006). Recruitment of SEP-GluR1 at the membrane as induced by the over expression of PSD-95 is not sufficient to induce spine enlargement (Kolec et al., 2007). These results suggest that spine enlargement and AMPA receptor recruitment at the membrane as induced by LTP are two separate mechanisms. The source of GluR1 at the synapse is provided by the dendrite. Thus, GluR1 is externalized in the dendrite and then laterally diffuses into the spine (Holmaat, Svoboda, 2009). The diffusion of AMPA receptors after LTP is similar to the processes described under baseline conditions, which is regulated by spine shape (Ashby et al., 2006). These in vitro examples demonstrate that the imaging of AMPA receptors in vivo is potentially a great tool to measure experience or learning-dependent potentiation of single spines.
1.4.1.2 Post synaptic density protein 95

PSD-95 is a major component of the post synaptic density. It is a member of the PDZ domain family (Kim, Sheng, 2004), which bind to a variety of synaptic partners including NMDA receptors (O’Brien et al., 1998) and indirectly to AMPA receptors (Ehrlich, Malinow, 2004). PSD-95 is therefore related to synapse strength. In the majority of the imaging studies, PSD-95 has been used as a synaptic marker to confirm the location of postsynaptic densities (Rose et al., 2009). Imaging studies in hippocampal slices using a photoactivatable GFP tagged PSD-95 show that PSD-95 is involved in the morphological plasticity of spines (Steiner et al., 2008).

Studies on PSD-95 mutants have provided insights into the role of its different domains in establishing stable post-synaptic structures (Sturgill et al., 2009). Experiments on organotypic hippocampal slices support a model in which the loss of PSD-95-GFP does not necessarily precedes spine loss (Woods et al.,...
Figure 1.4: Measuring the relationship between PSD-95 retention and PSD size. Two spines were photoactivated (square and circle). The fluorescence intensity within those spines was quantified after 60 min. At the end of the experiment the spines were reactivated to estimate the sizes of their PSDs (not shown). (Gray et al., 2006)
In vivo studies in the barrel cortex using photoactivatable GFP and GFP-tagged PSD-95 shows that the PSD-95 stability in spines is activity and experience-dependent. The retention time of PSD-95-paGFP in spines is higher for bigger spines (Fig. 1.4). It decreases with age and after sensory deprivation (bilateral whisker trimming) (Gray et al., 2006).

1.4.1.3 Calmodulin dependent protein kinase II

Glutamate uncaging (Bloodgood, Sabatini, 2005) is a technique based on the photo-stimulation of MNI-glutamate rendering it biochemically active. When glutamate is uncaged in the vicinity of spines it induces spine growth (Matsuzaki et al., 2004; Zhang et al., 2008). Studies in hippocampal neuron cultures and hippocampal slices show a correlation between spine growth and the recruitment of CaMKII at spines (Lemieux et al., 2012). The synaptic recruitment of CaMKII at spines is dependent on the NMDA glutamate receptors subunit GluN2B (She et al., 2012). The localization of CaMKII recruitment is not restricted to the activated spine, but propagates to neighboring spines as well (Rose et al., 2009), and even to some dendritic locations where their presence co-localizes spatially and temporally with Ca$^{2+}$ transients (Lemieux et al., 2012). CaMKII-based FRET ( Förster resonance energy transfer) sensors, which do not fluoresce under normal conditions but become green fluorescent when the protein is phosphorylated on a specific site, have shown that the spine specific activation of CaMKII (Fig. 1.5) is dependent on NMDA receptors and voltage sensitive calcium channels (Lee et al., 2009). Similar experiments based on FRET were performed with RhoA and Cdc42, two Rho GTPases acting downstream of CaMKII that
modulate the organization of the cytoskeleton. This demonstrated a correlation between the binding of these two proteins to their substrate (Rho GTPase binding domain (RBD) of Rhotekin/Pak3) and spine enlargement as induced by glutamate uncaging (Murakoshi et al., 2011). These examples show that CaMKII fluorescent probes can be used to track synaptic activity.

1.4.1.4 Gephyrin

Gephyrin is a scaffolding protein which is expressed only in inhibitory postsynaptic locations (Kneussel et al., 2001). It can therefore be used as a marker for inhibitory synapses. Recent in vivo studies on the mouse visual cortex revealed that gephyrin puncta are lost after monocular deprivation. On the other hand, their clustering is increased by experience (Chen et al., 2012a; Versendaal van et al., 2012). This is another example of how synaptic proteins can be a useful tool to further investigate synaptic plasticity.
Another way to link structural to functional plasticity is by the imaging of neuronal activity. Increased activity is accompanied by increases in intracellular Ca\(^{2+}\) concentration. Two main families of genetically encoded calcium indicators (GECIs) have been developed. One is based on a FRET sensor, in which the CaM-M13 domain is linked to two fluorescent proteins with different excitation and emission spectra. When Ca\(^{2+}\) binds to the complex the two fluorescent proteins come spatially closer which increases the efficacy of FRET between them (Miyawaki et al., 1997). The ratio between the fluorescence of the two protein can therefore be used as measure of the Ca\(^{2+}\) concentration. The other is based on a single fluorescent protein attached to the CaM-M13 complex. The binding of Ca\(^{2+}\) to the protein induces an increase of the fluorescent protein quantum yield (Nakai et al., 2001). One of the most widely used types is GCaMP3 (Tian et al., 2009). The majority of the in vivo studies focus on neuronal populations. For example, the activation pattern of a subpopulation of neurons in the barrel cortex relates to learning tasks (Huber et al., 2012). In vivo imaging of dendrites and single spine's calcium activity has been shown to be possible in vivo with OGB, a calcium sensitive dye. This technique lack the possibility to do chronic imaging (Chen et al., 2011b, 2012c). By using this approach in vivo studies on the auditory cortex shows that spines responding to the same tune are highly interspersed on the same dendrite (Chen et al., 2011b). Lately in vivo imaging of Ca\(^{2+}\) on awake mice expressing GCaMP3 under the control of Thy-1 promoter has been shown to be possible (Chen et al., 2012b).
The other possibility to link structural to functional plasticity is to detect changes in voltage. Genetically encoded voltage indicators (GEVIs) can be separated in three families. Similarly to GECIs, FRET based and quantum yield changes based GEVIs do exist (Knöpfel, 2012). A third family is composed of opsin based sensors where a change in fluorescence is associated with voltage-dependent protonation (Kralj et al., 2012). GEVIs have been successfully used in vivo at the population level (Akmann et al., 2010). The measurement of voltage changes in spines with ANNINE-6plus, a voltage sensitive dye, as been done in vitro (Pages et al., 2011). The future combination of GECIs or GEVIs with in vivo long term imaging of single spine could provide a fundamental tool to link spine functional and structural plasticity.

1.5 Objectives

Plasticity can be measured by changes in functionality and changes in connectivity, i.e. functional plasticity and structural plasticity respectively. The relationship between structural changes and functional changes (and vice versa) remains poorly understood. For example, when a neuron grows a new spine with a synapse, how does this affect its function? Neither is it known whether new synapses are formed within circuits that were not yet synaptically connected, or whether synapses are preferentially formed within pre-established synaptic circuits. The latter would simply involve a gain in synaptic input; the first option would result in a different configuration of synaptic activity patterns. It is also not clear what types of activity drive new synapse formation or synapse pruning.
To better understand the relationship between synapse formation, stability and long term functional plasticity, morphology and function need to be monitored in parallel. Synapses need to be monitored in real time in vivo, in relation to functional plasticity. The morphological appearance of a spine mostly signals the formation of a synapse. However, very young spines often do not carry a synapse. In addition, synaptogenesis on neurons without spines cannot be assessed by imaging of morphology alone.

In this thesis I aimed at direct probing of synaptogenesis in vivo. I used two techniques to express synaptic proteins that are tagged with a fluorescent probe in the adult neocortex. In utero electroporation allowed us to transfect large populations of L2/3 pyramidal cells with PSD-95-GFP. The drawback of this method is that neurons are faced with over-expression of physiologically important proteins during development. To overcome this potential issue we adapted a single cell electroporation method to express synaptic proteins in the adult somatosensory cortex, and to track single synapses over long times through a cranial window in vivo.

To probe synaptogenesis and synaptic plasticity in vivo, we used PSD-95-GFP and GFP-Gephyrin. In addition we tested the suitability of GFP-CamKIIα and SEP-GluR1 constructs for single cell electroporation in vivo.
Chapter 2

The relationship between PSD-95 clustering and spine stability in vivo

2.1 Introduction

The formation of new synapses at the expense of old synapses may underlie the experience-dependent and functional reorganization of cortical circuits in adults (Holtmaat, Svoboda, 2009; Fu, Zuo, 2011). Imaging studies in vivo have shown that proxies for synapses, such as dendritic spines and axonal boutons, appear and disappear over days to weeks, even in naïve mice. Under baseline conditions new dendritic spines rarely transform into persistent spines and usually retract within hours to days after they are formed (Holtmaat et al., 2005; Zuo et al., 2005a). These transient spines were hy-
pothesized to generate shortlived synaptic contacts. Indeed, in vitro studies have shown that new spines can display transmitter-evoked Ca$^{2+}$ transients within hours (Zito et al., 2009), and often rapidly express morphologically mature synapses (Nägerl et al., 2007; Zito et al., 2009). However, retrospective electron microscopy (EM) on dendrites that were imaged in vivo has shown that a large fraction of newly formed spines does not bear a morphologically defined synapse within the first day (Knott et al., 2006). Yet, new spines always contain a synapse after four days, and the size of the synapse positively correlates with the new spine’s lifetime. This suggests that synapse growth in vivo is slow and that spine stabilization and synapse growth are closely linked. It also implies that transient spines are perhaps new spines that fail to form a synapse. However, since the findings were based on endpoint measurements it remains uncertain whether synapse formation in vivo is a unique signature of new persistent spines. Neither do we know whether new spine survival in vivo is increased by the formation of a synapse, or whether the disappearance of spines in vivo results from synapse instability.

To start and answer these questions we expressed PSD-95-eGFP in L2/3 pyramidal cells using in utero electroporation, and imaged dendritic spines in the adult brain in vivo. PSD-95 is a postsynaptic scaffold protein that is present in the majority of excitatory synapses (Sheng, Hoogenraad, 2007). In single postsynaptic densities large numbers of PSD-95 molecules are linked to one another and to various other synaptic proteins (Craven et al., 1999; Chen et al., 2005). PSD-95 modulates postsynaptic function and maturation (El-Husseini et al., 2000; Ehrlich, Malinow, 2004). Auxiliary expression of labeled PSD-95 allows the visualization of postsynaptic densities in real time.
in vitro (Okabe et al., 1999; Sala et al., 2003; Sharma et al., 2006) and in vivo (Niell et al., 2004; Gray et al., 2006; Kelsch et al., 2008; Livneh et al., 2009), without disturbing gross synaptic network dynamics (Gray et al., 2006). Such experiments have indicated that the appearance and disappearance of fluorescently labeled PSD-95 puncta is likely to represent synapse formation and synaptic pruning respectively (Prange, Murphy, 2001; De Roo et al., 2008; Chen et al., 2011a).

Our data confirm that PSDs are dynamic in vivo, and imply that spine maintenance critically depends on PSD size. The accumulation of PSD-95 is a prerequisite for (new) spine survival. However, the presence of a PSD is not an exclusive attribute of persistent spines; a small fraction of transient spines may serve to produce short-lived synaptic contacts.

2.2 Methods

2.2.1 DNA constructs

The pCAG-PSD-95-eGFP-WPRE and pCAG-DsRedExpress-WPRE plasmids were obtained from K. Svoboda, Janelia Research Farms, USA (Gray et al., 2006). In the PSD-95-eGFP plasmid, the eGFP open reading frame was placed in frame at the 3’ translated region of PSD-95, which codes for the c-terminal part of the PSD-95 protein (Okabe et al., 1999). In both plasmids expression was driven by the CMV-enhanced chicken β-actin promoter (CAG). The 3’ untranslated regions contained the woodchuck hepatitis virus post-translational regulatory element (WPRE) and the bovine growth
hormone polyadenylation site.

### 2.2.2 **In utero electroporation**

This study was performed according to the guidelines of the Swiss Federal Act on Animal Protection and Swiss Animal Protection Ordinance. All experiments were approved by the ethics committee of the University of Geneva and the Cantonal Veterinary Office (Geneva, Switzerland).

In *utero* electroporations were performed as described (Saito, Nakatsuji, 2001; Tabata, Nakajima, 2001) (Fig. 2.1 A). In short, E16 timedpregnant C57BL/6J mice were anesthetized using an isoflurane-oxygen mixture (2% vol isoflurane/vol O\(_2\)). The uterine horns were exposed through a ∼1 cm incision on the abdominal wall. Approximately 1 µl of buffer solution containing ∼2 µg/µl of pCAG-PSD-95-eGFP-WPRE plasmid, a molar equivalent of pCAG-DsRedExpress-WPRE plasmid, and a trace of Fast Green (Sigma) was injected using a custom made intracellular microinjection dispense system that was controlled by a pulse stimulator (master-8, A.M.P.I.). The fluid was injected into the right lateral ventricle of each embryo through a pulled glass pipette. Subsequently, the head of each embryo was placed between custom-made tweezer electrodes, with the positive electrode contacting the right side of the head. Electroporation into the right cerebral cortex was achieved using five square pulses (duration = 50 ms, frequency = 1 Hz, 40V).
Figure 2.1: A, a mix of CAG-PSD-95-eGFP-WPRE and CAG-DsRedExpress-WPRE plasmids was injected in the lateral cerebral ventricle of E16 embryos in utero. Neurons were electroporated using electrode tweezers. B, coronal section showing a large group of labeled L2/3 pyramidal cells in the barrel cortex, located under the craniotomy. C, In vivo 2PLSM image of the transfected area (scale bar, 50 µm), showing a partial colocalization of the two fluorescent proteins. D, timeline of the imaging sessions. Boxes indicate days, ticks indicate multiple imaging sessions per day.
2.2.3 Cranial window

At postnatal day 42 mice were anesthetized with a ketamine (0.1 mg/g body weight) and xylazine (0.01 mg/g body weight) mixture. The cranial windows were implanted as described previously (Holtmaat et al., 2009) (Fig. 2.1 B). Prior to the surgery dexamethasone (∼2 µg/g body weight) was injected i.m. in order to reduce cerebral edema during the craniotomy. The craniotomy was located above the right somatosensory cortex (∼1.5 mm caudal and ∼3 mm lateral of bregma). The craniotomy was covered with a 5 mm dia meter cover glass, which was permanently glued to the skull using dental acrylic cement. The dura remained intact. After surgery the animals received buprenorphine (0.1 µg/g body weight; s.c.) and carprofen (5 µg/g body weight; i.p.) to reduce pain and inflammation. Imaging was started 2 weeks after cranial window implantation.

2.2.4 Imaging

In vivo images were acquired under isoflurane anesthesia (1% vol isoflurane/vol O₂) using a custom built twophoton laser-scanning microscope (2PLSM) (Holtmaat et al., 2009) controlled by custom software written in MATLAB (Scanimage, Janelia Research Farm) (Pologruto et al., 2003). As a light source we used a tunable Ti:sapphire laser (Chameleon ultra II, Coherent) running at 940 nm for simultaneous excitation of eGFP and DsRedExpress (typically 80-120 mW at the back focal plane of the objective). The microscope was equipped with a 40×, 0.8 NA water immersion objec-
tive (Olympus) and high quantum efficiency photomultiplier tubes (R3896, Hamamatsu). Emitted light was spectrally separated using a 565 nm dichroic mirror (Chroma) and two bandpass filters (510/50 nm and 620/60 nm, Chroma). Typically, the mice co-expressed PSD-95-eGFP and DsRedExpress in a large subset of L2/3 pyramidal cells under the cranial window (Fig. 2.1 B, C). Dendrites were imaged for 2 weeks over various time intervals (6 h, 18 h, and 96 h) (Fig. 2.1 D). Images were acquired at 2 ms/line (image size, 512 × 512 pixels; pixel size, 0.06 × 0.06 µm).

2.2.5 Image analysis

2.2.5.1 Scoring of spines and PSD-95-eGFP puncta

A custom written MATLAB program (Holtmaat et al., 2009) was used to analyze dendritic spines and PSD-95 puncta dynamics in three dimensions over the 13 imaging sessions. We collected data from 7 mice, including 1583 spines or PSD-95 puncta (a total number of ∼10000 spines or puncta were detected over the whole time course of the experiment, including gained and lost elements). Only spines that were emanating laterally from dendritic shaft were included. Protrusions or PSD-95 puncta were manually scored using strict criteria. In order to determine if a spine contained a PSD-95-eGFP punctum, a region smaller than the spine head was selected in the green channel (Fig. 2.2 B, gray arrowhead). Several (3 to 4) regions of the same size were selected in the dendritic shaft just below the spine (Fig. 2.2 B, black arrowhead), carefully avoiding unambiguous PSD-95-eGFP puncta (Fig. 2.2 B, open arrowhead), and averaged. Presumptive PSD-95 puncta within the
shaft were analyzed similarly. We considered PSD-95 concentrations to be clustered if the intensity of a presumptive punctum was 10% brighter than the average in the dendritic shaft, and $5 \times$ higher than the standard deviation of this average. This analysis revealed three different groups: spines with a PSD-95-eGFP punctum, spines without a PSD-95-eGFP punctum, and PSD-95-eGFP puncta without a discernible spine.

2.2.5.2 Quantification of fluorescence intensities.

Using 2PLSM and retrospective EM it has been shown that spine volumes are monotonically related to spine brightness (Holtmaat et al., 2005). Therefore, we used relative DsRedExpress and eGFP brightness in single two-photon optical sections to estimate relative spine volumes and PSD sizes respectively (Fig. 2.4 B). Spine fluorescence was calculated by integrating the pixel values of a selected area around the spine head of the brightest section (circle in Fig. 2.4 B). An average background pixel value was calculated from an area on the same section next to the spine (box in Fig. 2.4 B). The background pixel value was subtracted from each pixel of the spine area. Since dendritic shaft diameters were constant and relatively uniform, we used them to correct the spine fluorescence levels for possible variations in excitation and detection efficiencies. For each spine we selected a region (1-3 $\mu$m long) in the dendritic shaft. The average of the background-subtracted brightest pixels along this specified region was calculated using custom software in MATLAB (kindly provided by Sen Song) (Grillo et al., 2013), and used as a normalization factor. The spine and PSD-95-eGFP values were expressed as the background-subtracted spine and PSD-95-eGFP intensity divided by the
normalization factor. Although this analysis method proved to be generally sound for spines on big dendrites (Holtmaat et al., 2005), regions of interest in which the dendrite was very small could occasionally cause extreme spine brightness values. In general we assumed that the dendritic volume exceeds the point-spread function of the microscope, and that the maximum pixel values in the dendrite are linearly related to fluorescent protein expression levels. However, in some cases dendritic volumes were smaller than the point-spread function and presumably smaller than large spine heads. In these cases the maximum pixel values in the dendrite did not accurately relate to expression levels, which resulted in an overestimation of the relative spine volume. Similar errors occurred in the green channel. To minimize the influence of these outliers in the regression analysis we applied a robust least square regression analysis (Matlab).

2.2.5.3 Spine and PSD size changes over time

Relative spine and PSD size changes were expressed as the common logarithm ratio of the relative spine brightness on time point 2 over time point 1. Persistent spines were often sampled several times. To estimate the sampling error and the error due to movement noise we imaged 10 spines on various dendrites, 10 times (with 1 minute intervals), assuming that spine volume fluctuations are minimal over this time span. The log_{10} ratio between the highest and lowest value that was measured over this 10 minute period was 0.1.
2.2.6 Statistics

For structures identity analysis we pooled the spines of all animals and performed binary statistics (chi-square test). For regression analysis of new spines volumes we used a robust fitting method (Matlab). Comparison of fluorescence distributions was performed using a Kolmogorov-Smirnov test. Significance of volume changes was calculated using t-test.

2.3 Results

2.3.1 PSD-95-eGFP expression allows identification of PSD formation and pruning

Adult mice that were transfected at E16 expressed PSD-95-eGFP and DsRedExpress in L2/3 cortical pyramidal cells (Fig. 2.1 B, C). We observed co-expression in a large subset of neurons. Neurons that were sufficiently bright in both channels were selected for long-term imaging (2 weeks) of high-magnification regions of interest. (Fig. 2.1 D). The linear spine densities ($0.34 \pm 0.02$, $n = 39$ dendrites), as well as spine turnover (4d TOR: $0.2215$) were similar to previous observations (Holtmaat et al., 2005; Gray et al., 2006). We observed concentrations of PSD-95-eGFP in spines and dendritic shafts. Such puncta have been shown to represent postsynaptic densities (Okabe et al., 1999, 2001; Gray et al., 2006). In order to reproducibly discriminate between putative synaptic and non-synaptic PSD-95-eGFP concentrations we measured the ratio of eGFP fluorescence intensities per pixel in a presumptive PSD-95-eGFP punctum and the pixel intensity average of a
neighboring region in the dendritic shaft, avoiding clear puncta in the shaft (Fig. 2.2 B). We assumed that the dendritic shaft average represents the level of freely diffusing and unbound PSD-95-eGFP (Gray et al., 2006). We arbitrarily defined a PSD-95-eGFP punctum to represent a PSD if its fluorescence intensity was 10% higher than the average in the dendritic shaft, and 5x higher than the standard deviation of this average. This threshold resulted in three different types of structures (Fig. 2.2 A): protrusions without a PSD-95-eGFP punctum, which we termed ‘spines only’ (SpO; e.g. #1 in Fig. 2.2 A); protrusions with a PSD-95-eGFP punctum, termed ‘spines with PSD’ (SpP; e.g. #2 in Fig. 2.2 A); and puncta in the dendritic shaft, termed ‘PSD only’ (PO; e.g. #3 in Fig. 2.2 A). The latter structures may represent both shaft synapses and synapses on spines that could not be resolved along the optical axis of the microscope. At the start of the experiment 17.4% of all structures were classified as SpO, 62.5% as SpP, and 20.1% as PO (total n = 690 structures).

Time-lapse imaging revealed many protrusions and PSD-95-eGFP puncta that were present over the whole experiment (n = 302). However, as expected and similar to previous in vivo imaging studies, some structures appeared or disappeared during the imaging period. 1-5% of the structures that were present from the start until the end were classified as SpOs (1.7% at 0h, 5.0% at d17, Fig. 2.2 C), whereas 78-82% were SpPs (81.8% at 0h, 78.8% at d17) and 16% were POs (16.6% at 0h, 16.2% at d17). This indicates that persistent spines nearly always contain a PSD, confirming EM observations
Figure 2.2: A, dendritic region imaged at high magnification (scale bar, 5 \( \mu m \)). Three types of structure are visible: #1, spine without a PSD-95-eGFP punctum (SpO); #2, spine with a PSD-95-GFP punctum (SpP); #3, a PSD-95-eGFP punctum in the dendritic shaft (PO). B, procedure to determine the presence of PSD-95-eGFP puncta. PSD-95-eGFP fluorescence in the spine head was determined by integrating pixel values over an area smaller than the spine head (grey arrowhead). This value was divided by the average of the integrated pixel values of several (3-4) equally sized areas in the dendritic shaft, carefully avoiding apparent PSD-95-eGFP puncta (open arrowhead) in the shaft. C, fraction of spines with a PSD-95-eGFP punctum (SpP, black), spines without a PSD-95-eGFP punctum (SpO, gray), and PSD-95-eGFP puncta in the dendritic shaft (PO, light gray). D, fraction of new spines with a PSD-95-eGFP punctum (SpP, black) and new spines without a PSD-95-eGFP punctum (SpO), for spines appearing over 6, 18 and 96 h-intervals (left) and spines disappearing over 6, 18 and 96 h-intervals (right) (***, \( p < 0.005 \)).
(Knott et al., 2006). The majority of structures that were gained and/or lost comprised SpOs at the moment they were observed for the first and last time respectively (gains: 55.0% SpO, 27.2% SpP, 17.8% PO; losses: 50.5% SpO, 30.9% SpP, 18.6% PO). This indicates that a large fraction of spines that were newly formed did not immediately contain a PSD, confirming previous EM observations (Knott et al., 2006). Similarly, spines that were destined to be lost often did not contain a PSD shortly before they were pruned. However, it should be noted that a large fraction of gained and lost spines comprised transient spines that appeared and disappeared within the time frame of our experiment.

Since synapse formation may be considerably delayed as compared to the formation of a dendritic spine (Knott et al., 2006) we calculated the fraction of new and lost spines with a defined PSD, for various intervals (6, 18 and 96 h) over which we first or last observed them. It should be noted that here too both populations contained transient spines. 18.6% of the new spines that appeared over imaging intervals of 6 h contained a PSD. A significantly larger fraction (42.4%, p < 0.005, chi-square) of spines first seen over 96 h intervals contained PSDs. This suggests that although synapse formation may happen within hours after the growth of a protrusion, in some protrusions the accumulation of detectable PSD-95-eGFP puncta may take much longer (Fig. 2.2 D). Similarly, a significantly larger fraction of spines that were last seen before a 96h imaging interval contained a PSD than before a 6h interval (96 h, 48.4%; 6 h, 19.4%, p < 0.005, chi-square). This suggests that spines destined to disappear gradually lose their PSD over the course of days. Nonetheless, some spines contain a PSD shortly (hours) before they
are pruned, in accordance with recent *in vitro* data (Woods et al., 2011).

### 2.3.2 The relationship between PSD-95-eGFP clustering and spine stability

Throughout the imaging period we observed various combinations of spine and PSD stabilities. Most persistent spines continuously bore a PSD, but this did not always prevent them from being pruned (e.g. #1 in Fig. 2.3 A). Many large PSD-95-eGFP puncta in the dendrite were maintained over long time frames, but occasionally they were abruptly lost (e.g. #2 in Fig. 2.3 A) or gradually lost (e.g. #5 in Fig. 2.3 F). Many new spines failed to acquire a clear PSD-95-eGFP punctum on one of our imaging time points and were lost shortly after they were generated (e.g. #3 in Fig. 2.3 A). Some new spines that stabilized incorporated a distinct PSD-95-eGFP punctum (e.g. #4 in Fig. 2.3 A). Occasionally we saw new spines at least twice without a PSD-95-eGFP punctum (e.g. #6 in Fig. 2.3 G). To quantitatively investigate the relationship between PSD-95 clustering and spine stability we analyzed the fractions of new spines that accumulated PSD-95-eGFP and compared this to their survival rate (Fig. 2.3 B). First we measured the fraction of new spines with and without PSDs immediately after they were formed and reassessed this fraction 24 h later. We analyzed a subset of 193 new spines that were detected either on time point $t = 24$ h, $t = 168$ h or $t = 192$ h, and did not exist 24 h prior to these points ($t = 0$ h, 144 h, and 168 h respectively). 70.5% (136 spines) were devoid of a detectable PSD-95-eGFP punctum. Out of this pool of spines, 17.6% (24 spines) were still present after 24 h. In this subset of new spines none had accumulated
PSD-95-eGFP (but see spine #4 in Fig. 2.3 A for an example of a new spine with a delayed acquisition of a PSD). Conversely, 29.5% (57 spines) of the new spines contained a PSD-95-eGFP punctum, and 59.6% (34) of those were detected again 24 h later. All had maintained their synapses. As a result, the fraction of new spines with a PSD had significantly increased over 24 h (59% at t = 24 h, 30% at t = 0 h, p < 0.005, chi-square). To assess the relationship between the presence of PSD-95-eGFP and spine survival over longer time frames, we grouped new spines that were seen in at least two imaging sessions into two categories: those that were seen over less than 96 h, and those with life spans of more than 96 h. Out of the total number of new spines (882) that had appeared during the experiment, only small fraction survived for more than 96 hours (11.5%). 64.5% (69 out of 107) of the spines with life spans of less than 96 h displayed a PSD punctum at least once, whereas of the new spines with life spans of more than 96 h, a significantly larger portion contained PSD puncta (83.2%; 84 out of 101; p < 0.005, chi-square). Together, this indicates that the presence of a PSD enhances the probability of a new spine to stabilize. However, the acquisition of a PSD does not per se predict the stabilization of a spine for more than 4 days.

To investigate the interaction between the presence of PSD-95-eGFP puncta and spine loss we selected a population of SpPs that were present on the first imaging time point (t = 0 h) yet were lost over the course of the experiment (after t = 6 h). The lost spines were grouped into three categories: last seen
Figure 2.3: A, time-lapse image of a dendritic region containing: a SpP disappearing after day 13 (#1); a PO disappearing after the second imaging session on day 0 (#2); a transient SpO appearing and disappearing within 24 h after the first imaging session (#3); a new spine appearing as a SpO on day 6 and turning into a SpP on day 7 (#4). B, the fraction of new spines that survives over 24 h. The fraction of new SpPs that is still present after 24 h is larger than the fraction of SpOs (***, p < 0.005). C, new spines that show a PSD-95-eGFP punctum at least once during their lifetime have a higher probability of surviving for more than 96 h (***, p < 0.005). D, the fractions of lost SpPs that maintain or lose their PSD-95-eGFP punctum before disappearance. E, left, the fraction of new SpOs with lifetimes of more or less than 96 h. Right, the fraction of SpPs that continue as SpOs for more or less than 96 h before disappearing (***, p < 0.005). F, a disappearing spine that loses its PSD-95-eGFP punctum before pruning (#5). G, a new SpO that is present over two time points (#6).
before an interval of 6, 18 and 96 h. Although the majority of spines that disappeared still had a PSD-95-eGFP punctum on the last time point (6 h, 65%; Fig. 2.3 D), a substantial fraction had lost their puncta before their disappearance (6 h, 35%; e.g. #5 in Fig. 2.3 F). The fraction of spines that had lost their PSD-95-eGFP punctum prior to their disappearance tended to gradually decrease with an increase of the interval time, but we did not detect a significant difference (Fig. 2.3 D). This suggests that, in general, spine and synapse disappearance happens concomitantly (over the course of hours). Yet, some spines may persist for some time, even after they have lost their PSD (Fig. 2.3 F). 20% of the disappearing spines survived for more than 96 h after they had lost their PSD-95-eGFP punctum (Fig. 2.3 E). This fraction is significantly higher as compared to new spines that never accumulate a PSD-95-eGFP punctum (5%, p < 0.005 chi-square; Fig. 2.3 E). This suggests that once spines have acquired a PSD their survival rate increases even when they lose their synapse.

2.3.3 Spine volume and PSD-95 size are linearly related

EM studies have indicated that spine and synapse size are positively correlated (Harris, Stevens, 1989; Nusser et al., 1998; Takumi et al., 1999; Holtmaat et al., 2005; Knott et al., 2006). Electrophysiology and imaging studies in vitro have confirmed this finding (Matsuzaki et al., 2001; Zito et al., 2004). However, this relationship has not been confirmed using longitudinal imaging in vivo. In general, we observed that bright spines in the red channel also contained high green fluorescence intensity levels (Fig. 2.3 A and Fig. 2.4 A). Conversely, dim spines in the red channel contained low green fluorescence
intensities. To quantitatively investigate the relationship between spine size and putative PSD size we selected a random population of SpPs (n = 108) that were seen on the first imaging session. It was previously shown that spine brightness, as measured in single 2PLSM optical sections, is monotonically related to spine volume (Holtmaat et al., 2005; Mostany et al., 2013). Here we assumed that the brightness of PSD-95-eGFP reliably represents the PSD size. Since the absolute fluorescence levels differed between animals and different cells we normalized the integrated red and green fluorescence intensity in the spine head to the mean of the maximum pixel values that we encountered in a 1 to 3 µm stretch of parent dendrite just below the spine (Fig. 2.4 B). A regression analysis revealed a linear relationship between red and green intensities in the spine head ($R^2 = 0.79$, $r = 7.12$; Fig. 2.4 C). These data corroborate previous EM studies (e.g. (Harris, Stevens, 1989; Knott et al., 2006)). It also indicates that the auxiliary expression of PSD-95-eGFP does not disturb the relationship between spine and PSD size.

2.3.4 The relationship between spine formation and PSD size

To investigate the interaction between PSD size and new spine formation we measured the levels of PSD-95-eGFP and new spine volumes. We selected a random population of new spines that had appeared at any time point, and analyzed their normalized spine head volume and PSD sizes on the first time point of observation (Fig. 2.4 D, E). The population of new spines was divided into those that were classified as SpP (e.g. #2 and 3 in Fig. 2.4 D; blue dots in Fig. 2.4 E) and SpO (e.g. #1 in Fig. 2.4 D, red dots
in Fig. 2.4 E). As expected, in the SpO subpopulation the PSD-95-eGFP intensity modestly increased with spine volume. In new SpPs the slope of the interaction was much steeper ($r_{\text{spP}} = 2.38$ [range 2.31 - 2.46]; $r_{\text{spO}} = 1.18$ [range 1.14 - 1.22]; robust regression analysis), but still shallower than for randomly selected SpPs. Consequently, the cytosolic volumes and the PSD-95-eGFP levels of new spines that had acquired a PSD were significantly larger than the volumes of new spines without a PSD, and their respective normalized cumulative distribution was significantly different (Fig. 2.4 F; dotted lines vs dotted lines, $p < 0.005$; solid lines vs solid lines, $p < 0.005$; KS-test). These data indicate that on average young new spines contain smaller PSDs relative to their head volume, as compared to persistent spines. This may be indicative of a synaptic maturation process that lags spine enlargement.

### 2.3.5 PSD size changes correlate with spine volume changes

Spine head volumes have been shown to fluctuate *in vivo*, even in persistent spines (Holtmaat et al., 2005, 2006; Hofer et al., 2006; Loewenstein et al., 2011). Although spine volume and synapse size are tightly coupled, it could not be deduced from these experiments whether PSD sizes closely follow spine volume changes. We estimated spine head volume and PSD size alterations of persistent spines by measuring the log$_{10}$ ratio of fluorescence intensities between two consecutive time points (intervals of 6, 18 and 96 h) in both the green and red channel. This analysis confirmed that spine head volumes fluctuate continuously. PSD size changes and spine head volume changes
Figure 2.4: A, examples of SpPs with small (left) and large (right) volumes. B, procedure for measurement of spine and PSD fluorescence intensities. In brightest optical section of each channel, background-corrected pixel intensities are integrated over an area spanning the spine head (circles). Background pixel values are taken from nearby regions (rectangles). In each region of interest spine values are normalized to the average maximum pixel values of neighboring areas in the dendritic shaft (jagged lines). C, linear correlation between green and red fluorescence intensities in a random set of SpPs at $t=0$ h. D, new SpO (#1) and a new SpPs (#2 and 3). E, linear fits of red and green fluorescence for new SpPs (blue), new SpOs (red), and spines from C (black). Inset shows the smallest data points. F, cumulative distribution of red (continuous lines) and green (dotted lines) fluorescence intensities for all spines from E and C. In all panels scale bars, 1 $\mu$m.
could be fit with a linear function \( r = 0.6 \); Fig. 2.5 A), but the correlation between the magnitudes of the changes was poor \( R^2 = 0.26 \). In a small fraction (162 out of 1081, 15.0%) of sampled spines the PSD size and spine head volume dynamics were anisometric, i.e. the spine grew whereas the PSD shrunk or vice versa (quadrant II and IV in Fig. 2.5 A). These types may represent spines that had undergone synaptic weakening or strengthening shortly before they were imaged, since the initial phase (5-10 min) of LTP induction shows relatively poor interactions between spine head size and synapse strength (Matsuzaki et al., 2004; Lee et al., 2009).

To investigate the relationship between spine stability and PSD size and spine head volume changes, we compared the fold changes over two time points for new spines and lost spines, with persistent spines (Fig. 2.5 B). A random set of new SpPs was selected at various time points (from \( t = 6 \) h onwards) and their size was measured on the 1st and 2nd time point on which they were detected. Lost spines were defined as those that were detected with a PSD-95-eGFP punctum on the first imaging day \( t = 0 \) h and were subsequently lost over the time course of the experiment. Their sizes were measured on the last two time points at which they were detected. Despite numerous large fluctuations (e.g. more than 3-fold changes) on average growth and shrinkage canceled out one another in persistent spines (PSD-95-eGFP, \(-0.048 \pm 0.015\); DsRedExpress, \(-0.023 \pm 0.013\); Fig. 2.5 B). This may be indicative of homeostatic processes that keep synapse strength, and thereby PSD size, equal over time (Turrigiano, Nelson, 2004). On the other hand, the \( \log_{10} \) ratios were slightly skewed towards positive values for new spines, and towards negative values for lost spines (New: PSD-95-eGFP,
Figure 2.5: A, relation between changes in red and green fluorescence intensities. Dotted lines represent the measurement error. B, New spines display an increase in both green and red fluorescence over the first two time points after their appearance, as compared to persistent spines (***, p < 0.005, t-test). Lost spines display a decrease in both green and red fluorescence over the last two time points before their disappearance, as compared to persistent spines (***, p < 0.005, t-test). C, the absolute value of fluorescence intensity ratios over a random set of two consecutive time points, for persistent, new, and preexisting disappearing spines. New and lost spines display increased fluctuations (***, p < 0.01, t-test). D, example of a time interval used in B. E, example of time intervals used in C.

0.064 ± 0.042; DsRedExpress, 0.065 ± 0.033; p < 0.005, t-test between persistent and new; Lost: PSD-95-eGFP, -0.366 ± 0.093; DsRedExpress, -0.298 ± 0.068; p < 0.005, t-test between persistent and lost). New and lost spines displayed increased PSD size and spine head volume fluctuations, irrespective of the imaging time point or interval, indicating that their synapses are generally less stable than those of persistent spines (Persistent: PSD-95-eGFP, 0.393 ± 0.010; DsRedExpress, 0.311 ± 0.008; New: PSD-95-eGFP, 0.533 ± 0.058; DsRedExpress, 0.488 ± 0.047; p < 0.005, t-test; Lost: PSD-95-eGFP, 0.481 ± 0.027; DsRedExpress, 0.379 ± 0.024; p < 0.005, t-test; Fig. 2.5 C).

The increased PSD dynamics may be a signature of spines that are still in a growth process or destined to disappear.
Chapter 3

Pilot experiments and optimization of \textit{in vivo} labeling techniques

3.1 PSD-95 retention at synapses

The PSD-95-eGFP experiments in Chapter 2 showed that new spines have on average very small PSDs. In fact their PSD-to-spine head size ratio is positively skewed as compared to the ratio in persistent spines. This indicates that the PSD of a new spine is still in a maturation process and has not yet accumulated the proportion of PSD molecules that constitute a mature spine. Previous work by the Svoboda group (Gray et al., 2006) has shown that small spines (PSDs) have a lower retention of PSD-95 than large spines (PSDs). In other words, large PSDs tend to strongly absorb freely diffusing PSD-95
molecules, and may pull molecules away from small PSDs in their vicinity. This poses a contradiction: how do the small PSDs in new spines grow in the presence of nearby large PSDs that strongly pull at the PSD-95 resources? For the growth of a new PSD the incorporation and retention of PSD-95 molecules needs to be temporarily high in order to increase a new PSD above a critical size at which it can 'compete' with larger PSDs in its surrounding. Gray et al. (2006) showed that PSD-95 retention times vary with the age of the mouse and are modulated upon changes in sensory experience and input. Together, this predicts that new spines that are destined to stabilize will have much longer retention times than transient spines.

To test this hypothesis we set out experiments to measure PSD-95 retention dynamics in vivo. We used an approach identical to Gray et al. (2006), based on PSD-95 tagged with photoactivatable GFP (paGFP). In this construct, the replacement of the threonine at position 203 in ‘wild-type’ GFP with a histidine (T203H) results in an almost complete lack of absorption at wavelengths in between 450 and 550 nm for single-photon absorption and between 850 and 1040 nm for 2-photon absorption. Photoactivation with violet light or with 720 to 840 nm light in case of 2-photon excitation, results in a shift of the absorption peak. This dramatically increases green fluorescence when excited at 488 nm (or at 910 nm for 2-photon excitation) (Schneider et al., 2005). PSD-95-paGFP was expressed using in utero electroporation, similar to the PSD-95-eGFP experiments and as described in Gray et al. (2006). DsRedExpress was co-expressed to visualize dendrites and spines.

Since paGFP is non-fluorescent in its native state we searched for
DsRedExpress-positive spines and repeatedly (2-3 frames) scanned the excitation beam (at 810 nm) over a small region of interest including the spine head to activate paGFP. The laser was calibrated to a power of 150 mW at the back-focal plane of the objective. Immediately after photoactivation the laser was tuned to 940 nm and the spine was imaged in both the red and green channel every minute (until 10 minutes after photoactivation), and subsequently every 10 minutes (until 90 minutes after photoactivation). Fluorescence intensity was measured in both channels similar to the methods we used for the PSD-95-eGFP experiments. PSD-95-paGFP fluorescence was normalized to the red channel. The decay of fluorescence was plotted (Fig. 3.1 C). The retention times ($\tau_r$) were estimated using two rounds of exponential fitting (Gray et al., 2006). The first function contained a constant term ($I = ae^{(-t/b)} + c$) and was used to define the time frame over which $\tau$ was extracted. For the period $t = 0 - b$, the data were fit using a single exponential without a constant term ($e^{(-t/\tau_r)}$). $\tau_r$ reflects the time over which PSD-95-paGFP resides in the PSD.

In our pilot experiments we experienced various technical complications that precluded a more large-scale experiment at that point:

1. The unreliable co-expression that is obtained using in utero electroporation complicated our search for photoactivatable spines. The new spines that we were interested in are small. Therefore, the green fluorescence that was expected after photoactivation was low. Indeed in many cases we could not observe significant levels of paGFP (Fig. 3.1 B) after attempted activation. However, in most of these cases we were not certain that PSD-95-paGFP was
Figure 3.1: A, a spine displaying green fluorescence after photoactivation (photoactivation between $t = -1 \text{ min}$ and $t = 0 \text{ min}$). B, a spine lacking green fluorescence after photoactivation. Scale bar 1 µm. C, green fluorescence normalized over red fluorescence over time. Red line, fit with the first round of exponential ($t = 9.914$). $\tau_r$ is then extracted over the time interval $0 - 10 \text{ min}$.
expressed at sufficiently high levels for us to detect the PSD in small spines. The expression levels of PSD-95-paGFP could be confirmed by photoactivating neighboring large spines (Fig. 3.1 A), but after this control experiment we could not go back to the initial (small) spine since PSD-95-paGFP is exchangeable between spines, which ‘pollutes’ the GFP intensities in neighboring spines (Gray et al., 2006). We could sufficiently space large control spines from experimental small spines since the \textit{in utero} electroporation yielded dense expression patterns and many intermingled dendrites. However, this led to difficulties in tracing connected branches.

2. The second complication relates to fluorescence normalization over time. In contrast to our PSD-95-eGFP experiments we could not normalize photoactivated PSD-94-paGFP to its own dendritic background fluorescence. Thus, we normalized the signal to DsRedExpress. However, we noticed in our pilot experiments that DsRedExpress and PSD-95-paGFP may display small variations in expression levels which are independent of one another. Thus, the normalization itself led to variations in retention times over time that may not reflect real retention time changes. In addition, the DsRedExpress is more sensitive to bleaching than paGFP. Since we needed to image relatively often we may have overestimated PSD-95-paGFP retention times simply because red fluorescence was decaying. In order to perform reliable retention time measurements in small spines over longer time frames we would need to stably co-express both proteins and be able to normalize the green fluorescence independently (see Discussion).

At the time of these experiments we did not have clear and workable solutions...
at hand that would circumvent the problems sketched above. We therefore put the experiments on hold.

### 3.2 Rhythmic whisker-stimulation mediated plasticity

Our PSD-95-eGFP experiments showed that this recombinant protein is a good measure of synapse size. This tempted us to use the protein to monitor synapse size changes as part of synaptic potentiation and depression (LTP and LTD respectively) or other types of plasticity events. Some of the clearest examples of plasticity-related synapse size changes have been provided by LTP experiments \textit{in vitro} (Matsuzaki et al., 2004; Kopec et al., 2006; Harvey, Svoboda, 2007). These studies showed that the size and molecular constituents of spines that undergo LTP display significant alterations. It is tempting to speculate that the PSD-95-eGFP fluctuations that we observed in naïve animals reflect LTP and LTD-like processes. However, we had no direct access in these mice to measure whether a single cell was indeed potentiated or depressed. In addition, LTP and LTD may happen at different synapses on the same dendrite and thereby cancel out one another at the level of the whole cell. It is likely that experience-dependent plasticity paradigms increase LTP and LTD events. However, in these paradigms one faces the same temporal and spatial uncertainties. The best way to monitor synapse size changes \textit{in vivo} in relation to LTP would be through the application of a robust LTP paradigm. \textit{In vitro}, LTP is usually induced by high-frequency
afferent stimulation, by combining presynaptic input with backpropagating action potentials, or chemically. This requires electrode-based stimulation or pharmacological access. However, one cannot combine such invasive techniques with chronic in vivo imaging. Previous experiments have shown that rhythmic whisker stimulation can induce the potentiation of whisker-evoked local field potentials (Megevand et al., 2009). My colleague Frédéric Gambino verified this effect using whole cell recordings in vivo (Gambino-Holtmaat abstract SFN 2013). He showed that a 1 minute 8 Hz whisker stimulus increased subsequent whisker-induced post synaptic potentials (Fig. 3.2).

Based on these findings we set out experiments to measure spine size changes upon LTP induction in vivo (as a pilot to measuring LTP-related PSD-95-eGFP fluctuations). To asses if 8 Hz whisker stimulation had a structural effect on L2/3 pyramidal cells, labeling was performed with a GFP
plasmid (pGAG-GFP-WPRE) introduced by \textit{in utero} electroporation as previously described. Given our earlier experience with the confounding effect of dense expression patterns, we wanted to reduce the number of labeled cells. By increasing the time between plasmid injection in the ventricle and the actual delivery of the electrical train pulse, we were able to reduce the number of transfected cells. In contrast to earlier experiments we were not concerned by lack of co-expression. Therefore, we could reduce the number of transfected cells without repercussions. After cranial window implantation the region was screened for sufficiently isolated cells where the originating cell body and identity of the dendritic processes was unambiguous (Fig. 3.3).

If such a cell was found the barrel cortex was mapped using intrinsic optical imaging (for details see below). This allowed the identification of the home column of the cell of interest. High magnification images were then acquired.
Figure 3.4: Relative volume changes between the 4 imaging sessions. Whisker stimulation occurred just before timepoint 3 in experimental animals (right). Red lines represent the means of volume changes.

every hour for 4 hours (4 imaging time points). The principal whisker was stimulated for 10 minutes at 8 Hz (50 ms deflections) just before the third imaging session. Spine fluorescence intensities were measured as previously described, and volume changes were compared before and after whisker stimulation as well as to control animals (Fig. 3.4).

No major differences in volume changes were observed after whisker stimulation. The lack of detectable changes could be explained by the relative low number of spines that are likely to be involved in such a process. Since we could only image a limited number of spines per cell at high resolution (100-500 out of a total of thousands of spines per neuron), we could have easily missed the few spines that were potentiated. Even if LTP had occurred at many spines, it is likely that the changes were relatively small as compared to the in vitro experiments, and be at a level similar to the natural fluctuations that can be observed in vivo (the signal-to-noise ratio is too low). In
addition, *in vivo* imaging comes with considerable movement noise that may have blurred the effects on spine size.

These experiments were complicated by the following issues:

1. Low control over the density of labeling. Some mice had a relatively high density of labeling which resulted in difficulties to identify the cell body to which the imaged dendrites belonged. Therefore, we could not unambiguously identify the home barrel column of each cell.

2. In animals where the labeling was sufficiently sparse, the position of the neuron in the barrel field was highly variable. Therefore the home barrel column differed between animals. This potentially reduced reproducibility.

3. Point 1 and 2 would complicate even further the experiments using PSD-95-eGFP as a marker for synaptic potentiation (which was what we aimed for), since we would have to rely on co-expression of proteins.

Further experiments were put on hold until the 8 Hz whisker stimulation LTP protocol was better characterized – potentially revealing hot spots for LTP. In the mean time we worked on the development of a more suitable labeling technique (see below).

### 3.3 Is *in utero* electroporation the best method to label cortical pyramidal neurons?

The *in utero* electroporation has become a highly favored technique to express recombinant genes in somatic cells in the cortex, or even other parts
of the brain. The easiest way to perform this technique (as we do) is by injecting the plasmids into the embryo’s ventricle and applying electric pulses through electrode tweezers (Tabata, Nakajima, 2001; Saito, Nakatsuji, 2001). There are clear advantages associated with this technique. First, it has the potential to generate large fields of labeled neurons (see Fig. 2.1 C), which facilitates in vivo imaging experiments if high background and loss of cell identity is not a concern. Second, it has a high throughput potential provided that transfection efficiencies and newborn survival rates are high. It should be noted though that it nevertheless takes a considerable amount of time to generate an adult mouse with suitable expression for imaging: first, timed pregnancies should be produced, which on average takes 1 week; second, one needs to wait 3 more weeks for the delivery of the electroporated pups; third, one needs to wait until mice have reached the desired age (in our case 6 weeks) to perform the cranial window implantation; finally, one needs to wait another 10 days before the beginning of the imaging session. This accumulates to a total waiting time of 11-12 weeks. This limitation is negligible if the procedure yields large numbers of mice with suitable expression.

However, the technique has also quite strong limitations and drawbacks for high-resolution long-term imaging of neuronal structures and synapses in vivo. One of the major and more general concerns is the expression of recombinant proteins throughout development. The constructs are usually based on the chicken β-actin promoter to warrant strong and long-lasting expression. However, such an unconditional expression cassette likely starts to drive high levels of expression shortly after the cells get transfected. For regular, cytosolic markers this most likely does not pose a problem, but for
more physiologically relevant proteins it might. In our PSD-95-eGFP experiments we could confirm that the auxiliary expression had not heavily impacted synaptic development (Gray et al., 2006). However, when we experimented using Homer1C we could observe dendritic malformations (blobs; data not shown). The other drawbacks of the \textit{in utero} electroporation technique relate to the variable co-expression of fluorescent proteins, the level and stability of expression, and the poor spatial targeting. The latter three drawbacks were illustrated by the pilot experiments described in the previous paragraphs: 1. In our hands, the electroporation of two plasmids yielded only small populations of co-expressing cells. This strongly reduced the throughput, as the probability of finding suitable cells under the imaging window was low. 2. Expression levels and stability was unpredictable. Since the electrical pulses were applied through relatively large electrodes, which were manually positioned over the embryos’ heads, the transfection efficiency varied between embryos. In addition, we had very little control over the number of vectors that were transfected per cell. The slight instability of expression over time may have been due to fact that the expression vectors remained episomal. Therefore, the initiation of transcription is less well regulated and probably depends on the accidental encounter of transcription factors with the DNA. The inconstancy of expression levels complicated the ratiometric analysis of two imaging channels as well as comparisons between cells and animals. 3. Since the plasmids were injected into the ventricles without detailed visual guidance we had very limited spatial selectivity. Depending on the embryonic stage at which the electroporation was performed we could target expression to a particular cortical layer (in our case E16 and L2/3
respectively). Other than that we had a very low control over the number of cells that were transfected as well as their location.

To change the density of transfection one could adjust plasmid concentrations and the delay between the ventricular injection and the actual electroporation. However, in practice the method results in either a very high or a very low density of labeling. When the density is high it is very difficult to distinguish different dendrites and the cell bodies of origin. In addition, in densely labeled cortices the background (out of focus) fluorescence is high. When the density is low, the probability of finding transfected cells that co-expresses the two plasmids at levels allowing 2-photon imaging under the craniotomy region is dramatically low. In order to achieve a satisfactory density of transfected cells, plasmids expressing the fluorescent protein of interest under the control of cre-recombinase can be used. To achieve this the injection solution should contain the two floxed plasmids encoding the fluorescent proteins and a third plasmid that encodes cre-recombinase. By changing the concentration of the cre expressing plasmid one can titrate the density of labeling, without compromising the degree of co-expression (Chen et al., 2012a). However, it should be noted that co-expression would be as incomplete as for regular plasmids.

To achieve a higher spatial selectivity one could use viral vectors. This allows the targeting of layers and even specific barrel columns (Marik et al., 2010). Viral vectors would also circumvent the problem that the developmental expression of synaptic proteins may present. However, in utero electroporation alike, viral vectors have limitations regarding the temporal expression profile
and co-expression profile.

3.4 Single cell electroporation

To overcome the issues described above we decided to use single cell electroporation. This approach has been used first \textit{in vitro} in mammalian cells and \textit{in vivo} in Xenopus tadpoles. Recently this technique was applied successfully on living mice (Judkewitz et al., 2009). The protocol we conceived is based on a method published by the Hauser lab (Judkewitz et al., 2009) with adaptations for chronic 2-photon imaging.

3.4.1 Material and methods

3.4.1.1 Plasmids

pCl-SEP-GluA1 was kindly provided by R. Malinow. The SEP-GluA1 sequence was cloned in the pCAG-WPRE backbone. pCl-eGFP-CamKII\(\alpha\) was kindly provided by R. Yasuda. The eGFP-CamKII\(\alpha\) sequence was cloned in the pCAG-WPRE backbone. pCAG-eGFP-Gephyrin-WPRE was kindly provided by C. Levelt. In all the plasmids expression was driven by the CMV-enhanced chicken \(\beta\)-actin promoter (CAG). The 3’ untranslated regions contained the woodchuck hepatitis virus post-translational regulatory element (WPRE) and the bovine growth hormone polyadenylation site.

3.4.1.2 Solutions

\textbf{Cortex buffer}
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>125</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>1</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>2</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.1: Cortex buffer solution.

**Anesthesia mix (MMF)**

<table>
<thead>
<tr>
<th>Volume [µl]</th>
<th>Compounds</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Medetomidin</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>300</td>
<td>Midazolam</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>15</td>
<td>Fentanyl</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>210</td>
<td>H$_2$O</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2: Anesthesia solution.

**Antidote mix**

<table>
<thead>
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<th>Volume [µl]</th>
<th>Compounds</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Atipamezole</td>
<td>5 mg/mL</td>
</tr>
<tr>
<td>300</td>
<td>Flumazenil</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>15</td>
<td>Naloxon</td>
<td>0.4 mg/mL</td>
</tr>
<tr>
<td>255</td>
<td>H$_2$O</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.3: Anesthesia antidote solution.

Alternatively an antidote mix where the Naloxon is replaced by the Buprenorphin can be used to keep an analgesic effect after awakening.

**Alternative antidote mix with buprenorphin**
<table>
<thead>
<tr>
<th>Volume [μl]</th>
<th>Compound</th>
<th>Concentration [mg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Atipamezole</td>
<td>5</td>
</tr>
<tr>
<td>300</td>
<td>Flumazenil</td>
<td>0.1</td>
</tr>
<tr>
<td>20</td>
<td>Buprenorphine</td>
<td>0.0</td>
</tr>
<tr>
<td>25</td>
<td>H₂O</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.4: Alternative anesthesia antidote solution.

Intracellular solution

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMeSO₄</td>
<td>266</td>
</tr>
<tr>
<td>KCl</td>
<td>14</td>
</tr>
<tr>
<td>HEPES</td>
<td>20</td>
</tr>
<tr>
<td>MgATP</td>
<td>4</td>
</tr>
<tr>
<td>Na₂ATP</td>
<td>4</td>
</tr>
<tr>
<td>Na₂GTP</td>
<td>1</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 3.5: 2× intracellular solution solution.

The pH is adjusted to 7.2 with 2 M KOH and check that the osmolality of the 1× solution is 280-290 mOsm. The solution is then filtered using a 0.45 μm syringe filter and stored at -20 °C for up to 6 months.

3.4.2 Procedure

3.4.2.1 Anesthesia

The whole procedure takes between 2 and 3 hours. This time requirement exclude the use of the ketamin-xylazine mix usually used as anesthetic for cranial window implantation. The first option is to use isoflurane but considering that the mouse will need to move between three different setups we opted for the MMF mix. This mix also require an antidote for waking.
the mouse. The mouse is anesthetized with an intraperitoneal injection of anesthesia mix at 5 µl per gram of body weight after induction by isoflurane-oxygen mix at 4%.

3.4.2.2 Preparation for intrinsic optical imaging

The head of the mouse is carefully shaved and cleaned with a tissue sprayed with 70% ethanol solution. The mouse is then moved under the surgery optical microscope (Leica) and held in place by a nose-clamp holder and ear-bars. The skin on top of the skull is opened with surgical scissors exposing an area extending from the right temporal muscle to approximately 5 mm left of the sagittal suture and approximately 5 mm caudal to the lambda and 5 mm rostral to the bregma. After removing the periosteum and cleaning the surface of the skull the exposed region is dried by a gentle blow of compressed air. The region corresponding to the barrel cortex is identified (1.5 mm caudal to the bregma and 3.5 mm lateral). A custom made plastic ring of 7 mm diameter is centered on the identified region and two small drops of super-glue are used to keep it in place. The external side of the plastic ring is then covered with vaseline and the cavity is filled with cortex buffer to allow better transcranial visibility. A square of approximately 1 cm of side is cut from a cover glass to close the upper part of the plastic ring in order to avoid cortex buffer evaporation. It is important to remove all the bubbles from the solution since they may interfere with imaging. Finally the whisker of interest on the contralateral side, which in our case was C2, is painted
with nail polish to be easily identified.

3.4.2.3 Acquisition of Intrinsic optical signal images

The mouse was transferred on the intrinsic optical imaging setup and kept still with a nose clamp. The holder was then rotated in order to have the part of the skull corresponding to the barrel cortex as flat as possible. The camera was adjusted and focused to obtain a field of view as homogeneously sharp as possible. The focus of the camera was then lowered by 300 μm, corresponding to low L2/3 and L4 cells. The two optic fiber light sources where positioned as to have an equally distributed illumination of the surface. Finally the selected whisker was inserted into a glass capillary that was attached to a piezoelectric actuator (PL-140.11 bender controlled by a E-650 driver; Physik Instrumente) triggered by a pulse stimulator (Master-8, A.M.P.I.). Images were recorded by a VDAQ Imager 3001 system (Optical Imaging) equipped with a low read noise charge-coupled device (CCD) camera (1M pixel, 50 fps, 10 bit) using a stable halogen light source (100 W) in combination with a light guide system and a 700 nm interference filter (30 nm bandwidth) Accolla et al. (2007). Typically, 9 trials where recorded with 20s intervals: the first one as a control and lacking the actual whisker stimulation and the eight others with whisker stimulation. A trial consists of a total of 50 images. The first 10 images, spanning 1s, were used as a baseline period. This interval is then followed by 12 images, spanning 1.125 s, where the whisker is stimulated. Finally the post stimulus period ranges from image 23 to image
Figure 3.5: Thresholded intrinsic optical imaging signal overlapped to a transcranial recorded blood vessel pattern image. Scale bar 0.5 mm.

50. The visible response was usually observed in the range of frames 20 to 40. At the end of the imaging session, an image of the brain’s surface with the visible blood vessels pattern was taken using green light (546 nm interference filter). Intrinsic optical signal Images where imported in GIMP (GNU Image Manipulation Program) where after applying a Gaussian Blur (radius = 10) and an arbitrary threshold where overlapped to the blood vessels pattern to determine the location of the center of the selected barrel (Fig. 3.5).

3.4.2.4 Craniotomy

The mouse is then transferred back to the surgery setup. 20 µl of dexamethasone are delivered by IM injection. The glass coverslip and the plastic ring are gently removed. The surface of the skull is cleaned from the remnant of super glue and vaseline with a q-tip. The center of the barrel is labeled on the skull with a surgical pencil and the regions of the skull which will not host the craniotomy are delicately scraped with the tips of surgical forceps.
to remove the remaining dried periostium and increase the roughness of the surface. The temporal muscle is then circumspectly detached from the skull avoiding bleeding. A plastic ring similar to the one used for the intrinsic optical imaging is placed on top of the skull and centered on the barrel mark. The bottom part of the ring is glued to the skull. A thin layer of super glue is also applied on the remaining portion of exposed skull outside the ring and the space left after detaching the temporal muscle. Before the super glue has completely dried, dental acrylic is applied everywhere excepted inside the plastic ring. The ring along with the dental cement forms a well which is need for the imaging phase to have enough liquid between the objective and surface of the brain. A craniotomy of slightly more than 3 mm is performed using a dental drill. When the groove created by the drill is deep enough the remaining “island” of bone will shift downward if carefully pressed with surgical tweezers and the bone can be removed pulling with a syringe needle (30 G). The craniotomy is covered with gelfoam soaked in cortex buffer to stop any eventual bleeding and to protect the brain while the DNA mix is prepared.

3.4.2.5 DNA mix preparation

50 µl of the 2× stock solution are mixed with: plasmid(s) at a final concentration of 10 to 50 ng/µl, Alexa 488 at a final concentration of 50 mM and completed with milliQ water at 100 µl. The mix is filtered with a 45 µm centrifuge filter (Ultrafree centrifugal filters, Millipore).
3.4.2.6 Single cell electroporation

The mouse is then moved to the 2 photon laser scanning microscope, and fixed using a nose clamp. The holder is rotated in order to have the exposed portion of the brain as flat as possible and thus perpendicular to laser beam axis. A glass capillary (outer diameter 1.5 mm, inner diameter 0.86 mm, Harvard Apparatus GC150F-7.5) is pulled with a vertical puller (Narishige PC-100) programmed with a two stage pulling program and equipped with a combination of 2 “heavy weights” and 1 “light weight” to reach a resistance of 12 to 17 MOhms. The pipette is filled with 1 µl of DNA mix and is then mounted on the headstage (Axoporator 800A Headstage AP-1A X1MU, Molecular Devices) which is fixed at a 30° angle on micromanipulator (Luigs-Neumann). The visual field of the microscope is centered on the corresponding midpoint of the barrel signal using a 4x objective (Olympus). The tip of the pipette is first lowered to roughly 300 µm above the brain surface and then its tip is centered (Fig. 3.6). The pipette is subsequently shifted back by moving the micromanipulator X axis of 240 µm to then be approximately located in the center of the barrel once the tip reaches a depth of 130-160 µm (corresponding to L2 cells). After this adjustments the 40× objective (Olympus) is put in place and the needed amount of cortex buffer is added to the well and the pressure in the pipette is set to 150 mbar.

The pipette is thereafter slowly lowered vertically monitoring the resistance. Once the impedance increases, meaning that the pipette touches the dura, the movement of the manipulator is changed from vertical to diagonal (30°).
Figure 3.6: Positioning of the patch pipette in the center of the barrel IOI signal on the open craniotomy. Scale bar 0.5 mm.

Following the tip of the pipette on the green channel of the 2-photon microscope, the dura is pierced by a fast diagonal displacement until it reaches 60 µm of diagonal displacement. After the first pierce it is important to check that fluorescent dye is ejected from the pipette. If not exit the brain and reenter it as many time as needed to have a clear ejection of Alexa 488. Once the pipette enters the brain and has a constant flow of fluorescent dye lower the pressure to 40 mbar and continue the diagonal descent until 250 µm. The following step consist in identifying the shadow of a cell body and aim the tip of the pipette to it. The approach is performed at very slow speed. When the pipette is close enough to the cell a small increase of impedance is detected. The pressure is then released and a further increase in resistance is detected. The pulse train (10 pulses, -12 V, 500 µs, 20 ms) of the actual electroporation is triggered (Axoporator 800A, Harvard Apparatus) and an immediate filling of the cell with green fluorescence is detected (Fig. 3.7). This 'blind' transfection is likely to label pyramidal cells since they constitute the majority of the large cells in the cortex. Incidentally small interneurons may be targeted. The procedure can be repeated to electroporate other cells.
To avoid excessive brain damage we limited X and Y axis displacement to a 50 µm radius. Finally the pipette is completely removed from the brain by a slow diagonal retraction.

3.4.2.7 Cranial window

The mouse is moved for the last time to the surgery table. The craniotomy is carefully cleaned with cortex buffer and if needed small blood clots can be removed with soaked gelfoam. A 3 mm round glass coverslip is cleaned with ethanol and rinsed with cortex buffer before being placed on top of the craniotomy. The coverslip should fit inside the craniotomy and to avoid its movement and reduce to the minimum its distance from the brain surface it is kept in place by a thin metal bar fixed on a manipulator. The excess cortex buffer is removed and super glue is applied all around the coverslip and on its border carefully keeping the glue away from the center of the glass. The last step consists in filling the well and the borders of the coverslip with dental...
Figure 3.8: Left, blood vessels pattern over a 14 days interval. Scale bar 0.5 mm. 14 days interval maximum intensity projections of a electroporated cell viewed from the top (middle). 14 days interval lateral projection of an electroporated cell (right). R = rostral, C = caudal, M = medial, L = lateral. Scale bar 50 µm.

acrylic and eventually placing a custom made metal bar on the opposite side of the window for future imaging sessions.

3.4.3 Expression of fluorescent protein

After two days the fluorescence is already visible but we normally wait 10 day before starting the imaging session in order to reduce inflammation. The cell or cells can be easily tracked for several days to weeks with the help of the blood vessels pattern which remains pretty stable over time (Fig. 3.8). The overall appearance of the cell also looks stable over time excluding any alteration due to the overexpression of the proteins contained in the plasmids.
3.4.4 Debugging

The most important adjustments to previous protocols (Judkewitz et al., 2009; Holtmaat et al., 2009) are decreased plasmid concentrations and the complete insertion of the glass coverslip into the craniotomy rather than the placement on top of the skull edges. These adjustments proved to be key to successfully apply the single cell electroporation for long term imaging.

3.4.4.1 Plasmid concentrations.

The original single cell electroporations protocol (Judkewitz et al., 2009) suggests plasmid concentration of up to 200 ng/µl. In our case, the use of such concentration, not only reduced the number of fluorescent cells at 10 days after the procedure but also produced abnormal cells with blobs at the extremity of dendritic branches. The cells displaying this kind of structural anomalies showed an uncommon rate of spine elimination which finally resulted in their death. The reduction of plasmid concentration, typically to a concentration of 10-50 ng/µl, resulted in an increased number of fluorescent cells at 10 days after electroporation and reduced the number of cells displaying the blobs abnormalities at the tip of dendritic processes (Fig. 3.9).

The other points highlighted in the original single cell electroporation protocol remain important to achieve a successful procedure. The most important being the distance of the pipette tip from the cell body. If too far, the result will be a lower amount of plasmids entering the cell finally leading to an insufficient fluorescence for 2-photon imaging. On the other hand, if too close,
Figure 3.9: Success rate of single cell electroporation versus plasmid concentration. Black, fraction fluorescent cells after 10 days from electroporation. Gray, fraction fluorescent cells with blobs after 10 days from electroporation.

it can lead to membrane damage and, eventually, to cell death.

3.4.4.2 Cranial window implantation

A change in the cranial window implantation, compared to the original protocol used for the PSD-95 in utero electroporation experiment, was to sink the coverslip into the craniotomy instead of placing it on top of the bone edges (see Fig.1 of the annex Holtmaat et al. 2013 and compare to Fig. 2.1 B). This new method allowed to reach a success rate of more than 90%. The resulting windows were, at 10 days after the implantation, transparent, devoid of any tissue growth and could be imaged for periods of time ranging from weeks to months.
3.4.5 Examples

To test the potential of this technique we set out pilot experiments using various synaptic protein expression vectors. First we used the CAG-PSD-95-eGFP-WPRE plasmid to directly compare the results of the single cell electroporation with the experiments described in chapter 2. The distribution of PSD-95-eGFP within the transfected cell was comparable to what was observed the in utero experiments (Fig. 3.10). Clear puncta of various sizes could be observed in dendritic spines and dendritic shafts. Qualitative visual inspection learned that the density of puncta and their size distribution were more or less similar between both types of experiments. We also tested Gephyrin, a postsynaptic scaffolding protein in inhibitory synapses that interacts with glycine and GABA-A receptors (Fritschy et al., 2008). We transfected CAG-eGFP-Gephyrin-WPRE plasmids in L2 pyramidal cells. This resulted in a punctate staining similar to the distribution of PSD-95-eGFP, with one main difference. In contrast to PSD-95-eGFP, eGFP-Gephyrin puncta were found predominantly on dendritic shafts and to a much lesser extend in dendritic spines (Fig. 3.10). This fits with the idea that in pyramidal cells most inhibitory synapses are found on dendritic shafts. In addition, eGFP-Gephyrin appeared to form larger clusters in dendritic shafts and be more prominently present outside of the puncta (please note that the example in Fig. 3.10 is the result of a high offset and therefore does not show the non-punctate inhomogeneity in the dendrite). This might have been caused by overexpression, but could also represent the true distribution of Gephyrin in dendrites. Control experiments using lower expression levels and careful
comparisons with immuno-staining of native Gephyrin will need to be performed to investigate this (retrospective EM could also be used, see General Discussion). In our pilot experiments using CAG-eGFP-CaMKIIα-WPRE and CAG-SEP-GluA1-WPRE we observed substantially different distribution patterns. eGFP-CaMKIIα was spread out through the whole dendrite at different levels (Fig. 3.10). Dense and bright clusters (usually long-stretched) could be observed in dendrites, similar to previous experiments in vitro (Lemieux et al., 2012). Dendritic spines displayed punctate labeling. The distribution of eGFP-CaMKIIα may be less static as compared to Gephyrin and PSD-95, and drastically vary between two time points. CaMKIIα is thought to translocate to dendritic areas on which synapses are activated (Lee et al., 2009; Lemieux et al., 2012), which may vary over time. If eGFP-CaMKIIα reliably represents native CaMKII distributions it is tempting to speculate that the high intensity of labeling in Fig. 3.10 (white arrowheads) depicts synapses in parts of the dendrite that were active during or just prior to imaging. Transfection of SEP-GluA1-WPRE yielded very low levels of expression, and resulted in poor image qualities. We exclude the possibility that the green fluorescence was the result of crosstalk from the red channel, since cells in which we expressed DsRedExpress only never showed such intensities in the green channel. The SEP-GluA1 recombinant protein contains a mutant version of GFP that is very sensitive to the pH (super-ecliptic pHluorin or SEP) (Miesenböck et al., 1998). The strong pH dependence of the fluorescent protein renders it non-fluorescent in acidic secretory vesicles and fluorescent in the more neutral extracellular milieu. Since SEP was cloned at the N-terminal region of the glutamate receptor the
protein remains generally non-fluorescent and only turns fluorescent upon externalization of the receptors (Ashby et al., 2004; Kopeck et al., 2006). Thus, the low levels of fluorescence were not unexpected, similar to previous in vitro experiments. Despite the low intensities of SEP-GluA1 the results were far superior as compared to earlier pilot experiments that we performed using in utero electroporation. Thus, although the use of SEP-GluA1 will need to be optimized to make it useful for long-term imaging in vivo, we believe that single cell electroporation will be the method of choice. This produces labeling with the lowest possible background fluorescence, which is needed for recombinant proteins that yield low-intensity labeling. At last we used the single cell electroporation technique to specifically label inhibitory cells.

3.4.6 Pilot experiment

Previous studies from our lab (Gambino, Holtmaat, 2012) have shown that dual whisker pairing, i.e. the clipping of all but two whiskers (dual whisker experience, DWE), increases the whisker-induced post-synaptic potential (PSP) amplitude ratio between the surround and principal whisker (SW/PW; both spared) (Fig. 3.11 A). DWE also facilitates surround-whisker driven STD-LTP in L2/3 pyramidal cells (Fig. 3.11 B). The facilitated LTP and increased SW/PW ratio are likely related to metaplastic events, such as decreased SW-evoked inhibitory conductances (Fig. 3.11 C). Since the SW-evoked excitatory conductance remains unaltered, the E/I ratio increases.
Figure 3.10: Dendric branches of L2/3 pyramidal cells coexpressing DsRedExpress along with, from top to bottom: PSD-95-eGFP, eGFP-Gephyrin, eGFP-CaMKIIα and SEP-GluA1. From left to right: red channel, green channel and merge. White arrowheads indicate high fluorescence regions. Scale bar 5 µm.
These findings led to the hypothesis that DWE induces the loss or shrinkage of inhibitory synapses on L2/3 cells. To test this hypothesis we set out experiments using single-cell electroporation, aiming at the longitudinal tracking of eGFP-Gephyrin.

Four week old C57/BL6J male mice were single cell electroporated in the C2 barrel using a mix of DsRedExpress and eGFP-Gephyrin. A cranial window was implanted immediately after electroporation. Ten days after the procedure the imaging begun. Four mice were imaged once per day during the first three days and then all but the C1 and the C2 on the contralateral side of the window were clipped. The mice were then imaged again once per day one, two, five, seven and fourteen days after the clipping. Whiskers were clipped again every two days. Three mice were used as control in which whisker clipping was performed.

3.4.6.1 Scoring and tracking of eGFP-Gephyrin puncta

Unlike PSD-95-eGFP, eGFP-Gephyrin has a less inhomogeneous dendritic background fluorescence. Thus when fluorescence values between time points change because of variation in the optical properties of the cranial window or laser intensities, we lack a constant normalization factor that allows the unambiguous scoring of fluorescent protein puncta. We tried various normalization methods. First we tried to use a normalization factor based on red fluorescence intensities to determine the lookup table settings in the green channel at different time points. For this we assumed that both proteins are
Figure 3.11: A, Ratio of PSP peak amplitudes for principal (PW) and surrounding (SW) whiskers (***, p < 0.001, t-test). B, Mean of PSP peak amplitudes normalized to baseline for principal (PW) and surrounding (SW) whiskers in control and DWE mice (***, p < 0.001, t-test). C-D, Left, averaged PW and SW-evoked synaptic inhibitory conductances over time in control and DWE mice. Right, mean integrated conductance (**, p < 0.01, t-test). Adapted from Gambino, Holtmaat (2012).
stably expressed over time. We took the first time point as the reference point for the detection of puncta. In the green channel, the minimum value of the lookup table was set such that it rendered a clear and punctuate pattern when binarized (Fig. 3.13, top image). Please note that these settings were arbitrary. We did not know what level of Gephyrin clustering represented the physical presence of inhibitory synapses. In order to standardize the lookup table settings that needed to be applied to the green channel on the next day, we used the red channel. On a maximum projection of the red channel, fluorescence intensities were integrated over a line passing through the core of dendrite in between two distant spines that were present in all imaging sessions. This was repeated on all time points. For each time point the lookup table minimum value was adjusted by dividing it by the ratio between the red channel intensities on time point X and time point 0. After this was done for all time points, eGFP-Gephyrin puncta were scored if the green fluorescence was detected over at least 4 pixels and two consecutive sections. The puncta were tracked over time and the number of gained and lost puncta between each time point were calculated (Fig. 3.12). It is important to note that scoring of puncta is performed on stacked images and not in maximum intensities projections images which are used for sake of simplicity in the examples (Fig. 3.13 and Fig. 3.14).

This normalization method resulted in dramatic fluctuations in gains and losses over time. Since such fluctuations have not been described before (Chen et al., 2012a; Versendaal van et al., 2012) we were uncertain about our results. We also noted a strong coincidence between the increase in gained puncta and the decrease in lost puncta and vice versa. Furthermore,
Figure 3.12: Increase of gained eGFP-Gephyrin puncta numbers (blue line) corresponds to a decrease of lost eGFP-Gephyrin puncta numbers (black line) and vice versa. The increase and decrease in puncta correlates with the changes in red dendritic average fluorescence (red line).

the fluctuations in gained and lost puncta correlated with changes in the normalization factor that was based on the red channel (Fig. 3.12). Retrospective visual inspection of the images learned that a large portion of the puncta that were scored as gained (open yellow arrowhead, Fig. 3.13) or lost (yellow arrowhead, Fig. 3.13) were likely to be artifacts stemming from the different thresholds on each time point.

We concluded that perhaps the intensities in the red and green channels are not constant enough to warrant this type of normalization. In a second attempt we tried to find normalization factors based on the green channel. Because it is difficult to define the regions in the dendrite where eGFP-Gephyrin is likely to represent the concentration of freely diffusing protein, we decided to average the green fluorescence over the whole dendrite. The assumption behind this normalization is that although puncta may appear and disappear, the total amount of eGFP-Gephyrin is likely to be constant or to fluctuate minimally. The details of the normalization procedure, using
Figure 3.13: Series of thresholded (based on the red channel) green channel images. Puncta losses due to a decrease in fluorescence (yellow arrowhead). Puncta gains due to a decrease in fluorescence (open yellow arrowhead). Scale bar 5µm.

An ImageJ macro (annex ImageJ threshold macro), are detailed hereafter. A gaussian blur filter (sigma=2) was applied to each section of the green channel stack to lower the noise, by reducing high frequency components (low pass filter) and to enhance image structures. The slices were then registered to minimize any possible artifact created by lateral movement during image acquisition. The maximum intensity projection of the registered stack is then created. To define the area for normalization, a line is drawn on a maximum projection following the core of the dendrite. Two distant spines were used as landmarks, in order to keep the normalization area constant over time. A 10 pixels diameter circle then travels, pixel by pixel, along the traced line recording mean pixels values and standard deviations (over the area of circle). The average of the mean pixels values (finalmean) and the
average of the standard deviations (finalstd) were computed. A threshold was computed based on these two factors: $I_{\text{Threshold}} = \text{finalmean} + (2 \times \text{finalstd})$. $I_{\text{Threshold}}$ was then applied to the filtered stack, after which the image was binarized. eGFP-Gephyrin puncta were scored if the green fluorescence was detected over at least 1 pixels in 1 section. Some puncta disappeared and reappeared over time in the same position. In these cases they were considered to be present during the intermediate time point(s), similar to other studies (Chen et al., 2012a). The reason for this assumption is that very often these puncta have pixel values near the threshold. Another important consideration regarding the arbitrary thresholding is that we do not know which threshold renders puncta that represent inhibitory synapses. It is very likely that our thresholds led to an underestimation of the number of inhibitory synapses. One way to answer this question is to perform EM reconstructions of eGFP-Gephyrin expressing dendrites, and determine the relation between the fluorescence levels and the actual presence of inhibitory synapses. In the example in Fig. 3.14, to compare the relative reproducibility of the thresholding method, the thresholded images are next to the green channel images with the same LUT applied. We can observe an increase in fluorescence on day 5 which then slightly decreases on day 7 and day 14. This change is not significantly affecting the number of puncta in contrast to the method seen on Fig. 3.13.
3.4.6.2 Preliminary results

Preliminary results based on the analysis of 7 ROIs (100 puncta on day -2) from 1 DWE mouse and 3 ROIs (83 puncta on day -2) from 2 control mice are hereafter reported. The first observation is the relatively low survival fraction at 16 days (DWE = 0.55, control = 0.59, Fig. 3.15 A) as compared to previously published data (0.85) (Versendaal van et al., 2012). Similarly we found a slightly lower spines-to-shaft puncta ration (0.27 versus 0.4). As previously described, the spine puncta are displaying a lower fluorescence in-
tensity. Thus the arbitrarily high threshold may have been set to high and cut off some of the low fluorescence eGFP-Gephyrin-containing spines, thereby lowering the ratio. Moreover the high threshold could also be an explanation for the lower survival fraction at 16 days. By lowering the threshold we might be able to decrease the number of losses between time points, and thereby increase the survival fraction.

Regarding the effect of DWE we can not draw any conclusion at this point. The number of mice and puncta are too low to calculate any meaningful standard deviation, or to perform statistics. As an example of possible data to be analyzed we can think of the total number of puncta over time (Fig. 3.15 B). Moreover we can look at the gained (Fig. 3.15 C) and lost (Fig. 3.15 D) puncta and classify these changes on spine puncta or shaft puncta (dashed vs. continuous lines).
Figure 3.15: A, survival fraction of eGFP-Gephyrin puncta (black = DWE, blue = control). B, total eGFP-Gephyrin puncta over time. C, gained eGFP-Gephyrin puncta in spines (dashed line) and gained shaft eGFP-Gephyrin puncta (continuous line). D, lost eGFP-Gephyrin puncta in spines (dashed line) and gained shaft eGFP-Gephyrin puncta (continuous line).
Chapter 4

Discussion

4.1 The relationship between PSD-95 clustering and spine stability *in vivo*

We have characterized the relationship between PSD dynamics and spine stability in real time *in vivo*. Our data show that persistent spines almost always bear a PSD (98%; Fig. 2.2). New spines and spines that are destined to be lost often lack a clear PSD (new, 67%, lost, 62%; Fig. 2.2 and Fig. 2.3). New spines that acquire a PSD are more likely to become persistent (Fig. 2.3). However, most PSD-bearing new spines are transient. Persistent spines that lose a PSD are likely to be pruned (Fig. 2.3). Furthermore, PSD size scales linearly with spine head volume (Fig. 2.4). The PSD to head size ratio is positively skewed in new spines, suggesting that they slowly acquire the PSD levels that are required in mature and stable spines. Spines that are destined to be lost on average lose PSD content over hours to days before
they are finally pruned (Fig. 2.5).

4.1.1 Auxiliary expression of PSD-95 and scoring of PSD-95-eGFP puncta

Although we cannot completely exclude the possibility that the auxiliary expression of PSD-95-eGFP influences synaptic physiology and synaptic structural dynamics, several observations indicate that the disturbing effects are minimal. We expressed PSD-95-eGFP in a similar way to the study by Gray et al. (2006). This study found spine densities and synaptic connectivity not to be affected upon in utero transfection of PSD-95-eGFP. We found spine densities to be similar to Gray et al. (2006), and other in vivo imaging studies (Holtmaat et al., 2005; Hofer et al., 2009). Similarly, spine turnover fell into the same range as observed in GFP-M mice (Holtmaat et al., 2005; Wilbrecht et al., 2010). Furthermore, spine sizes and PSD sizes followed a log-normal distribution (data not shown), similar to a recent imaging study in vivo (Loewenstein et al., 2011), and individual spines and PSDs alternately grew and shrank over time. Altogether, this suggests that the auxiliary expression of PSD-95-eGFP does not cause the continuous growth of PSDs, and does not heavily affect the turnover of PSD-95 molecules within PSDs (Gray et al., 2006). Many new spines never acquired PSDs or only expressed them transiently. Many previously persistent spines lost their PSDs. Thus, expression of PSD-95-eGFP did not massively stabilize spines, and still allowed continuous spine and synapse pruning. This also suggests that the synaptic incorporation of PSD-95 is a regulated process that is not readily inflated by auxiliary expression, which holds true for for various other synaptic pro-
teins (Kessels, Malinow, 2009). In our study we erred towards conservative estimates of PSD formation, i.e. clear clustering of PSD-95, with fluorescence intensities of 10% above dendritic background levels. These strict criteria may have resulted in an underestimation of the number of spines with PSDs (see #1 in Fig. 2.2). A for an example of PSD-95-eGFP enrichment that did not survive our criteria). For very small synapses the number of PSD-bound PSD-95 molecules may barely exceed the amount of freely diffusible PSD-95 in the spine head and neck. In addition, the microscope may not spatially resolve the PSD. Indeed, we found that about 21% of the spines did not contain a distinct PSD-95-eGFP punctum, a fraction that is higher than reported in EM studies (Knott et al., 2006; Arellano et al., 2007). Though, it should be noted that a large proportion of the spine population without a PSD represented very thin transient spines, which may often be overlooked in static histological preparations. Furthermore, the spine population investigated in the other studies was different from previous EM studies and in vitro studies (predominantly L5 in (Knott et al., 2006), L2/3 basal dendrites in visual cortex in (Arellano et al., 2007), and hippocampal pyramidal cells in the in vitro studies).

4.1.2 The relationship between spine and PSD formation

The majority of gained spines did not stabilize and did not acquire a distinct PSD. This corroborates EM studies that have detected a lack of PSDs and/or synapses in a large fraction of newly formed spines (Knott et al., 2006; Nägerl et al., 2007). New spines that did not display a PSD-95-eGFP punctum at
the first point of observation, rarely acquired it later on. However, we also observed an increasing fraction of new spines with PSDs over increasing time intervals. This indicates that PSD-95 clustering is not always immediate, but occurs over hours to days after the growth of the parent spine. PSD growth showed similarities to in vitro studies. However, they were quantitatively different from the fast and functional synapse formation in those experiments (Nägerl et al., 2007; Zito et al., 2009). There are several explanations. First, our PSD scoring criteria may have resulted in an underestimation of synapse formation (see above). Second, synaptic-like activity may occur even when PSDs are small or absent, through non-clustered NMDA and AMPA receptors that are juxtaposed to presynaptic boutons. Third, PSD formation may be slower in vivo as compared to organotypic cultures. This could be due to differences in circuit activity, synaptic inhibition and neuromodulation. In our experiments, the dendrites were positioned in L1, a region of the cortex that receives many modulatory and long-range connections, whereas most slice experiments focused on hippocampal pyramidal cells that lacked long-range modulatory inputs and hormonal regulation. Hormonal oscillations and modulatory inputs can have profound effects on synaptic plasticity and spine maturation (Jones et al., 2009; Liston et al., 2013).

Some new spines displayed a PSD yet did not stabilize for long periods of time. However, they still persisted longer than spines without PSDs (Fig. 2.2 D and Fig. 2.3 B). This suggests that in addition to the PSD other factors determine the stabilization of the spine. This is not inconceivable since spine morphology is regulated through a vast signaling network (Patterson, Yasuda, 2011). Because we did not have insight into axonal bouton dynamics
we can neither exclude the possibility that some PSDs lacked a presynaptic counterpart. Spine and PSD formation might be induced by large quantities of presynaptic glutamate release or spillover (Richards et al., 2005; Kwon, Sabatini, 2011). However, these spines may not always be able to contact the presynaptic site from which glutamate was released. Alternatively, synapses in those spines may not be potentiated and therefore fail to stabilize, which may cause the spines to retract (Matsuzaki et al., 2004; Kopec et al., 2006; Harvey, Svoboda, 2007; Hill, Zito, 2013; Oh et al., 2013). Nevertheless, our data imply that transient spines with a PSD may serve to provide shortterm synaptic connections and could thereby temporarily influence network activity. This may be important for learning and downstream neuronal network plasticity (Xu et al., 2009; Yang et al., 2009).

4.1.3 The relationship between spine loss and PSD dynamics

PSD-95-eGFP levels in persistent spines fluctuated. We found a concomitant decrease in spine volume and PSD-95-eGFP levels before spines were pruned (Fig. 2.5 F). This indicates that before a spine is lost, it gradually loses its PSD. A slow and gradual reduction of spine volumes before spine pruning was also observed during the experience dependent loss of persistent spines (Holtmaat et al., 2006). These observations fit well with intrinsic spine volume changes in organotypic brain slices (Yasumatsu et al., 2008) and multiplicative dynamics in vivo (Loewenstein et al., 2011). The results may indicate that the maintenance of a spine relies on a critical PSD size. New spines that do not acquire enough PSD molecules may disappear, and
old spines that lose too many PSD components will be pruned. The loss of PSD components likely depends on long-term depression (Nägerl et al., 2007; Zhou et al., 2004; Oh et al., 2013). Synaptic adhesion molecules that are linked to PSDs may be key in the maintenance of a synapse. Once the PSD size is reduced below a critical point adhesion to the presynaptic bouton may become insufficient to maintain the synaptic connection. Spines without a synapse may spontaneously disappear due to a lack of filamentous actin anchoring points (Sekino et al., 2007; Soria Fregozo, Pérez Vega, 2012). On the other hand, we did observe persistent spines and spines destined to be pruned that temporarily existed without a PSD (Fig. 2.3 F). We cannot exclude the possibility that in some cases the spine was lost and replaced by a new spine that had not (yet) acquired a PSD. Nonetheless, the result may indicate that some, perhaps non-synaptic, adhesion molecules temporarily keep the spine head linked to its presynaptic element after the complete removal of the PSD.

Similar to the study by Woods et al. (2011) we found that the loss of PSD enrichment is not a prerequisite for spine loss. Many spines displayed normal PSD-95-GFP levels at the last time point of observation. This may indicate that spines can be very rapidly pruned after the loss of a synapse. An interesting alternative is that active spine pruning in some cases strips the synaptic contact, a process that could be modulated by non-synaptic mechanisms. However, it should be noted that most of our time intervals were much longer than in the in vitro studies, and we may not have captured rapid PSD decline before spine pruning.
4.1.4 Future experiments

Our data indicate that PSD-95-eGFP can be used to probe synapse dynamics in real time in vivo. This is a considerable advantage over tracking of spine or axonal boutons alone, since many new protrusions are (initially) devoid of synapses. In the future this recombinant proteins could be used to estimate synapse dynamics in relation to learning or changes in experience (see 4.2.5). Various parameters could be deduced from such experiments. First, one could monitor ‘synapse’ turnover. Second, one could monitor ‘structural synaptic strength’. Both parameters have been suggested to change in relation to experience and learning (Holtmaat et al., 2009; Fu, Zuo, 2011). Such experiments could also be combined with the use of SEP-GluA1 and eGFP-CaMKIIα (provided that PSD-95 is tagged with a red fluorescent protein, e.g. mCherry). This could reveal a direct relationship between PSD-size changes and synaptic activity. Since the in utero electroporation technique resulted, in our hands, in long turnaround times, I recommend performing such experiments using single cell electroporation (see 4.2). Another line of experiments could be aimed at understanding the role of the transient spines bearing a synapse. The functionality of this spines could be also assessed by in vivo glutamate uncaging experiments (Noguchi et al., 2011).

4.2 Optimization of in vivo labeling techniques

4.2.1 Considerations on in utero electroporation

Although in utero electroporation is a robust and easy to learn technique,
we observed some limitations. The main drawbacks of the \textit{in utero} electroporation method are: a potential impact on neuronal development due to overexpression in embryonic and postnatal stages, co-expression variability, low control of the density of labeling, low spatial control, a long time to produce adult mice with labeling, and the relatively low throughput. In particular, these limitations turned out to be problematic in two of our pilot experiments (see 3.1 and 3.2). In case of our PSD-95-paGFP experiments the time that was needed to find sufficiently labeled spines (paGFP) was impractical, due to variable co-expression of PSD-paGFP and DsRedExpress. In the case of our 8 Hz whisker stimulation experiments the low control of the density and area of labeling resulted in difficulties in finding suitable fluorescent cells and in variability in the principal whisker identity. Moreover not all the mice expressing fluorescent proteins in L2/3 pyramidal cells had a region that was suited for this experiment, which decreased the throughput even more.

4.2.2 Single cell electroporation

In order to improve the methods to study synaptic protein dynamics \textit{in vivo}, we have tried and combined single cell electroporation techniques with long-term 2PLSM \textit{in vivo} (see 3.4). This approach overcame some of the problems that we encountered with other existing labeling methods such as \textit{in utero} electroporation or viral vector injections. The main advantages of the technique are:

1. Low background fluorescence, which allows imaging of proteins that yield very low fluorescence levels, such as SEP-GluA1.
2. High co-expression efficiency (in our cases, 100% of the fluorescent cells expressed both constructs at similar levels).

3. The high spatial control, which potentially allows one to select not only a specific cortical layer and specific regions such as a single barrel column, but also a specific neuronal subtype (e.g. targeted electroporation of fluorescently labeled PV interneurons).

4. Finally the amount of time between the labeling and the start of imaging is short.

Pilot experiments in which we used eGFP-Gephyrin to explore structural changes related to dual whisker experience, confirmed the usefulness of single cell electroporation. We showed that dually labeled L2/3 pyramidal cells that are suitable for imaging could be produced in less than 2 weeks. Moreover, we could reproducibly target cells within an identified barrel and layer, and express two fluorescent proteins at relatively stable levels.

4.2.3 Normalization of fluorescence

One of the major problems that we faced during the analysis of both our PSD-95-eGFP and eGFP-Gephyrin experiments was the normalization of the green channel over time. Especially for *in vivo* experiments this normalization is crucial, since expression levels, excitation conditions, fluorescence detection efficiency and movement artifacts may somewhat fluctuate over time.

In our PSD-95-eGFP experiments the low and relative homogeneous background labeling of PSD-95-eGFP along the dendritic shaft proved to be a
good source for normalization. Small areas of the low-background PSD-95-eGFP, which presumably reflect freely diffusing protein, could be selected to normalize the intensity levels that were found in puncta. This provided a means to set a threshold for puncta detection. On the other hand, in case of eGFP-Gephyrin, the fluorescence pattern in the dendrite was rather inhomogeneous. Therefore, background could not be selected from small regions near a presumptive punctum. Under ideal circumstances, such a normalization factor could be derived from the other channel (which represents a freely diffusing cytosolic protein). However, small and differential changes in this channel may disturb the normalization. This is not unlikely in vivo.

A way to get around this problem is to use the average dendritic fluorescence over the whole dendrite, ignoring the fact that this contains puncta. We successfully applied this, assuming that the overall number of eGFP-Gephyrin molecules/puncta will remain relatively stable over time. In this case, new and lost puncta will have only a small effect on the average dendritic fluorescence. It is important for all of these measurement that one makes sure, during the recording, to have a similar distribution of pixel intensities at different time points (this can be quickly checked using the histogram function in the acquisition software). This is needed to reduce the errors in the normalization factor. The ideal way to normalize the fluorescence over time would be to introduce a stable source of fluorescence. Such a source of fluorescence could be fluorescent beads (that are normally used for microscope calibration). A small amount of beads could be injected in the region close to transfected cell, for example following single cell electroporation.
4.2.4 Biological significance of fluorescence levels

When imaging auxiliary expressed fluorescent proteins in vivo one can never be completely certain of which intensities relate to a biological significant signal. For example, in case of synaptic scaffold proteins, the arbitrary selection of the threshold above which fluorescence is considered to represent a ‘synaptic punctum may lead to under or overestimation of the number of synapses. In both the PSD-95-eGFP and eGFP-Gephyrin experiments we probably misinterpreted the actual number of synapses. In some of our experiments we used the puncta-threshold to estimate the turnover of synapses. Since, new synapses (both excitatory and inhibitory) are likely to be small, their respective puncta may be very small. Therefore, the threshold will have a major impact on the dynamics that are finally reported. It is important in such experiments to apply consistent thresholds over time, between animals and conditions. In addition, it is desirable to also apply control and test conditions to the same animal. The ultimate proof for ‘correct’ thresholding may come from retrospective serial section EM reconstructions of imaged structures. The EM reconstruction will reveal the presence of structural synapses (the actual synapses). By comparing the in vivo images with the profile that is seen in the serial EM sections, one could calibrate the thresholds that are needed in the in vivo images to render the correct amount and location of synaptic puncta.

4.2.4.1 Considerations on protein expression levels

The use of vectors coding for recombinant proteins may lead to the overexpression of the protein. Therefore, the level of protein expression may remain
above the levels that are normally reached by the native protein. This may result in changes in the cell’s physiology. For example, it has been reported that overexpression of PSD-95 occludes LTP (Ehrlich, Mallinow, 2004). A way to overcome this issue one solution is to use conditional knock-in mice. In case of PSD-95-eGFP we could think of inserting a sequence containing a lox-STOP-lox cassette followed by the GFP (or other spectral variants) coding sequence downstream of the PSD-95 region on the chromosome. The expression of PSD-95 will then continue to be driven by its native promoter, and the expression of the labeled version can be induced by Cre recombinase.

4.2.5 Potentiation protocols

Fluorescently tagged proteins would be ideal substrates to probe synaptic plasticity in vivo. Especially it is tempting to use them in order to find correlations between LTP and structural synapse growth. However, we currently lack paradigms in which we have temporal and spatial control of the potentiation events. Electrophysiological studies in our lab have shown that rhythmic whisker stimulation can potentiate neurons with a high spatial selectivity (targeting a single barrel column). Unfortunately and despite this selectivity, there is still only a small fraction of the vast number of synapses on each cell that are actually potentiated. Since it is nearly impossible to image all spines with sufficient resolution, it remains a challenge to find the ones that undergo LTP – and correlate the structural changes. Ideally we would like to know which spines are most likely going to be affected by the protocol and to be able to directly target them for imaging. One option could be to use GEClIs to identify the spines that are regularly activated by
whisker deflections (as an example see Chen et al. (2013)), and target them for imaging in the rhythmic stimulation paradigm. The limiting factor of such an approach is the need of three different colors of fluorescent protein: one for the GECI, one for the protein of interest and one for the cytosolic labeling of the structures. Another alternative is the use of channelrhodopsin to specifically activate presynaptic cells. In this case it would be preferable to turn on ChR2 expression only in the cells that are presynaptically coupled to the cell of interest. Such a method of viable trans-synaptic labeling has recently been published (Gradinaru et al., 2010). In this method, the authors used wheat germ agglutinin (WGA) coupled Cre to trans-synaptically switch on Cre-dependently rhodopsin expression. For our studies we could use a transgenic mouse that conditionally expresses ChR2 (e.g. Cre-dependent) in all cells. We could single cell electroporate a L2/3 pyramidal cell with two plasmids: one expressing the protein of interest and the other one expressing the WGA-Cre fusion protein. Thus far, the experiences with this approach are not very promising. Preliminary reports (the Petersen lab at the EPFL) show that WGA-Cre has a very limited potential to switch on expression in the presynaptic cell. Perhaps this could be improved by engineering the transfection vectors. Alternatively, other trans-synaptic vectors could be tried, and by using a similar approach (e.g. the c-fragment of tetanus toxin (Cordero-Erausquin et al., 2009); or recombinant rabies virus based vectors (Wickersham et al., 2007). If such an approach is successful we could then use an illumination protocol to induce LTP and at the same monitor variations in the synaptic protein levels.
Annex

Holtmaat et al. 2013
Optical imaging of structural and functional synaptic plasticity in vivo

Anthony Holtmaat a,n, Jerome Randall a,b, Michele Cane a,b

a Department of Basic Neurosciences, Faculty of Medicine, and the Center for Neuroscience, University of Geneva, Switzerland
b Lemanic Neuroscience Doctoral School, Switzerland

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ABSTRACT

The adult brain has long been viewed as a collection of neuronal networks that maintain a fixed configuration of synaptic connections. Brain plasticity and learning was thought to depend exclusively on changes in the gain and offset of these connections. Over the last 50 years, molecular and cellular studies of neuroplasticity have altered this view. Brain plasticity is now viewed as a continuum of structural changes that could vary from long-range axon growth to the twitching of dendritic spines and synaptic receptor composition dynamics. Plasticity proteins similar to those that drive neuronal development may underpin brain plasticity, and consequently could regulate adaptations to new experiences and learning. In vivo imaging has confirmed that neuronal plasticity in the adult brain involves subtle structural changes at synaptic connections, including synapse formation and pruning. Synaptic structural changes are associated with experience-dependent plasticity, learning, brain traumas and neurodegeneration. Owing to the expanding toolbox of in vivo imaging we have come to the brink of understanding the causal relationship between structural synaptic network dynamics and functional brain plasticity. This review summarizes the technical developments in the imaging of laboratory animals’ brains in vivo and the insights they have provided into the mechanisms of brain plasticity and learning.

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1. Neuronal plasticity

Since Santiago Ramón y Cajal’s first observations of neuronal (synaptic) networks and the subsequent development of the 'neuron doctrine', scientists have been intrigued by the stability and dynamics of the brain's neuronal connections. A long-standing dogma stated that synaptic networks are flexible during pre- and postnatal development, stabilize during adolescence, and become structurally inert in adulthood. According to this idea, new experiences produce permanent and extensive modifications in brain circuits during early-life periods. These are known as critical periods during which life-long memories are created and...
particular skills are engrained (Hensch, 2005). Consequently, during later phases in life, the communication between neurons can only be sculpted through alterations in synaptic strength rather than through the addition or removal of synaptic connections. It was also thought that this would benefit the retention of important memories and skills, and provide a modest capacity for learning, which declines with age.

The idea that neuronal networks are ‘stable’ in the adult brain was supported by clinical observations in people with brain trauma. Insults to the adult brain usually result in devastating effects, impairing mobility or behavior of patients for a lifetime. Thus, understandingly it was thought that the central nervous system (CNS) is unable to compensate for a major loss of function, let alone to repair itself; i.e. CNS neurons do not regrow their axons and dendrites and do not reconstitute synaptic connections (e.g., Akbik et al., 2012).

The notion that neurons in the central nervous system have a poor capacity to regenerate whereas damaged peripheral nerves do regrow and to find their target prompted a quest in the 1980’s to characterize molecules and cellular features that could underlie this difference (Richardson et al., 1980). At the same time it was recognized that the limited growth capacity of CNS neurons paralleled the reduced synaptic plasticity in adults. This posed the interesting option that by boosting plasticity processes in the adult brain one could also revoke the potential for growth or regeneration. Research was initiated with the aim to find factors that regulate plasticity in an otherwise rigid CNS. A group at the University of Utrecht, led by W.H. Gispen, conducted a line of research that was lying at the heart of these endeavors. They discovered a protein (B-50) that plays a role in membrane modulation and was enriched in growth cones, yet still expressed in the CNS at low quantities and in a location specific manner (Jolles et al., 1980). The same protein was independently characterized in other laboratories and would become best known under the name GAP-43 (Benowitz and Routtenberg, 1997). When B-50/GAP-43 was overexpressed the neurons’ growth potential was enhanced (Aigner et al., 1995; Holtmaat et al., 1995), and when the gene was mutated axon growth was stalled at particular places in the CNS during development (Strittmatter et al., 1995). Around the same time many other plasticity proteins were discovered and characterized, setting the stage for an era in which intrinsic neuronal growth mechanisms were meticulously characterized, linked to synaptic plasticity and subsequently to learning. The direct extracellular environment of neurons turned out to be important too. For example, myelin-components and some extracellular matrix molecules were shown to exhibit potent growth inhibitory and plasticity regulating activity in the adult brain (Schwab, 1996). More recently, it was shown that developmental axon growth repellents are continuously expressed in the adult central nervous system, and may thereby contribute to the CNS axons’ failure to regenerate (Giger et al., 1998; Pasterkamp et al., 1998). They may also be involved in synaptic plasticity (Pasterkamp and Giger, 2009). The list of developmentally important growth factors that continue to play a role in adult CNS plasticity keeps growing. Altogether, the cloning and characterization of growth and guidance proteins embodied a new trend in the thinking about neuronal plasticity: perhaps the growth-related molecules that are abundant during development continue to modulate plasticity in the adult brain, albeit in a more subtle way. Experiments in laboratory animals confirmed that plasticity and regeneration might represent replays of events that normally occur abundantly during development (Aubert et al., 1995).

Clinical data too started to point towards quite robust types of plasticity within the adult CNS. Improved imaging techniques and electrophysiological measurements indicated that functional adaptations occur in patients and experimental animals when relatively small parts of the brain were damaged (Ward and Cohen, 2004). This work showed that compensatory responses and ectopic brain region activation occur quite soon after the trauma and during the recovery or rehabilitation. Thus, when the damage is relatively mild, adaptations do occur in the CNS, even in the absence of large-scale regeneration. How are adaptations brought about without the regeneration of damaged neurons? It is plausible that parts of the neuronal network adjust to the loss of function elsewhere by altering the gain and offset of their synapses, thereby allowing information to be routed through pre-established yet previously silent synaptic connections. However, it is also possible that vacated synaptic space—due to the dieback of degenerating axonal projections, is reconstituted by surviving neurons that have axons in the vicinity. In this case, axons and dendrites that were not connected before the trauma would now become connected, for example through the growth of dendritic spines or axonal boutons, or perhaps through axonal or dendritic sprouting (Jones, 2000).

Thus, adult brain plasticity may encompass more than just changes in synaptic strength. Based on post-mortem analysis of experimental brain tissue, a plethora of studies has concluded that synaptic structures change, and might even appear and disappear after alterations in experience and during learning. For example, dendritic spine densities increase in enriched environments, after local brain damage and in learning paradigms (Holtmaat and Svoboda, 2009). Some of these in situ experiments have also provided evidence for large scale remodeling of axons and dendrites. The outcome of those studies has been highly variable. First, this is due to the capriciousness of in situ labeling techniques, and second because the cell types that were analyzed were often not homogeneously distributed and therefore not comparable between studies or even between groups within studies. Third, these studies could only analyze quantities and shape difference between groups and did not readily capture dynamics, i.e. fluctuations of shape and synapse numbers over time. Longitudinal optical imaging studies of neuronal structures in vivo have greatly increased and detailed our insights into the processes of structural synaptic plasticity (Fu and Zuo, 2011; Holtmaat and Svoboda, 2009; Lichtman and Fraser, 2001; Purves and Hadley, 1985).

2. The in vivo imaging toolbox

2.1. 2-Photon laser scanning microscopy

A turning point for studying functional and structural plasticity at the cellular level in the live mouse brain came with the development of 2-photon laser scanning microscopy (2PLSM) (Denk et al., 1990) combined with transgenic mice expressing high levels of fluorescent proteins in neuronal subpopulations (De Paola et al., 2003; Feng et al., 2000). 2PLSM, which is based on long-wavelength excitation in a diffraction-limited focal spot, allows imaging in vivo at intermediate depths (up to ~500 μm into the brain) with a reduced risk for photo-toxicity (Helmchen and Denk, 2005; Kerr and Denk, 2008; Svoboda and Yasuda, 2006; Zipfel et al., 2003). Optical imaging of superficial structures in vivo can be achieved either through thinned skull or through a cranial window (Fig. 1). Both methods have distinct advantages and disadvantages (for a comparison between the two types of preparations, see (Holtmaat et al., 2009; Holtmaat and Svoboda, 2009)). The cranial window technique has the advantage that it allows frequent imaging of large cortical areas, and it provides temporary access for infections of viruses or dyes. The brain surface vasculature can be used as a guide to find back axons and dendrites for longitudinal imaging (Fig. 1). This technique can also be used to image fluorescent axons in the spinal cord (Farrar...

Fig. 1. High-resolution two-photon laser scanning microscopy of cortical neurons. (A) Schematic of the preparation (based on a plate from the Paxinos mouse brain atlas). A small piece of the skull is removed and replaced by a small cranial window. The dura remains intact. In this example, the window is placed above the somatosensory cortex representing the mouse’s mystacial vibrissae. (B) The cranial window provides long-term optical access to the cortex. The brain’s superficial vasculature can be used as landmarks to locate regions of interest in long-term imaging experiments (e.g., compare arrowheads in (B) and (C)). (C) 2PLSM images of the vasculature and neuronal structures. The vascular system can be visualized using an injection of fluorescent dextran in the tail vein. In this example, the dye was injected in a transgenic mouse expressing GFP (GFP-M line (Feng et al., 2000)). The red and green fluorescent molecules are both excited at the same wavelength (another distinct advantage of 2PLSM) and recorded in two channels using dichroic filters. (D) The dendrite (in the green channel) is seen from the top in a maximum intensity projection of a stack of 125 focal planes. Dendritic spines (yellow arrow) and axonal boutons (green arrow) can be resolved at high magnifications. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2012; Kerschensteiner et al., 2005). Long-term optical imaging in vivo has painted a picture of ongoing structural dynamics in the adult cerebral neocortex (e.g., Grutzendler et al., 2002; Majewska et al., 2006; Trachtenberg et al., 2002), cerebellum (e.g., Nishiyama et al., 2007) and olfactory bulb (e.g., Mizrahi and Katz, 2003). More recently, deep brain imaging techniques, using endoscopes (Wilt et al., 2009), regenerative amplification of laser pulses (Mittmann et al., 2011), or 3-photon excitation (Horton et al., 2013) have emerged. These will ultimately allow the assessment of structural dynamics in deeper brain areas, such as deep cortical layers and the hippocampus (Barretto and Schnitzer, 2012). Miniaturized portable microscopes, and fiber optics now allow imaging in awake and behaving animals (Helmchen et al., 2001; Wilt et al., 2009). Some studies have indicated that super resolution imaging techniques, such as 2P-STED, can also be applied to study dynamics of microstructures (Bethge et al., 2013; Takasaki et al., 2013). In short, the toolbox for longitudinal imaging in vivo continues to expand and is expected to provide us with an increasingly detailed picture of the structure and function relationships in various regions of the brain.

2.2. Expression of fluorescent proteins in vivo

Transgenic mice in which GFP or spectral variants thereof are expressed under the Thy-1 promoter were the first to be utilized for in vivo imaging and have set the standard for the potential of this technique. These mice are exquisitely suitable for 2PLSM since the GFP expression levels are high, albeit in a small number of neurons (De Paola et al., 2003; Feng et al., 2000). The cause of the sparse, Golgi-like, labeling of neurons in these mice is not well understood, but it proved to be key to their success. The sparse patterns result in low background fluorescence and distinct and identifiable structures. This allows high-resolution imaging (close to the point spread function of the diffraction-limited spot of the microscope). In addition, GFP variants have a relatively large 2-photon crosssection and are resistant to bleaching, hence scanning can be relatively slow and repeated over relatively short time scales without causing photo-toxicity (Tsien, 1998). The most popular transgenic lines include GFP-M, YFP-H, YFP-S and YFP-G expressing cytosolic fluorescent proteins (Feng et al., 2000), and mGFP-L15 and mGFP-L21 expressing membrane-tethered GFP (De Paola et al., 2003).

The disadvantage of the transgenic mice mentioned above is the low level of control over the temporal and spatial expression patterns. The expression in some transgenic lines is too dense to allow imaging and analysis of single cell structures. Yet in other lines the expression is very sparse, making it hard to predict were fluorescent cells will be found. Labeling may also comprise various cell subtypes; hence the variability in some parameters may be high in some imaging experiments (e.g., compare spine densities and dendritic spine turnover rates between studies using densely expressing YFP-H mice and sparsely expressing GFP-M mice (Fu and Zuo, 2011; Holtmaat and Svoboda, 2009)). Recently, the Allen Institute for Brain Science (http://www.alleninstitute.org) has provided the neuroscience community with several new knock-in mouse lines that express fluorescent proteins at high enough levels for imaging in vivo (Madisen et al., 2012). In these mice, an amplified expression cassette within the ROSA locus drives fluorescent protein expression, which is dependent on the Cre-mediated excision of a STOP-cassette flanked by loxP sites.
(hereafter referred to as ROSA-LSL). These lines can be crossbred with Cre-recombinase-expressing lines to render cell-type specific labeling (Fig. 2). The GENSAT Program at the Rockefeller University (www.gensat.org) provides BAC-transgenic mouse lines that express Cre-recombinase or fluorescent proteins in cell-type specific manners.

Targeted expression of fluorescent proteins can also be obtained using recombinant viral vectors. The most useful vectors, currently, are lentiviral (LV) vectors and adeno-associated viral (AAV) vectors. The latter has become the vector of choice for many studies that aim at long-term expression and longitudinal imaging in vivo. AAV has a limited packaging size, but in most cases this suffices to harbor the coding region of GFP or its spectral variants. Even genetically encoded biosensors can be expressed (see below). Expression usually takes a few weeks to sufficiently build up, but once plateau expression levels are reached they remains stable for many months (Tenenbaum et al., 2004). AAV-mediated or LV-mediated expression levels are generally high enough (depending on the promoter) to allow 2PLSM in the cortex, cerebellum or olfactory bulb in vivo (Mizrahi, 2007; Nishiyama et al., 2007; Stettler et al., 2006). Cre-recombinase-bearing viral vectors can be used in combination with the ROSA-LSL-GFP lines (see above) to spatially and temporally control the expression. Viral vectors also allow expression and imaging in other species than rodents (e.g., zebra finches (Roberts et al., 2010) and monkeys (Stettler et al., 2006)).

In utero electroporation is another, relatively straightforward technique to drive expression of recombinant proteins in vivo (Fig. 2). ‘Naked’ expression vectors are injected in the lateral ventricle of embryos, varying in age from E12 to E18. Injections are performed through the uterine wall, the skull and the cortex, after which a few pulses of a strong electrical field are applied using a set of electrodes. Primordial neurons within the ventricle wall and within the electrical field take up plasmids through a process that is not well understood, but likely to be equivalent to mechanisms of electroporation and chemical transfection in vitro. Although the vectors remain episomal, expression levels are relatively stable. For studies in cortex the advantage of this technique is that expression can be targeted to a layer depending on the age of the embryo at the time of injection. For example, L2/3 cells are ‘born’ at around E16, and therefore electroporation at this age will predominantly target cells that end up as pyramidal cells in L2/3 (Fig. 2).

More precise targeting of cells can be obtained through single cell electroporation (Kitamura et al., 2008). Our own lab has recently optimized this technique for long-term imaging in vivo.

2.3. Fluorescent probes

Proteins such as GFP lend themselves very well for cytosolic labeling. The protein readily diffuses from the cell body into dendrites and the axoplasm. Membrane-tethered GFP has also been used. This has proved to be most useful for revealing...
structures that have a relatively large surface-to-volume ratio such as axons, and small dendritic compartments such as spines (De Paola et al., 2006; Portera-Cailliau et al., 2005). To specifically label neuronal microstructures in vivo, cell-compartment-specific proteins have been tagged with GFP or variants. These methods have been used for a long time in organotypic slice cultures and have now made their way into in vivo imaging studies. For example, GFP-tagged PSD-95 has been used to label excitatory post-synaptic densities (Fig. 3) (Gray et al., 2006); gephyrin-GFP for inhibitory post-synaptic clusters (Chen et al., 2012; Van Versendaal et al., 2012); CamKII-GFP and GluR-SEP (glutamate receptors tagged with pH sensitive GFP that allow exclusive visualization of surface receptors), possibly mark regions with synaptic activity (M.C. and A.H., unpublished observations) (Makino and Malinow, 2011; Mower et al., 2011). Synaptophysin-GFP labels transmitter vesicle-containing postsynaptic components (Holtmaat and Svoboda, 2009). Actin-GFP can provide insights into cytoskeletal dynamics of post-synaptic compartments, such as spines (Matus, 2000). Thy-1-mito-CFP-S transgenic mice have been used to visualize mitochondria in vivo (Misseld et al., 2007). In all cases, the auxiliary expressed proteins join or substitute the endogenous proteins. This includes a risk. In order to sufficiently visualize these microstructures transgene expression may need to be driven at high levels, which possibly induce dominant-positive effects. To avoid as much as possible the disturbance of the cell’s physiology it is best to keep expression levels relatively low and to avoid expression during developmental stages during which neurons massively form and prune synapses.

Another upcoming application for 2PLSM in vivo is biosensor-based optophysiology (Knopfel et al., 2006), i.e. the assessment of cellular function through optical microscopy. Among the most popular proteins for in vivo imaging of neuronal activity are the genetically encoded Ca$^{2+}$ indicators (GECI) (Grienberger and Konnerth, 2012). These are often based on compound proteins in which a GFP-like fluorophore is linked to a Ca$^{2+}$ sensitive biomolecule, such as the calmodulin–M13 complex. The binding of Ca$^{2+}$ tightens the interaction between calmodulin and M13, which in turn leads to conformational changes in the fluorophore–calmodulin complex. In single-fluorophore based sensors (e.g., GCaMP (Akerboom et al., 2012)) the binding of Ca$^{2+}$ forces the conformation of GFP into a higher fluorescence emitting state. In dual fluorophore-based sensors (e.g., Yellow Cameleon [YC] (Nagai et al., 2004)), the conformational change brings together two different fluorophores, close enough for the Förster resonance energy transfer (FRET). The signal is generated through the enhanced fluorescence of the FRET acceptor (venus in case of YC). Another popular FRET-based Ca$^{2+}$ sensor is TN-XXL, consisting of the Ca$^{2+}$ binding protein Troponin C fused to GFP and Citrin (Mank et al., 2008). GECIs are used to detect action potentials at neuronal somata or axon terminals (Grienberger and Konnerth, 2012). They can also detect dendritic Ca$^{2+}$ spikes and even single synapse Ca$^{2+}$ entry through NMDA receptors or from intracellular stores. However, not all of the electric activity in neurons involves large Ca$^{2+}$ responses. A more direct way to sense activity changes in neurons can be achieved using voltage sensitive proteins (Dimitrov et al., 2007; Fink et al., 2012). Unfortunately, cells do not generally tolerate chronic expression of these proteins very well; hence we are still awaiting their application for long-term imaging in vivo. Direct imaging of synaptic activity is possibly achieved using genetically encoded glutamate sniffers (Marvin et al., 2013).

Thus, optophysiology is an exquisite tool to longitudinally probe neuronal network function during changes in experience and learning, and will ultimately provide deeper insights into the relation between functional and structural synaptic plasticity. It can be used in head-fixed animals that navigate in a virtual world or are trained to engage in a behavioral task for which head movements are not required. Portable microscopes and fiber optics allow the application of optophysiology in freely behaving animals.

3. Longitudinal imaging of synaptic plasticity

3.1. Dendrite and axon stability

Classic Golgi studies have indicated various types of structural plasticity: large-scale morphological changes, such as neurite sprouting or pruning, and small-scale changes, such as the addition and pruning of synaptic structures, or synaptic shape changes. However, from longitudinal imaging experiments in vivo it became clear that large-scale structural dynamics are not abundant in the upper layers of the cortex and cerebellum of naïve animals. Dendrites almost never grow or lose branches, and even length changes of dendrite endings are minor in adults (Chow et al., 2009; Holtmaat and Svoboda, 2009; Trachtenberg et al., 2002). Axon endings are slightly more dynamic (De Paola et al., 2006). However, in naïve mice the growth and retraction amount only to a few percent of the total length of the axon terminal arbor. The large-scale dynamics of dendrites and axons upon learning or...
changes in experience have only recently begun to be studied in a quantitative fashion. There are indications that supragranular cortical axons sprout upon peripheral sensory deprivation (Mark et al., 2010). In contrast, dendrites remain largely stable, even after permanent peripheral nerve deafferentation (Schubert et al., 2011), and stroke (Mostany and Portera-Cailliau, 2011).

3.2. Dendritic spine and axonal bouton plasticity

In cortical pyramidal cells the majority of excitatory synapses are found on dendritic spines. Therefore, dendritic spines have been used as proxies for synapses in imaging studies in vivo. The appearance and disappearance of spines are assumed to reflect synapse formation and pruning respectively. This relationship was confirmed using retrospective serial section electron microscopy (SSEM, see below). From cell filling and Golgi studies it was concluded that synapses may appear and disappear in adulthood. In vivo imaging studies have nuanced and enriched this view: synapses may appear and disappear in a homeostatic way, leaving the total number of synapses unchanged. Such subtle changes could have never been detected using static measurements. Similarly, axonal boutons, the presynaptic elements in various types of synaptic connections continuously turn over in the naïve brain. Spines can be classified in at least two dynamic groups: transient and stable spines (Holtmaat et al., 2005). Transient spines generally live for only a couple of days, whereas stable spines are often persistent for many months and sometimes even for the lifetime of the animal (Holtmaat et al., 2005; Yang et al., 2009). Not surprisingly, dendritic spine and axonal bouton turnover is highest during cortical development. In neonates and young adults, spines and boutons are short-lived, and new spines increasingly stabilize, contributing to a growing pool of long-lived dendritic spines. This process continues far into adulthood (Holtmaat et al., 2005; Mostany et al., 2013; Zuo et al., 2005). Surprisingly, a recent study found that in old adult mice dendritic spine and axonal bouton turnover starts to increase with age (Mostany et al., 2013). Although spines readily stabilize in those mice, they generally do not live as long as in midlife adults. This suggests that the synaptic network becomes slightly destabilized in old mice, a phenomenon that could be linked to a reduction in the capacity to learn and store long-term memories during aging.

Spines come in various forms. Large, mushroom-like spines are most stable, and have been shown to always carry synapses and often a spine apparatus. Small, thin spines (including filopodia-like structures) are most dynamic. They are often transient, with life times in the order of days to weeks. Small spines can be precursors of big spines, with their transformation leading to increased stability. A large fraction of small and thin spines often do not bear a synapse immediately after they are formed (Knott et al., 2006). However, upon stabilization they always bear synapses. It is unclear what the function is of transient spines. They may simply act as intermediate (silent) synaptic structures, probing putative presynaptic partners and generate a long-lived connection upon long term potentiation (LTP)-like strengthening and growth of its synapse. They could also have a more distinct role in synaptic network function, by transiently mediating connectivity changes that may be needed to support short-lived memories or short-term adaptations to new inputs.

3.3. Imaging of synapse formation

In most imaging studies synapse formation and pruning is inferred from the appearance and disappearance of proxies, such as spines and boutons. However, this provides an incomplete picture. First, synapses come in various sizes and their size may be dynamic; and second, synapses also occur on dendritic shafts and small axonal varicosities, which are not easily tracked by virtue of structure alone. This is especially a problem for many inhibitory neurons, whose morphology is smooth, with most synapses occurring on the dendritic shaft. The direct probing of synapses using fluorescently tagged synaptic proteins can circumvent this problem. Two of such probes that have successfully been used in vivo are PSD-95-GFP (Cane et al., 2011; Gray et al., 2006) and gephyrin-GFP (Chen et al., 2012; van Versendaal et al., 2012) to label excitatory and inhibitory synapses respectively. The stability of synaptic structures can be tested using photo-activatable GFP (paGFP) tags (Fig. 3). paGFP is ‘dark’ in its native form, but becomes green fluorescent upon photo-activation. The PSD-95 content of single synapses can be activated using 2PLSM, and their diffusion or trafficking can be monitored (Gray et al., 2006). The retention dynamics of PSD-95 may be measures of synapse size and activity.

3.4. High-resolution reconstruction of structures imaged in vivo

Synapse formation can also be assessed using retrospective SSEM (Knott et al., 2009) or array tomography (Micheva and Smith, 2007). In the EM method, a previously imaged dendrite or axon is labeled in the perfusion-fixed brain using antibodies (e.g., anti-GFP) and an electron dense product (e.g., DAB). Regions of interest are then identified and embedded in resin. Serial ultrathin sections are collected and photographed in an electron microscope. Labeled structures can be reconstructed from the serial sections. Retrospective SSEM has been successfully used to investigate the relationship between spine maturation and synaptogenesis (Knott et al., 2006). The ultrastructure profiles of spines of different ages that were tracked in vivo were compared. Young spines often did not have a detectable synapse, whereas spines of a few days old always did. These results can now be confirmed using imaging of PSD-95-GFP (see above). Those experiments indicate that new thin spines do sometimes transiently form synapses before they disappear (Fig. 3). Recently, the laborious technique of serial sectioning has been replaced by block face scanning microscopy (Denk and Horstmann, 2004; Knott et al., 2008). Using focused ion beam milling, optical sections can be even thinner and previously imaged neuronal structures can be resolved without the need for electron-dense labeling of fluorescent proteins (Maco et al., 2013).

3.5. Experience-dependent structural synaptic plasticity

The dynamism within the synaptic network may be a basis for experience and learning-dependent remodeling of network function (Caroni et al., 2012). Indeed, imaging experiments in vivo have shown that somatosensory deprivation (Holtmaat et al., 2006; Trachtenberg et al., 2002), visual deprivation (Hofer et al., 2009; Keck et al., 2008), motor learning (Fu et al., 2012; Xu et al., 2009; Yang et al., 2009), and fear conditioning (Lai et al., 2012) evoke robust changes in spine turnover, often without or only transiently changing spine densities. The formation and pruning of synapses are balanced. New spines are increasingly stabilized at the expense of some old spines that are pruned. New spine formation often occurs in clusters (Fu et al., 2012; Lai et al., 2012), suggesting that, upon learning, particular circuits are preferably selected as pre-synaptic partners. Retrospective SSEM showed that, upon whisker deprivation, many new spines contact axonal boutons that already maintained synapses with other dendrites (Knott et al., 2006). This is atypical since most boutons and spines usually maintain only one excitatory synapse. Thus, the formation of multi-synaptic boutons may be a characteristic intermediate of synapse stabilization, i.e. one or more synapses will be pruned in due time; or they

occur because spine stabilization preferentially happens on axons that have a high incidence of multi-synaptic boutons.

Thus far, our understanding of the relationship between structural changes and functional network alterations is only circumstantial. We do not know exactly what is the effect of a new spine, simply because imaging studies do not reveal the presynaptic input that impinges on this spine nor do they assess how and when the synapse on a new spine is recruited relative to network function. The same holds true for the formation and pruning of axonal boutons. Transsynaptic tracing or retrospective EM will need to be combined with long-term imaging and optophysiology to better probe the relationship between structural changes in the synaptic network and alterations in network activity. Some of our own work suggests that synaptic LTP influence structural plasticity (Fig. 4). For example, sensory deprivation has been shown to cause disinhibition, which in turn facilitates spike-timing dependent LTP (Gambino and Holtmaat, 2012). In vitro studies have indicated that LTP increases the size of synapses, and even promotes the formation of new synapses (De Roo et al., 2008; Harvey and Svoboda, 2007). The subsequent stabilization of new synapses may also depend on LTP-like processes (Wilbrecht et al., 2010). Thus, once the input/output balance of a network has changed, metaplasticity events (such as disinhibition) may evoke a cascade of structural and functional changes. If the events continue over long times, e.g. upon long-term sensory deprivations or CNS lesions, the interplay between synaptic potentiation and synapse formation may lead to

configuration changes in the neuronal network. Some studies have started to assess long-term changes in network function using GECIs (e.g., Margolis et al., 2012). Such studies combined with the imaging of structure may confirm that the functional-structural synaptic plasticity cascade (Fig. 4) is at play upon learning and changes in experience.

3.6. Imaging of neuronal degeneration and regeneration

Long-term 2PLSM in vivo is starting to provide insights into the process of neurodegeneration and regeneration after neuronal damage and in models for neurodegenerative diseases. Axons or dendrites can be damaged using the 2-photon focal volume to locally cause photo-toxicity with microprecision in fluorescent structures (e.g., Allegra Mascaro et al., 2010; Sacconi et al., 2007) (Fig. 5). This work has started to reveal that axons and dendrites in the CNS do not readily regenerate, even when their surrounding is largely free of scar tissue. We have experimented with this technique to transsect dendrites and axons or to ablate single spines (Fig. 5). These structures remain remarkably stable, even over long times after the transsection. Interestingly, single spine ablation is incidentally followed by increased new spine formation in the proximity of the ablated spine. Axon and dendrite restructuring has also been studied in the context of larger, more clinically relevant lesions such as stroke (Brown et al., 2007; Mostany and Portera-Cailliau, 2011) and spinal cord injury (Kerschensteiner et al., 2005). These studies have shown

Figure 4. Schematic of the interplay between functional synaptic plasticity and structural synaptic network changes. Deprivation-mediated disinhibition (1) facilitates spike-timing-dependent LTP (2), which strengthens synapses (3) (Gambino and Holtmaat, 2012). The continuous interplay between these processes may lead to synapse growth (4) and new synapse formation (5) (Holtmaat et al., 2006; Wilbrecht et al., 2010). Upon long-term sensory deprivation disinhibition may become structural (6) (Chen et al., 2012; van Versendaal et al., 2012), which continues to drive the processes above (2)–(5). Ultimately, this may lead to formation of synaptic connections with neurons that were not initially part of the network (7), and recruitment of their activity (8).

Figure 5. Laser-mediated lesions of single dendrites and axons. (A) Low magnification of a dendritic arbor in a GFP-M mouse. (B) A dendrite was cut using the pulsed laser light of the 2PLSM. Shortly after the transsection the severed distal dendrite tip disappears and the proximal part slightly retracts. In this example the dendrite does not display any growth over weeks. (C) The transection of an axon results in similar phenomena as the cutting of a dendrite. These experiments demonstrate that damaged axons and dendrites in the CNS do not readily regrow. (D) Using similar techniques as in (B) and (C), single spines can be ablated (arrow). The vacated space on the dendrite remains empty over the first day. However, in the vicinity of the ablation site new spines are formed, suggesting that the dendrite is attempting to reconstitute its synaptic inputs.

once again that dendrites are remarkably stable. Though, the spines on dendrites that are spared from the ischemic damage follow the increased stabilization of new spines, suggesting that synaptic circuits structurally adapt to the loss of function. Studies in models for Alzheimer’s disease (Spires-Jones et al., 2007; Tsai et al., 2004), prion disease (Fuhrmann et al., 2007) and fragile X syndrome (Cruz-Martín et al., 2010; Pan et al., 2010) have painted a similar picture: remodeling of circuits occurs locally through the turnover of presynaptic and postsynaptic elements. Spine dynamics often increase in these models, leading to a decrease in spine stability. Our review has almost exclusively focused on structural plasticity of synapses. However, it has become evident that non-neuronal cells display forms of plasticity or might be involved in the modulation of synaptic activity (Wang et al., 2006). Giall cells also play an important role in neuroinflammatory responses (Davalos et al., 2005; Ding et al., 2009; Nimmerjahn et al., 2005). Thus, to fully understand the synaptopathies in diseases it will be important to image the interplay between glial cells and structural remodeling (Misgeld and Kerschensteiner, 2006; Zhuo et al., 1997).

4. Conclusions

Studies of adult brain plasticity have come a long way since the formulation of the neuron doctrine. From the initial purification of the proteins that may be involved in learning and neuronal growth, and the subsequent characterization of those proteins in cell cultures and finally in transgenic mice, we have entered an era in which we can monitor synaptic plasticity in real time in awake and behaving animals.

Those studies have expanded our view of the mechanisms behind brain plasticity and learning. They have indicated that synapses are continuously formed and pruned, even in old adults. This dynamic process is modulated by changes in experience or learning. The formation and subsequent stabilization of new synaptic contacts likely optimize neuronal network function to support the execution of new programs or store new information. This knowledge has provided a framework for more detailed studies of plasticity gene function. It has become possible to study the consequences of the elevation or knockdown of ‘plasticity’ protein expression patterns for ongoing structural synaptic plasticity, (e.g., Akbik et al., 2013; Chow et al., 2009) and to relate these to learning and memory formation. Yet, there is still a long way ahead of us. The currently available microscopy techniques have only provided a limited high-resolution access to superficial parts of the rodent brain. Recently developed deep brain imaging techniques will be needed to complete and extend our understanding of brain plasticity towards other, perhaps more primal brain areas. In addition, a full understanding of the relationship between basic neuroanatomical changes and functional brain plasticity is still lacking. Real-time imaging studies will be needed in which functional plasticity measurements and animal behavioral readouts are combined with the assessment of structural dynamics within the same animal, and correlated over time and space. Once we have a full understanding of plasticity protein function in ongoing and evoked plasticity, we may be able to employ them to increase plasticity after brain trauma such as stroke, in neurodegenerative disorders such as Alzheimer’s, and in spinal cord injury.

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References


ImageJ threshold macro

dir1 = getDirectory("Choose Source Directory");
dir2 = getDirectory("Choose Destination Directory");
list = getFileList(dir1);
//setBatchMode(true);
for (j=0; j<list.length; j++) {
    showProgress(j+1, list.length);
    filename = dir1 + list[j];
    if (endsWith(filename, "max.tif")) {
        
    }
    else if (endsWith(filename, "tif")){
        open(filename);
        stackname=getTitle();
        run("DeInterleave ", "number=2");
        selectWindow(stackname+" #0");
        n = nSlices;
        run("Duplicate ...",
        "title=[MAX_mc099d003.tif #0-1]
duplicate range=1-n");
        run("Gaussian Blur ...",
        "sigma=2 stack");
        run("Align Slices in Stack",
        "transformation=[Rigid Body]");
    }
run("Z Project ...", "start=1 stop=n
projection=[Max Intensity ]");
setMinAndMax(0, 50);
getRawStatistics(nPix, mean, min,
max, std, hist);
maxthreshold=max;
setTool("freeline ");
waitForUser("Make selection ");
getSelectionCoordinates(x_, y_);
means=newArray(x_.length);
stds=newArray(x_.length);
for (i=0;i<x_.length;i++) {
    xvalue=x_[i];
yvalue=y_[i];
run("Specify ...",
"width=10 height=10
x=xvalue y=yvalue slice=1
oval centered ");
getRawStatistics(nPix,
mean, min, max,
std, hist);
means[i]=mean;
stds[i]=std;
}

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Array.getStatistics(means, min, max, mean, std);
finalmean=mean;

Array.getStatistics(stds, min, max, mean, std);
finalstd=mean;

thresholdvalue=finalmean+
(2*finalstd);

setAutoThreshold(
"Default dark stack ");
setThreshold(thresholdvalue, maxthreshold);
run("Convert to Mask");
selectWindow(stackname+#0);
run("Gaussian Blur...", "sigma=2 stack");
setAutoThreshold(
"Default dark stack ");
setThreshold(thresholdvalue, maxthreshold);
run("Convert to Mask", " black");

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saveAs("Tiff",
    dir2+substring(stackname,0,9)+
    "mean2sd ");
run("Close All");
Bibliography


Glazewski Stanislaw, Barth Alison L., Wallace Helen, McKenna Mervyn, Silva Alcâno, Fox Kevin. Impaired Experience-dependent Plasticity in Barrel Cortex of Mice Lacking the Alpha and Delta Isoforms of CREB // Cerebral Cortex. IV 1999. 9, 3. 249–256. PMID: 10355905.


147
Hensch Taka o K. Critical period plasticity in local cortical circuits // Nature


Hofer Sonja B, Mrsic-Flogel Thomas D, Bonhoeffer Tobias, Hübener Mark.

Hofer Sonja B., Mrsic-Flogel Thomas D., Bonhoeffer Tobias, Hubener Mark.


Lemieux Mado, Labrecque Simon, Tardif Christian, Labrie-Dion Étienne, LeBel Éric, Koninck Paul De. Translocation of CaMKII to dendritic mi-

Li Lu, Bender Kevin J., Drew Patrick J., Jadhav Shantanu P., Sylwestrak Emily, Feldman Daniel E. Endocannabinoid signaling is required for development and critical period plasticity of the whisker map in somatosensory cortex // Neuron. XI 2009. 64, 4. 537–549. PMID: 19945395 PMCID: PMC2796273.


Loewenstein Yonatan, Kuras Annerose, Rumpel Simon. Multiplicative Dynamics Underlie the Emergence of the Log-Normal Distribution of Spine


Marik Sally A., Yamahachi Homare, McManus Justin N. J., Szabo Gabor, 


Noguchi Jun, Nagaoka Akira, Watanabe Satoshi, Ellis-Davies Graham C. R, Kitamura Kazuo, Kano Masanobu, Matsuzaki Masanori, Kasai Haruo. In


Pagès Stéphane, Côté Daniel, Koninck Paul De. Optophysiological approach to resolve neuronal action potentials with high spatial and temporal reso


Rao Yan, Fischer Quentin S., Yang Yupeng, McKnight G. Stanley, LaRue Adrienne, Daw Nigel W. Reduced ocular dominance plasticity and long-term potentiation in the developing visual cortex of protein kinase A RI-Ialpha mutant mice // European Journal of Neuroscience. 2004. 20, 3. 837–842.


Zito Karen, Scheuss Volker, Knott Graham, Hill Travis, Svoboda Karel.
