Two-photon imaging of structural plasticity underlying classical eyeblink conditioning in mouse barrel cortex

Dissertation

zur Erlangung des Grades eines
Doktors der Naturwissenschaften

der Mathematisch-Naturwissenschaftlichen Fakultät
und
der Medizinischen Fakultät
der Eberhard-Karls-Universität Tübingen

vorgelegt
von

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aus Memmingen, Deutschland

Juni 2014

Tag der mündlichen Prüfung:		02.12.2014
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Dekan der Medizinischen Fakultät:		Prof. Dr. I. B. Autenrieth
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Danksagung

Ich möchte an dieser Stelle den Menschen danken, die mich während meiner Zeit als Doktorandin auf vielfältige Weise unterstützt haben.

Mein besonderer Dank geht an Herrn Prof. Cornelius Schwarz, der mir die Möglichkeit gegeben hat, dieses spannende Projekt in seiner Arbeitsgruppe durchzuführen. Ich danke ihm für die freundliche und engagierte Betreuung meiner Arbeit. Die anregenden Diskussionen und Hilfestellungen waren von großem Wert für mich.

Weiter danke ich Herrn Prof. Mathias Jucker und Herrn Dr. Marcel Oberländer für ihre beratende Tätigkeit im Advisory Board.

Ich bedanke mich bei dem Deutschen Zentrum für Neurodegenerative Erkrankungen (DZNE) in der Helmholtz-Gemeinschaft für die finanzielle Unterstützung der Doktorarbeit durch ein dreijähriges Stipendium.

Weiter bedanke ich mich besonders bei folgenden Arbeitsgruppenmitgliedern: Ursula Pascht, für die Hilfe bei der Anfertigung histologischer Schnitte und allen organisatorischen Angelegenheiten, Dr. Alia Benali für die gute Zusammenarbeit beim Aufbau des Intrinsic Imaging Setups und bei Dr. Dominik Brugger für seine intensive Unterstützung bei der Programmierung der Auswertungssoftware. Der gesamten Arbeitsgruppe Systems Neurophysiology danke ich für die tolle Arbeitsatmosphäre und den Zusammenhalt auch außerhalb des Labors.

Many thanks to Dr. Angelos Skodras for his invaluable technical help with the two-photon microscope.

Ein besonderer Dank geht an Dr. Jasmin Hefendehl. Sie hat mich auf die Arbeitsgruppe und das Hertie Institut für klinische Hirnforschung aufmerksam gemacht. Sie hat mir die Implantationstechnik gezeigt und war von Anfang an ein wichtiger Diskussionspartner. Aber vor allem ist sie seit über zehn Jahren eine gute Freundin.

Mit ganzem Herzen bedanke ich mich bei meinen Eltern, Dorothea und Helmut Joachimsthaler, bei meiner Tante, Gerda Amman, und meinem Onkel, Walter Amman, die mich uneingeschränkt während meines Studiums und meiner Doktorarbeit unterstützt haben. Abschließend möchte ich meinem Freund Dr. Timo Schuster danken, der mir stets den nötigen Rückhalt und ein liebevolles Zuhause geschenkt hat.

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1. Summary

Sensory maps of the neocortex are constantly updated to adapt the individual to changes in the outside world that require the association of new sets of stimuli to adequate behavior. It is long known that such adaptation involves macroscopic changes of body representations in sensory maps. The emerging technology of two-photon microscopy together with the availability of transgenic mice that express fluorescent proteins in cortical neurons made it possible to monitor the postsynaptic cell compartments in vivo which are affected by experience dependent structural plasticity: dendritic spines.

In the present work I combined classical trace eyeblink conditioning in awake head-fixed mice with two-photon imaging of dendritic spines. Classical conditioning that involves mnemonic processing, i.e. a 'trace' period between conditioned and unconditioned stimulus, requires awareness of the association to be formed, and is considered a simple model paradigm for declarative learning. The whisker representation of primary somatosensory cortex, named barrel cortex, is required for the acquisition of the tactile variant of trace eyeblink conditioning. To obtain insight into the cellular mechanisms underlying memory storage I monitored daily performance levels and plastic spine turn processes in test animals which underwent conditioning and in control animals which underwent pseudo conditioning.

I showed that one cellular expression of barrel cortex plasticity during learning is substantial spine elimination on layer V neurons' apical dendrites in layer I. The number of eliminated spines and their time of elimination were tightly related to the observed learning success. Pseudo conditioned animals on the other hand showed low baseline spine turnover rates. Moreover, I found that spine plasticity induced by

learning was highly specific for the barrel column receiving signals from the stimulated vibrissa - spines located in an adjacent column were unaffected. The fact that layer I spines receive neuronal signals from associative thalamo-cortical and cortico-cortical circuits, together with the finding of column specific spine elimination observed in this study suggests that spine plasticity may arise via an interaction of ascending sensory (therefore spatially precise) and top-down associative signals.

Zusammenfassung

Sensorische Karten des Neokortex werden ständig aktualisiert um das Individuum an Umweltveränderungen, welche die Assoziation neuer Stimuli mit geeigneten Verhaltensweisen erfordert, anzupassen. Es ist lange bekannt, dass eine solche Form der Anpassung mit makroskopischen Veränderungen von Körperrepräsentationen in den sensorischen Karten einhergeht. Die neue Technologie der Zwei-Photonen-Mikroskopie zusammen mit der Verfügbarkeit von transgenen Mäusen, die fluoreszierende Proteine in ihren kortikalen Nervenzellen exprimieren, machte es möglich, die postsynaptischen Zellkompartimente in vivo zu beobachten, die von erfahrungsabhängiger struktureller Plastizität beeinflusst werden: die dendritischen Dornfortsätze.

In der vorliegenden Studie kombinierte ich die klassische Konditionierung des Lidschlussreflexes in nicht narkotisierten, kopffixierten Mäusen mit der Bildgebung von dendritischen Dornfortsätzen mit Hilfe des Zwei-Photonen-Mikroskops. Klassische Konditionierung, die mnemonische Verarbeitung beinhaltet, also eine Gedächtnisspur zwischen konditioniertem und unkonditioniertem Stimulus, erfordert die Erkenntnis über die zu formende Assoziation und wird als einfaches Model deklarativen Lernens

angesehen. Die Vibrissenrepresentation des primären somatosensorischen Kortex, der Barrel Kortex genannt wird, wird für die Aneignung der taktilen Variante der klassischen Konditionierung benötigt. Um Einblicke in die zellulären Wirkmechanismen zu erhalten, die der Gedächtnisbildung zu Grunde liegen, habe ich täglich die Trainingsleistungen und die plastischen Umbauvorgänge der Dornfortsätze sowohl innerhalb einer Testgruppe als auch in einer Kontrollgruppe, die pseudo-konditioniert wurde, verfolgt.

Ich konnte zeigen, dass Barrel-Kortex Plastizität während des Lernvorgangs auf der zellulären Ebene zu einem beträchtlichen Verlust an Dornfortsätzen auf Schicht-V-Nervenzellen, deren apikalen Dendriten sich in Schicht I befinden, führte. Die Anzahl der eliminierten Dornfortsätze und der Zeitpunkt der Beseitigung standen in direktem Zusammenhang mit dem beobachteten Lernerfolg. Pseudo-konditionierte Tiere wiederum zeigten ein geringes Maß an Umbauprozessen wie es im nicht trainierten Tier zu finden war. Ferner konnte ich feststellen, dass lerninduzierte Dornfortsatz-Plastizität hoch spezifisch für die Barrel-Kolumne war, die Signale von der stimulierten Vibrisse erhielt. Dornfortsätze, die sich in der benachbarten Kolumne befanden blieben unbeeinflusst. Die Tatsache, dass Dornfortsätze der kortikalen Schicht I Signale von assoziativen thalamo-kortikalen und kortiko-kortikalen Netzwerken erhalten, deutet zusammen mit der hier gefundenen kolumnen-spezifischen Dornfortsatzeliminierung darauf hin, dass Dornfortsatz-Plastizität durch eine Interaktion aufsteigender, sensorischer (und daher räumlich präziser) und absteigender assoziativer Signale entsteht.

2. Introduction

Every organism needs to adapt to changes in its outside world to survive. Adaptation requires the association of new stimuli to new adequate behavior.

The brain is plastic and can undergo various modifications as a consequence of experience. Highly specialized regions of the brain contain representations of the outside world for each of the sense modalities: the sensory maps. Sensory maps in the cortex are constantly updated to account for changes in the environment. A substantial component of such adaption is the dynamic character of neuronal interconnectivity realized either by changes in synaptic gains or the wiring scheme of local networks. These connection changes are typically referred to as ‘weight changes’ or ‘wiring changes’ (Chklovskii et al., 2004). Physiological alterations in synaptic transmission and their underlying cellular mechanisms known as long-term potentiation (LTP) and long-term depressions (LTD), have extensively been investigated in brain slice preparations (Bear and Malenka, 1994; Feldman et al., 1999; Feldman, 2000). However, in addition to this first expression of plasticity on the synaptic level, interneuronal connections can also be altered as a result of structural plasticity. Here individual connections of local networks are modified, removed or recreated, expressed by morphological changes of axons, axonal boutons, dendrites or dendritic spines.

In the past, numerous deafferentation and lesion experiments were performed in vivo, which induced massive subcortical and cortical reorganization processes in the adult brain. In 1999 Glazewski and Fox, for instance, found, that single whisker deprivation in mice resulted in an enhancement of neuronal responses representing intact whiskers (Glazewski et al., 1999). Barrel representations of intact whisker additionally

showed a notable expansion in size (Diamond et al., 1994). Wallace and Fox also reported the opposite phenomenon: the active shrinkage and the suppression of responses in barrels which received inputs from deprived whiskers (Wallace and Fox, 1999). Deafferentation and lesion experiments can powerfully induce plasticity in various brain regions (Robertson and Irvine, 1989; Gilbert and Wiesel, 1992; Eysel and Schweigart, 1999; Keck et al., 2008; Oberlaender et al., 2012; Glazewski and Fox, 2013), but these rather coarse manipulations of the sensory input do not reflect environmental changes which are relevant for an animal in its daily life. Therefore, several research groups started to investigate the impact of more naturalistic changes in the sensory input and plastic changes of cortical circuits induced by learning. Galvez and colleagues, for example, discovered first in the rabbit (Galvez et al., 2006) and later in the mouse (Galvez et al., 2011) that classical conditioning of eyeblinks using whisker deflection as conditioned stimulus leads to map plasticity in the barrel cortex. They observed a widening of barrel columns which were involved in the processing of the conditioned whisker stimulus; the mean barrel area in the horizontal plane was significantly increased for the trained animal group compared to a pseudo conditioned control group.

The aim of my doctoral study was to address the question, whether the map plasticity observed by Galvez et al. (2011), is also expressed by structural plasticity on the level of dendritic spines. By combining an in vivo imaging technique with a behaviorally relevant learning task, I aimed to examine the extent and time course of structural spine plasticity underlying learning mechanisms in layer I of the mouse barrel cortex.

In the following sections of the introduction I will describe the anatomical substrate for memory storage, the dendritic spine. Furthermore, I will give an overview over experiments performed in the past to investigate experience-dependent structural

synaptic plasticity in the cortex of the rodent brain. After the description of the experimental approach I will present the model system of the barrel cortex. In this context I will explain why the cortical layer I is a very interesting locus to investigate structural plasticity. Finally, I will introduce the classical conditioning paradigm used in the study and summarize the goals of my doctoral work.

2.1 Dendritic spines

Dendritic spines were first described in 1888 by the famous neuroanatomist Santiago Ramón y Cajal (García-López et al., 2007). A spine is a small morphological protrusion emanating from a neuron's dendrite. The size of a spine can range in volume from less than 0.01 μm^3 to 0.8 μm^3 (Harris, 1999). The spine head contains the so-called postsynaptic density (PSD), which appears as an electron-dense, dark area under the electron microscope and comprises receptors, ion channels and adjacent intracellular signalling cascades. Most proteins in the PSD are involved in synaptic transmission and the regulation of synaptic strength. Dendritic spines typically receive input from a single synapse of an axon. They can be found on various neuronal cell types, including pyramidal neurons of the neocortex, medium spiny neurons of the striatum and Purkinje cells of the cerebellum (Rochefort and Konnerth, 2012). The morphology of spines can be highly variable, but typically they are classified into five categories: thin, stubby, mushroom shaped, cup shaped spines and filopodia (Fig.1).

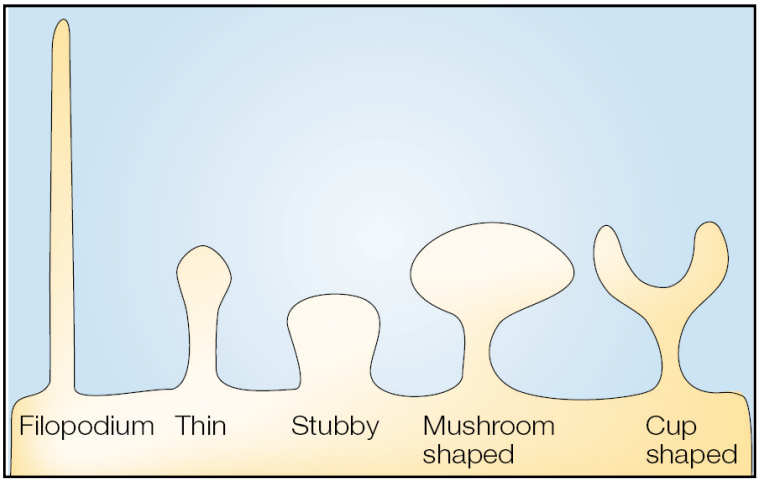

Figure 1: Morphological classification of dendritic spines (Hering and Sheng, 2001).

Filopodia are thin protrusions, which lack a bulbous head and which are often found on dendrites of developing neurons. They sometimes receive synaptic input and they are thought to be precursor structures which can eventually evolve into mature spines (Fiala et al., 1998). The morphology of a spine can undergo many types of transformations on a timescale of seconds to minutes due to a very dynamic actin cytoskeleton. Glutamate uncaging experiments demonstrated, that *de novo* spine growth from the dendritic shaft of cortical layer II/III pyramidal neurons can be induced in a location specific manner within the short time of 20 seconds (Kwon and Sabatini, 2011).

Various studies suggest a close relationship between the spine morphology and its function. AMPA glutamate receptors, for instance, are numerous in mushroom spines, but they are sparsely distributed in thin spines or filopodial structures (Matsuzaki et al., 2001). Furthermore, the narrow spine neck seems to play a physiological key role as it allows the compartmentalization of calcium. Several studies strongly suggest that spine calcium dynamics are likely to be involved in computational tasks (Yuste and Denk, 1995; Koester and Sakmann, 1998; Yuste et al., 2000). A third example in which

morphological features of dendritic spines seem to relate to function was found in glutamate uncaging experiments: long-term potentiation (LTP) correlates with spine enlargement (Matsuzaki et al., 2004).

Dendritic spines are plastic structures the lifespan of which is influenced by input activity (De Roo et al., 2008). In mammals, a net loss of spines occurs toward the end of childhood, due to an increase in spine elimination. Figure 2 shows measurements in brains of healthy human individuals (black) vs. brains affected by some pathological conditions (colored). Different dynamics of spine turnover during development in different cortical areas indicate varying critical periods for specific brain regions (Zuo et al., 2005a). In adulthood, however, net spine elimination slows down and synaptic circuits appear more stable (Nimchinsky et al., 2002; Holtmaat et al., 2005).

Disruptions in dendritic spine number are linked to various brain disorders, especially to those that involve deficits in information processing like autism spectrum disorders (ASD), schizophrenia and Alzheimer's disease (Glantz and Lewis, 2000; Spires-Jones et al., 2007; Hutsler and Zhang, 2010; Penzes et al., 2011). Post-mortem ASD human brain tissue revealed an increase in spine density on apical dendrites of pyramidal neurons in different cortical layers of various brain regions (Hutsler and Zhang, 2010). Spine density was inversely correlated with cognitive function. This finding is consistent with the emerging hypothesis that the brains of individuals with ASD are characterized by hyperconnectivity in local circuits (Geschwind and Levitt, 2007). Spine dysmorphology due to pruning deficits is thought to contribute to abnormalities in specific circuits, which in turn may underlie the socio-cognitive impairments characteristic for ASD. Individuals with schizophrenia, on the other hand, show a profound reduction in spine density, for example in the primary auditory cortex (Sweet et al., 2009) or the hippocampus (Kolomeets et al., 2005). Here, exaggerated spine

pruning during late childhood or adolescence is thought to lead to the emergence of symptoms during these periods (Penzes et al., 2011). In Alzheimer's disease, spines are rapidly lost in late adulthood, suggesting perturbed spine maintenance mechanisms that may underlie cognitive decline. Although the detailed mechanisms that cause spine degeneration in Alzheimer's disease are still mostly indistinct, recent findings show evidence that Aβ oligomers disrupt synaptic plasticity mechanisms and induce spine dysgenesis by interfering with the NMDAR-dependent regulation of the spine cytoskeleton, causing synapse loss and decreased connectivity with nearby axons (Snyder et al., 2005).

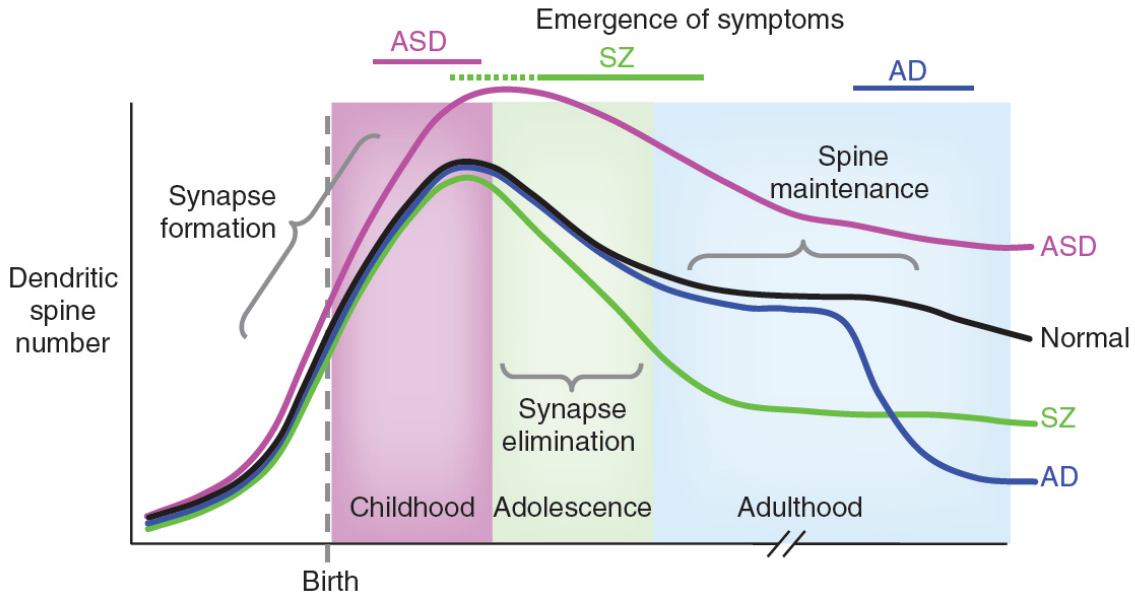

Figure 2: Dendritic spine pathology in neuropsychiatric disorders (ASD=autism spectrum disorders, SZ=schizophrenia, AD=Alzheimer's disease, Penzes et al. 2011).

Plastic changes in spine number and increased spine turnover (STO) also play an important role in the context of epilepsy (Wong, 2005) and stroke (Brown et al., 2007).

Under the latter pathological condition a 5-8-fold increase in spine formation rate can be monitored two weeks after the cerebro-vascular accident.

In the healthy subject structural plasticity occurs predominantly in response to neural activity as demonstrated by electrical stimulation in hippocampal slices: the induction of LTP leads to the appearance of new spines on the postsynaptic dendrite (Engert and Bonhoeffer, 1999), whereas LTD leads to substantial decrease in spine volume (Zhou et al., 2004).

Finally, structural plasticity and changes in dendritic spine turnover can be discovered after new experiences and learning events. Despite the stability in net spine numbers in adults, spines are known to exhibit life-long structural plasticity and therefore they are likely to play a role in learning and memory (Hübener and Bonhoeffer, 2010). The following section shortly describes the history of in vivo experiments conducted in rodents to elucidate the role of structural spine plasticity in the context of experience and learning.

2.2 Experience-dependent structural synaptic plasticity in the neocortex

A decisive feature of a neuronal network is its capacity to exhibit plasticity in response to experience and learning. While some emphasize the diminishing of the brain´s capacity to rewire and point to the relatively stable synaptic contacts over time (Grutzendler et al., 2002), others, using manipulations of sensor or brain function find that plasticity can attain high levels also in the adult animal (Feldman and Brecht, 2005; Keck et al., 2008).

In the extreme case, where massive lesions of afferent neuronal structures are induced, even rearrangement of whole dendritic branches can be found (Hickmott and Steen, 2005). With less severe manipulations, e.g. preventing sensory input to reach the brain (e.g. plucking or trimming of whiskers), changes in the dendritic arborisation are found to be widely absent. Instead, substantial changes in the number and turnover rates of dendritic spines can be detected (Trachtenberg et al., 2002; Wilbrecht et al., 2010; Schubert et al., 2013).

Trachtenberg et al. (2002), one of the pioneering studies investigating the effect of sensory deprivation on synaptic structures of layer V pyramidal neurones in the mouse barrel cortex, reported increased STO rates after a period of 2 - 4 days after chessboard trimming of whiskers. By combining two-photon imaging with electron microscopy the authors showed that newly formed spines in fact bore functional synapses, linking anatomical structure to physiological function.

In sensory deprivation experiments, Zuo and colleagues (2005b) found that long term whisker deprivation in mice prevents the net loss of spines normally occurring during adolescence by reducing the rate of spine elimination rather than increasing spine formation (Zuo et al., 2005b). This study clearly emphasises the important role of experience on synaptic plasticity in primary sensory systems in the adult brain.

Yang and colleagues (2009) chose in some ways the opposite approach - increasing the richness of incoming sensory. They investigated spine plasticity in the barrel cortex of mice while exposing the animals to novel experience in an enriched environment. This opposite modulation resulted in an significant increase in spine formation (Yang et al., 2009).

Deprivation, lesion and enrichment of the sensory input powerfully stimulate the reorganization of the adult brain, but these coarse manipulations are likely to induce gross differences in neural activity between affected and non-affected sensory structures. In contrast, everyday learning requires the individual to differentiate between relevant and irrelevant stimuli which may evoke about the same net amount of neural activity. Also in these situations, structural spine plasticity can be a valid readout and a correlate for learning and memory.

The first studies which monitored plasticity in response to learning investigated spines in the primary motor cortex (M1) (Yang et al., 2009; Xu et al., 2009; Fu et al., 2012). Here, animals acquired specialized motor skills by learning a forelimb reaching task or by practicing to run on an accelerated rotarod. All three studies report an increase in spine formation in the supragranular layers of the motor cortex after motor learning. Two of the studies, in fact, even show specificity of spine location for a certain motor task (Xu et al., 2009; Fu et al., 2012).

In 2012, Lai and colleagues tested the effect of fear conditioning on spine dynamics in the mouse frontal association cortex. After animals reliably showed freezing responses, indicating the association of the sound stimulus with the highly aversive foot shock, a significant increase in spine elimination was observed (Lai et al., 2012). In contrast, fear extinction increased the rate of spine formation. The authors were able to show a high correlation between the spine turnover and behavior. Spine elimination and formation induced by fear conditioning and extinction occurred on the same dendritic branches in a cue- and location-specific manner.

The most recent in vivo studies investigating structural synaptic plasticity as a result of learning were carried out in the primary auditory cortex (Moczulska et al., 2013) and the barrel cortex (Kuhlman et al., 2014). In the first study auditory cued fear

conditioning induced the opposite effect compared to what was reported for the prefrontal cortex in the study of Lai et al. (2012): a small but significant increase in spine formation was observed two hours after conditioning. In the second study initial training to an active, whisker dependent object localization task led to enhanced spine formation suggesting rapid changes in connectivity between sensory cortex and motor centers.

All mentioned studies so far indicate that learning leaves a distinguishable and lasting trace in cortical networks of the adult brain. The aim of this research project was to investigate whether structural spine plasticity in vivo occurs in the primary sensory system of the mouse when the animal is trained to an associative learning paradigm, namely trace eyeblink conditioning. This form of classical conditioning has been used extensively to study neural structures and mechanisms that underlie learning and memory. Prior to the description of the used model system barrel cortex (section 2.4) and the conditioning task (section 2.5), the technical principles of two-photon imaging of labelled dendritic spines in transgenic mice are introduced in the following chapter.

2.3 Two-photon imaging and transgenic mice

Since the early work of Ramón y Cajal, dendritic spines have been studied in fixed tissue using light and electron microscopy (Gray, 1959) or in slice preparations using confocal microscopy (Moser et al., 1994). Unfortunately, these ex vivo approaches do not allow following structural plasticity of individual spines in the same animal over time. A major step towards imaging the living brain was taken by the implementation of two-photon laser scanning microscopy (2PLSM) by Winfried Denk and colleagues in 1990 (Denk et al., 1990). Nowadays, this method is widely used to image structural

and functional properties of cortical neurons on the cellular and sub-cellular level in highly scattering tissue of the living animal (Helmchen and Denk, 2005; Rochefort and Konnerth, 2012).

In this imagining technique a pulsed laser generates a very high local intensity of photons, increasing the probability that two photons of low energy are simultaneously absorbed by a fluorescent molecule (delay < 0.5 fs), which is therefore excited to a higher energetic state (Fig. 3). Subsequently, the excitation results in an emission of one fluorescence photon, typically of higher energy compared to one excitatory photon. Because the probability of two-photon absorption is limited to a very small volume around the focus, photo-damage and photo-toxicity outside the focal plane is strongly reduced compared with standard one-photon microscopy and a high degree of rejection of out-of-focus objects can be attained. The method of two-photon microscopy does also have a second advantage compared to linear microscopy: long wavelength (near infrared) excitation light penetrates deeper into tissue with much less scattering. Fortunately, in most of the tissues endogenous absorbers are greatly absent; photo-toxicity is therefore reduced significantly in comparison to linear microscopy (Svoboda and Block, 1994). The advantages of two-photon microscopy make it possible to optically access cortical tissue of several hundred micrometers depth in the living brain (Mittmann et al., 2011).

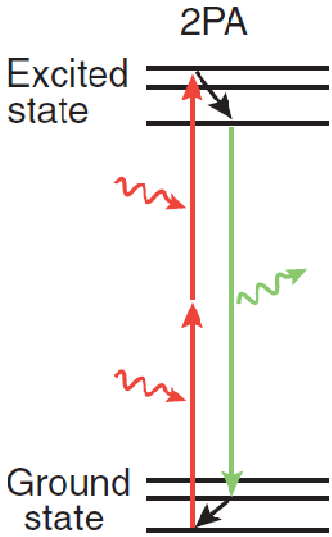

Figure 3: Schematic diagram of the principle of two photon absorption (2PA) (from Helmchen & Denk (2005)).

In order to carry out two-photon imaging experiments in a living system fluorescent molecules must be introduced into the tissue or structure of interest. Neurons and their sub-cellular compartments are typically labelled using the fluorescent protein GFP (green fluorescent protein) or its derivatives like YFP (yellow fluorescent protein). The introduction of these proteins can thereby be accomplished using viral transfection or by using transgenic mouse lines carrying the labelling gene in their genome under a neuron specific promoter. To reliably follow dendritic spine dynamics in vivo over a long experimental period I chose to use the transgenic mouse line YFP-H which expresses the yellow fluorescent protein under the Thy-1 promotor in the membrane of layer V pyramidal neurons (Fig. 4: YFP-H line, Feng et al., 2000). These cells send their apical dendrites to superficial layers of the cortex (see Fig. 4B), where 2PLSM can be performed to tackle dendritic spine plasticity. The applicability of this mouse line for repeated in vivo 2PLSM over months was shown by various former studies

(Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005b; Xu et al., 2009; Lai et al., 2012).

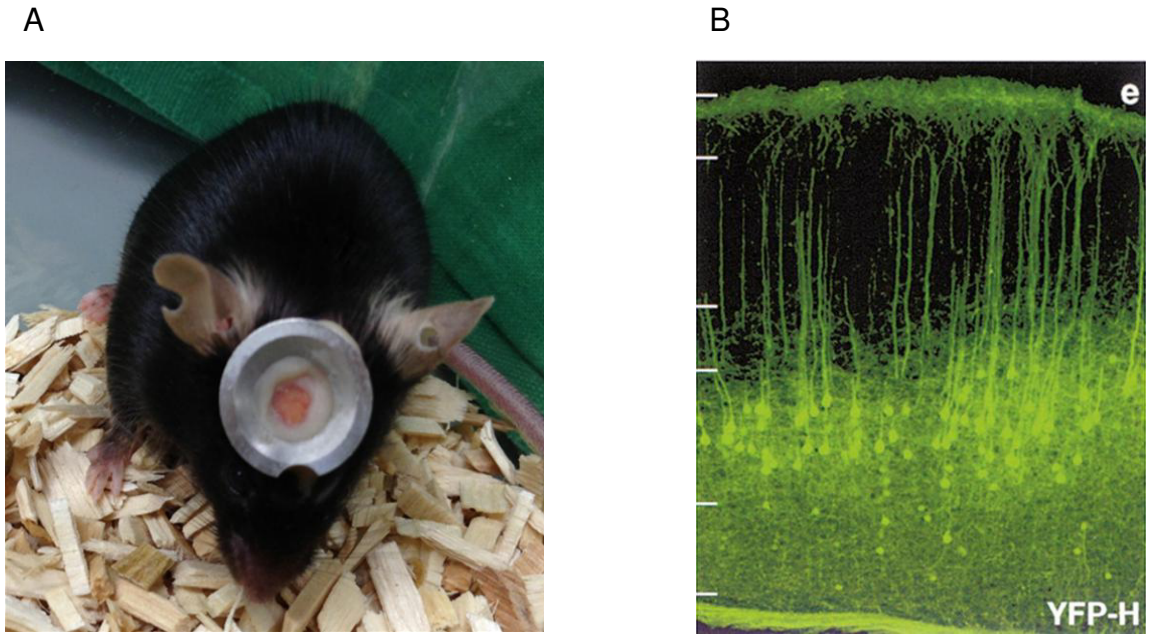

Figure 4: Expression of the yellow fluorescent protein in the cortex of YFP-H transgenic mouse line. A) YFP-H mouse implanted with chronic cranial window over the right barrel cortex. B) YFP expressing pyramidal neurons of layer V sending their apical dendrites to superficial layers (adapted from Feng et al. (2000), lines mark cortical layer borders).

Besides the fact that the largest and most established body of genetic tools is available for the mouse, this easy to handle experimental animal is also steadily trainable on a variety of behavioral tasks.

The following section comments on why I chose the mouse barrel cortex as a model system to study the effects of classical trace eyeblink conditioning on structural synaptic plasticity in vivo. Subsequently, I will argue why the cortical layer I plays an important role in learning and information processing, and therefore is a very interesting locus to investigate structural plasticity.

2.4 The barrel cortex and the importance of layer I

In this study I used the mouse barrel cortex as a model system. Since the first detailed investigations of the barrel cortex (BC) in the 1960s and 1970s (Welker, 1964; Zucker and Welker, 1969; Woolsey and Van der Loos, 1970; Welker, 1971), it has widely gained importance as a model system for map and experience-dependent plasticity. One reason for its attractiveness in research on neocortex is its well-defined and strictly topographical representation of the ca. 28 movable whiskers on the animal's snout, which is preserved throughout the sensory pathway. Tactile information acquired by an individual whisker is sent to the primary somatosensory cortex (S1) via brainstem and thalamus within a precisely defined cortical column of ~ 300 µm width (Feldmeyer et al., 2013). In layer IV of the cortex these columns show a morphological specialization resembling the shape of a barrel (hence the term 'barrel cortex'). The arrangement of whiskers on the snout is precisely matched by the layout of the barrels in S1 (Fig. 5, Aronoff and Petersen, 2008).

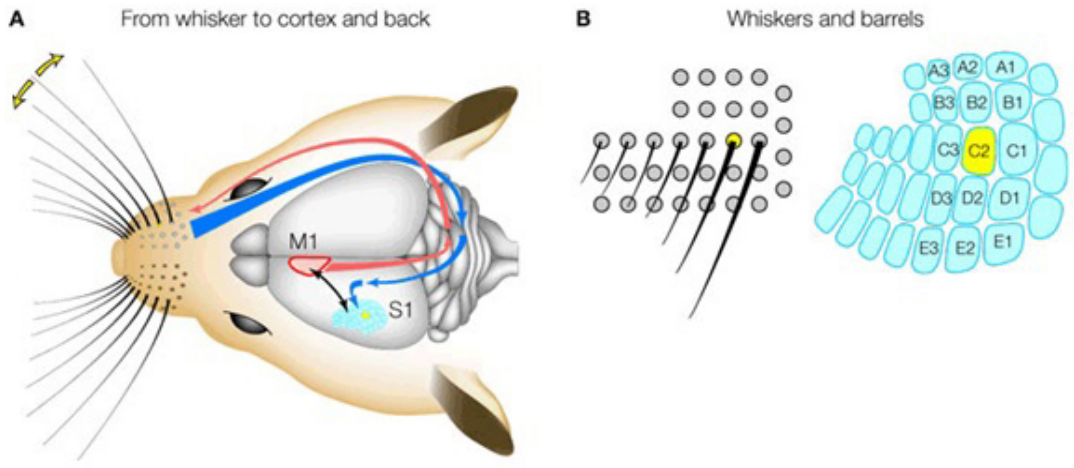

Figure 5: The mouse barrel cortex (adapted from Aronoff and Petersen, 2008).

Another convenient aspect for studying map and experience dependent plasticity is the fact that the sensory input to the system can be readily manipulated. Synaptic plasticity was induced in S1, for example, by simple plucking or trimming of individual whiskers (e.g. Van der Loos and Woolsey, 1973; Fox, 2002; Feldman and Brecht, 2005). Overstimulation of a single whisker and exposure of the animal to an enriched environment, on the other hand, are two additional means to induce plasticity in the barrel cortex (Welker et al., 1992; Polley et al., 2004). Due to the spatial layout of barrels in layer IV structural changes in map plasticity can be easily visualized by standard histochemical procedures (e.g. cytochrome oxidase staining). In 2011 Galvez and colleagues, for example, showed that classical conditioning of the eyelid response using whisker deflection as a conditioned stimulus (CS) results in map plasticity in the mouse barrel cortex. These researchers found a significant increase in size of the conditioned barrel columns using optical density measurements (Galvez et al., 2011).

Another reason for the popularity of the model system barrel cortex is its superficial and easy to access location, allowing the use of in vivo imaging techniques like intrinsic optical imaging (Schubert et al., 2013; Langer et al., 2013), two-photon imaging (Trachtenberg et al., 2002), or electrophysiological techniques (Crochet and Petersen, 2006; Stüttgen and Schwarz, 2008; Sachidhanandam et al., 2013).

The rodent barrel cortex is highly structured in the horizontal as well as in the vertical direction. In the horizontal direction barrels in layer IV comprising a cortical column are separated from each other by septal columns (Alloway, 2008). Due to their ascending input neurons located in a specific barrel column respond predominantly to the stimulation of one specific whisker, the so-called principle whisker. Neurons residing in septal columns, on the other hand, are activated by multiple whiskers (Chapin, 1986). In the vertical direction the barrel cortex is organized in six layers.

The pathway carrying the information of whisker movements from the periphery to the barrel cortex starts in the trigeminal ganglion. The ganglion contains the cell bodies of neurons which send axons towards the whisker follicle and towards the trigeminal nucleus in the brainstem. From the trigeminal nucleus originate four parallel thalamo-cortical pathways. Two 'lemniscal' pathways signal different types of vibrissal information to S1. The first lemniscal pathway is thought to carry mono-whisker information. Here, axons from the ventral posterior medial (VPM) nucleus terminate predominantly in individual layer IV barrels (Petersen, 2007). Cortico-thalamic layer VI neurons in turn provide reciprocal feedback to the VPM. The second lemniscal pathway carries multi-whisker signals via VPM to septal regions in the barrel cortex (Veinante and Deschênes, 1999). The extralemniscal pathway sends multi-whisker signals via VPM to the secondary somatosensory area (S2). Finally, the paralemniscal pathway carries multi-whisker information to septal regions in barrel cortex. Here, axons of the posterior medial (POm) thalamic nucleus target primarily layer I and Va (Feldmeyer et al., 2013). Cortico-thalamic neurons in layer V in turn provide strong input to POm.

What is the function of the rodent barrel cortex? Compared to the trigeminal ganglion at the periphery where whisker stimuli are encoded with high reliability, the neocortex shows a high trial-to-trial variability (Jones et al., 2004; Arabzadeh et al., 2005). Receptive fields in the trigeminal ganglion are tuned to one single whisker, in contrast to that neurons in neocortex have broad receptive fields (Simons, 1978; Brecht et al., 2003). These observations suggest that a primary function of the neocortex is to generate associations of different sensory inputs (Petersen, 2007).

To elucidate the role of the barrel cortex in information processing it is important to investigate its connectivity on a microscopic (columnar microcircuitry) as well as on a

macroscopic level (e.g. long range projections) (Feldmeyer et al., 2013). Looking at the microscopic columnar organization layer IV is thought to be the main input layer (Douglas et al., 1989). Whisker related information from layer IV spreads mostly vertically within the column to cells in the superficial layers II and III, where the information is further processed or distributed to neighboring cortical regions via horizontal transmission. Subsequently, information is sent down to deep cortical layers to activate neurons in layer V, which represent the main output of the cortical column. In a recent study Constantinople and Bruno (2013) propose a new model which stands in contrast to the classical view of a canonical cortical circuit (da Costa and Martin, 2010) with two streams of sensory input entering superficial and deep cortical layers separately (Constantinople and Bruno, 2013).

On the macroscopic level barrel cortex is highly interconnected with other brain regions via cortico-cortical, cortico-thalamic and other cortico-sub-cortical pathways, as well as via modulatory systems (Feldmeyer et al., 2013). I will introduce some examples of cortico-cortical connections, which are important for context-dependent information processing. Within the barrel cortex horizontal short projections originating from supra- and infragranular layers target neighboring septal domains and neighboring barrels (Kim and Ebner, 1999; Adesnik and Scanziani, 2010). Outside the barrel cortex barrel column projections terminate in S2 as well as in M1 (Koralek et al., 1990; Chakrabarti and Alloway, 2006; Chakrabarti et al., 2008). Additionally, reciprocal cortico-cortical connectivity exists, suggesting a bi-directional flow of information. Petreanu and colleagues (2012), for instance, report cortical-feedback projections from M1 to primary sensory areas terminating in layer I where they connect with tuft dendrites of pyramidal neurons. The authors suggest that layer I input provides contextual information about active whisker touch (Petreanu et al., 2012). Using anterograde and

retrograde tracers Cauller and colleagues (1998) further identified reciprocal cortico-cortical connections between S1 and secondary cortical areas. Thereby, input fibers to S1 are concentrated in layer I, where they extended horizontally across several S1 barrels (Cauller et al., 1998).

The above mentioned studies show that layer I of the barrel cortex is of great interest because it receives long range projections from cortical areas providing feedback information essential for cognitive processes. The general idea of top-down influence is that complex information (experience, attention, expectation and brain state information) represented at higher stages influences simpler processes occurring at lower stages. Modulatory effects can range from sharpening of tuning curves to the modulation of plasticity (Gilbert and Sigman, 2007).

Characteristic for layer I is its very low cell density; virtually all cells located in this cortical layer are inhibitory neurons categorized into four classes (Kubota et al., 2011). These GABAergic (γ-aminobutyric acid) interneurons are positioned such that they directly contact dendritic tufts of layer II/III and V pyramidal neurons (see Fig. 4B) and are therefore able to modulate the firing of the majority of excitatory neurons in the cortex (Palmer et al., 2012). In 2013, Jiang and colleagues were able to identify two main classes of layer I inhibitory neurons, single bouquet cells (SBCs) and elongated neurogliaform cells (ENGCs), which have competing influences (disinhibitory versus inhibitory) on the coupling between tuft and basal region of layer V neurons (Jiang et al., 2013; Larkum, 2013b). These two separate circuits can lead to inhibition or disinhibition, suggesting that the cortex can be regulated in both directions. The existence of these highly regulatory GABAergic interneurons together with the convergence of projections from higher cortical areas make layer I a very interesting locus to investigate higher cognitive function and plasticity.

In 2011, Letzkus and colleagues published a study showing that layer I interneurons play a central role in conveying information about an aversive stimulus (Letzkus et al., 2011). Using targeted recordings from identified populations of the mouse auditory cortex in combination with pharmacological and optogenetic manipulations, they were able to identify a disinhibitory microcircuit required for fear conditioning. During foot shock presentation basal forebrain cholinergic projections acutely activate the majority of layer I interneurons which in turn inhibit parvalbumin (PV) positive basket cells. These fast spiking interneurons form strong synapses with high release probability on the perisomatic region of pyramidal neurons and can therefore control their firing (Markram et al., 2004). The authors suppose that in the end the observed disinhibition of pyramidal neurons can gate the induction of activity-dependent plasticity.

The mentioned studies certainly demonstrate the importance of layer I circuits. Because of its apparent role in feedback information processing the present study examines learning induced spine plasticity in layer I of the somatosensory cortex. In the following section of the introduction I state why I chose trace eyeblink conditioning as learning paradigm.

2.5 Classical eyeblink conditioning

Pavlovian eyeblink conditioning is a very intensively studied model system for associative learning and memory. Classical or Pavlovian conditioning describes learning that occurs through associations between a neutral signal and a naturally occurring reflex. In the classic experiment of the physiologist Ivan Pavlov with dogs the neutral signal was the sound of a tone and the naturally occurring reflex was salivating in response to food. By associating the neutral stimulus with the environmental

stimulus (the presentation of food), the sound of the tone alone induced salivation (Pavlov, 1927). In contrast to classical conditioning, operant conditioning first described by Burrhus Frederic Skinner focuses on using either reinforcement or punishment to increase or decrease a behavior (Skinner, 2005). Therefore an association is formed between the behavior and its consequences. A major difference between the two concepts, central to behavioral psychology, is that operant conditioning requires the learner to actively participate or to perform some type of action in order to be rewarded or punished. Classical conditioning, on the other hand, is dependent on involuntary, reflexive behaviors. In the context of learning often two additional terms are mentioned: habituation and sensitization. Habituation is a form of learning in which a subject stops to respond to a stimulus after repeated presentation (Thompson and Spencer, 1966). In contrast to habituation, sensitization leads to an increase in elicited behavior after repeated presentation of a stimulus (Thompson and Glanzman, 1976).

In the present study I investigated structural spine plasticity induced by classical trace eyeblink conditioning in the primary sensory cortex of the mouse. Acquisition and extinction of conditioned nictitating membrane movements were first described in albino rabbits (Gormezano et al., 1962). Since then, eyeblink conditioning has been widely used to explore the neuronal circuitry involved in the acquisition of the associative learning behavior. Often tones or somatosensory stimuli serve as the conditioned stimulus (CS), which are paired with an unconditioned stimulus (US) such as a peri-orbital shock or a corneal air puff to the eye. With ongoing pairing of the two stimuli the animal establishes an association which results in an eye lid closure occurring before the onset of the US, the conditioned response (CR).

Eyeblink conditioning comes in two major variants: the ‘delay’ and the ‘trace’ paradigm. In the delay paradigm the association between CS and US is accomplished by pairing the stimuli in a temporally overlapping way; usually the longer CS co-terminates with the shorter US. In the trace paradigm the CS and US are delivered in a sequential way with a stimulus-free time interval between presentations, in which a memory trace is supposed to be formed to bridge the temporal gap, allowing the animal to associate the behaviorally relevant inputs. Whereas the acquisition and retention of the delay variant is dependent on the cerebellum and associated brainstem structures (Thompson and Krupa, 1994), the trace paradigm additionally requires the functioning of the forebrain (e.g. hippocampus and neocortex). In rabbits, for example, trace conditioning was severely disrupted when hippocampus or the prefrontal cortex were damaged (Solomon et al., 1986; Moyer et al., 1990). Additionally, the primary sensory cortex seems to play an important role during trace eyeblink conditioning using whisker deflections as CS. By performing cortical barrel lesions prior to and following trace conditioning, Galvez and colleagues showed in 2007 that the barrel cortex is necessary for the acquisition of the task and still plays a role during retention (Galvez et al., 2007). In agreement with a study published a year earlier, in which the group reported learning-specific expansions of whisker-related cortical barrels after trace eyeblink conditioning in rabbits (Galvez et al., 2006), these results suggest that the barrel cortex is a potential site for long-term storage of the trace eyeblink association. Another important observation in the field was that motor cortex also plays a critical role in trace eyeblink conditioning. Animals in which motor cortex function was blocked could no longer elicit CRs, learning and extinction of trace eyeblink conditioning was inhibited (Woody et al., 1974; Krupa and Thompson, 2003). In 2013 Magal therefore proposed the hypothesis that the cerebellum might rather detect the coincidence of CS and CR signals, than the occurrence of CS and US (Magal, 2013).

I chose to use trace eyeblink conditioning to investigate structural plasticity in the barrel cortex because trace conditioning critically involves the neocortex. Therefore, synaptic reorganization processes are to be expected as animals improve their performance. This hypothesis is already supported by the finding of map plasticity observed by Galvez and colleagues (Galvez et al., 2006). Even more decisive to choose this behavioral task is the fact that trace eyeblink conditioning is known as a model paradigm for declarative learning. It has been shown that trace conditioning is associated with the acquisition of declarative knowledge about the CS-US contingency, which is demonstrably dispensable for delay conditioning (Clark, 1998; Clark et al., 2001, 2002). Further, trace eyeblink conditioning is an eligible paradigm to study the interplay between primary sensory and prefrontal areas during learning. The cerebellum is not able to maintain a stimulus presentation across the trace interval. However, if the cerebellum receives processed information about the CS such that CS and US information is sent to the cerebellum in a temporally overlapping fashion, the formation of a conditioned response in trace eyeblink conditioning is possible. Various studies suggest forebrain regions like the medial prefrontal cortex (mPFC, homologous to the dorso-lateral prefrontal cortex in primates) to generate activity bridging the trace period between CS and US (Weible et al., 2000, 2003, 2007; Kalmbach et al., 2009, 2010; Siegel et al., 2012; Siegel and Mauk, 2013). This preserved activity would allow a signal transmission to pontine nuclei leading to a coupling of both stimuli in the interposed nucleus (IP), a deep nucleus of the cerebellum (Fig. 6, from Woodruff-Pak and Disterhoft, 2008). Given that the barrel cortex is necessary for the tactile variant of the trace conditioning task and that the mPFC shows persistent activity during the trace period creates the possibility that there is a critical interplay and a recurrent direct or indirect connection between the two involved structures.

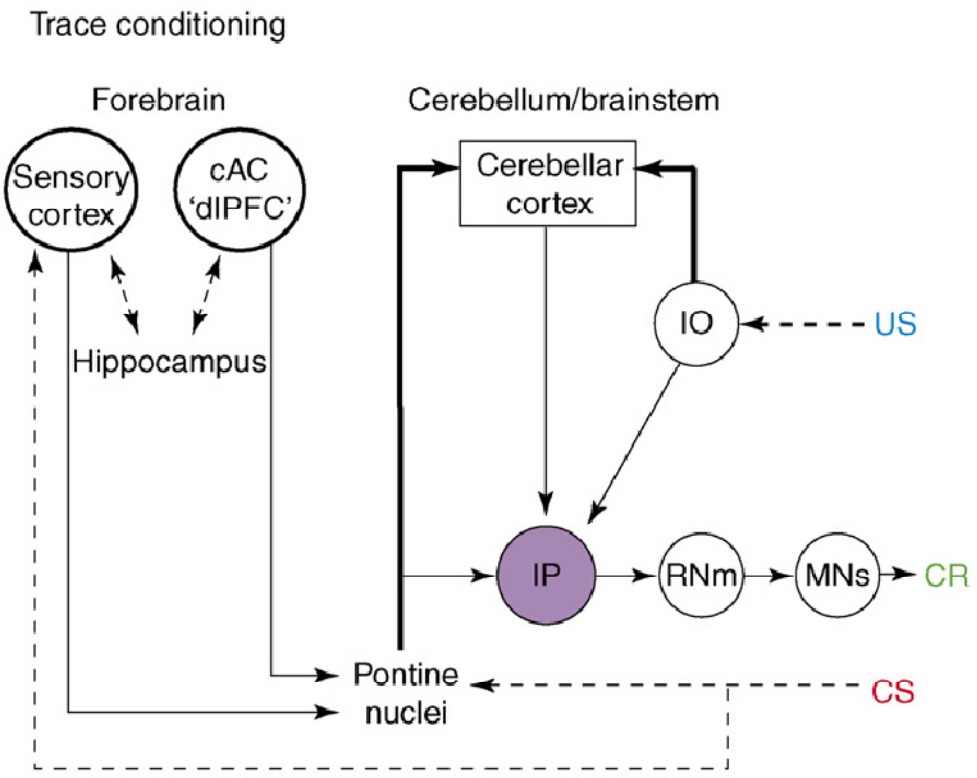

Figure 6: Forebrain and cerebellum dependent circuit underlying trace eyeblink conditioning. cAC: caudal anterior cingulated; dlPFC: dorso-lateral prefrontal cortex; IO: inferior olive; IP: interposed nucleus; RNm: medial red nucleus; MNs: motor neurons (from Woodruff-Pak and Disterhoft, 2008).

Taken together, trace eyeblink conditioning makes it possible to investigate the interesting relationship between primary sensory and prefrontal areas during learning. Finally and even more importantly, given the connectivity pattern of layer V pyramidal neurons in layer I of the barrel cortex, this classical conditioning task makes it possible to directly investigate the impact of cognitive processes and feedback information on the cortical microcircuit.

2.6 Aim of the study

The aim of this doctoral work was to elucidate the functional role and the cellular mechanisms of primary sensory cortex plasticity during trace eyeblink conditioning in mice.

In this study, an in vivo imaging technique was combined with a behaviorally relevant learning task engaging the primary sensory cortex. For the first time the extent and course of learning induced structural spine plasticity in layer I during trace eyeblink conditioning was examined in the mouse barrel cortex. To my knowledge this is also the first study to investigate structural synaptic plasticity in vivo in the context of a classical conditioning task in which the awareness of the stimulus contingency plays a central role. In general, the following three main questions are supposed to be answered:

1) Is the map plasticity after whisker trace eyeblink conditioning, observed by Galvez and colleagues in 2006, also expressed by structural plasticity on the level of dendritic spines in layer I?

2) What is the time course of learning induced spine plasticity?

3) Is spine plasticity specific and limited to the barrel column receiving the CS or are neuronal circuits of neighboring barrels affected as well?

3. Material and methods

3.1 Animals

All experimental and surgical procedures were performed in accordance with guidelines of animal use of the Society for Neuroscience and German Law (approved by the Regierungspräsidium Tübingen). The study was carried out using adult male transgenic mice expressing yellow fluorescent protein (YFP) in cortical pyramidal neurons. More specifically, animals originate from a YFP-H transgenic line generated on a C57BL/6J background. The fluorescent protein is expressed under the Thy1 promoter, which provides a high level of neuron specific labeling of a subset of layer V pyramidal cells in the cortex (Feng et al., 2000). Animals were bred and group-housed under pathogen-free conditions. After surgery animals were housed individually with food and water ad libitum under an inverted 12 hour light/dark cycle.

3.2 Cranial window implantation

To obtain permanent optical access, optimized for intrinsic optical imaging and two-photon imaging in the awake behaving mouse, a round coverslip (Thermo Scientific, Menzel GmbH, Braunschweig, Germany) with a diameter of 4 mm was implanted over the right barrel cortex under general anesthesia (3 component anesthesia: fentanyl 0.05 mg/kg, Ratiopharm GmbH, Ulm, Germany; midazolam 5 mg/kg, Ratiopharm GmbH, Ulm, Germany; medetomidine 0.50 mg/kg, Sedator, Eurovet Animal Health B.V., Bladel, Netherlands). Animals were fixed in a stereotactic frame while body temperature was maintained at ~ 37 °C with the help of a warming pad (Havard Apparatus, Holliston, MA, USA). Throughout the surgery eyes were covered with ointment to prevent them from drying out. After the removal of hair, skin

and periosteum the skull surface was cleaned with H_2O_2 (3 %). Afterwards the contours of a template coverslip were carefully drawn over the region of the craniotomy. This area was spared when a two-component bonding agent (Optibond FL, Kerr Corporation, Orange, CA, USA) and a first layer of light curing dental cement (Tetric EvoFlow, Ivoclar Vivadent AG, Schaan, Liechtenstein) was applied to the skull. Gently a small line was drilled along the outlined trepanation border. Care was given not to apply to much pressure to prevent heat generation. When the bone was thin enough to gently remove the portion of skull over the barrel cortex, great care was taken not to injure the *dura mater*. A sterile coverslip was then slowly pushed onto the brain tissue by means of a custom made post mounted onto a micromanipulator, until it formed a plane with the surrounding bone and was sealed to the skull with dental cement. Subsequently, a custom made titanium ring (0.7 g, 14 mm diameter, (Hefendehl et al., 2011)) was attached over the cranial window, with the help of which the animal could be head-fixated in a horizontal fixation plate under the two-photon microscope. After the skin was sutured rostrally and caudally such that it enclosed the titanium ring, a mixture of antidotes reversing the action of the three components of the anesthesia was injected subcutaneously (naloxon 1.20 mg/kg, Hameln Pharma PL GmbH, Hameln, Germany; flumazenil 0.50 mg/kg, Fresenius Kabi Deutschland GmbH, Bad Homburg v.d.H., Germany; atipamezol 2.50 mg/kg, Atipam, Eurovet Animal Health B.V., Bladel, Netherlands). To keep the animal free of pain, carprofen (0.05 mg/kg, Rimadyl, Pfizer GmbH, Berlin, Germany) was injected subcutaneously for three days. After surgery animals were allowed to recover for at least one week before acute optical imaging experiments were performed.

3.3 Intrinsic signal imaging

Intrinsic imaging was used to functionally map the location of the CS-activated barrel column and to identify neighboring whisker representations. The procedure for optical imaging has been described in detail previously (Grinvald et al., 1986; Peterson and Goldreich, 1994). Images were captured using a CCD camera (Teli CS3960DCL, Thoshiba Teli Corporation, Tokyo, Japan; 12 bit depth resolution, 300 x 300 pixel) equipped with a macro adapter (6.5 mm), a macro extensor (25 mm) and a macro iris (Schneider Kreuznach, Bad Kreuznach, Germany) and controlled by the software Helioscan ((Langer et al., 2013), http://www.helioscan.org) via a camera link card (PCI-1426, National Instruments, Austin, Texas, USA). First, green light (570 nm) from a custom made ring with two types of LEDs, mounted around the camera was used in order to illuminate the exposed somatosensory cortex. The blood vessel pattern was captured to serve as a reference to localize the intrinsic optical signal. For the acquisition of the intrinsic optical signal I switched to monochromatic red light (630 nm) and focused the CCD camera onto a tissue depth of approximately 200 - 250 µm. A measurement (sweep) consisted of three image acquisition phases each lasting for 5s. Whisker stimulation was applied exclusively in the last phase. Frames were acquired over 20 sweeps (frame rate: 20 Hz, 300 x 300 pixel, spatial resolution: 17.4 µm/pixel, field of view area: 5.23 x 5.23 mm^2). For each time-point relative to the start of a phase, an image with the relative difference between the first and the last two phases were calculated. Difference images were averaged over the duration of a phase and over sweeps resulting in a control image and an image carrying the intrinsic signal (Langer et al., 2013).

During the optical imaging procedure, animals were sedated using the three component anesthesia. Mice were secured in a custom-made restrainer specifically

designed for intrinsic optical imaging (Fig. 7). A titanium-ring compatible head-fixation plate ensured the rigid stabilization of the animal, which is essential for motion free image acquisition. Single whisker stimulation was conducted with the help of a small glass capillary (0.58 mm inner diameter, Science Products GmbH, Hofheim, Germany) glued to a piezo bender (Physik Instrumente, Karlsruhe, Germany), in which the selected whisker was carefully inserted. 60 Hz sinewave stimuli were delivered at 7 -10 V resulting in approximately 0.7 mm rostro-caudal deflections of the selected whisker. Voltage commands were generated using the software Helioscan and delivered via an amplifier for piezo bender actuators (E-650.00 LVPZT, Physik Instrumente, Karlsruhe, Germany). I positioned the capillary tip 5 mm from the skin by means of an adjustable holding system (Strato Line 3D Articulated Gauging Arm, Baitella AG, Zurich, Switzerland) and ensured that no neighboring whisker was accidentally co-stimulated.

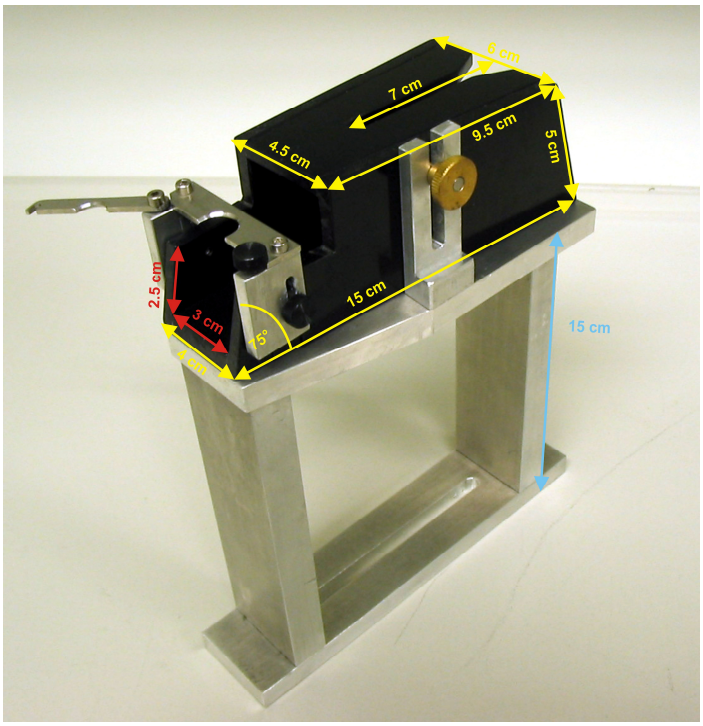

Figure 7: Restrainer with head-fixation plate designed for intrinsic optical imaging of anesthetized and awake mice (measures in yellow mark outer diameter, in red inner diameter, blue measure indicates height).

For each animal I typically located the following whisker representations: ɣ-Straddler, E1, E2 and D1. Usually, this mapping procedure was completed one hour after initial anesthesia application. Initially, I generated a first approximate overlay map of located barrel representations with blood vessels serving as land marks. Under the two-photon microscope dendrites near the center of the CS-activated area determined via intrinsic imaging were chosen to be imaged. After completion of the experiments imaging sites were verified by alignment of dendritic locations with the intrinsic signal (see also section 3.8). Therefore, the extracted intrinsic signal was automatically traced by boxcar filtering the image (kernel 10 x 10 pixel) followed by normalization of the range of captured gray values to [0, 255] and adjusting a threshold of gray values to capture an activation area roughly the diameter of a barrel (300 µm). It is noteworthy to remember that outside layer IV no histological criterion of horizontal barrel borders exist. Histological reconstruction therefore depends on projection of the stained layer IV barrel onto the surface of the cortex, a procedure that is influenced by the accuracy of vertical cortical vasculature and by compression and shearing of the tissue during fixation and preparation for horizontal sectioning. I therefore consider the physiological approach using intrinsic signals originating in upper cortical layers the most direct and best available method to obtain surface maps of barrel columns.

3.4 Handling and head-fixation under the two-photon microscope

In order to be able to train the animals directly under the two-photon microscope, I undertook a two week handling procedure to accustom the animals to the experimental setup and the head-fixation. I was determined to train the animals directly under the microscope and to disclaim the administration of anesthetics, because several studies

indicate, that the use of anesthetic agents itself induces changes in spine turn over (Tan et al., 2009; Yang et al., 2011).

In order to reduce stress two conditions had to be met – first, it was essential that the mouse gets accustomed to the experimenter´s smell, touch, and to being picked up. Second, it was important that the animal was trained to be head-fixed in a restrainer in which it felt secure. Similar observations were made in our laboratory for rats (Schwarz et al., 2010).

The handling procedure started by simply placing the hand in the mouse cage, allowing the animal to explore it and to familiarize with the experimenter for two days. Next, the animal was accustomed to being picked up for another two days, thereby care was taken not to catch hold of the mouse by seizing its tail, rather it was approached with the opened palm of the hand.

Subsequently, I introduced the restrainer-box in the animal's cage. The restrainer was similar to the one used for intrinsic imaging (see Fig. 7), except for the head-fixation plate, which was removed because the counterpart of the titanium ring was mounted on a separate stage under the microscope. The black restrainer box had a trapezoid base area which effectively narrowed the width of the box toward the front end to prevent turning of the mouse after entering from the open back. The restrainer made from aluminum had dimensions enclosing the body of the animal snugly, what turned out to have a calming effect. After the mouse ran through the restrainer within its cage several times, the restrainer was placed on a 15 cm high mounting table (Fig. 7). Mice tend not to jump from this height. I then trained the animals to enter the restrainer from the back and to leave the restrainer in the front by climbing onto the hand of the experimenter without turning inside. At the back a little door could be slit in and be fixed

by a screw, to keep the animal from leaving the restrainer on the wrong side (Fig. 8, left).

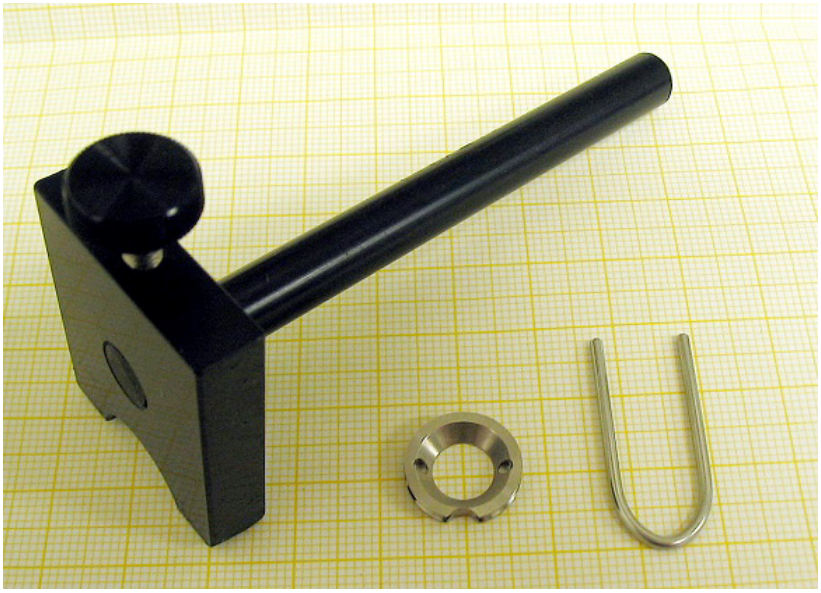

Figure 8: Handling and head-fixation equipment. From left to right: back door, titanium ring with two drillings, ring holder.

To finally fixate the animal in a stress-free manner on approximately the seventh day of training, a small holder was plugged into two small holes of the implanted titanium ring to be able to gently move the animals head towards the counterpart closing (Fig. 8, right). On the first day of head-fixation mice stayed 1 min in the restrainer, 5 min on the second day. With ongoing training duration of head-fixation was increased gradually until animals were habituated to stay in the restrainer for 45 min after the second week. I rewarded animals after completed training sessions with yoghurt drops. Special care was given never to overburden the animal during habituation. The training session would have been aborted if mice emitted auditory signs of distress or if they generated secrets of the Harderian gland (a white substance covering the eye). With the habituation procedure described above these signs were never observed.

3.5 Trace eyeblink conditioning

I used trace eyeblink conditioning to investigate learning induced spine plasticity (Fig. 9). Animals were trained on five consecutive days directly under the two-photon microscope, one session lasting for approximately 30 min. Classical conditioning was conducted using a 250 ms long whisker stimulus (60 Hz sine wave, 0.7 mm rostro-caudal deflection) as CS. For this purpose, the left E1 whisker was inserted into a small glass capillary glued to a piezo bender (Physik Instrumente, Karlsruhe, Germany). The CS was followed by a 250 ms stimulus free trace interval before the US in form of a corneal air puff (Picospritzer III, Parker, Bielefeld, Germany) was applied to the center of the right eye for 50 ms. 75 dB white noise was present during the training to mask potential sound artifacts of the whisker stimulator. Eyeblinks were monitored using a custom made infrared reflective optic sensor translating the closure of the eyelid into a positive signal amplitude (Weiss and Disterhoft, 2008). At the beginning of each training session the eyeblink sensor was calibrated by setting its voltage signal to a reference value when the animal's eye was completely open. Up to five CS-US pairings were delivered before the start of the session to adjust the position of the optic sensor and the air-puff outlet. A conditioned response (CR) was defined as a voltage increase of the optic sensor's output (i.e. closing eye) exceeding 3 standard deviations of the baseline signal (10^{th} percentile, open eye) measured for each trial during the pre-stimulus period of 500 ms. Only responses that persisted until the last 15 ms before US onset were accepted as CR. Trials in which animals elicited spontaneous blinks which reached > 1/5 of the later air puff response amplitude (closed eye, 80^{th} percentile of all measured air puff responses) during the 150 ms prior to the CS stimulation were excluded from the analysis. One group of mice was trained on paired CS-US presentation (called 'test mice', n = 6 in which only the E1 barrel column was

imaged, and additionally n = 3 in which E1 and E2 barrel columns were imaged). Here, one session consisted of 60 trials (53 paired presentations of CS and US and 7 trials with CS alone) separated by a mean inter-trial interval of 30 s (random pick from a flat probability distribution ranging from 20 - 40 s). A second group of mice underwent pseudo conditioning (tagged 'control mice', n = 6). They received 60 whisker deflections and 53 US air puff presentations exactly as the test mice, but in random sequence with a mean inter-trial interval of 15 s (random pick from a flat probability distribution ranging from 10 - 20 s). Both groups of animals were trained once a day on five subsequent days. Individual sessions lasted for about 30 min. Eyeblink traces were analyzed using a self written Matlab script (Version R2010b, MathWorks, Natwick, MA, USA).

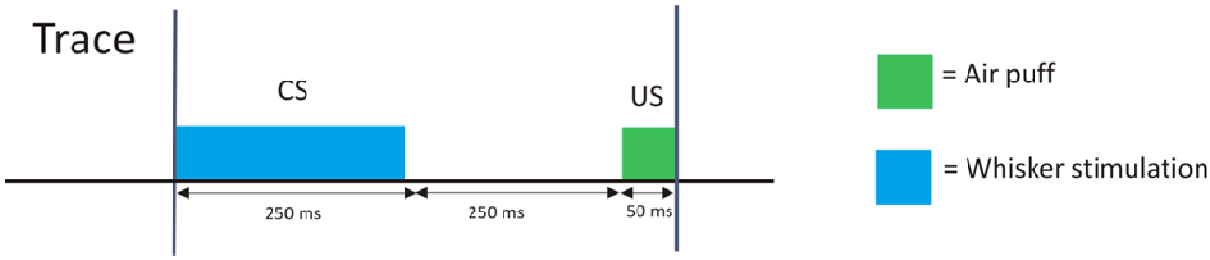

Figure 9: Trace eyeblink conditioning (CS = whisker stimulus, US = air puff).

3.6 In vivo two-photon imaging

Awake mice entered a restrainer box and subsequently were head-fixed by fitting the implanted titanium ring in a clamp connected to a motorized XY-stage (Luigs & Neumann, Ratingen, Germany). The head-fixation system made it possible to rapidly re-localize previously imaged regions of interest with a precision of a few micrometers (Hefendehl et al., 2012). Two-photon imaging was carried out using a Leica DMLFS

microscope equipped with a TCS 238 SP2 scan head (Leica Microsystems, Wetzlar, Germany) and a 239 Spectra Physics (San Jose, CA, USA) Mai-Tai BB laser. Neuronal structures were imaged using an excitation wavelength of 910 nm. Detection of the YFP signal was performed using non-descanned detectors (R6357 P.M.T., Hamamatsu, Bridgewater, NJ, USA) through a 40 HCX APO water-immersion objective (0.8 numerical aperture; Leica Microsystems, Wetzlar, Germany). Less than 45 mW of laser power was delivered to the brain to avoid laser-induced photo-toxicity.

Apart from baseline sessions, imaging was carried out immediately after the training sessions in awake animals. Z-stacks of areas containing dendrites and dendritic spines of interest (apical tuft within the patch of intrinsic optical signal) in layer I (median depth: 60 µm, range; 49 - 87 µm) were acquired at high resolution (1024 x 1024 pixels, pixel size: 0.098 µm, 0.5 µm z-step size, stack size: 20 – 30 µm).

3.7 Experimental procedure

Figure 10 gives an overview over the experimental procedure and the time required for discrete steps. The surgery was performed within 2 - 3 hours. After surgery animals were allowed to recover for at least one week, before the barrel cortex was functionally mapped with intrinsic optical imaging. For intrinsic imaging the animals were kept under general anesthesia and placed in the restrainer box for about 1 hour. On the following day, a two week handling protocol described above was started (section 3.4). When the mouse was successfully accustomed to the experimental setup and to the head-fixation under the microscope, I collected cortical stacks on seven consecutive days to follow learning induced spine dynamics. Stacks were aquired on two daily baseline sessions, before the animals were trained to trace eyeblink conditioning and

on five daily sessions immediately after the training session. The imaging sessions were always perfromed on seven subsequent days without gap.

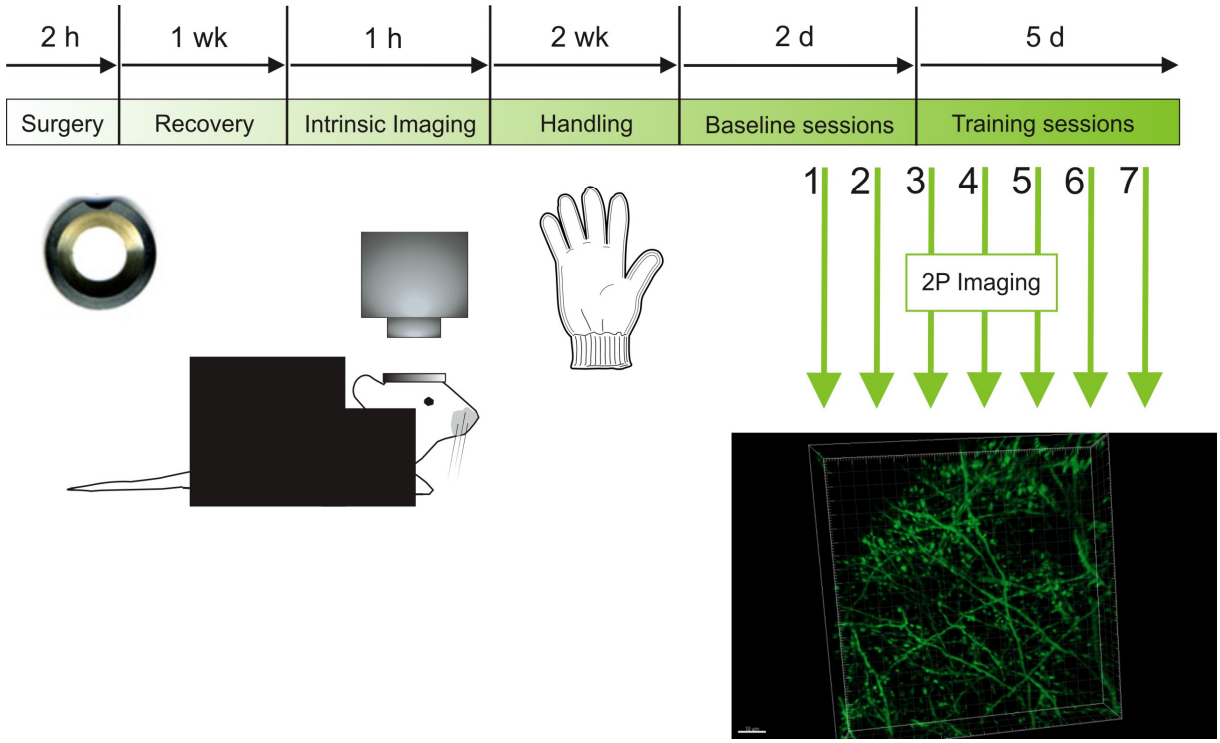

Figure 10: Overview over experimental procedure (timeline).

3.8 Alignment of dendritic locations with intrinsic signal

To verify imaging sites after the training procedure, I imaged dendrites of interest and the surrounding blood vessel system at various magnifications (40 x, 10 x objectives, Leica Microsystems, Wetzlar, Germany). Animals were anesthetized (fentanyl 0.05 mg/kg, midazolam 5 mg/kg, medetomidine 0.50 mg/kg) and Texas Red dextran (70,000Da molecular weight; 12.5 mg/ml in sterile PBS (Invitrogen, Carlsbad, CA, USA) was injected intravenously, providing a fluorescent angiogram (Bacskai et al., 2002). The localization of examined dendritic branches and spines with respect to the

CS-activated cortical area indicated by the intrinsic signal was aligned using the surface vessels imaged during the two-photon and during the intrinsic imaging session.

3.9 Data analysis

Data analysis was performed blind to the training condition and the barrel representation. For image processing, first, a motion correction algorithm was applied using a Hidden Markov Model (adapted from Dombeck et al., 2007), to compensate for minor xy-displacements induced by movements and breathing of the animal. Furthermore, imaged stacks were deblurred using Autoquant X (Media Cybernetics, Rockville, MD, USA). Image J software (NIH, Bethesda, MD, USA) was used to measure background fluorescence. Protrusions with fluorescence intensities smaller than 5-fold the standard deviation of the background fluorescence and smaller in length than 0.5 µm were excluded from scoring.

Spines were counted using a custom-written Matlab (MathWorks, Natwick, MA, USA) script, which allowed semi-automated alignment of individual dendrites of the three-dimensional stacks for each imaging session and therefore facilitated to follow spines across imaging days. Spines emanating laterally from the dendritic shaft were counted irrespective of their shapes, including filopodia-like structures. For image display of example dendrites, fluorescent structures near and out of the focal plane were removed manually from image stacks and adjusted for contrast and brightness using Image J. The Pearson correlation coefficient was used as a measure of the strength of linear dependence between spine changes and behavioral responses.

4. Results

4.1 Dataset

In this study a total of 15 adult male transgenic mice were used to investigate spine plasticity in the barrel column during the acquisition of a trace eyeblink conditioning task in vivo. In 6 test mice I combined chronic two-photon imaging of dendritic spines with eyeblink conditioning to an E1 whisker stimulus. In a second group of 6 control mice daily imaging sessions were performed after the animals underwent pseudo conditioning with incoherent E1 whisker stimulus presentations. Unfortunately, one animal of the control group suffered from an enteral infection and had to be taken out of the training after the third session. Finally, a third group of 3 additional animals were used to test the column specificity of possible learning induced plasticity effects. The latter three animals were conditioned to trace eyeblink conditioning of the E1 whisker, and dendritic spines were imaged in the E1 barrel column, exactly as done with the 6 previous test animals. Two-photon imaging of dendritic spines, however, was in addition carried out in the adjacent neighboring E2 column. To verify whether imaged dendrites were located in the CS-activated E1 and the neighboring E2 barrel column reconstructions of imaging sites were performed for the third animal group by aligning the surface blood vessel patterns imaged through the two-photon microscope with surface vessels imaged during the intrinsic imaging session.

4.2 Intrinsic signal imaging

In cooperation with Dr. Dominik Langer (Laboratory of Neural Circuit Dynamics, University of Zürich) and Dr. Alia Benali (Systems Neurophysiology, CIN, Tübingen) I successfully set up the technique of intrinsic signal imaging in the laboratory. With the

help of this method I was able to reliably map the activation areas and cortical representations of single barrel columns (confirmed by standard electrophysiology during establishment). By performing CO control stainings of the cortical tissue Dominik Langer and coworkers showed that in the context of the present experimental setup the centre of a barrel column can be determined with a precision of roughly 100 µm (Langer et al., 2013).

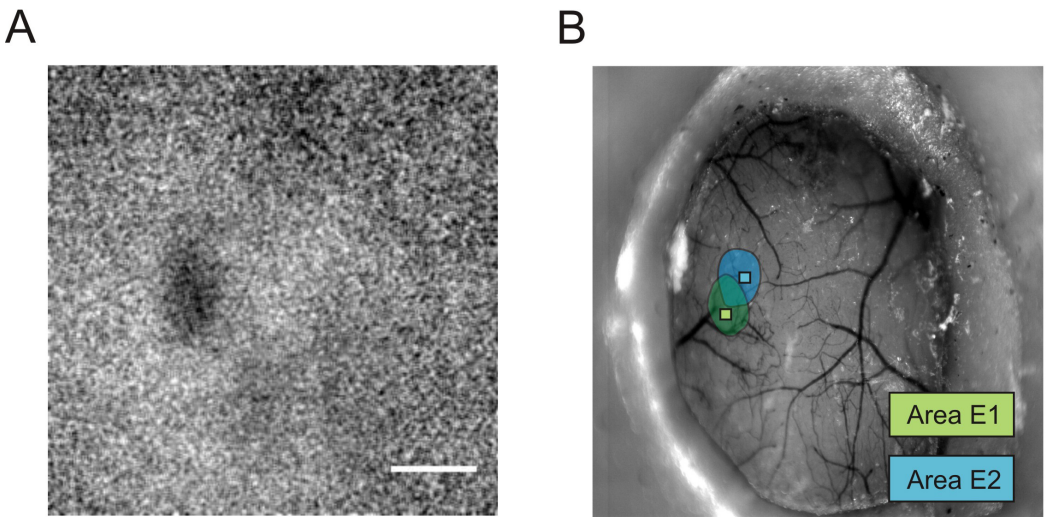

Figure 11: Intrinsic optical signal imaging. A) Intrinsic optical signal extracted by tissue light reflectance changes during single whisker stimulation of E1 (scale bar: 1 mm). B) First approximate overlay map of activated barrel representations E1 and E2 with blood vessels pattern imaged through cranial window (in green: E1, in blue: E2; light colors indicate location of cortical volume imaged with two-photon microscopy: area E1, area E2).

Figure 11 A shows an example image of the intrinsic optical signal extracted by tissue light reflectance changes during single whisker stimulation of E1. Typically, the intrinsic signal is very small, on the order of 0.1 % reflectance change (Langer et al., 2013). The imaging technique utilizes the optical absorption properties of hemoglobin to detect changes in the local cortical blood supply that occurs when neuronal activity increases

due to stimulation (Frostig et al., 1990). Deoxygenated hemoglobin is more absorbent in the wavelength of red light than is oxygenated hemoglobin. Therefore, the hypothesis is that the main intensity change of reflected light is due to an increase in the relative amount of deoxygenated hemoglobin. Reduced blood-oxygenation, on the other hand triggers an increase in local blood flow by vasodilatation of capillaries, which in turn also affects light absorption.

Interestingly, I found during the establishment of the technique, that the size of the activated region (dark area in Fig. 11 A) is highly dependent on the anesthetic agent used to sedate the animal. The average size of the optical signal observed was considerably larger when I anesthetized the animal with isoflurane (approximately 350µm - 400 µm) compared to, for example, the three component anesthesia (approximately 300 µm). Strikingly, I observed that the most locally defined activation areas were obtained in the awake animal (< 300 µm). In an ongoing study I try to systematically investigate this difference in tissue activation by evaluating the optical signal properties under urethane, isoflurane, ketamine and the three component anesthesia.

I decided to functionally map the barrel cortex under the three component anesthesia and not to perform intrinsic imaging in the awake animal to exclude negative effects on the subsequent conditioning success as animals were not yet handled at this point of the experimental procedure. Signal quality and size under the three component anesthesia however allowed the precise localization of the CS-activated and neighboring barrel columns. To correctly locate the activated brain regions under the two-photon microscope, for each animal I generated a first approximate overlay map of activated barrel representations and blood vessels (Fig. 11 B). Under the two-photon microscope dendrites near the center of the CS-activated area were chosen to be

imaged. After completion of the experiments imaging sites were verified by alignment of dendritic locations with the intrinsic signal (see Fig. 22). The method of intrinsic imaging turned out to be a helpful tool to locate cortical whisker representations, making the use of invasive electrophysiology dispensable.

4.3 Trace eyeblink conditioning

In the course of this doctoral thesis work I trained in total nine animals to trace eyeblink conditioning. To my knowledge it is the first time that mice were trained to this classical conditioning task using single whisker stimulation during head-fixation under a microscope. I trained the animals directly under the two-photon microscope, being fully awake and head-fixed in order to exclude possible effects of anesthesia on the spine turn over. Intensive handling was therefore of great importance and the basis for successful learning.

I used a 250 ms long 60 Hz sine wave deflection of the E1 whisker as CS followed by a 250 ms long stimulus free trace interval. The US in form of a corneal air puff was subsequently applied for 50 ms. The closure of the eye was monitored via an infrared reflective optic sensor and translated into a positive signal amplitude. With ongoing training animals learned that the CS predicted the US and therefore responded to the CS by eye closure, the conditioned response (CR) (Fig. 12). All mice responded with CRs already during the CS presentation and kept their eye half-way closed until the US occurred and reflexively evoked full eye closure (UR) after air pressure caused a brief reopening of the eyelid.

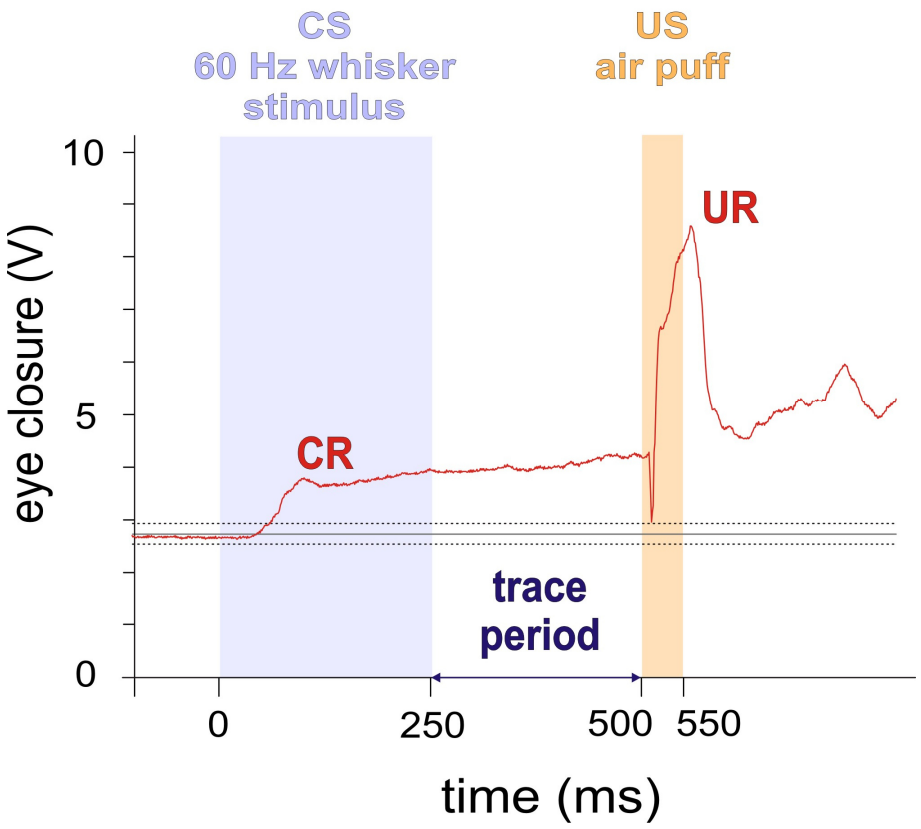

Figure 12: Example of classical conditioned response (CR). The red curve presents the voltage trace depicting the eye closure measured via an infrared reflective optic sensor of a trained animal across one trial. The CS and US presentation intervals are indicated (filled light violet and orange boxes). The trace period is interspersed between CS and US. The conditioned response (CR) consists of a partial eye closure during the CS which is kept until the US reflexively evokes full eye closure (UR). A CR is defined as an eye closure above 3 SD (broken lines) of baseline (full line).

A second group of additional six mice underwent pseudo conditioning. This control group received the same number of whisker and air puff stimuli in a random, temporally incoherent manner.

While the test group showed CRs which passed the criterion (see material and methods section) typically between 50 and 100 ms, the control group exhibited

spontaneously occurring blinks more equally distributed in the time window of 50 - 250 ms after CS onset. Figure 13 shows the latency histogram of conditioned responses observed in the test (red) and control (blue) group averaged across mice.

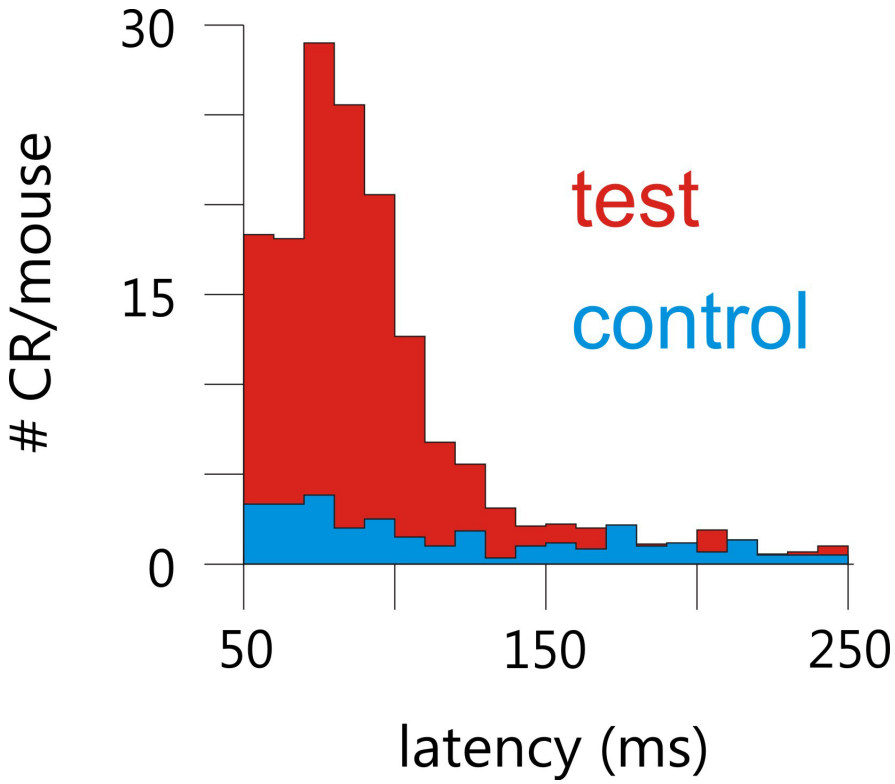

Figure 13: The distribution of CR latencies after CS onset observed in test (red) and control animals (blue) is plotted as a histogram.

Comparison of test and control group revealed that all mice, which underwent trace eyeblink conditioning were able to acquire the task within five training sessions (Fig. 14). Most animals showed first CRs already after the first training session and further improved performance during the following days. One individual even showed stable CRs right from the start indicating that learning already took place during a small

number of trials (< 5) that were presented for calibration purposes before a session was started. Control mice, on the other hand, did not learn, showing only few correct responses due to spontaneous blinking.

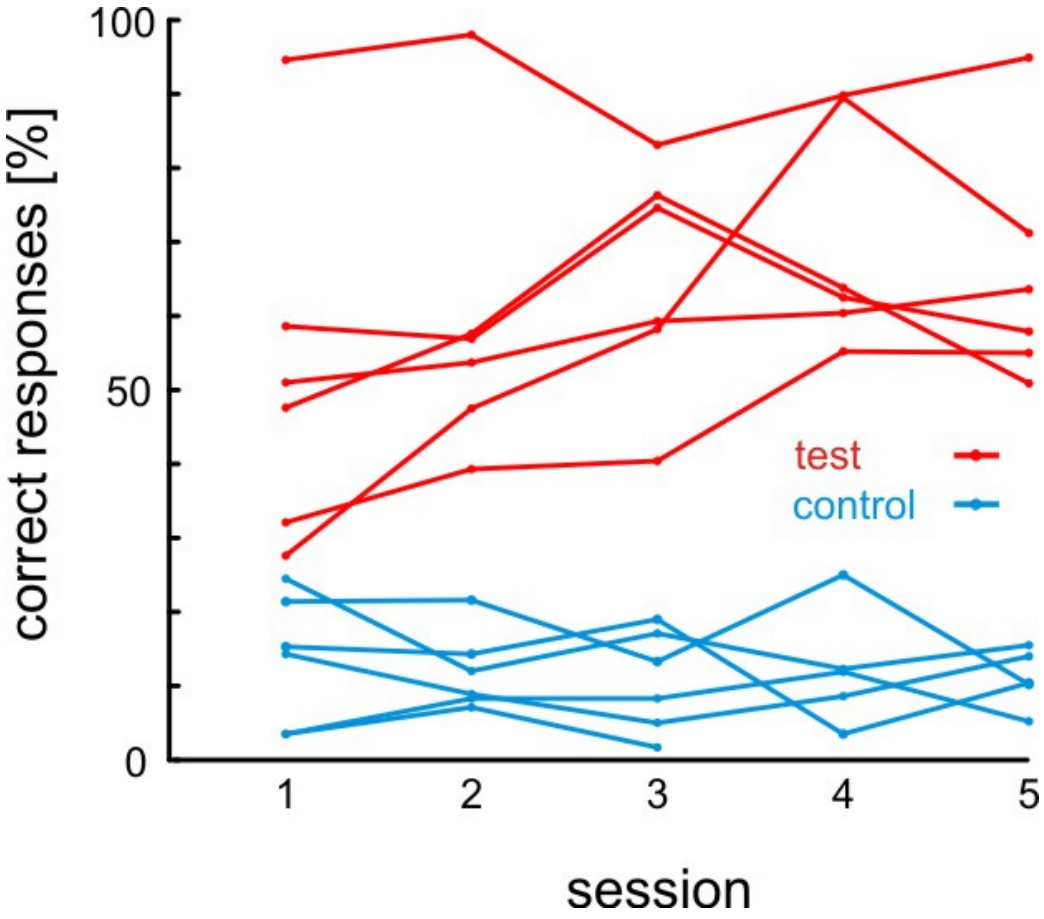

Figure 14: Learning curves measured over five training sessions for six test (red) and six control (blue) animals that received imaging of barrel column E1. One control animal was only investigated until the third training session because of an acute enteral infection.

Figure 15 shows that the learning success measured for all animals trained to trace eyeblink conditioning varied to some degree, but all subjects showed cumulative CRs that quickly separated from those observed in the control group. In fact, many test mice

showed a clear separation from the control group data already during the first 60 trials (i.e. the first session).

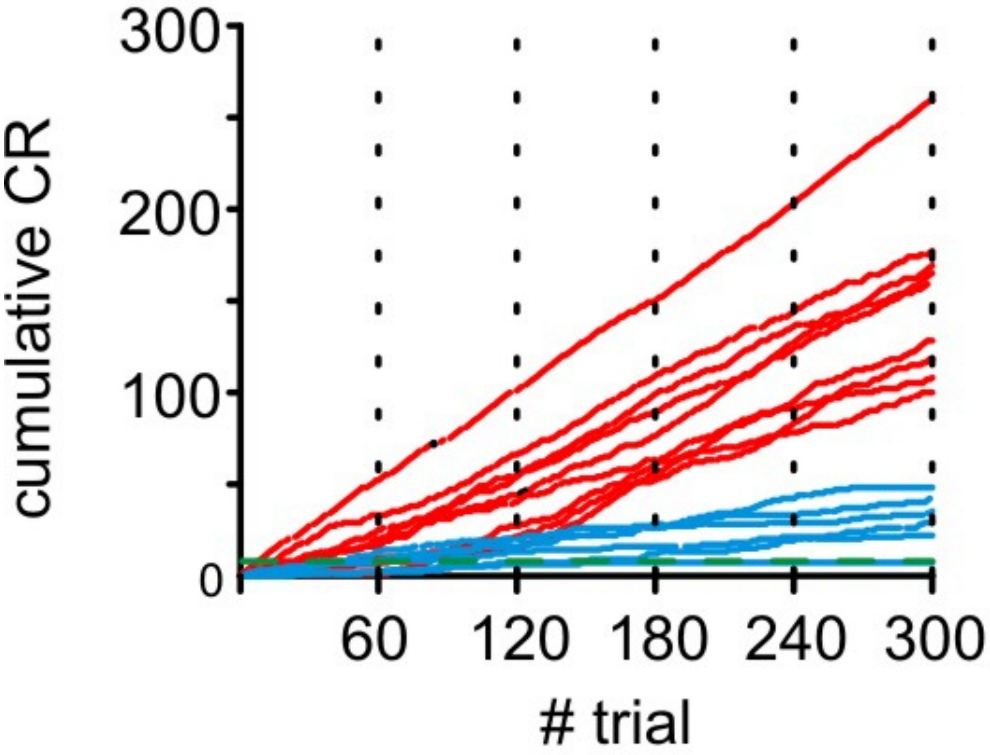

Figure 15: Learning curves of test (n = 9, red) and control animals (n = 6, blue) plotting cumulative CRs across the total number of 300 trials. Green dotted line indicates learning criterion used later in Fig. 19. Vertical dotted lines mark the end of a training session.

4.4 Spine plasticity induced by learning

To investigate, whether trace eyeblink conditioning is expressed by structural plasticity on the level of dendritic spines in mouse barrel cortex, I combined the training on the classical conditioning task with chronic two-photon imaging of dendritic spines in mice expressing YFP in cortical layer V pyramidal neurons. Dendritic spines on apical tufts of the labeled cells, located in the CS-activated cortical representation via intrinsic

optical imaging, were imaged for seven consecutive days, on two daily baseline sessions (B1, B2) and then after five daily conditioning sessions (S1-S5).

Figure 16 shows an example dendritic branch imaged in an animal which underwent trace eyeblink conditioning. On the first day of baseline imaging the dendrite carries three spines. Over the course of the training all original stable spines are eliminated (red arrows).

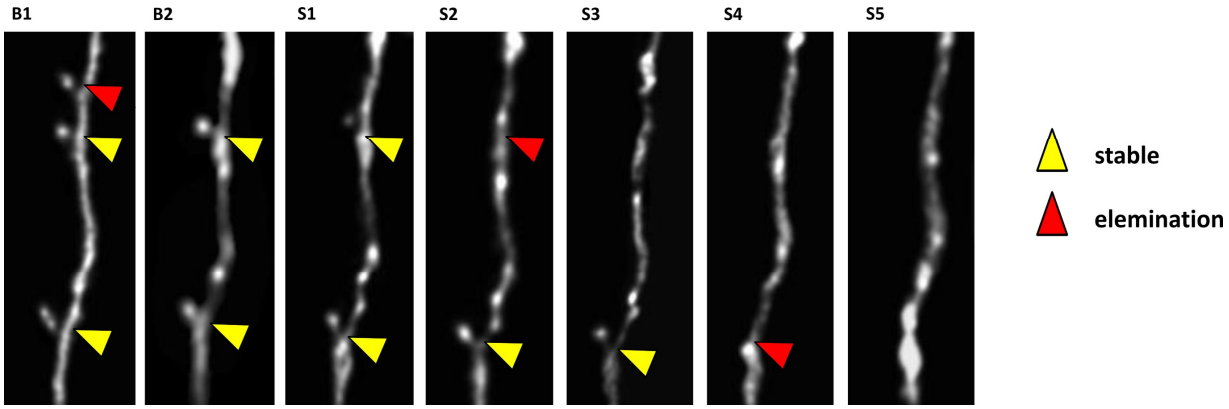

Figure 16: Example dendrite imaged in a test animal on seven consecutive imaging sessions (yellow arrows mark stable spines, red arrows mark spine elimination, B1-B2: baseline session, S1-S5: imaging sessions after training).

A main finding of the study is that for all mice which underwent trace eyeblink conditioning a strong decrement in absolute spine number was observed. In contrast to that no change in spine number was detected for the control group which underwent pseudo conditioning. Figure 17 shows an example dendrite which was imaged over two baseline imaging sessions and five sessions after pseudo conditioning. The dendritic branch carries five stable spines on the first baseline session as well as on the last day of pseudo conditioning.

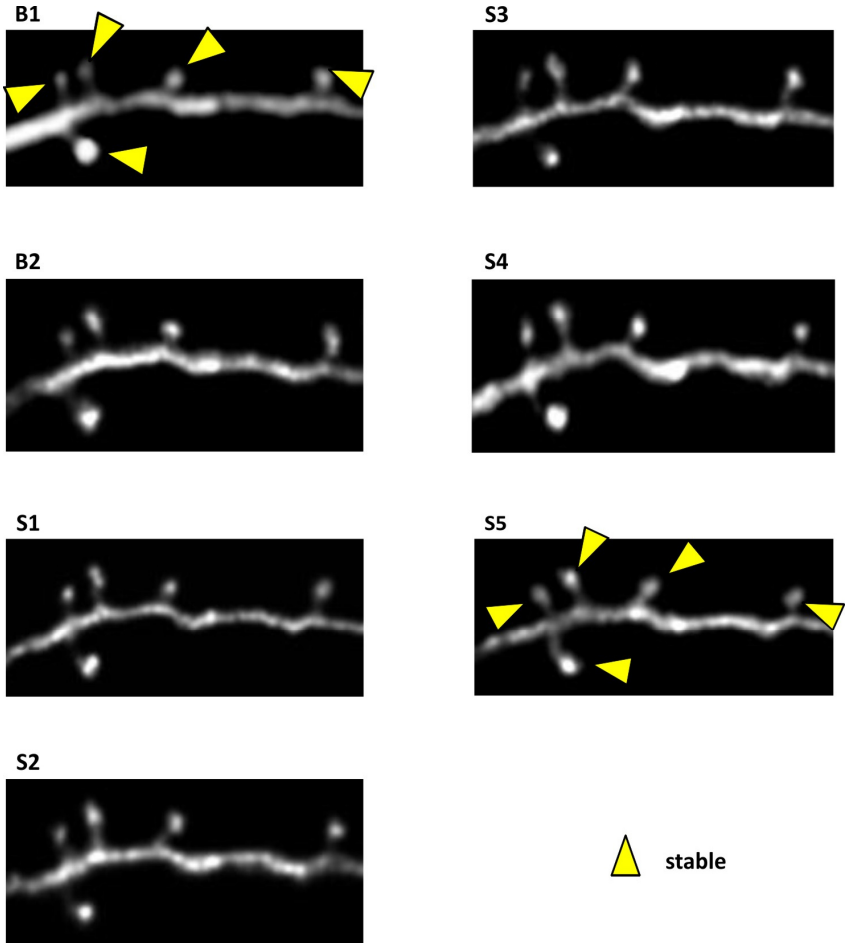

Figure 17: Example dendrite imaged in a control animal on seven consecutive imaging sessions (yellow arrows mark stable spines, B1-B2: baseline session, S1-S5: imaging sessions after pseudo conditioning).

The extent of the learning induced reorganization processes became obvious when following the change in spine count relative to the baseline sessions (B1/B2) over the course of the five training sessions (Fig. 18). The spine count in test animals systematically descended to -8 % to -20 % after the last training session while the percentage of spine number change observed in control animals fluctuated around baseline (0 % change). Importantly, after the last day of training the two distributions of relative spine numbers were completely non-overlapping.

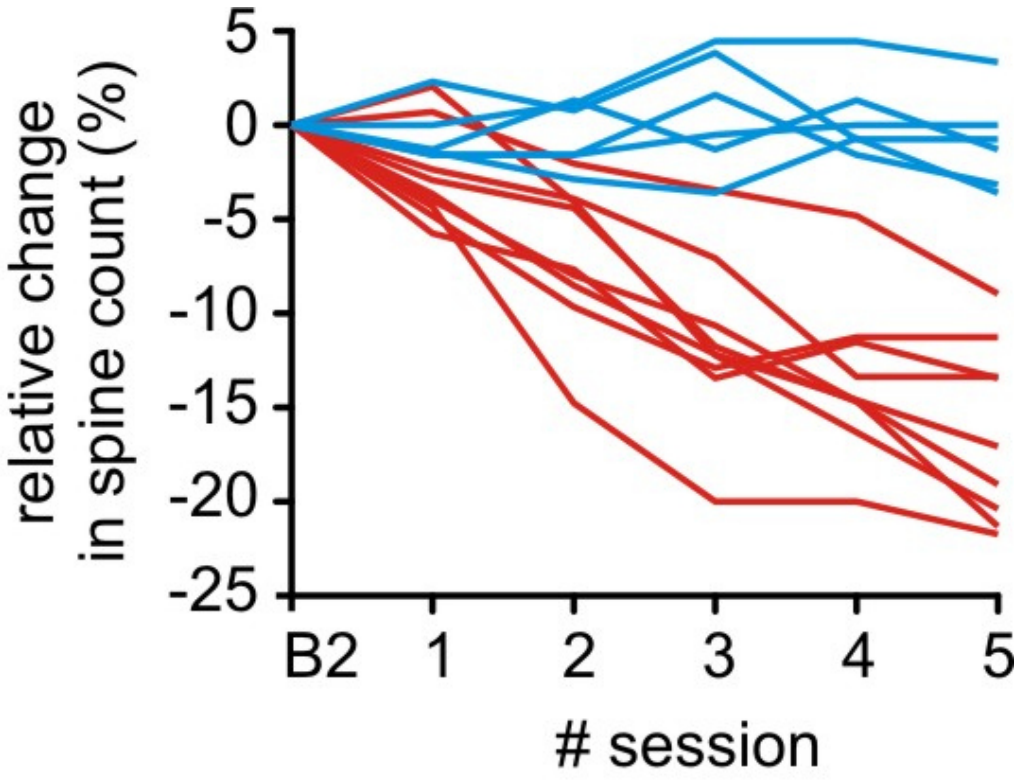

Figure 18: Relative change in spine count across the five training sessions plotted with reference to the baseline sessions B1/B2 (in red= test group, n = 9; in blue= control group, n = 6).

To extract the relationship of learning and spine loss, I had to cope with the problem that the two processes did not proceed simultaneously. Rather, spine loss seemed to follow learning with a certain delay. As learning occurred within the first two days in all test mice, I measured when the cumulative CR curves crossed a threshold ('learning criterion', green dotted line in Fig. 15) that was adjusted to yield crossing times within the first two sessions (60 - 120 trials). This measure can be interpreted as the 'speed' of learning. I regressed speed of learning with the number of the session at which the maximum spine reduction was observed (peak spine elimination) and found a strong correlation ($r = 0.84$, $p = 0.005$) shown in the left panel of figure 19. The crossing point

of the linear fit with the ordinate suggests a delay of learning and spine loss between one and two sessions (60 - 120 trials).

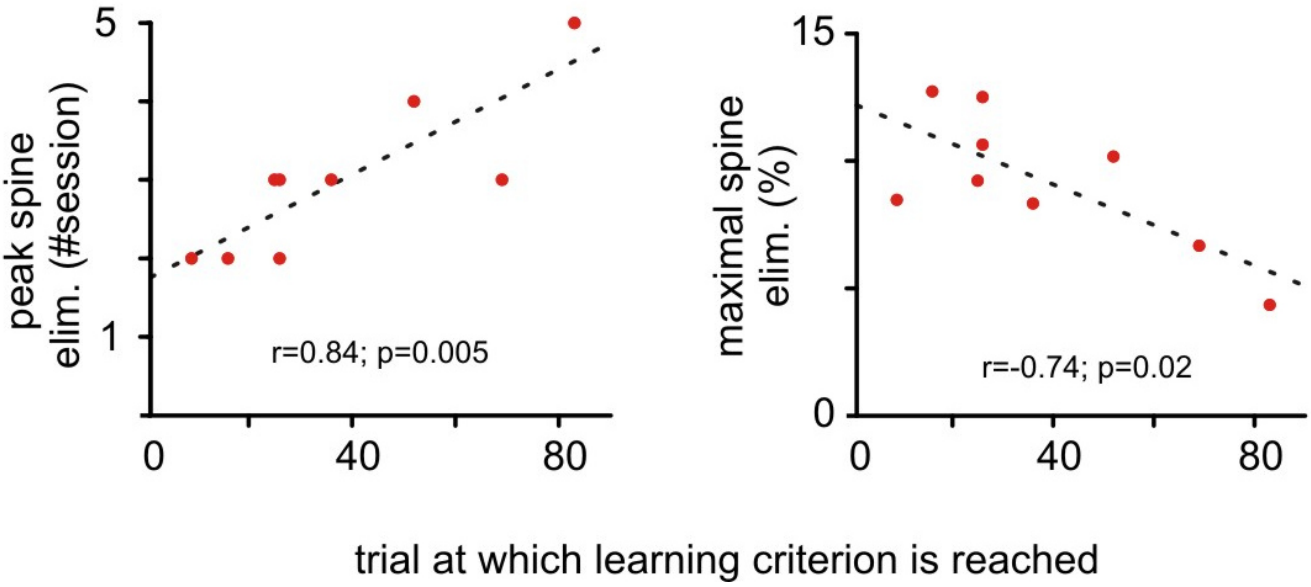

Figure 19: Relationship between learning and spine loss for each of the nine animals trained on trace eyeblink conditioning. Left panel: trials at which criteria of learning was reached plotted against the number of the session in which the peak of spine loss was observed. Right panel: trials at which criteria of learning was reached plotted against the maximum spine elimination per session encountered. In both panels broken lines represent the best linear fit. Pearson correlation coefficient and significance level are indicated in both plots.

The right panel of figure 19 shows the maximal percentage of spine reduction (measured to the day before) regressed with speed of learning. Again, a strong correlation was found ($r = 0.74$, $p = 0.02$). Similar results were obtained with varying learning criteria as long as they yielded crossing times within the first two sessions.

In summary, these results provide a strong indication that task acquisition and spine loss are tightly related. Good learners showed fast and high spine loss while these processes were lower and more sluggish for slow learners. There is a delay between learning and spine loss of about 1 - 2 sessions (60 - 120 trials) which indicates that learning is unlikely to account for the initiation of learning, but it may well underpin the consolidation of learned behavior.

4.5 Column specificity of learning induced spine plasticity

Next, I was interested whether the decrement in absolute spine number observed in test animals is specific for and limited to the barrel column receiving the CS. Therefore, I monitored the spine turn-over not only in the receiving barrel column E1 (as before), but additionally in the neighboring E2 barrel column in a subset of three trained test mice.

Figure 20 presents an example of the striking finding that spine elimination was very specifically limited to layer I in the receiving E1 barrel and was entirely absent in the neighboring E2 barrel.

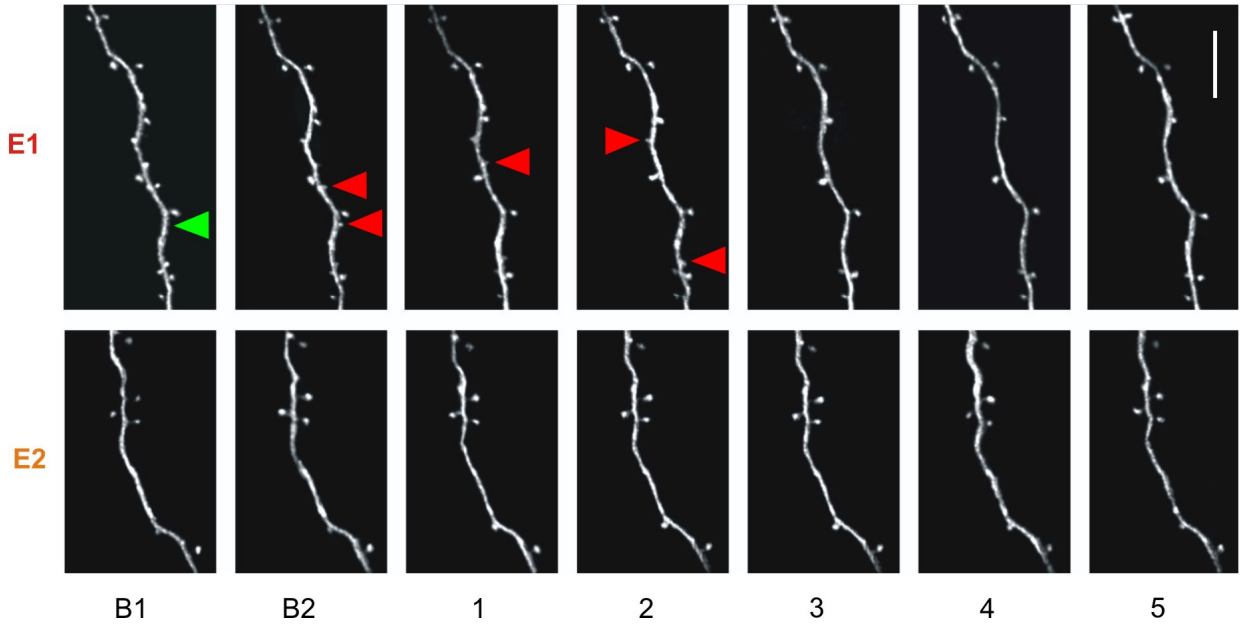

Figure 20: Example dendrites imaged in the CS receiving barrel column E1 (upper panels) and the neighboring E2 barrel column (lower panels) on seven consecutive imaging sessions (red arrows mark spine elimination, green arrow marks spine formation, B1-B2: baseline sessions, 1-5: imaging sessions after training). Scale bar: 10 µm.

The quantification of the relative reduction of spine numbers confirmed the impression given in figure 21. Dendritic spines located in the barrel column E1 were significantly reduced (as shown before, Fig. 18) while spines imaged in the neighboring E2 column showed relative counts matching those found in control animals. Figure 21 illustrates the finding.

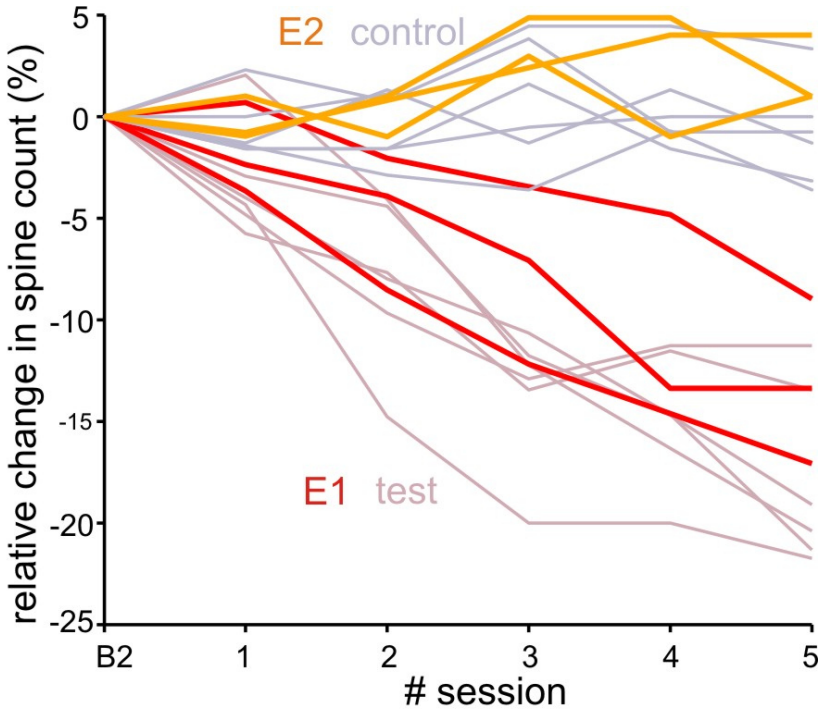

Figure 21: Relative change in spine counts observed in the CS receiving barrel column E1 (red) and the neighboring column E2 (orange) in three animals which underwent trace eyeblink conditioning. The light colored lines plot the data of all test and control mice as reference. Spine loss is present in barrel column E1 but not in column E2.

To verify whether I performed two-photon imaging of dendritic segments and spines in the intended whisker representations of E1 and E2, I reconstructed the imaging sites using the surface vessels imaged with the two-photon microscope and during the intrinsic imaging sessions. Figure 22 shows that virtually all of the imaging sites (white squares) are located within the intrinsic signal induced by single whisker stimulation. Dendrites within the extracted intrinsic signal were chosen such that they did not overlap with the one found for the neighboring barrel column. In conclusion, the reconstruction reassures that the learning induced decrement in spine number is specific for and limited to the CS receiving barrel column.

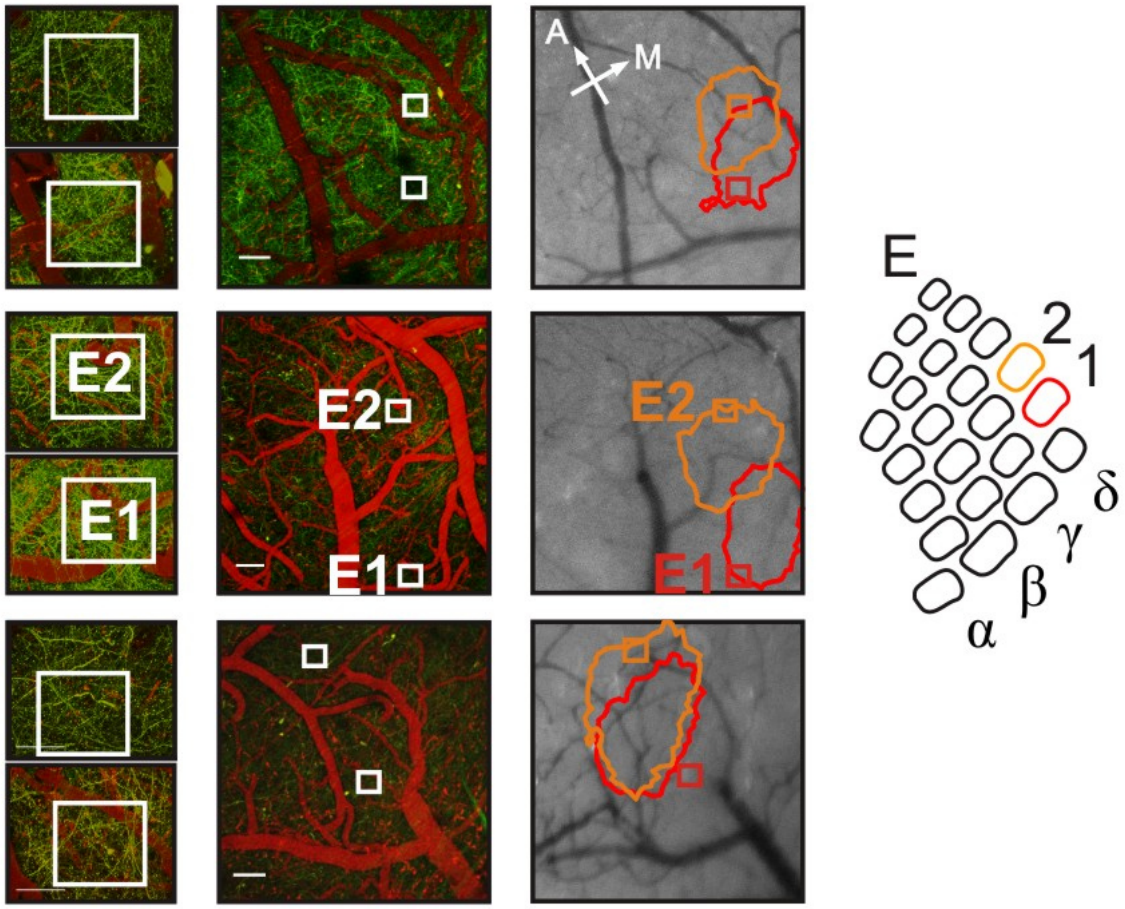

Figure 22: Reconstruction of imaging sites with respect to the barrel field in three mice which underwent trace eyeblink conditioning (upper, middle and lower row). Left: two-photon imaging fields of view used to count spines (40 x magnification, scale bar: 50 µm) framed by white boxes; center: same but 10 x magnification; scale bar: 100 µm; right: surface picture and circumference of intrinsic imaging signal obtained with stimulation of whisker E1 (red) and whisker E2 (orange). The correspondence of E1 and E2 in the three images is given in the second mouse. In the other cases correspondence is equivalent. Left and center are maximum projections. Orientation is the same for all mice: A: anterior, M: medial. A schematic depicting the two barrels within the barrel field is shown on the right.

5. Discussion

In this doctoral work I was able to establish a preparation that allows the monitoring of structural adaptive changes at the level of dendritic spines located in layer I in the primary sensory cortex. Classical trace eyeblink conditioning successfully induced memory based learning mechanisms which resulted in a substantial reorganization of circuits involved in the processing of the conditioned stimulus. The acquisition of the tactile variant of trace eyeblink conditioning was correlated with spine elimination on layer V pyramidal neuron's apical tuft in layer I. The number of eliminated spines and their time of elimination were tightly related to the learning success of an individual. Furthermore, learning induced spine dynamics were highly specific for the barrel column which was activated by the whisker deflection; dendritic spines located in neighboring barrel columns were not affected.

In the following sections of the discussion I want to elaborate methodological considerations important for in vivo experiments examining structural spine dynamics in response to learning. Further, I discuss cellular processes which could underlie the observed decrement in spine number. Additionally, I examine possible mechanisms which might be responsible for the spatial specificity of observed spine dynamics. Finally, I want to address the question: what is the role of the primary sensory cortex in trace conditioning?

5.1 Methodological considerations

In the following section of the discussion I want to work out some methodological considerations, which might be important for in vivo experiments, which concentrate on examining synaptic plasticity in response to learning. As already mentioned in the

introduction, several recent studies used two-photon imaging to investigate dendritic spine dynamics in the neocortex during learning (Yang et al., 2009; Fu et al., 2012; Lai et al., 2012; Moczulska et al., 2013; Kuhlman et al., 2014). This trend is important, as sensory deprivation or enrichment are likely to be accompanied by gross differences in neural activity – at least in initial phases – between the affected and non-affected sensory structures. In contrast, the every-day task of a sensory system to differentiate between relevant and irrelevant stimuli deals with representations that contain equal amounts of neural activity, but differ in contingencies to relevant instances in the outside world, and therefore must trigger useful behavior.

Most of the studies follow structural synaptic plasticity over several days or even weeks while the animal is acquiring a certain task or skill. Whenever the experimenter wants to get information about the current state of spine plasticity dynamics he needs to anesthetize the animal to put it under the microscope. Unfortunately, there is evidence suggesting that anesthesia can have an effect on intrinsic spine dynamics (Tan et al., 2009; Yang et al., 2011). The present study is the first study, which examines structural plasticity induced by learning while animals are trained directly under the two-photon microscope in a head-fixed preparation without the use of anesthetic agents. Therefore, I was able to exclude potential anesthesia influences on the synaptic spine turn over.

An important precondition for the successful training directly under the two-photon microscope was that animals were extensively well habituated to the head-fixation and the experimental procedure with the help of a two week handling protocol (see section 3.4). Animals were habituated and trained on a daily basis, at a time when animals are known to be most active (1 - 3 hours after sunset (Weinert and Waterhouse, 1998), inverted 12 hour light/dark cycle). Special effort was taken never to overburden the

animal and to reduce stress in any possible way. In this context, I think the fact that every single animal was able to acquire the conditioning task within few training sessions shows that animals actively participated in the task and were able to concentrate on the CS presentation. In contrast to that Weiss and colleagues report in 2008 that they could not detect stable conditioned eyeblink responses in mice under a head-fixed preparation when they used an air puff as a US (Weiss and Disterhoft, 2008). This observation suggests that an extensive handling procedure might be decisive for successfully eyeblink conditioning during head-fixation.

A major methodological consideration concerning in vivo experiments investigating structural synaptic plasticity in general should be to minimize potential factors which could intrinsically affect the spine turn over. Although several studies show the vast effect of sensory deprivation and trimming of whiskers on cortical spine plasticity (Lendvai et al., 2000; Holtmaat et al., 2006; Wilbrecht et al., 2010; Oberlaender et al., 2012), a recent study performs extensive trimming of whiskers two weeks before the beginning of experiments (Kuhlman et al., 2014). In my opinion this experimental procedure may have led to a misinterpretation of reported results. In my study I was determined to preserve the intactness of all whiskers on the pad to prevent manipulations of the sensory input.

Studies interested in structural synaptic plasticity induced by learning nowadays mostly use two-photon imaging as their method of choice. However, spine dynamics can also be followed using the ex vivo approach of Golgi stained neurons. Golgi's method is a silver staining first described by Camillo Golgi in 1873 that is used to visualize the soma, dendrites and dendritic spines of neurons under light microscopy. A study which was published very recently by Chau and colleagues in 2014, for instance, examines the effect of trace eyeblink conditioning on Golgi stained neurons

in layer IV of the mouse barrel cortex (Chau et al., 2014). Both techniques, the classical light microscopy of Golgi stainings and the two-photon imaging of living tissue, come with certain advantages and disadvantages. Golgi stainings can be analyzed in great detail at high magnification (resolution in light microscopy is limited to approximately 0.2 µm). When examining fixed thin samples, imaging can be optimized to achieve the highest resolution possible, by choosing high numerical aperture (NA) objectives and matched refractive indices between immersion medium and fixed sample. However, in vivo two-photon microscopy imposes further limitations to the resolution, stemming from the nature of the experiment. Long-distance objectives must be used for deep tissue imaging, that come with inherently limited NA. Moreover, suboptimal correction for refractive index mismatch between immersion medium and live tissue causes a reduction in the image contrast. Additionally, light wavefront aberrations inside the tissue, due to light scattering, further contributes to the distortion of the structures. However, the most significant impact on the resolution is the localization precision, negatively affected by the animals breathing and movement. Chau and colleagues (2014) were able to assign monitored spines to one of the five described spine classes (see section 2.1), whereas due to the technical limitations inherent to two-photon in vivo imaging I was not able to reliably distinguish for example between a mushroom shaped and a stubby shaped spine. Further, the image resolution acquired during two-photon imaging in awake mice is not high enough to reveal whether dendritic spines change their shape or size in response to learning. Another advantage of the Golgi method is that dendritic spines located in all cortical layers can be visualized; standard two-photon microscopy is limited in depth to a few hundred micrometers, providing access only to the superficial layers of cortex. Further, Golgi stained fixed tissue can be visualized under a light microscope many times without perturbing the specimen. In contrast to that, in vivo two-photon imaging involves photo-toxicity as the

brain tissue is exposed to the focused excitation light over several minutes. However, two-photon imaging holds major advantages over the ex vivo approach of Golgi stainings. To investigate synaptic plasticity with the ex vivo method the animal has to be anesthetized and sacrificed. Therefore, information about spine plasticity in one animal is available only at one single point in time. This is why for an individual structural spine plasticity dynamics cannot be followed during the process of learning. Important parameters like peak spine elimination or formation stays unobserved. Data has to be averaged over animal groups, although performance can vary a great deal between subjects. By using two-photon imaging in the living brain I was able to monitor in detail the extent and the time course of learning induced spine plasticity in individual animals. Hence, in my opinion two-photon imaging is the method of choice to follow temporally defined synaptic reorganization processes which coincide with the individual learning progress of an animal.

Today we know that dendritic spine modifications play a critical role in learning and memory consolidation (Kasai et al., 2010; Fu and Zuo, 2011; Rochefort and Konnerth, 2012). So far, this conception is based upon experiments which often used general learning paradigms, in which a subject undergoes multiple different learning events over several days of training (Yang et al., 2009; Xu et al., 2009; Kuhlman et al., 2014). Under these circumstances it is difficult to determine what kind of learning and association steps were mastered by the animal. Which acquired learning contents lead to the observed plasticity event and what was time course of synaptic remodeling mediated by each learning step? In the following I will discuss the advantages and disadvantages of different behavioral tasks which were used in the past to induce synaptic plasticity and I will argue why I think that trace eyeblink conditioning is an eligible paradigm to investigate the time course of neocortical reorganization induced

by a defined learning content. In Xu et al. (2009), for instance, mice were trained to reach for a food reward through a small slit in the housing. The authors claim that motor skill learning induced synaptic reorganization in the motor cortex (Xu et al., 2009). However, it does not arise from the study which motor skill caused the observed spine plasticity and when. Did the animal acquire an efficiency to grasp and hold the food pellet or did it develop a strategy how to position its body in a way so it can conveniently reach for the reward? Maybe the mouse also learned with ongoing training how to navigate the food pellet through the thin slid in the wall. I think that the advantage of the learning paradigm in the study of Xu et al. (2009) is that it critically involves the target structure, the motor cortex, which is without doubt the main processing unit of the learning content. The disadvantage of the learning task on the other hand is that the observed spine turnover might reflect the effect of multiple learning events, therefore insights into mechanistic processes of single learning events and their time course cannot be obtained. Similar arguments speak against the learning paradigm used by Yang et al. (2009). Here, mice underwent an accelerated rotarod training over two days which resulted in significant spine turn-over changes (Yang et al., 2009). Multiple learning and association steps may have lead to the observed result. One learning event might have been that animals changed their gait pattern to stay on the accelerated rod. Additionally, animals might have learned to use their tail to balance on the rotarod. Again, one cannot disentangle single learning event effects.

I further want to discuss the learning paradigm used by Kuhlman et al. (2014). Here, head-fixed mice learned an active, whisker-dependent object localization task. Animals responded with licking when they located a descending pole at a defined position. The authors found enhanced spine growth in layer II/III neurons of the barrel

cortex during initial skill acquisition (Kuhlman et al., 2014). An advantage of the learning paradigm is that the animal actively moves its whisker to locate the object, a behavior that naturally occurs when the animal explores its environment and therefore must be of great relevance. The disadvantage of the learning task is again that multiple learning events occur in parallel, so that the time course of induced neocortical reorganization cannot be followed for as single learning content. First, the animal has to learn to move its whiskers to receive information. It can then learn to adjust its whisking strategy to increase the number of active touches. Further, the animal learns to distinguish between at least two pole positions and to associate the correct position with reward. It stays unclear which learning process contributed to reported spine plasticity and at which time during the acquisition.

In the present study I used a simple association task, namely classical trace eyeblink conditioning, to precisely investigate the effect of a single learning event on the spine turn-over. In trace eyeblink conditioning animals have to make one single association: a whisker stimulus (CS) predicts a corneal air puff (US); therefore they reflexively close their eye (CR). Studies which use fear conditioning as a learning paradigm to induce structural spine plasticity also overcome the problem of generating multiple learning aspects (Lai et al., 2012; Moczulska et al., 2013). Here, animals have to associate a tone (CS) with a foot shock which reflexively induces freezing behavior (CR). In my opinion, classical fear conditioning and classical eyeblink conditioning are learning paradigms which are well suited for the investigation of synaptic plasticity underlying a single learning event and therefore they can be used to determine the time course for neocortical learning.

5.2 Mechanisms of cellular processes underlying spine loss

The main result of my doctoral work is that during the acquisition of the trace eyeblink conditioning task a substantial loss of dendritic spines can be observed in the cortical layer I of the barrel cortex. Previous work studying in vivo spine dynamics during learning described net spine formation in layer I of the motor cortex during motor learning (Xu et al., 2009; Fu et al., 2012), in auditory cortex during fear conditioning (Moczulska et al., 2013) and in barrel cortex during a whisker-dependent object localization task (Kuhlman et al., 2014). As already mentioned a recent ex vivo study by Chau and colleagues (2014) further reports a training-dependent spine proliferation in layer IV during trace associative learning. This result stands in contrast to my finding of substantial spine loss in cortical layer I. A possible explanation for this discrepancy might be that reorganization of cortical connections in response to learning is layer dependent. Net spine elimination in layer I during learning has so far only been observed in prefrontal association cortex during fear conditioning (Lai et al., 2012).

In the following sections of the discussion I will present explanations for the observed column specific spine loss. First, I will discuss possible mechanisms of cellular processes underlying the detected spine loss. Subsequently, I will uncover possible mechanisms involved in the columnar specificity of learning induced spine plasticity and finally I will discuss the role of the primary sensory cortex for trace conditioning.

There are several cellular processes that could be involved in the observed decrement in spine number. First of all, layer I synapses may have been affected by long term depression (LTD). LTD is an activity-dependent reduction in the efficacy of neuronal synapses in response to a strong or long lasting stimulus pattern. LTD is thought to result mainly from a decrease in postsynaptic receptor density (Ogasawara et al.,

2008). LTD is one of several processes that serves to selectively weaken specific synapses in order to make constructive use of synaptic strengthening caused by long term potentiation (LTP), the opposing process to LTD; which leads to long-lasting increase of synaptic strength (Massey and Bashir, 2007; Engert and Bonhoeffer, 1999). LTD plays an important role in preserving the brains plasticity because, if allowed to continue increasing in strength, synapses would ultimately reach a ceiling level of efficiency, which would inhibit the encoding of new information. Using two-photon imaging Zhou et al. (2004) showed that the induction of LTD is accompanied by spine shrinkage in acute hippocampal slices. The authors speculate that over a protracted time course, spine shrinkage and LTD may lead to synapse elimination mediated by continuous loss of α-amino-3-hydroxy-5-methylisoxazole-4-propionicacid (AMPA) receptors (Carroll et al., 1999) and to the reduction of the size of the post synaptic density (PSD) (Zhou et al., 2004).

In this context, another cellular process may also have contributed to the observed spine loss: spike timing-dependent plasticity (STDP). STDP is an expression of plasticity, in which a millisecond-scale change in the timing of presynaptic and postsynaptic action potentials leads to changes in postsynaptic calcium signaling, inducing either LTP or LTD. Jacob and colleagues, for instance, found in 2007 that LTD occurs in the rat barrel cortex when postsynaptic spikes precede presynaptic spikes by up to 20 - 50 ms (Jacob et al., 2007). Postsynaptic activation preceding specific inputs onto dendritic spines in layer I may have given rise to LTD dependent spine loss.

Another factor that might have had an impact on the observed elimination of synapses is intracellular signaling. A valid speculation is that plasticity of synapses connecting

the apical dendrites of layer V pyramidal neurons is related to prominent calcium plateau potentials generated by the apical dendrites of these cells (Varga et al., 2011; Xu et al., 2012; Larkum, 2013a; Hill et al., 2013). Using two-photon calcium imaging in mice performing an object-localization task Xu et al. (2012) recorded dendritic activity in layer V pyramidal neurons. The authors observed global large- amplitude signals throughout the apical tuft dendrites when the animal actively touched an object at a particular location or whisker angle (Xu et al., 2012). Xu and colleagues claim that the global calcium (Ca^{2+}) signals are produced by dendritic plateau potentials that require both vibrissal sensory input and primary motor cortex activity. These results provide evidence of nonlinear dendritic processing of correlated sensory and motor information in the neocortex. The pyramidal neuron is able to detect coincident input to proximal and distal dendritic regions. In this context, Larkum (2013b) suggests an associative mechanism at the cellular level for combining feed-forward and feedback information (Larkum, 2013b). In the present study dendritic calcium signaling might have played an important role in modulating the synaptic input of monitored layer V pyramidal neurons in layer I.

Ca^{2+} signaling does not only occur in dendrites, several studies show that Ca^{2+} currents can be monitored within single dendritic spines (Denk et al., 1996; Takechi et al., 1998; Koester and Sakmann, 1998; Higley and Sabatini, 2012). One of the first studies which reported this new synaptic response was Takechi et al. in 1998. Using high-resolution cellular imaging, these authors identified this class of postsynaptic response which consists of a transient increases in Ca^{2+} concentration and which is apparent while changes in somatic membrane potential are absent. The authors speculate that the Ca^{2+} signal might be one of the critical cues, which determines the input specificity of LTD (Takechi et al., 1998). Imaging studies further revealed that

Ca^{2+} can enter spines through voltage-sensitive and ligand-activated channels, as well as through Ca^{2+} release from intracellular stores. The relationship between Ca^{2+} signals and induction of various forms of synaptic plasticity are beginning to be elucidated. Ca^{2+} signaling within the dendritic spine might have been an additional cellular process underlying the spine loss observed in the present study. To answer the question whether Ca^{2+} signaling in dendrites or spines plays an important role in the induction of structural synaptic plasticity during trace eyeblink conditioning in the future behavioral training needs to be combined with calcium imaging of apical dendrites of layer V pyramidal neurons.

5.3 Mechanisms of columnar specificity

In my doctoral work I show that spine elimination occurs exclusively at the site within primary sensory cortex at which the conditioned stimulus is represented. Structural plasticity in primary sensory cortex related to trace eyeblink conditioning therefore might be strictly limited to the representation of the memorized sensory context. But how is this spatial specificity achieved?

The lack of spine loss in the neighboring column E2 observed in the present study, is the first evidence for strict columnar specificity outside the layer IV barrel structures and their associated thalamo-cortical afferents (Welker, 1976; Wimmer et al., 2010; Oberlaender et al., 2011). It strongly suggests that the pruned synapses must originate from cellular elements carrying distinct column specific information and that these elements project to layer I. Cortico-cortical terminals originating from the posterior-medial thalamic nucleus (POm), the motor cortex (M1), and association tactile areas, which are known to project to the barrel cortex layer I are unlikely to fulfill

this requirement (Cauller and Connors, 1994; Cauller et al., 1998; Oberlaender et al., 2011; Petreanu et al., 2012). Also infragranular layer neurons in the home column are unlikely candidates, as they display the largest receptive fields within the column (Ghazanfar and Nicolelis, 1999; de Kock et al., 2007). Stronger contenders are spiny stellate and pyramidal cells in layers IV and II/III - both cell types project to layer I and have receptive fields largely restricted to one or very few whiskers (de Kock et al., 2007). Fittingly, dendrites of layer II pyramids, which are assumed to receive inputs comparable to that of layer V apical tufts, have been demonstrated to house a distinct subset of spines which are activated only by one whisker (Varga et al., 2011).

I think that the synaptic plasticity revealed in this study is involved in modifying column specific processing which may arise via an interaction of intercolumnar spatially precise ascending and top-down associative signals transmitted via horizontal inputs to layer I. In this context, the pruning of synapses could mean that weak connections are recycled while strong connections are strengthened. Unfortunately, image resolution acquired during two-photon imaging in awake mice was not high enough to reveal changes in spine shape or size. Eventually, locally defined disinhibitory microcircuit comparable to those identified in the study of Letzkus et al. (2011) (section 2.4) may have also contributed to the observed spine decrement. For now several questions stay unanswered: what was the presynaptic input to the spines which were lost over the course of the behavioral training and where do the involved neurons originate? To answer these questions in the future viral injection of anterograde and retrograde tracers could useful to identify neurons which contact layer V pyramidal neurons in layer I.

5.4 Role of primary sensory cortex for trace conditioning

The present study shows that classical trace eyeblink conditioning results in a column specific loss of dendritic spines in the cortical layer I of the barrel cortex. Spine elimination seems to be a layer specific phenomenon as Chau et al (2014) report a training-dependent spine proliferation during trace associative learning in layer IV (Chau et al., 2014). These observations lead to the questions: what is the critical role of the primary sensory cortex in the tactile variant of the trace eyeblink conditioning task and what happens in different cortical layers?

To answer this question it is vital to know which brain structures are critically involved in the association task and in which sequence and under which hierarchy do they communicate with each other. There is strong evidence that trace eyeblink conditioning is dependent on the cerebellum and associated brainstem structures (McCormick and Thompson, 1984; Thompson and Krupa, 1994; Boele et al., 2010), the hippocampus (Solomon et al., 1986; Moyer et al., 1988; Moyer et al., 1990; Tseng et al., 2004) and the medio-prefrontal cortex (mPFC) (Weible et al., 2000; Leal-Campanario et al., 2013). Additionally, the primary sensory cortex seems have an important role for the tactile variant of trace eyeblink conditioning. By performing chemical lesions prior to learning Galvez and colleagues showed that subjects were unable to acquire trace conditioned response (Galvez et al., 2007). The same study shows that barrel cortex is still important during the retention of the learning content as its blockade significantly reduces the animals' performance, yet the learning behavior is not entirely abolished.

Many studies were performed to identify the brain structures involved in the classical conditioning task, but very little is known about the interplay between the mentioned key structures. A very important insight into the functional mechanisms underlying the communication between structures was given by the study of Siegel and colleagues (2012). They showed that the mPFC exhibits persisting neuronal activity throughout the trace period, potentially bridging the time gap between the CS and the US (Siegel et al., 2012). Woodruff-Pak and Disterhoft (2008) suggest that this preserved activity could allow a signal transduction to pontine nuclei leading to a coupling of the CS and the US in the interposed nucleus, a deep nucleus of the cerebellum (Woodruff-Pak and Disterhoft, 2008). There is further evidence that the cerebellum provides the basis for the association (Delgado-García and Gruart, 2006; Woodruff-Pak and Disterhoft, 2008; Kalmbach et al., 2010). Using electric stimulation of mossy fibers Kalmbach et al (2010) found that CS-driven and mPFC-like inputs are necessary and sufficient for the cerebellum to learn well-timed trace conditioned responses.

At first glance the above mentioned studies seem to support the hypothesis that the barrel cortex takes over a mere assisting role, especially in the acquisition phase, providing a sensory throughput towards higher association or communication centers like the mPFC, which exhibits persisting neuronal activity throughout the trace period. This point of view, however, does not explain the spatial specificity of observed map plasticity expressed as a widening of layer IV barrels after trace eyeblink conditioning in rabbits and mice (Galvez et al., 2006, 2011). The column specific learning induced spine loss found in the present study also strongly speaks against the assumption that barrel cortex is just a sensory throughput station, especially as pseudo conditioned mice, receiving the same sensory input do not show the two aspects of plasticity. Therefore, I suggest an interaction of the barrel column with downstream association

centers during early learning phases, allowing the selection of cortical columns which are enlarged and in which spine plasticity guided by the learning success occurs. An interaction of intercolumnar spatially precise ascending and top-down associative signals transmitted via horizontal inputs to layer I may explain why spine loss is detected in layer I and not in layer IV. Reciprocal modulations in neuronal activity between barrel cortex and other neuronal structures involved in the association task may have lead to a sensory tagging process facilitating the processing of incoming stimuli information. A study which hints to this direction was published by Ward et al. (2012). Authors report learning-related changes in firing rates of infragranular neurons in the somatosensory cortex of rabbits recorded during task acquisition. Neurons in layers V and VI in both conditioned and pseudo conditioned animals robustly responded to whisker stimulation, but exclusively neurons recorded in conditioned animals showed a significant enhancement in responsiveness in concert with learning (Ward et al., 2012). If barrel cortex contributes to sensory tagging, I expect its impact to be highest during initial learning, as spine turnover rates are mainly elevated during the acquisition phase and its role during retention becomes less important (Galvez et al., 2007).

In conclusion, primary sensory cortices are critical for trace conditioning, but their exact mechanistic role in the declarative learning task is still elusive. To investigate whether there is some kind of interaction between barrel cortex and medio-prefrontal cortex throughout CS, trace and US periods (via firing rate or neuronal oscillations) further electrophysiological experiments investigating supragranular layers are needed.

6. References

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