MECHANISMS
OF VISUAL CORTICAL PLASTICITY
INDUCED BY FOCAL RETINAL LESIONS

by

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Statement

I certify herewith that the dissertation at hand was completed and written independently and without outside assistance. The “Guidelines for Good Scientific Practice” according to § 9, Sec.3 were adhered to. This work has never been submitted in this or similar form at this or any other domestic or foreign institution of higher learning as a dissertation.

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II. ABBREVIATIONS

- (d)LGN – (dorsal) lateral geniculate nucleus
- AMPA - $\alpha$-amino-3-hydroxyl-5-methyl-4isoxazole-propionate
- AP – action potential
- BCM – Bienenstock Cooper Munro (theory)
- BDNF – brain-derived neurotrophic factor
- CA1/CA3 - hippocampal subfields 1 & 3 of the ammonus horn
- CaM - calmodulin
- CAMK – calcium/calmodulin dependent kinase
- CaMKII - Ca2+/calmodulin dependent kinase II
- cAMP - cyclic adenosine monophosphate
- CB1 – cannabinoid receptor 1
- CCK – cholecystokinin
- Ch – chandelier cell
- CNS - Central Nervous System
- CREB – cAMP response element binding protein
- DB – double bouquet cell
- eEPSC - evoked excitatory postsynaptic current
- eIPSC - evoked inhibitory postsynaptic current
- EPSP - excitatory postsynaptic potential
- GABA – $\gamma$-aminobutyric acid
- $\text{GABA}_A$ - $\gamma$-aminobutyric acid receptor type A
- GAD – glutamic acid decarboxylase
- IPSP - inhibitory postsynaptic current
- LPZ – lesion projection zone
- LTD – long-term depression
- LTP – long term potentiation
- mEPSC - miniature excitatory postsynaptic current
- MHC-I – major histocompatibility complex class I
- min - minute
- mIPSC - miniature inhibitory postsynaptic current
- mPSC - miniature postsynaptic current
- NMDA – N-methyl-D-aspartate
- PBS – phosphate buffer saline
- PFA - paraformaldehyde
- PV – parvalbumin
- RMP – resting membrane potential
- STDP – spike-timing dependent plasticity
- TBS – theta-burst
- TNF-α – tumor necrosis factor $\alpha$
- TrkB - tropomyosin-related kinase B
- VSD (I) - voltage sensitive dye (imaging)
III. COMPOUNDS

- **DNQX** - 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione
- **TTX** – tetrodotoxin
- **PTX** – picrotoxine
- **D-AP5** - D-2-Amino-5-phosphonovaleric acid
- **RH – 1838**, oxonol-based voltage-sensitive dye
- **EGTA** - Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
- **HEPES** - N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)
- **QX-314** – N-(2,6-dimethylphenyl)acetamide-2-triethylammonium bromide
IV. ABSTRACT

Focal visual deprivation resulting from retinal lesion can trigger functional reorganization in the initially silenced area of primary visual cortex. It is proposed that intrinsic cortical connectivity can be a substrate enabling recovery of function within such a lesion-projection zone (LPZ). This work addressed retinal lesion-induced changes in the function of intrinsic cortical connections in the layer 2/3 of primary visual cortex of rats. First, the spread of synaptic activity in the LPZ in millisecond time resolution using voltage-sensitive dye imaging was visualized. Shortly after the lesion, the majority of the neurons within the LPZ were subthresholdly activated by delayed propagation of activity that originated from unaffected cortical regions. With longer recovery time, latencies within the LPZ gradually decreased and activation reached suprathreshold levels, indicative of possible involvement of Hebbian mechanisms in reorganization. The involvement of different plasticity mechanisms in reorganization was further addressed using patch-clamp recordings in vitro in slices of primary visual cortex. 8 – 10 days after the lesion bidirectional scaling of excitatory and inhibitory synapses in the LPZ was observed, leading to dramatic shift of the excitation/inhibition ratio in layer 2/3 pyramidal cells towards excitation and increased levels of spontaneous firing in the LPZ. A potentiation of eEPSCs and depression of eIPSCs coming from long-range horizontal inputs were noted, while both excitatory and inhibitory transmission in layer 4 inputs to layer 2/3 pyramidal cells were depressed. Overall, the excitation/inhibition ratio shifted strongly for horizontal inputs, but remained close to control values in layer 4 to layer 2/3 inputs. However, no enhancement of LTP could be observed in the intra-LPZ pyramidal cells in layer 2/3. On the contrary, most cells did not express LTP at all or even displayed a depression of EPSPs. Therefore, lesion-induced cortical reorganization appears to involve complex interplay between homeostatic and Hebbian mechanisms.
1. INTRODUCTION

Sensory and motor cortical areas display a remarkable degree of plasticity during development and are readily modifiable by experience. Although some aspects of cortical processing units, like ocular dominance columns, are plastic only during a narrow time window during development and hard-wired after the closure of critical period, other properties of cortical maps, like orientation and retinotopic maps remain dynamic through the lifespan of animals. For example, inputs that are most active and used gain larger cortical territory during and after application of perceptual learning protocols, peripheral injury or intracortical microstimulation (Godde et al., 2002; Gilbert et al., 1998; Buonomano & Merzenich, 1998). The adult visual cortex, for example, is capable of plastic reorganization that can partially recover function lost as a result of retinal lesion (Kaas et al., 1990; Gilbert & Wiesel, 1992; Dreher et al., 2001; Heinen & Skavensky, 1991; Schmid et al., 1996). In primary visual cortex, focal retinal lesion cuts-off retinal input leading to a cortical region in which no visually evoked spikes can be detected. However, after a given period of recovery, lesion-affected neurons regain responsiveness with receptive fields shifted towards positions represented by neurons at the border of the lesion projection zone (LPZ) (Gilbert & Wiesel, 1992; Heinen & Skavenski, 1991; Das & Gilbert, 1995; Calford et al., 2000; Giannikopoulos & Eysel, 2006), even within hours after lesion (Schmid et al., 1995; Calford et al., 1999). Although a lot is known about synaptic and network changes underlying ocular dominance shifts during the critical period, much less is known about synaptic and network mechanisms coming into play during visual cortical plasticity induced by retinal lesions. Below I review the current state of the knowledge about lesion-induced plasticity of sensory cortices and known forms of synaptic plasticity which may be relevant to them.

1.1. Plasticity of cortical sensory maps

The initial evidence for plasticity of cortical maps in adult animals came from studies in the somatosensory system of monkeys (Merzenich et al., 1983a; Merzenich et
al., 1983b; Merzenich et al., 1984). In monkeys removal of a finger causes alteration of the body surface's map in S1: cortical area originally receiving input from the amputated finger switches to representing adjacent fingers. Similar observations were later found in both sensory and motor areas after different types of peripheral lesions and even after change in usage mode of limb muscles in motor cortex (Gilbert, 1998; Kelahan & Doetsch, 1984; Calford & Tweedale, 1991; Calford & Tweedale, 1988; Rasmusson, 1982; Donoghue et al., 1990; Sanes et al., 1992). In the auditory system, for instance, partial removal of the cochlea leads to changes in tonotopic auditory map so that the representation of frequencies taken up by the destroyed part of the cochlea shrinks and an expansion of representations of neighboring frequencies occurs (Kamke et al., 2005; Rajan et al., 1993; Robertson and Irvine, 1989).

In the visual system, described form of reorganization has been demonstrated with focal retinal lesions leading to removal of visual input to a localized portion of primary visual cortex (Heinen & Skavenski, 1991; Gilbert & Wiesel, 1992; Giannikopoulos & Eysel, 2006; Kaas et al., 1990; Chino et al., 1992). Following introduction of lesions in some cases it was possible to follow the effect of the lesion over a time course of minutes to many months after the lesion was actually made. In cats and monkeys, matched binocular lesions resulted in formation of anatomical and functional scotoma in primary visual cortex - a zone receiving input from the lesioned part of the retina. Initially this area, referred to as “lesion projection zone (LPZ)” is silenced, but over a period of a few months recovers visually driven activity. Immediately after lesion the receptive fields of the cells in the vicinity of the LPZ's border expanded. Over a time of several weeks to several months the neurons inside the LPZ switched from representing the visual space affected by the lesion to representing the area surrounding the lesion. Thus, an effective remapping of the cortical topography occurred, leading to shrinkage of the representation of the retinal lesion and expansion of representation of lesion's surround. The reorganization has been observed also with monocular lesions (Schmid et al., 1995, 1996). Interestingly, in this model it was reported that focal central monocular lesions lead to altered topographic maps in response to stimulation of the lesioned eye and normal maps in response to unaffected
eye (Schmid et al., 1996; Calford et al., 2003; Young et al., 2007). The extent of the reorganization was shown to be quite substantial, up to several cortical millimeters in cat and monkey (Gilbert, 1998; Buonomano & Merzenich, 1998). However, the degree of functional recovery was found to be different in various models. For example, matched bilateral lesions resulted in an almost normal orientation tuning, direction selectivity and spatial frequency tuning in intra-LPZ neurons (Chino et al., 1995), while in monocular lesion model the post-reorganization responses in contrast to unaffected units were substantially weaker and displayed rapid habituation when subjected to repetitive stimulation (Schmid et al., 1996). However, the contrast thresholds were found to be elevated in intra-LPZ neurons also in matched binocular lesion models (Chino et al., 1995). Finally, results obtained in monkey visual cortex with metabolic markers and fMRI pointed to the limited extent of reorganization even after one year post lesion (Horton & Hocking, 1998; Smirnakis et al., 2005). Measurements in human subjects, often combining measures of neuronal activity, like fMRI or EEG with psychophysics, showed a degree of reorganization occurring in the primary visual cortex, together with a perceptual fill-in, in course of degenerative disease of retina. The perceptual fill-in manifests in the fact that the „blind“ area, initially appearing in a part of visual field affected by the lesion disappears, and the stimuli passing through the retinal scotoma start to appear complete, albeit distorted (Gilbert, 1998). For example, a square-shaped stimulus presented in the vicinity of the lesion-affected part of visual field would appear as a rectangle elongating towards lesion-affected area (Dilks et al., 2007).

Mechanisms underlying cortical reorganization, the role of subcortical structures in it and potential for reorganization in different sensory modalities and different species appear to rely on different substrates depending on modality. For example, extent of reorganization observed in primary somatosensory cortex after digit amputation in the monkey led to the suggestion that the changes responsible for the functional recovery were intrinsic to the cortex. However, in other studies in the somatosensory cortex of rodents reorganization was also demonstrated at much earlier stages of processing - in spinal cord and nucleus gracilis. Sprouting of afferent terminals in the spinal cord and brainstem after peripheral nerve damage was indicative of the potential for
reorganization at early levels in the somatosensory pathway (Cameron et al., 1992; Doubell et al., 1997; Florence et al., 1993, 1995; Woolf et al., 1992). Reorganization has also been observed in the somatosensory thalamus after downstream lesions in primates and rodents alike (Garraghty & Kaas, 1991; Rhoades et al., 1987). Some of the changes at the cortical level can also reflect reorganization of the peripheral innervation, when axons that originally controlled the lost digits rewired to control the edge of the stump (Manger et al., 1996). Such early changes may affect reorganization at consequent points in somatosensory pathway. Extent of reorganization, initially measured to be about 2 mm in primate and feline sensory cortex was indicative of involvement of plasticity of thalamocortical arborizations (Rajan et al., 1993; Merzenich et al., 1983b; Calford & Tweedale, 1988). However, with increase in post lesion times and extent of initial deafferentation the reorganization occurred as far as 10 mm of cortical surface inside the LPZ (Pons et al., 1991).

Adult visual system generally has minimal involvement of subcortical mechanisms in reorganization. In visual pathway, unlike in somatosensory pathway, the subcortical structures do not show pronounced lateral connectivity. In most experiments in cats and primates the extent of reorganization was found to be about 6 – 8 mm, which is much larger than the extent of thalamocortical arbors in these species (about 2 mm) (Blasdel & Lund, 1983; Freund et al., 1985, 1989; Ferster & LeVay, 1978). Some degree of reorganization was observed in lateral geniculate nucleus, but it never exceeded 500 micrometers, even when the cortex showed almost complete filling-in (Eysel et al., 1980, 1981; Gilbert & Wiesel, 1992), indicative of profound involvement of intracortical mechanisms (Darian-Smith & Gilbert, 1994). The extent of reorganization, ~6-8 mm in diameter, could not be explained by the lateral spread of thalamic afferents, which spread ~1.5-2 mm laterally in monkey and cat visual cortex, unless they increased their projection pattern into the center of the reorganized region. In addition, no sprouting of thalamocortical axons inside the LPZ was found (Darian-Smith & Gilbert, 1994; Gilbert & Wiesel, 1992).

Importance of intracortical connectivity also comes from a group of studies, performed in a monocular retinal lesion model in a cat. In this model, a monocular retinal lesion is made in one eye, while leaving other eye intact. This offers an opportunity to measure the properties of neuronal responses in the LPZ and changes in
neuronal receptive fields of "reorganized" inputs (coming from the periphery of the LPZ) and baseline inputs, coming from a normal visual pathway of the unaffected eye. While studying this model several important findings were made. First, while "reorganized" receptive fields underwent a substantial spatial shift towards LPZ's border after the lesion, the unaffected eye's receptive fields did not change their position. Second, the activity, elicited in the intra-LPZ neurons via stimulation of the lesioned eye was greatly affected by application of blockers of glutamatergic synaptic transmission in the cortex outside the LPZ lateral to the recording site, while it was not the case with unaffected eye stimulation (Calford et al., 2003). These observations point to the role of intrinsic cortical connectivity in reorganization.

The likely sources of the reorganization are represented by intrinsic cortical connectivity: intra-areal long-range horizontal connections and top-down connections from the higher visual areas. A number of results from different studies can be interpreted in support of involvement of long-range connections in visual cortical reorganization. The first consideration is anatomy of long-range connections. In monkey V1 long-range connections normally extend about 6-8 mm - the same distance over which the cortical reorganization has been observed, and are capable of providing cells in the LPZ with input from parts of the visual field surrounding that covered by the retinal lesion. Importantly, long-range lateral connections were implicated in shaping orientation selectivity columns during early development and also preferably link groups of neurons that share ocular dominance and orientation preference (Malach et al., 1993; Lamme et al.,1998; Shouval et al., 2000). A study in cat, employing a combination of intrinsic optical imaging and single-unit recordings, showed that the pattern of orientation columns emerging inside the lesion-projection zone after recovery crudely matches the one seen before the lesion, despite the fact that the receptive fields of the cells in this region shift considerably in visual space towards the lesion's border (Das & Gilbert, 1995). Since horizontal connections preferentially link neurons of similar orientation preference, the involvement of a pre-existing framework of horizontal connections in the reorganization would cause the reorganized cortex to recover its original pattern of orientation columns.
Little is known about cellular and network mechanisms that might underlie the possible increase in strength of horizontal connections. Changes in expression and modification of a number of molecular substrates known to play a role in triggering and maintenance of various forms of synaptic plasticity were documented in the LPZ (Obata et al., 1999; van den Bergh et al., 2003; Arckens et al., 2000a & 2000b). Those range from immediate early genes, like zif268 and c-fos, mediators of LTP, like CaMKII, factors involved in developmental maturation of visual cortical circuitry, like BDNF, to GABA-synthesizing enzymes, components of synaptic vesicle recycling machinery and other molecules, commonly associated with plasticity and growth (Obata et al., 1999; van den Bergh et al., 2003; Arckens et al., 2000b; Berardi et al., 2003; Leysen et al., 2004). Despite all those studies, the exact role of each factor remains unknown as well as particular changes that they take part in. Of note are a group of studies, pointing at possible change in balance between excitation and inhibition in the primary visual cortex after the lesion (Massie et al., 2003a; Massie et al., 2003b; Qu et al., 2003; Rosier et al., 1995; Arckens et al., 1998; Arckens et al., 2000a). Iontophoretic measurements showed, for instance, that glutamate concentration was increased in the area at the border of LPZ over a time of several weeks after the lesion, and a matching decrease in GAD65 expression and drop in GABA immunoreactivity was also observed. Moreover, in cat, the area of supposed hyperexcitability, thought to result from described effects, was changing its position from the border of the LPZ to deeper inside the LPZ with increasing post lesion time (Arckens et al., 2000a). It is known, that under normal conditions, the lateral spread of activity in the visual cortex and the function of long-range connections is under strict regulation of excitatory-inhibitory balance and changes of this balance partly mediate modulatory effects of long-range connections in respect to extraclassical receptive fields (Gilbert, 1998). An immediate expansion of receptive fields of neurons located at the border of the LPZ directly after performing a lesion in the retina, is thought to result from disinhibition, caused by removal of dominant vertical input. Disinhibition also may be responsible for short-term perceptual effects brought about by presentation of „artificial scotoma“, which blocks a part of visual input during stimulus presentation (Kelly et al., 1999; Gilbert, 1998; Eysel et al., 1999; Buonomano & Merzenich, 1998). To study the contribution of different plasticity mechanisms and role of excitatory-inhibitory balance further experiments are needed. Below I discuss the mechanisms of changes in synaptic strength, shown to be of importance in the visual cortex and the possibility of their involvement in cortical
1.2. Long-term potentiation and long-term depression

Long-term potentiation and long-term depression were described in hippocampus first and studied afterwards in a multitude of excitatory and inhibitory synapses, including those in the primary visual cortex (Daw et al., 2004; Wang & Daw, 2003; Malenka & Bear, 2004). Possible involvement of LTP and LTD in retinal lesion-induced plasticity is indicated by several lines of evidence. First, expression of several molecules involved in induction, expression and maintenance of LTP and LTD changes in V1 as a result of retinal lesion. One of such molecules, whose role as an LTP mediator is backed up by an overwhelming body of experimental evidence is calcium/calmodulin-dependent kinase II, which appears to be central to LTP induction, despite of the particular experimental protocol used, except for the time very early in postnatal development (Malenka & Bear, 2004; Lisman et al., 2002; Malenka & Nicoll, 1999; Kirkwood et al., 1997). Another example is BDNF, which was proposed to trigger presynaptic changes during plasticity, acting as a retrograde messenger (Poo, 2001). Both CaMKII autophosphorylation levels and expression of BDNF in V1 are strongly affected by retinal lesion (Obata et al., 1999; van den Bergh et al., 2003). While initial phase of LTP (“early“ phase) depends on a rapid change in dynamics of postsynaptic AMPA-receptors and is not transcription dependent (Malenka & Bear, 2004; Malinow & Malenka, 2002), ongoing strengthening of synapses (“late phase“), occurring after the initial potentiation and lasting for as long as weeks, depends on production of new proteins and expression of certain genes. Again, calcium-calmodulin dependent kinases, such as CaMKII & CaMKIV were implicated as messengers who link the initial rise in intracellular calcium to transcriptional activation of genes in the nucleus via regulation of transcription factor CREB and immediate early gene products, like zif268 and c-fos. Long-lasting change in expression of those immediate early products was observed after retinal lesions in both LPZ and distant parts of V1 (Arckens et al., 2000b).

Second, potentiation and depression of stimulus-evoked responses, sharing some properties with LTP or LTD can take place both in adult and developing sensory cortex as a result of manipulations with sensory input. One situation where a role for LTP in visual cortical development was implied is ocular dominance plasticity, induced by
monocular deprivation (Smith et al., 2009; Gordon et al., 1996; Taha et al., 2002). In rat visual cortex, a homosynaptic NMDA-receptor dependent LTP can be induced in layer 4 to layer 2/3 inputs with a conventional tetanic stimulation protocol and also in layers 3 & 4 via application of tetanus in white matter and LGN. Such LTP is also enough to sustain the increase in the magnitude of visually-driven responses (Kirkwood & Bear, 1994; Heynen & Bear, 2001). In mice and cats monocular deprivation elicits bidirectional effects – while responses of visual cortical neurons to deprived eye fade already after 3 days of deprivation (in some cases as early as 12 hours after start of deprivation), the responses to open eye grow in strength with a slower timecourse, change becoming evident after 5 days of deprivation (Smith et al., 2009; Frenkel & Bear, 2004; Mioche & Singer, 1989; Sawtell et al., 2003). However, in mice with genetically reduced expression of NMDA receptors in the superficial layers of visual cortex the potentiation of responses to open eye failed to occur (Sawtell et al., 2003). In barrel cortex it was shown that sensory experience drives recombinant GluR1 into the postsynaptic sites in layer 2/3 neurons. Moreover, this experience-dependent potentiation could be prevented by expressing a cytoplasmic tail of GluR1, which prevents delivery of GluR1 subunits and their insertion to the postsynapse during LTP induction (Takahashi et al., 2003).

Ocular dominance plasticity was also proposed to rely on homosynaptic LTD. It was suggested by BCM theory that depression of deprived inputs in developing visual cortex was triggered by presynaptic activity consistently failing to produce a a strong evoked postsynaptic response (Bienenstock et al., 1982). Indeed, certain level of presynaptic spontaneous activity was needed to induce an ocular dominance shift: an injection of tetrodotoxin in the deprived eye caused much more modest depression of deprived eye responses than lid suture, as it abolished the spontaneous activity waves in the retina (Rittenhouse et al., 1999). In visual cortex two forms of NMDA-receptor dependent LTD were described. First one shares properties with a classical hippocampal LTD at CA1 synapses – it can be induced by long-term low frequency stimulation, depends on protein phosphatase activation, is expressed postsynaptically and involves dephosphorylation of ser-845 of the GluR1 subunit (Heynen et al., 2003; Kirkwood et al., 1993; Dodt et al., 1999). This form of LTD actually leads to an alteration of number and phosphorylation levels of postsynaptic AMPA receptors (Heynen et al., 2003). Similarly to deprivation-induced depression this LTD can be induced only during critical period for ocular dominance plasticity and fails to occur after intraocular
tetrodotoxin injection. The second form depends on spike-timing mechanism – it involves precise pairing of presynaptic stimulation and postsynaptic spikes, requires coincident activation of presynaptic NMDA and CB1 receptors, and a rise in postsynaptic calcium. This LTD is expressed presynaptically (Sjostrom et al., 2003).

Evidence for involvement of LTD-like mechanisms with deprivation-induced changes of synaptic transmission also comes from ex vivo studies in cortical slices. Such studies follow the assumption that if deprivation uses the same mechanisms and molecular pathways as LTD, then LTD should be occluded in visual cortex by prior deprivation. Deprivation protocols used for such studies were the classical monocular deprivation for visual cortex and removal of whisker rows to induce plastic changes in rat barrel cortex (Heynen et al., 2003; Allen et al., 2003; Crozier et al., 2007). Analysis of single-unit recordings in partially deprived barrel cortex showed reversed order of spiking of units in layer 4 and superficial cortical layers in the deprived barrel, with layer 2 units spiking before layer 4 units after removal of principal whisker (Celikel et al., 2004). Analogous combination of pre- and postsynaptic spikes in layer 2/3 neurons, with postsynaptic firing occurring before presynaptic layer 4-originating spikes was also shown to induce LTD in vitro (Feldman, 2000, Feldman & Brecht, 2005; Bender et al., 2006a, 2006b). In visual cortex monocular deprivation was shown to occlude both CB1 receptor – dependent LTD in layer 2/3 and NMDA receptor – dependent CA1 area – like LTD occurring in layer 4 (Crozier et al., 2007). It was also shown, that monocular deprivation affects the same molecular mechanisms that are engaged during classical CA1-like LTD in visual cortical layer 4, altering the internalization of AMPA-receptors (Yoon et al., 2009).

1.3. Spike-timing dependent plasticity and cortical reorganization

Important feature of Hebbian forms of plasticity is their dependency on the order of firing of post- and presynaptic neurons. Presynaptic firing preceding postsynaptic depolarization is known to drive the synapses to express LTP, while reversal of the spiking order or asynchronous random firing leads to LTD (Debanne et al., 1994; Abbott & Nelson, 2000). Spike-timing dependent plasticity was shown to
have a role in development and plasticity of the sensory cortices, especially rodent barrel and visual cortex (Feldman & Brecht, 2005; Dan & Poo, 2006). As a result of large number of studies in various preparations STDP was established as a highly temporally asymmetrical phenomenon and temporal windows during which LTP and LTD could be induced by pairing spikes in various preparations were defined (Bi & Wang, 2002; Abbott & Nelson, 2000). Generally, LTP is induced if a postsynaptic spike follows a presynaptic within a period of 0 to about 20 – 40 ms, while a reversed order of spikes with postsynaptic firing taking place as much as 200 ms before presynaptic leads to induction of the LTD. Thus, absence of stimulus-driven firing and spontaneous random activity occurring during different sensory deprivation protocols can trigger net spike-timing dependent changes in many synapses across the deprived area (Feldman & Brecht, 2005). Presynaptic spikes in this case arrive at random intervals in respect to postsynaptic firing and both LTP and LTD can occur as a result of this situation. In layer 2/3 pyramidal cells random pairing of pre- and postsynaptic spikes is known to reduce the overall strength of synapses (Feldman, 2000).

Spike-timing dependency of Hebbian plasticity is thought to prevent a neuronal network capable of such plasticity from firing-rate instabilities, regulating both the rate and variability of postsynaptic firing (Song et al., 2000; Abbott and Nelson, 2000). When a neuron gets a strong multisynaptic drive, leading to high firing frequency, summed input from many synapses causes cell to fire regularly as if the current step was delivered through the electrode. In such a case the timing of postsynaptic spikes does not depend on the timing of presynaptic spikes. Such random pairing leads to overall expression of LTD in the synapses of this neuron, leading to eventual reduction of firing. On the other hand, if average synaptic current is only barely enough to make the neuron fire, than postsynaptic firing can be generated by coincidental arrival of several action potentials, making synapses prone to expressing LTP rather than LTD. STDP also may regulate the average firing rate of a neuron by introducing competition into Hebbian plasticity (Zhang et al., 1998; Abbott & Nelson, 2000). Eventually, groups of synapses, that are effective at causing postsynaptic firing will strengthen further, while other inputs, whose timing is random in respect to spikes caused by these dominant inputs will display LTD (Abbott & Nelson, 2000; Feldman et al., 2000, Dan & Poo, 2006).

Importance of the spike-timing dependent mechanisms and their possible role in reshaping the cortical maps was demonstrated by several studies in vivo. Manipulating
firing order of visual cortical neurons can induce bidirectional shifts in strength of intracortical connections and position of receptive fields of those neurons (Fu et al., 2002). In cat visual cortex repetitive pairing of the briefly shown oriented visual stimulus with electrical stimulation of visual cortex for several hours caused paired orientation to gain cortical territory from other orientations, when visual stimulus preceded electrical stimulus by 10 – 20 ms. However if the stimulation order was changed, the responses to paired orientations were depressed at the site of stimulation, consistent with spike-timing plasticity of intracortical connections (Schuett et al., 2001). Pairing of two visual stimuli with different orientations for several minutes produced a shift in the orientation tuning of cortical neurons in adult cat V1, the direction of which depended on the temporal order of presentation of the paired stimuli (Yao & Dan, 2001). In rat visual cortex, whole cell recordings in intact animal showed that synaptic responses to a flashed bar stimulus can be potentiated or depressed by pairing the visual stimulation with postsynaptic spiking via current injection through the whole-cell electrode (Meliza & Dan, 2006). In rat barrel cortex, a spike-timing dependent mechanism, depending on temporal order of firing displayed by pyramidal cells in layer 4 and layer 2/3 was proposed to drive LTD of deprived inputs in barrel cortex after whisker deprivation (Allen et al., 2003; Celikel et al., 2004). An indication for the role of STDP in reorganization of visual cortex after monocular retinal lesion was pointed out by Young et al. (2007), based on the observation that in their lesion model the “reorganized” receptive fields were always clustered in one spot instead of being evenly distributed around the border of lesion projection zone. An attractive feature of this proposed STDP involvement is the directional specificity of the change, coming from temporal asymmetry of STDP. This means, that a neuron, that leads the firing order exerts a „teaching“ influence on its targets, while the „followers“ can not exert such influence on the leading neuron. As a result, the connections from leading neuron to follower neurons strengthen, while connections from follower neurons to leading neuron tend to become depressed, causing a spread of LTP or LTD in the neuronal chain, which can result in formation and changes in retinotopic and orientation maps. This spatiotemporal asymmetry of STDP also suppresses strong recurrent excitatory loops, that can form as a result of correlation-based plasticity in a chain of reciprocally connected neurons (Abbott and Nelson, 2000; Turrigiano, 1999).
1.4. Homeostatic plasticity and synaptic scaling

In contrast to LTP and LTD, that are local synapse-specific forms of plasticity, homeostatic scaling is a global plasticity mechanism, operating on multiple levels – from synapses to networks - to keep the firing rates of neurons in the network constant in face of ongoing synaptic modifications (Turrigiano & Nelson, 2004; Turrigiano, 2008). "Firing rate homeostasis“ was first described in cultures of neocortical neurons (Turrigiano et al., 1998). Such culture is a network of excitatory and inhibitory neurons, that display regular spontaneous firing. Perturbing the firing rates in such a network leads to reinstallation of a previous value of firing frequency after an initial drop or increase (Turrigiano, 2008). “Firing rate homeostasis” was shown to occur irrespective of the manipulation used to induce a change in neuronal firing. For example, firing rates would change during chronic application of blockers of GABAergic transmission or when neurons were transfected with inwardly rectifying potassium channel, causing hyperpolarization and reduction of firing rates. Over time period of several days the firing rates returned to those seen before treatment, even though the neurons continued to express the channel in the second case (Turrigiano, 2008; Burrone et al., 2002).

A number of ways that are used by neurons to achieve this result were documented. First, both in cultured neocortical neurons and in vivo, altered firing rates result in bidirectional changes in the number of excitatory and inhibitory synapses (Kirov et al., 2004) and their strength (Kilman et al., 2002 , Turrigiano & Nelson, 2004). The latter is achieved by regulation of the amount of neurotransmitter receptors, clustered at postsynaptic sites (Ibata et al., 2008; Kilman et al., 2002) or influencing signaling pathways, that control function, insertion, trafficking and removal of receptors and their maintenance in PSD (Ehlers, 2003; Chowdhury et al. 2006, Cingolani et al., 2008, Ju et al., 2004, Okuda et al., 2007, O'Brien et al., 1998). Second, homeostatic regulation also may affect presynaptic sites - by changing the patterns of expression of vesicular glutamate and GABA transporters, thus affecting the size of readily releasable pool of vesicles and release probability of neurotransmitters (Erickson et al., 2006; De Gois et al., 2005; Murthy et al., 2001). Also, a threshold for induction of either LTP or LTD can be changed after a neuron's firing rate exceeds or drops below a certain value as a result of prior synaptic modification. Such metaplasticity has been described in both hippocampus and visual cortex (Abraham & Bear, 1996; Kirkwood et al., 1996;
Smith et al., 2009). Finally, intrinsic excitability of the neuron may undergo homeostatic adjustment via manipulation of balance between competing voltage-depending conductances, that determine spiking threshold and other intrinsic properties of the neurons (Turrigiano, 2008; Marder & Goaillard, 2006; Zhang & Linden, 2003; Turrigiano, 1999; Marder & Prinz, 2003).

Synaptic scaling of excitatory and inhibitory synapses in the primary visual cortex resulting from altered visual experience was documented by several studies in the last 5 years. In cultures of visual cortical neurons pharmacological modulation of activity leads to change in amplitude of mPSCs, that represent postsynaptic response to release of single vesicles of neurotransmitter and can be indicative of unit strength of the synapse. Study of the mEPSCs arising from total synaptic input onto the recorded neuron (Turrigiano et al., 1998) showed that change in network activity shifted the entire amplitude distribution of mEPSCs in a uniform manner, scaling global synaptic strength of excitatory transmission up or down. Interestingly, scaling of mPSCs appears to be bidirectional – in many cases properties of inhibitory currents are scaled in opposite manner to properties of excitatory currents (Kilman et al., 2002; Swanwick et al., 2006; Dani et al., 2005; Maffei and Turrigiano, 2008). However, synaptic scaling and regulation of firing rates in vivo is different from the neocortical cultures: the „firing rate homeostasis“ of neurons in the visual cortex could not be demonstrated. For instance, TTX injections and lid suturing in one eye always result in moderate but stable increase of firing rates of pyramidal cells in the affected visual cortex, presumably because they also lead either to scaling up of excitatory synapses and subsequent long-term change of excitatory-inhibitory balance or intrinsic neuronal excitability. Those effects are layer-unspecific – they occurred in layer 4, layer 2/3 and 5 pyramidal cells (Maffei & Turrigiano, 2008; Maffei et al., 2004). Importance of homeostatic mechanisms for plasticity in adult visual cortex is indicated by the fact that, in contrast to developmental downregulation of some forms of LTP and LTD after the end of critical period (Jiang et al., 2007), scaling of excitatory synapses has a different developmental regulation, persisting through adulthood in superficial layers of visual cortex (Goel & Lee, 2007). Interestingly, in layer 4 the potential to express synaptic scaling is greatly diminished during second and third postnatal week, while starting to be expressed in layer 2/3. Those observations suggest, that homeostatic regulation of plasticity is a subject to multiple layer-specific critical periods (Desai et al., 2002).

Most studies of scaling in visual cortex made use of monocular deprivation to
produce an input reduction somewhat analogous to blockade of activity in culture. In rodents, a deprivation of one eye through lid suture during classical critical period for ocular dominance plasticity leads to an ocular dominance shift within binocular part of the primary visual cortex. Binocularly driven cells in the deprived cortex loose responsiveness to the deprived eye within a period of a few days and then, over approximately a week, increase the strength of their responses to the intact eye. Those processes have mostly been attributed to LTD and LTP of excitatory inputs to the deprived cells (Smith et al., 2009; Malenka & Bear, 2004), and the role for developmental change in cortical inhibition was implicated (Hensch, 2005; Fagiolini & Hensch, 2000). However, it is possible, that the potentiation of responses to the non-deprived eye is established through homeostatic scaling mechanisms that increase the excitability of cortical neurons in response to partial loss of input (Mrsic-Flogel et al., 2007; Kaneko et al., 2008). For example, it was shown, that after a period of monocular deprivation the neurons, that were driven only by the deprived eye had stronger responses, the same as neurons in binocular portion of cortex after binocular deprivation, which is more consistent with a role for homeostatic mechanism, than with Hebbian mechanism (Mrsic-Flogel et al., 2007).

There is also a high chance of a complex interplay between different mechanisms of plasticity during experience-driven changes in circuit operation. For example, recent study in rat visual cortex demonstrated that the mode of homeostatic plasticity may change depending on the method of visual deprivation. Both dark rearing and removing a part of visual drive through injection of TTX into one eye induce synaptic scaling of mEPSCs in visual cortical layer 2/3 (Desai, 2002; Goel & Lee, 2007, Maffei & Turrigiano, 2008). Lid suturing, on the other hand, leads to homeostatic increase of intrinsic excitability of layer 2/3 pyramidal cells, but not to scaling of mEPSCs in those neurons; although both TTX injection and lid suturing cause systematic increase in firing rates of neurons in layer 2/3. This observation has been attributed to the well-known ability of lid suture protocol to induce a profound LTD of layer 4 to layer 2/3 excitatory synapses, presumably via decorrelation of inputs, depending on spontaneous retinal waves (Rittenhouse et al., 1999). Lid suturing produces an incomplete block of light-induced activity and leaves retinal waves intact, while TTX injection leads to total blockade of activity in the retina and much more profound break down of residual activity in vertical inputs to upper cortical layers. Therefore, lid suturing engages the affected cortex into expression of LTD together with
homeostatic upregulation of intrinsic excitability, happening at the same time (Maffei & Turrigiano, 2008).

What is the nature of a mechanisms that allow the neurons to detect the change in their firing rates and produce a scaling output in response? First, a global compensation of firing rate changes may take place after critical number of neurons underwent such change, resulting in a widespread change of activity in the network, that could in turn lead to activity-dependent simultaneous production of a soluble factor by many neurons or glial cells (Turrigiano, 2008; Stellwagen & Malenka, 2006). Local changes in synaptic signaling induced by changes in firing could turn on local homeostatic regulation of presynaptic neurotransmitter release and postsynaptic maintenance, subunit composition and biophysical properties of postsynaptic receptors (Hou et al., 2008, Sutton et al., 2006). Finally, a mechanism of firing rate sensing operating on a single-neuron level is also possible (Turrigiano, 2008). The best candidate signal for activity detection is the level of the intracellular calcium, being ubiquitous in the nervous system and depending on the amount of firing. Changes in intracellular calcium are directly linked to neural activity and calcium inflow is critical for many forms of both synaptic plasticity and regulation of intrinsic excitability (Lisman et al., 2002; Malenka & Bear, 2004; Zhang & Linden, 2003). Computational studies point that integrated calcium level may represent an activity set point of a homeostatic feedback mechanism (Marder and Prinz, 2003). It was shown that a drop of calcium influx through voltage-gated channels leads to scaling-up of mEPSC amplitudes and accumulation of AMPA-receptors in the postsynaptic sites (Ibata et al., 2008, Thiagarajan et al., 2005). Change in calcium influx and concentration is sensed by multiple calcium-dependent proteins, among them the calcium/calmodulin dependent protein kinases (CaMK). Interestingly, intracellular block of CaMKs with KN93 abolishes the effect of activity blockade on mEPSC amplitude, preventing synaptic scaling (Thiagarajan et al., 2002). A fair amount of evidence implicates CaMKIV to have a major role in synaptic scaling of glutamatergic synapses. During normal neuronal activity there is a high concentration of activated CaMKIV, but even a transient TTX treatment in neuronal culture makes those levels drop. Additionally, a transfection with a dominant-negative form of CaMKIV, that can not be any longer phosphorylated by CaM kinase kinase to become activated, and blocking of CaMKK activity leads to scaling up of the mEPSC amplitude and expression of dominant-negative CaMKIV also occludes the effect of tetrodotoxin application in culture.
A number of molecules were suggested to have a role in synaptic scaling, including TNF-α, BDNF, MHC-I, integrins, Arc, intracellular calcium and calmodulin-dependent kinases (Turrigiano 2008; Turrigiano & Nelson, 2004; Swanwick et al., 2006). BDNF, for instance, is known to have a role in development of cortical inhibition, especially in rodent primary visual cortex and it is one of the molecules, whose levels in the visual cortex are profoundly affected by retinal lesions (Obata et al., 1999). BDNF is thought to be released in activity-dependent manner from pyramidal cells and has an important role in expression of different forms of cortical plasticity (Abidin et al., 2006; Lu, 2003). Application of BDNF in the culture medium blocks the scaling-up of mEPSC amplitudes, induced by chronic blocking of activity with TTX in cultures of neocortical neurons. Moreover, the scaling-up of mEPSCs can be induced in culture even in absence of activity blockade by scavenging endogenous BDNF with high-affinity BDNF TrkB receptor wash-in (Rutherford et al., 1998). Those observations suggest, that synaptic scaling of mEPSCs may be mediated in part by reduction in BDNF release caused by a drop in firing after, for example, visual deprivation. (Rutherford et al., 1998). Moreover, BDNF may be the factor influencing the strength of inhibition and excitation in opposite directions, and it also was shown to prevent TTX-induced down-scaling of mIPSCs in culture, and, in vivo, to enhance the inhibitory drive and to promote the development of parvalbumin-positive interneurons in the visual cortex (Rutherford et al, 1997; Huang et al., 1999; Turrigiano, 2008; Swanwick et al., 2006).

1.5. Role of inhibition in development and plasticity of cortical circuits

Importance of the strength of inhibitory drive for the development of visual cortex and maturation of the properties of visual cortical neurons is addressed by several studies dissecting the role of inhibition in ocular dominance plasticity and regulation of column development. For example, it was shown, that infusion of benzodiazepine
agonists, that enhance local inhibition, in kitten visual cortex during critical period leads to widening of ocular dominance columns, while infusion of inverse agonists induces formation of thinner columns (Hensch & Stryker, 2004). The broad range of column sizes observed in various species naturally could be explained by differences in the span of cortical inhibition and length of long-range inhibitory connections (Horton & Hocking, 1996; Hensch, 2005), providing a scaffold for discrimination of competing sensory input. Studies making use of GAD-65 knockout mice, that have poor GABA release upon strong repetitive stimuli, showed, that visual cortical neurons of those mice had normal receptive field properties, but could not undergo an ocular dominance shift, unless a chronic infusion of diazepam in the visual cortex was performed to restore inhibitory drive to normal levels. Such „rescue“ of the critical period was possible throughout lifespan of the mouse, indicating that a certain level of inhibitory strength was necessary for expression of critical period plasticity (Fagiolini & Hensch, 2000; Hensch et al., 1998; Tian et al., 1999). In line with this, manipulations that speed up maturation of inhibitory circuits, such as injection of benzodiazepine agonists in the visual cortex and transgenic overexpression of BDNF, that promotes maturation of GABAergic interneurons (Fagiolini & Hensch, 2000; Iwai et al., 2003; Fagiolini et al., 2004; Hanover et al., 1999; Huang et al., 1999) accelerated the onset of critical period. Delay of critical period by dark rearing can thus be explained by the fact that dark rearing also reduces the levels of BDNF in the visual cortex and prevents maturation of inhibition (Morales et al., 2002; Iwai et al., 2003; Fagiolini et al., 2003). Dependence of critical period onset on expression of BDNF and correspondence of normal onset of critical period to the time of emergence of parvalbumin-expressing inhibitory interneurons, implicated a specific role for parvalbumin-positive interneurons in regulation of critical period plasticity. The parvalbumin-positive large basket cells also may be implicated in regulation of the ocular dominance column size, as they form a plexus of long-range inhibitory axons, spanning the extent of individual ocular dominance columns (Buzas et al., 2001).

A number of studies in retinally lesioned cats and monkeys point to possible role of inhibition in retinal lesion induced reorganization. For instance, immediate expansion of receptive fields of neurons, located at the border of the LPZ and inside the LPZ immediately after the lesion or during first several hours after the lesion can be attributed to disinhibition (Buonomano & Merzenich, 1998, Gilbert & Wiesel, 1992; Calford et al., 1999; Dreher et al., 2001; Schmid et al., 1995; Eysel et al., 1999). Change
in balance of excitation and inhibition coming from altered transport and production of glutamate and GABA after retinal lesion is suggested by several studies of extracellular neurotransmitter concentrations, expression of GAD65 & GAD67, glutamate transporters and GABA receptors in the LPZ and perilesional non-deprived primary visual cortex (Massie et al., 2003a & 2003b, Qu et al., 2003; Rosier et al., 1995; Arckens et al., 2000a; Arckens et al., 1998).
2. Aim of the study

Although the study of both mechanisms of synaptic plasticity in developing visual cortex and visual cortical reorganization has enjoyed a rapid advancement in the last several years, little is known about synaptic and network mechanisms which come into play in primary visual cortex during the course of retinal lesion-induced reorganization in V1. Existing histological, single unit recording and intrinsic imaging data suggest an important role for long-range horizontal connections and possible contribution of Hebbian forms of plasticity and excitatory-inhibitory balance changes during reorganization. The aims of the present work are:

1. First, to address the proposed role of long-range horizontal connections in reorganization.
2. Second, to undertake a study of intrinsic properties of intra-LPZ neurons and their basic synaptic transmission during reorganization.
3. Third, to test the role of Hebbian and homeostatic forms of plasticity in reorganization.

To study the role of lateral connectivity in reorganization, a combination of multiunit recordings with a fast imaging technique was applied, employing voltage-sensitive dyes and allowing to image a population neuronal activity over large area of V1 with millisecond time resolution (Spors & Grinvald, 2002; Jancke et al., 2004; Ferezou et al., 2007; Derdikman et al., 2003). This approach enabled the study of spatiotemporal dynamics of activity in the V1 of retinally lesioned rats in vivo and exploration of lateral spread during and after reorganization.

For the study of synaptic transmission in V1 after retinal lesions and the role of LTP of horizontal inputs in reorganization patch-clamp recordings were made in the slices of primary visual cortex taken from animals with retinal lesions. This approach allowed to study the properties of excitatory and inhibitory inputs in the same cell and to apply a spike-timing dependent protocols for the study of LTP in horizontal inputs to layer 2/3 pyramidal neurons.

3.1. METHODS

All surgical and experimental procedures were approved by the German Animal Care and Use Committee (AZ 50.8735.1 Nr. 105/12) in accordance with the Deutsche Tierschutzgesetz and the NIH guidelines. 18 healthy adult (9–14 weeks old) Agouti Brown rats were used for the experiments.

3.1.1. Recording of neuronal activity with voltage-sensitive dyes

Structure of voltage-sensitive dyes. The molecules of the voltage sensitive dye (VSD) consist of a long hydrophobic tail and a fixed charge, making up the hydrophilic kernel. The hydrophobic part anchors the dye in the plane of a membrane, while the charged part prevents the molecule from crossing the plasma membrane of a cell. Such structure provides the molecule with a large dipole moment, that makes it sensitive to electrical field changes across the neuron's membrane that occur during activity. The sensitivity of dye's optical properties to voltage changes can be the result of direct electrochromic effect or the movement of the molecule in and out of the membrane depending on the strength and direction of the electric field. Therefore, the molecules of the VSD transform changes in membrane potential of the excitable cells into changes in absorption or emitted fluorescence of the dye, that occur in microseconds and can be optically registered (Fig. 3.1.1, Grinvald et al., 1999; Grinvald & Hildesheim, 2004). In single-cell preparations the amplitude of the signals, detected with VSDs, displays linear correlation with changes in membrane potential and with membrane area of the stained cells, located in each pixel of the produced image (Salzberg et al., 1973; Grinvald & Hildesheim, 2004).

Applications of VSDs. VSDs were first used to record electrical activity in preparations of nerve trunks of lobsters, spider crabs and fin nerves of squids (Tasaki et
al., 1968) and also individual leech neurons (Salzberg et al., 1973); later they were adapted to study electrical events on a single-cell scale in cell culture (Grinvald & Hildesheim, 2004). Application of VSDI to population activity started in mammalian brain slices (Grinvald et al., 1982), frog tectum (Grinvald et al., 1984) and salamander olfactory bulb (Orbach & Cohen, 1983; Cinelli et al., 1995a; Cinelli et al., 1995b). Usage of VSDs for the study of cortical neuronal populations in intact animals is complicated by considerable biological noise, arising from heartbeat and respiratory pulsation. These respiratory and heartbeat artifacts can be removed by synchronizing the recording with electrocardiogram and subtraction of a blank trial. Another set of potential problems in VSDI imaging arises from contamination of the fluorescent signals with slow intrinsic signals. Hemodynamic artifacts can be diminished by using dyes, that can be excited outside of the absorption band of hemoglobin. Using such „blue“ dyes (Shoham et al., 1999), activity in the neocortex evoked with sensory stimuli can be recorded in vivo with high signal to noise ratio (Peteresen et al., 2003, Derdikman et al., 2003; Spors et al., 2002; Jancke et al., 2004; Ferezou et al., 2007). The highest possible spatial resolution of the VSDI is limited by optical hardware and light scattering, comprising about 50 μm².

**Experimental setup for in vivo population recording (Fig. 3.1.2).** To expose the cortex for staining and subsequent imaging during in vivo experiment, the animal is undergoing a craniotomy, after which a chamber is fixed on the skull. The chamber can be filled with solutions and sealed with a coverslip. After staining with the dye the cortex is illuminated with a wavelength corresponding to VSD's excitation peak value. A series of images of the cortex is than taken at high temporal resolution (up to 500 frames per second) using a fast camera coupled with a macroscope. Marcoscope provides considerably larger numerical aperture for low-magnification compared to standard microscope objectives, making it possible to sample fluorescent signals over large areas (in the present study, about 5 mm²) with acceptable resolution, as illumination intensity and amount of collected fluorescence depend on the square of numerical aperture (Grinvald et al., 1999).

**Nature of the VSD population signals recorded in intact brain.** In single-cell preparations, where dendrites and cell bodies can be visualized, the dye signal coincides with electrical signal taken with intracellular electrode (Salzberg et al., 1973; Grinvald & Hildesheim, 2004). During in vivo measurements, however, both neurons and non-neuronal membranes are stained by the dye, which leads to the question - what is
Figure 3.1.1. Scheme of VSD interaction with the membrane. A. The molecules of the dye harbor a fixed charged group and hydrophobic tail, that is responsible for interaction with the membrane. Only the membrane-bound dye is fluorescent. The intensity of fluorescence may depend on the extent of immersion of hydrophobic part of the molecule into the lipid bilayer, that is dependent on electric field strength and direction across the membrane. B. During an action potential a sequence of fast changes of the electric field across the bilayer occurs. This leads to rapid movement of the dye in and out of the membrane, leading to changes in the fluorescence. (Vo – potential outside the cell, Vin – potential inside the cell, E – strength of electrostatic field, d – thickness of membrane). (Adopted and modified from Grinvald et al., 1999).
Figure 3.1.2. Setup of voltage-sensitive dye imaging experiment. During acquisition of the images the rat views a visual stimulus (moving grating). Acquisition of cortical images is performed with fast camera operating through the macroscope. After acquisition the data are analyzed offline with Matlab. Multiunit recordings can also be performed before and after imaging, and also simultaneously with imaging (Jancke et al., 2004). (Adopted with modifications from Grinvald & Hildesheim, 2004).

membranes within that pixel. Because the VSD signal is linearly related to the stained membrane area, the signal mainly originates from cortical dendrites and non-myelinated axons, since their areas are significantly larger than those of cell bodies. Additional question concerns the distribution of the recorded signals among cortical layers, which depends on the properties of optical hardware and efficiency of dye staining in individual layers, as well as cortical anatomy of the used animal species. The depth of dye penetration in the cortex is about 1.5 mm, but the upper layers are stained much more profoundly than the lower layers, with most of the dye located in layer 2/3 (Grinvald & Hildesheim, 2004; Petersen et al., 2003). However, neurons in layers 5 and 6 of rodent neocortex have thick apical dendritic tufts and extensive arborizations in upper cortical layers contributing to the recorded signals. Since the dendrites of cortical cells are much more densely packed than axons, VSD signals mainly come from dendritic postsynaptic potentials, with dendrites of layer 5 and 6 neurons generating a large portion of recorded signals. In case of primates, however, the contribution of dendritic tufts of neurons from layers 5 and 6 can be less prominent, than in rodents, as most of layer 5 pyramidal cells in the primary visual cortex of macaque monkey, for
example, typically extend their basal and apical dendritic branches only to the top of layer 5 (Callaway, 1998; Callaway & Wieser, 1996).

3.1.2. Retinal lesions

The left retinae of ketamine/xylazine anesthetized adult rats were focally photo-coagulated by a high-intensity laser lesion (1 mm, 1000 mW, 200-300 ms), operating through a laser-adapted microscope. The resulting round lesions were localized in the retina dorsal to the optic disc, and typically extended about 1 mm horizontally (corresponding to 15°-20° along the horizontal meridian in visual space). The lesion destroyed all retinal layers including axonal fibers of passage leading to retrograde degeneration of all retinal ganglion cells peripheral to the lesion, which extends the scotoma from the lesion proper towards the far periphery of the monocular lower visual field (Fig. 3.2.1 A, B and D). Measurements were performed in three groups of animals: two groups of rats were lesioned at P65 and recorded either at P69–P72 (acute lesion) or after a longer period of recovery, at P92–P105. Unlesioned rats of matching ages were used in control experiments to delineate normal retinotopy.

3.1.3. Animal preparation

Experiments were performed under general anesthesia. After premedication with 0.05 mg kg⁻¹ atropine sulphate, anesthesia was induced with Chloralhydrate (4%, 1 ml/100 g). Xylocaine (4%) was used for additional local anesthesia. Animals were tracheotomized and artificially ventilated (0.8-1.1% Isofluorane, 40% O₂, 60% N₂O, 1–1.2 Hz). End-tidal CO₂ was measured constantly and kept at 3.8–4.2 %. Heart rate and body temperature (37–38°C) were monitored during the entire experiment. Craniotomy was performed over the right primary visual cortex (3–9 mm posterior to Bregma, 1-5 mm lateral). Subsequently a metal chamber was attached to the skull. During imaging sessions, the chamber was filled with artificial CSF and sealed with a coverslip. During the experiments, animals received an intravenous infusion of electrolytes (Sterofundin®, Braun), 2.5% glucose, and alcuronium chloride (Alloferin®) to block eye movements.
(0.06 mg x kg⁻¹ x h⁻¹). The eyes were regularly flushed with hyperosmotic saline. Before the measurements the pupils were dilated with atropine.

3.1.4. Visual stimulation

Visual stimuli were generated by VSG (Cambridge Research Systems Ltd., UK), controlled by custom-written Matlab© routine, and displayed on a 24" Sony monitor (GDM-FW900, 100 Hz). Stimuli were presented at 30 cm distance to the contralateral eye, covering ~60x60°. The position of the papilla projection was mapped onto the screen using ophthalmoscope backprojection, and was repeatedly measured to check for residual eye movements. Full-field stimuli consisted of vertical and horizontal square wave gratings (0.02 cycles/°; 2 cycles/s). For retinotopic measurements grating size was 20x20°, presented within a 3x3 grid. Mean luminance of the stimuli was 57 cd/m². Each stimulus (750 ms presentation time) was alternated with an interstimulus period (15s) in which an isoluminant gray background (blank) was shown. To measure baseline activity, two blanks were included in each trial consisting of all stimulus conditions presented in pseudorandom order.

3.1.5. Multiunit recordings

Prior to imaging, electrophysiological recordings (glass-coated tungsten, 1–2 MOhm) were performed through the intact dura. Recording depth was 300–500 µm, corresponding to units located in layer 2/3. Receptive field positions were either hand-mapped or quantified by retinotopically arranged moving gratings. Histograms were calculated by averaging (20–60 stimulus repetitions). To measure orientation/direction selectivity index the mean of initial 300 ms of response to each grating was taken, and after identifying the preferred and non-preferred direction/orientation the selectivity index (SI) was calculated using these means according to the formula:

\[ SI = \frac{(preferred - non-preferred)}{preferred} \]
3.1.6. Voltage-sensitive dye imaging

After the dura was carefully removed, the cortex was stained for 2 hours with blue voltage-sensitive dye (RH–1838), applied at 0.2 mg/ml in Ringer's solution. Subsequently, unbound dye was washed out with artificial CSF. Imager 3001 (Optical Imaging Inc, NY) was used with a tandem lens macroscope, 85 mm/1.2 toward camera and 50 mm/1.2 toward subject (Ratzlaff & Grinvald, 1991). The camera was focused ~400-500 microns below cortical surface. The cortex was illuminated with 630 ±10 nm light and emitted light was high-pass filtered with cutoff at 665 nm using a dichroic filter system. Frames were collected at 100 Hz.

3.1.7. Data analysis

Processing of raw imaging data. Normalization was performed for each pixel by its DC level during pre-stimulus period (200 ms). Next, heart-beat and respiration-related artifacts were removed by dividing by the average of blank signals recorded in absence of stimulation. These steps were applied for each trial separately and then average across trials (n=20-30) was taken.

Determination of the lesion projection zone (LPZ) and velocity of horizontal spread. First, in every trial, activity of all grating conditions was averaged. Significance of stimulus evoked responses was then estimated using bootstrap with replacement over single trials. For each image pixel and each time frame, p-values were derived by comparison to pre-stimulus conditions using ANOVA. The resulting p-value maps were smoothened (Butterworth, filter size 270 microns) and latencies of pixels were calculated by determining the time of significant response (p<0.05). The unaffected cortical region was characterized by earliest latencies and included pixels whose activity crossed significance threshold in three consecutive time frames. The border of the lesion projection zone (LPZ) outlines pixels with longer latencies. For the calculation of propagation velocities profiles parallel to the LPZ border were created (using unfiltered p-value maps). Velocity was calculated perpendicular to these strips (width 4 pixels=215 microns). The slope of the curves from linear regression of latencies is equal to speed.
3.2. RESULTS

3.2.1. Location of the lesion projection zone (LPZ)

Visual input to the medial monocular part of rat primary visual cortex was removed by laser coagulation of a small ~1 mm diameter patch of the upper retina just dorsal to the optic disc. Retinal lesion resulted in formation of a scotoma of 15-20° temporo-nasal width extending from above the optic disc to the far periphery of the upper retina, as shown in Fig. 3.2.1. A. Therefore, the visual input to the medial monocular part of primary visual cortex was removed and a functional scotoma extending from below the optic disc's projection to the far periphery of the monocular lower visual field was formed (Fig. 3.2.1.D, right panel). To determine the initial position and extension of the lesion projection zone (LPZ) functionally, retinotopic mapping was performed using VSDI (Fig. 3.2.1. B). Apart from the affected lower central position (red), each stimulated locus in the visual field was represented by a local spot of activity. Unlike the lesioned animals, the same retinotopic stimulation in normal age-matched control animal evoked robust local activation for every stimulus, as shown in Fig. 3.2.1.C. Fig.3.2.1.D summarizes the cortical retinotopic organization after lesion. The colored contours outline the portions of cortex, showing significant activation in response to stimulation of corresponding retinotopic location. Due to the cortical point-spread function, activated regions were partially overlapping, in sum representing ~60x60 degrees of the visual field excluding the projection of the lesion that was cortically neglected. Stippled line in Fig. 3.2.1.D marks border of the LPZ (shaded in gray), as determined by procedure described in Methods, section 3.1.7.

3.2.2. Horizontal spread of activity into the LPZ

Next, we measured responses to full-field stimuli that entirely covered the sampled visual field including the projection of the lesion. The gradual spread of activity into the LPZ is shown in 20 millisecond time frames in Fig. 3.2.2.A, which depicts the response dynamics for two different cases, 6 and 28 days after lesion (shown in the upper and lower rows, respectively). At the onset of response both animals
revealed an instantly activated cortical region (greenish areas in 60 ms frames) reflecting early vertical input to the lateral part of the cortex unaffected by the lesion. At longer post stimulus times, while activity continued rising at initial input regions, low-level activity crossed the LPZ border (black line) at which horizontal input starts dominating thalamic contributions (see also Fig.3.2.9, in which horizontal spread was uncovered by using an artificial scotoma). Timecourses of single pixels, located in intact cortex, border of LPZ and inside the LPZ are shown for both cases: 6 days post lesion (Fig. 3.2.2.B) and 28 days post lesion (Fig. 3.2.2.C). Propagation of activity between chosen pixels is slower in the animal 6 days post lesion compared to the case 28 days post lesion, as evident in the initial part of activity timecourses – in animal 28 days post lesion the timecourses appear more compressed together (Fig. 3.2.2. B and C, right panels). An increase in relative amplitude of fluorescent signal for the intra-LPZ pixels is also evident in animal 28 days post lesion compared to 6 days post lesion. Those observations occurred across all pixels: amplitudes reached within the LPZ relative to unaffected regions of V1 were lower after brief recovery (Fig. 3.2.3.A). Fig. 3.2.3.C shows mean timecourses of activity across pixels representing unaffected regions (green), LPZ border (red), and the LPZ (gray). The gradual shift of the curves shows the systematic increase in latencies across the LPZ. Importantly, the slope of the latency gradient across the LPZ was significantly steeper in the acutely lesioned animal compared to 28 days post-lesion (Fig. 3.2.3.B and 3.2.3.C). For example, in Fig. 3.2.3.C in lower plots curves appear compressed demonstrating a rapid subsequent activation in comparison to upper plots, mirroring the situation for arbitrarily chosen single pixels. Fig. 3.2.3.B shows the corresponding latency maps in which earliest responses can be seen along the lateral posterior-anterior axis (green colors). Starting from this region both examples (upper panel – 6 days post lesion, lower panel – 28 days post lesion) showed a gradual increase in latencies towards medial (red – gray) indicating delayed propagation of activity into the LPZ.
Figure 3.2.1. Monocular retinal lesion produces a circumscribed loss of input to primary visual cortex. A. Photograph from a Nissl stained retinal wholemount from the left eye. Note the optic disc (asterisk), the adjacent direct laser lesion in the dorsal retina, and the dorsally extending region of retrograde ganglion cell degeneration that adds up to the complete area of visual loss in the retina (broken line). B. Cortical retinotopy 6 days after lesion. VSDI signals were averaged over the first 50 ms of responses. Colorbar indicates levels of activity ($\Delta F/F$). Black line marks the border of the LPZ. Retinotopic stimuli ($20^\circ$) were arranged within a 3x3 grid (D, right panel). Color codes stimulus identity; stimulus position: ut/lt=upper/lower temporal, n=nasal. Gray patch sketches lesion-affected location in visual field coordinates. Asterisk marks projection of the papilla. C. Cortical retinotopy in the non-lesioned control. Note that the lower central stimulus (red) evoked early activity in those regions that were unresponsive in (B). D. Retinotopic cortical map of significantly activated regions derived from the data shown in (B). Due to the retinal lesion, cortical regions beyond the LPZ border (stippled line) do not receive direct input. Position of the recording chamber indicated by coordinates: P=posterior relative to Bregma, L=lateral from midline. V1M – monocular V1, V1B – binocular V1.
**Figure 3.2.2. Horizontal spread of cortical activity across the lesion projection zone (LPZ).**

**A.** Timecourse of evoked VSDI signals changes with post lesion time. Colorbars show activity levels ($\Delta F/F$), horizontal bar=1 mm. Upper row: 6 days after lesion; Lower row: 28 days after lesion. Black line in images marks LPZ border. High amplitude activity propagates beyond the border of the LPZ after 28 days of recovery and stays confined to unaffected cortex after 6 days.

**B & C.** Timecourses of activity in single pixels located in unaffected primary visual cortex (green), on the border of the LPZ (red) or deep inside the LPZ (black). Locations of pixels are marked with asterisks of corresponding colors in left panels of B (6 days post lesion) and C (28 days post lesion). Activity frames in right panels of B and C are taken 120 ms post stimulus. Propagation of activity between those pixels is slower in animal 6 days post lesion (B, right panel) than in animal 28 days post lesion (C, right panel), where the initial parts of timecourses appear more compressed (stippled blue square). The later phase of the responses often varied unsystematically across animals, and was independent of post-lesion times. Blue panels represent ± 2 SD of mean fluorescence level during frame zero for those pixels. Solid lines represent stimulus-driven responses; stippled lines show activity without stimulation.
3.2.3. Changes in amplitude and latency of activity after different post lesion times.

For statistical analysis, we divided all treated animals in two groups: those measured shortly after lesion (I: 4-7 days, n=6) and those that had recovered for a longer time (II: 28-44 days, n=6). For both groups unaffected cortical regions had similar latencies (I: 98.8; II: 97.2 ms after stimulus onset) (Fig. 3.2.4.A). For animals measured several days after lesion, latencies within the center of the LPZ (860 µm from the border) were ~28 ms delayed (126±5 ms) compared to unaffected regions. In contrast, after longer recovery, latencies within the LPZ were delayed by only ~12 ms (108±2 ms) and were almost similar across the entire LPZ (Fig. 3.2.4.A, compare dark (28 – 44 days post lesion) and light (4 – 7 days post lesion) gray bars). This reduction of latency with longer post lesion times was paralleled by a significant increase in amplitudes within the LPZ, reaching ~80% of values obtained across intact regions (Fig. 3.2.4.B). Both progressive shortening of latencies and increased maximal amplitudes point to increased efficacy in synaptic transmission within the LPZ after prolonged recovery. The VSDI signal originates from superficial layers (>80%) due to focal depth, penetration depth of the dye, and light scattering (Grinvald et al., 1994; Peteresen et al., 2003). Neurons in layers 5/6 contribute to the signal as well, since they possess dendritic arbors in layers 2/3. Therefore, the locus of the described changes is represented by cortical sites, that harbor majority of synaptic contacts formed by horizontal inputs.
Figure 3.2.3. Latencies of appearance of subthreshold activity and maximal levels of activity in each pixel depend on cortical site. White bar=1mm, P – posterior to bregma, L – lateral from midline. A. Relative activity levels within LPZ and over unaffected primary visual cortex. Values were normalized to maximum value reached across all pixels (see colorbar). Black line – border of the LPZ; LPZ is always located medially from the border, with unaffected V1 being lateral to the border. Upper plot: 6 days after lesion, most of the pixels within the LPZ reached values of only ~60% of unaffected regions. This value increased to ~80% with longer recovery (bottom image). B. Each pixel's latency was evaluated by determining the time at which activity crossed pre-stimulus levels (see Methods, Section 3.1.7.). Note that after 28 days of recovery (lower panel) latencies within the LPZ were decreased compared to 6 days after lesion (upper panel). C. Time courses averaged across pixels outside (green), border (red), and inside the LPZ (gray). Solid lines depict mean, colored contours show ± 1 SD. Blue lines mark ±2 SD from baseline, stippled lines depict activity without stimulation.
Figure 3.2.4. Summary of VSDI experiments: effect of post-lesion time on changes in synaptic cortical activity at different cortical regions (color code as in Fig. 3.2.3.C). A. Latencies at different cortical recording sites, colorcode: 4–7 days post-lesion - light gray, 28-44 days post-lesion - dark gray. Both groups had similar latencies across cortical regions, that were unaffected by lesion (-0.43 to 0 mm) and up to 0.2 mm from the LPZ border (red). Inside the LPZ, latencies increased differently for short recovery as compared to the group with longer recovery times (Asterisk, p<0.05). Propagation velocity of activity: for short recovery, 0.03–0.05 m/s, for long recovery, 0.08–0.11 m/s. B. Effect of post-lesion times on response amplitudes (stark colors denote 4-7 days post-lesion; darker colors mark 28-44 days post lesion). For each individual experiment, amplitudes of activity over unaffected cortex (green) was normalized to one. Error bars are SE.
3.2.4. Multiunit recordings in primary visual cortex after the lesion

Even though the voltage-sensitive dye signal reports changes in synaptic potentials across several millimeters of cortex with high temporal accuracy, spike events are not directly detected by the signal (Sterkin et al., 1998; Petersen et al., 2003; Jancke et al., 2004). Thus, in order to test whether higher fluorescence levels correspond to suprathreshold activity after longer post lesion times, we performed additional multiunit recordings in the LPZ and unaffected cortex.

Fig. 3.2.5 shows PSTHs and corresponding receptive fields in animal 7 days after the lesion. No sustained firing could be evoked in units located inside the LPZ (Fig. 3.2.5.C, gray panel), while units in unaffected cortex displayed normal firing and receptive field layout (Fig. 3.2.5. B & C, white panel). However, in animals with longer post lesion times (28 – 44 days), visually evoked multiunit activity could be found as far as 0.8–1 mm within the LPZ, starting from the border of the LPZ (Fig 3.2.6.C & 3.2.7.C, red-orange-black PSTHs, gray panels). As expected, the corresponding “reorganized” receptive fields (Fig. 3.2.6.B & 3.2.7.B, bold outlined rectangles) were shifted away from their normal retinotopic positions and clustered at the border of the lesion-affected visual space (Fig. 3.2.6.B & 3.2.7.B, gray shaded areas). Moreover, we found that some intra-LPZ units showed bias to stimulus orientation and direction after long-term reorganization (polar plots in Fig. 3.2.6 & 3.2.7). Although orientation tuning varied across a broad range, selectivity of units within the LPZ was not significantly different from units located in unaffected cortex (Fig. 3.2.8). Also, broad variation of direction and orientation selectivity, as well as receptive field size of neurons in rat primary visual cortex was reported before (Girman et al., 1999). Hence, in animals that were allowed to recover for several weeks, multiunit recordings proved that the increased amplitudes of the dye signals within the LPZ indeed reflect suprathreshold activity.
Figure 3.2.5. Unresponsive units within the LPZ of acutely lesioned animals. Multiunit activity was recorded 7 days after the lesion was introduced. A. Recording sites (color coded) relative to LPZ border (red line). The recordings were made before imaging. B. Positions of receptive fields corresponding to recording sites in (A). While there is gradual progression of receptive field positions as the position of recording electrode was moved towards the LPZ, no receptive fields could be mapped for intra-LPZ penetrations. C. PSTHs of activity evoked by presentation of moving grating of preferred orientation (mean of 20 to 40 trials). Substantial modulation of spiking activity was observed when neurons were located in unaffected cortical regions, in contrast to neurons inside the LPZ (orange, black PSTH, gray panel). Notably, some intra-LPZ neurons showed high spontaneous activity (black) and a small increase in firing at stimulus onset, presumably due to weak horizontal input from neurons outside the LPZ.
Figure 3.2.6. Electrophysiological confirmation of functional recovery 32 days post-lesion. A. Vascular image and recording sites prior to imaging session. Red line marks LPZ border; P – posterior to bregma, L – lateral to midline. Positions of recording sites and corresponding receptive fields in B are color coded. B. Receptive fields of neurons within the LPZ (outlined bold) appeared clustered at the border of the lesion’s projection (gray; asterisk indicates projection of papilla). Neurons outside the LPZ showed normal retinotopic arrangement of receptive fields (thin-outlined rectangles). C. Post-stimulus time histograms (PSTH) of neurons recorded at locations shown in (A), colors match recording sites. Lower panels present spontaneous activity. Neurons within the LPZ (gray background) were responsive to drifting gratings and showed various degrees of direction and orientation tuning (see polar plots). Each histogram displays activity evoked by preferred grating orientation. See Fig. 3.2.7 for another example, 29 days post-lesion.
Figure 3.2.7. Electrophysiological confirmation of functional recovery 29 days post-lesion. A. Vascular image and recording sites prior to imaging session. Red line marks LPZ border; P – posterior to bregma, L – lateral to midline. Positions of recording sites and corresponding receptive fields in B are colorcoded. B. Receptive fields of neurons within the LPZ (outlined bold) appeared at the border of the lesion’s projection (gray; asterisk indicates projection of papilla). Neurons outside the LPZ showed normal retinotopic arrangement of receptive fields (thin outlines). C. Post stimulus time histograms of neurons located in the LPZ. Neurons within the LPZ (gray panel) were responsive to drifting gratings and showed various degrees of direction tuning, comparable to those of units in the unaffected cortex (PSTHs in D) (see polar plots). Each histogram displays activity evoked by preferred grating orientation. Lower panel represent spontaneous activity in the absence of visual stimulation.
Figure 3.2.8. No differences in orientation selectivity between neurons inside and outside the LPZ of recovered animals (28-44 days post-lesion). Upper graph: Distribution of orientation selectivity indices \( ((\text{preferred}) - (\text{non-preferred}))/ (\text{preferred}) \) among recorded units. Orientation tuning showed a large variance in selectivity as typically found in rat visual cortex (Girman et al., 1999). Lower graph: Mean index of orientation selectivity, error bars are SE. Difference was not significant \((p>0.05; \text{Selectivity index}: \text{LPZ}, 0.51\pm0.06, \text{controls}: 0.54\pm0.05)\).
3.2.5. Horizontal spread: artificial scotoma

To investigate properties of horizontal spread under baseline conditions we made use of “artificial scotoma” stimuli. In this case a part of visual field is covered by gray mask during presentation of the full-field grating. Luminance of the mask is equal to the mean luminance of the grating. Such stimulus is called “artificial scotoma”, since it is thought to produce substantial decrease in vertical input of affected neurons, while neighboring neurons receive normal vertical input. Thus, “artificial scotoma” is somewhat analogous to focal retinal lesion. Indeed, it was possible to observe a wave of activity spreading horizontally into scotoma-affected primary visual cortex, in contrast to fairly uniform activation resulting from presentation of the grating alone (Fig. 3.2.9.D, upper panel). However, the firing of the units located inside the scotoma was diminished to baseline levels if the scotoma covered the receptive field of the recorded unit, while same units displayed prominent modulation during presentation of the grating alone (Fig. 3.2.9.A – C). These results show, that under normal conditions horizontal inputs are mainly subthreshold and contribute sparsely to driving neuronal firing, in contrast to postreorganizational cortex, where they become suprathreshold.
Figure 3.2.9. Unmasking of horizontal spread by introducing an artificial scotoma.

A. Vascular image (after staining) with 2 penetration sites. Positions of recording electrodes and corresponding receptive fields in B are colorcoded. Blue stippled line – border of scotoma's projection. B. Corresponding receptive field positions, produced by hand-mapping. The receptive fields did not overlap. Asterisk marks papilla projection. Gray – mask position. C. PSTHs derived from electrical recordings at locations in (A). Upper row: At both penetration sites spiking activity was evoked while the gratings covered the full screen. Middle row: activity evoked by full-field gratings with an artificial scotoma - identical to the background in luminance and covering the “green” RF (see B) - the affected neuron (green) showed reduction in firing, now receiving only lateral input. Bottom PSTHs depict spontaneous activity when only the background was presented. D. VSDI, 20 ms image frames are shown starting at activity onset. Full-field stimulation without artificial scotoma activated large portions of visual cortex nearly simultaneously (upper row). Introducing an artificial scotoma causes a lack of direct input within the scotoma projection zone (see arrows at early response times; black line marks the border of the scotoma’s projection). However over the time course of the response, this region is “filled-in” by horizontally propagating activity from surrounding regions.
3.3. DISCUSSION

Using voltage-sensitive dye imaging, we explored the plasticity of the adult visual cortical circuitry triggered by monocular retinal lesions in the rat. As a consequence of removal of dominant subcortical input, intracortical horizontal spread was unmasked, originating from parts of V1 unaffected by the lesion. Within a short period of recovery, remote inputs from neurons approximately 1 mm outside the LPZ remained mainly subthreshold. After several weeks of recovery, however, the horizontal inputs from lesion-unaffected neurons were capable of driving stimulus-related suprathreshold activity as far as 1 mm inside the LPZ. These observations suggest gradual reinforcement of horizontal inputs that compensate for lesion-induced loss of function in vertical projections.

3.3.1. Challenging findings

Using metabolic markers such as cytochrome oxidase (Horton & Hocking, 1998) or hemodynamic signals (Smirnakis et al., 2005), previous investigations in monkey V1 could not confirm cortical reorganization after binocular retinal lesions, as opposed to studies, employing single-unit recordings or intrinsic optical imaging (Gilbert & Wiesel, 1992; Heinen & Skavenski, 1991; Das & Gilbert, 1995; Calford et al., 2003; Keck et al., 2008) and present results. Two explanations may exist for this discrepancy between results, obtained with metabolic markers and fMRI and results obtained in this work.

First, inter-species differences in cortical magnification factors could account for various extents of filling-in in the LPZ. In the cited studies (Horton & Hocking, 1998; Smirnakis et al., 2005), retinal lesions affected visual cortical representations covering 4-12° or 4-13° of visual space. Thus, full recovery of the LPZ in monkey would have to involve cortical regions of many square millimeters, demanding far-reaching remodeling of connectivity. Here, as in (Keck et al., 2008), rodents were used in which each millimeter of visual cortex contains neurons with dense overlap of dendritic and axonal trees covering a much larger visual space compared to primate V1. Therefore, strengthening of horizontal connectivity within a millimeter of rodent V1 leads to functional recovery of up to 20° in visual field coordinates. In addition, spine
turnover, indicating potential for plasticity, was found to be ~8 times higher in mouse primary visual cortex compared to macaque V1 (Keck et al., 2008; Stepanyants et al., 2002). Second, metabolic markers do not report cortical activity directly but generate signals that are slow compared to the underlying neuronal events. Therefore, the position of the actual border of the LPZ might be underestimated based on fMRI signals that dominantly reflect integration of input rather than spiking output (Logothetis et al., 2001). The time-averaged hemodynamic signal may include both components, summing lateral input into the LPZ as well as retinotopically evoked spikes, eventually smearing the exact position of the LPZ. In our study the high spatiotemporal resolution of VSDI allowed separation between lateral and direct cortical input by detecting latency differences of synaptic activity (Bringuier et al., 1999).

Ultimately, more studies are needed that describe “filling-in” after loss of retinal input to investigate the degree and the quality of functional recovery at behavioral levels (Zur & Ullman, 2003; Baker et al., 2005; Dilks et al., 2007) in parallel with physiological measurements.

3.3.2. Reinforcement of lateral activation: structural and functional synaptic changes

Lesion-induced strengthening of lateral activation inside the LPZ after prolonged post lesion time (> 3 weeks), as observed in our work, must be reflected by structural changes at the single neuron and network level. Several studies have addressed the structural effects that dominant subcortical input removal has onto morphology and structure of neuronal dendrites and axons in several species. Using two-photon imaging in the visual cortex of mice expressing a green fluorescent protein in the subset of neurons of layer 5, a recent study demonstrated a threefold increase in dendritic spine turnover several days after monocular retinal retinal lesion was introduced in the contralateral eye (Keck et al., 2008). This study also employed intrinsic optical imaging to follow a time course of reorganization and to make targeted exploration of intra-LPZ layer 5 neuronal dendrites, fine dendritic branches and dendritic spines. Immediately after the lesion the spine turnover rate underwent a 3.5-fold rapid increase, and spine dynamics remained elevated the first month after the lesion and returned to baseline
levels only after two months. The period of highest spine turnover rates was paralleled by the period of fastest dynamics of recovery of intrinsic signals inside the LPZ.

During 2 months of recovery, ~90% of spines at apical dendrites of layer 5 neurons were replaced, while in normal age-matched mice, only 38% of spines were replaced with new stable ones. The authors also showed that this remodeling of synaptic contacts inside the LPZ depended on input from neighboring cortical regions, as animals with complete binocular lesions revealed only little increase in spine dynamics. Importantly, increased spine dynamics during first two weeks post lesion was accompanied by remarkable instability of newly formed spines and the increased turnover rate was a result of equal levels of spine formation and removal. In addition, the percentage of original spines that were lost and replaced with new ones during these two months of recovery revealed a positive correlation with extent of functional reorganization. The cells located at the border of the LPZ displayed only moderate increase in spine dynamics, compared with cells located in the center of the LPZ. In contrast to those dramatic changes in spine dynamics, no marked changes in the structure of dendrites occurred and dendritic endings remained stable during reorganization. Formation of new stable dendritic spines and their enlargement is known to represent an anatomical basis for LTP-related strengthening and maturation of synapses (Murphy & Segal, 1997; Lynch, 2004; Jedlicka et al., 2008). Increased amplitudes of neuronal responses inside the LPZ and shortening of latencies of those responses, observed during recovery in our data most likely reflect both improved integration properties of the neurons and strengthening of excitatory synaptic drive coming from lateral inputs onto the intra-LPZ neurons. Sprouting of intrinsic axons, as an additional anatomical substrate for reorganization, was found in layer 2/3 of cat visual cortex after retinal lesions (Darian-Smith & Gilbert, 1994) and in mouse barrel cortex after vibrissectomy of individual whiskers (Kossut & Juliano, 1999). A similar structural signature of reorganization was observed in monkey somatosensory cortex, where deafferentation led to expanded and more widespread lateral connections in areas 3b and 1 compared to normal controls (Florence et al., 1998). Notably, in study by Florence et al., the distribution pattern of thalamocortical connections remained unchanged compared to control animals. Another structural substrate for reorganization is represented by alterations in dendritic field morphology. Changes in dendritic complexity of neurons in primary sensory areas were observed in various experimental conditions after manipulating the properties of sensory input through environmental
change or injury. For instance, complex large-scale changes in structure of dendritic arbors occurred in supragranular neurons in rat S1 after forepaw denervation and in barrel cortex after deafferentation (Hickmott & Steen, 2005; Hickmott & Ethell, 2006). However, during visual cortical reorganization in mouse no changes in dendritic morphology, as well as no sprouting or formation of new axons could be observed (Keck et al., 2008). Therefore, to which extent axonal sprouting and dendritic growth and elimination take part in rat visual cortex remains largely unknown.

In our study strengthening of lateral activation, occurring in LPZ over first 28 days of recovery also coincided with reappearance of visually driven activity in the intra-LPZ neurons and shifts of their receptive fields towards the lesion's border. In experiments using an artificial scotoma – a gray mask, obscuring the receptive field of the neuron, the lateral spread also occurred during presentation of artificial scotoma, but it remained largely subthreshold. In contrast, after reorganization the lateral inputs went from subthreshold to suprathreshold driving influence and could evoke firing with properties close to normal values in terms of direction/orientation selectivity and receptive field's sizes (Girman et al., 1999; Fig. 3.2.6, 3.2.7 & 3.2.8). Therefore in our study we could observe functional reorganization after 28 days post lesion, while early after the lesion (first week) the units inside the LPZ showed mainly spontaneous activity and the amplitude of laterally spreading activity wave remained low compared to amplitude of activity in the spared visual cortex. Similarly, shortening of latencies inside the LPZ was observed after longer time post lesion compared to acutely lesioned cases. Therefore, changes in strength of lateral connections leading to recovery happen during 2-4th week post lesion. This is consistent with results of optical imaging study in mice (Keck et al., 2008), showing that fastest recovery of visually-evoked signals takes place between day 7 and day 20 post lesion, although spine turnover rates become increased immediately after the lesion and then gradually reduce, until they return to baseline levels after 2 months when recovery is complete.

At the synaptic level, increased glutamate concentrations (Arckens et al., 2000a; Massie et al., 2003b), decreased GABA concentrations and altered GAD expression (Massie et al., 2003a; Rosier et al., 1995) at the border of LPZ early after lesion may all contribute to net hyperexcitability as revealed around the border of the LPZ in cat visual cortex immediately after the lesion. During the recovery process, this region of hyperexcitability propagates into the LPZ paralleled by a constant shrinkage of the initially non-responsive area (Giannikopoulos & Eysel, 2006; Arckens et al., 2000a).
Although we could not find hyperexcitability reflected in the dye signal, possibly due to low signal-to-noise close to baseline, our electrical recordings frequently revealed units with high spontaneous activity (Fig. 3.2.5.C, black PSTH). Hyperexcitability due to lesion-induced shifts in the excitation-inhibition balance (Benali et al., 2008) may create the basis for enhancement of synaptic transmission via horizontal fibers through LTP-like mechanisms (Hirsch & Gilbert, 1993). Interestingly, in both ICMS-induced cortical reorganization (Benali et al., 2008) and retinal-lesion induced cortical reorganization (Massie et al., 2003a) the regions of the visual cortex, located away from the LPZ or ICMS electrode displayed increase in the expression of glutamic acid decarboxylase and GABA. Possibly those changes are designed to prevent the net level of network excitability from reaching pathological levels (Benali et al., 2008).

In contrast to normal V1, the main source of activation of neurons inside of the LPZ is wave of activity traveling from the parts of V1 unaffected by the lesion (Fig. 3.2.2, 3.2.3.B & 3.2.4.A). Therefore the L2/3 neurons in unaffected V1 lead the firing of intra-LPZ neurons. Such firing order during the recovery process may trigger spike timing dependent alterations in synaptic transmission. Indeed, computational considerations implicate STDP-dependent reorganization after monocular retinal lesion in cat or after whisker-trimming in rat barrel cortex (Young et al., 2007; Celikel et al., 2004). An elegant approach towards exploring possible role of STDP mechanisms in vivo is presented by monocular retinal lesions in cat. In this model (Calford et al., 2003; Calford et al., 1999; Calford et al., 2000; Young et al., 2007) the lesion is introduced into one eye, while another eye is left intact. This leads to eventual shift of receptive fields of binocular cells, when the affected eye is stimulated, but leaves receptive fields intact during stimulation of the intact eye. This way it becomes possible to know the exact position of intra-LPZ neuron's receptive field before the lesion and after lesion-induced reorganization and to measure exact direction of the shift. It was found that most receptive fields of intra-LPZ neurons in the same animal shifted to one preferred position after reorganization. STDP-like process acting in a network of reciprocally connected cells would: a) operate in the network, displaying specific firing order, which has neurons, that lead the firing and neurons, that fire after the leading neurons (followers) b) enhance the connections from neurons who lead the firing to follower neurons and depress the connections of follower neurons to leading neurons, and c) enhance strong inputs to the follower neurons and depress weaker inputs to the follower neurons. Therefore, such directional clusterization of receptive fields would be expected.
if the shift was driven by STDP-dependent process. On the contrary, the correlation-dependent mechanism of reorganization would have the reorganized receptive fields be evenly distributed around the border of the LPZ (Young et al., 2007; Abbott and Nelson, 2000). In somatosensory barrel cortex of rodents the importance of STDP-like mechanisms was studied with single-unit recordings. When principal whisker was removed the order of firing of units in different layers was reversed during deflection of all whiskers: in normal rats layer 4 units led the firing of layer 2/3 units, while in whisker-deprived animals layer 2/3 units led the layer 4 units. Importantly, such shift in firing order was due to increase in latency of firing in layer 4 units and shortening of firing latency in layer 2 units as a result of loss of principal whisker-induced inhibition in neighboring columns, that led to more efficient propagation of secondary whisker input to the layer 2 of deprived column via dense intercolumnar lateral connections (Celikel et al., 2004). In all, we were able to demonstrate analogous effects in V1 of a rat 8 – 10 days after retinal lesion: reduction in inhibitory drive in the LPZ was accompanied by increase of the amplitude of horizontally-mediated EPSCs, evoked by stimulation of inputs coming from outside of LPZ (see Section 4). At the population level, these plastic changes in synaptic transmission are reflected by reduction in the latency of activity traveling from unaffected V1 into the LPZ and increase in amplitude of lateral activation after prolonged reorganization as observed in our in vivo study. At the same time the distributions of origins of activity in V1 would be expected to change, reflecting the change in firing order of units in the LPZ relative to unaffected cortex (Fig. 3.2.2.A & 3.2.3.B).
3.4. CONCLUSIONS – 1

Marked reduction in the latency of horizontal spread in the LPZ after prolonged recovery, accompanied by increase in signal's amplitude, may be interpreted as a result of potentiation of long-range horizontal inputs to the intra-LPZ neurons from the neurons of V1 unaffected by the lesion. Under baseline conditions postsynaptic activity induced via horizontal long-range inputs is mainly subthreshold and fails to cause robust stimulus-driven firing, the same being true in the LPZ after acute retinal lesion. Partial removal of input by retinal lesion possibly triggers gradual potentiation of long-range horizontal inputs to intra-LPZ neurons. This potentiation represents a basis for reestablishment of visually driven activity in intra-LPZ neurons and emergence of shifted receptive fields of those neurons observed after prolonged reorganization.
4. Excitatory-inhibitory balance in the neurons of the LPZ and properties of LTP of horizontal inputs during reorganization.

4.1. METHODS

4.1.1. Animals

Experiments were conducted in a pigmented strain of rats, Long Evans (n = 104). For the experiments, we used two groups of rats. Lesioned animals received the retinal lesion at the age of 21 days (P21), were returned to their home cage and used for experiments at P28 – P30. Control animals, receiving no treatment were recorded at P28-P30. Animals were housed in controlled conditions: constant temperature 28°C, humidity 50-60%, 12:12 dark-light cycle. Standard food pellets and water were freely available. All animal procedures were performed in accordance with the guidelines of the local animal research committee and with the laws of Germany the EU.

4.1.2. Retinal lesions

The left retinas of ketamine/xylazine anesthetized P21 rats were focally photo-coagulated by a high-intensity lesion laser (1 mm, 1000 mW, 200-300 ms, Iris Medical) through a laser-adapted operating microscope. The resulting rectangular lesions were localized in the retina dorsal to the optic disc, and typically extended about 1 mm horizontally. The lesion destroyed all retinal layers including axonal fibers of passage leading to retrograde degeneration of all retinal ganglion cells peripheral to the lesion, which extends the scotoma from the lesion proper towards the far periphery of the monocular lower visual field. Essentially, the lesioning procedure was the same as for rats used in VSDI experiments (see Section 3.2.1.).
4.1.3. Slice preparation

Control and lesioned rats aged 28-30 days were deeply anesthetized by ether inhalation and decapitated. Post lesion time of lesioned rats was 8 – 10 days. Brain was rapidly placed into oxygenated and cooled artificial cerebrospinal fluid (ACSF) containing in mM: 125 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 25 D-Glucose, 2 CaCl$_2$, and 1.5 MgCl$_2$ bubbled with 95 % O$_2$ and 5 % CO$_2$ to pH 7.4). The temperature of ACSF during slice production was kept at 1-4 ºC. Coronal slices of 350 μm thickness containing the visual cortex were prepared using a vibratome (Leica, VT 1000S, Germany). The slices were placed in the storage chamber and incubated in ACSF at room temperature. The cortical tissue was allowed to recover at room temperature for at least 1.5 hours before individual slices were transferred to a submerged recording chamber. During recordings the slices were superfused with oxygenated ACSF at 36 ± 1ºC. The recording chamber was mounted on the stage of an upright microscope (Olympus BX50-WI, Olympus, Japan) equipped with 2.5x and 40x submerged objectives. The cell bodies and proximal dendrites were visualized with differential infrared contrast.

4.1.4. Patch-clamp recording

**Whole-cell recording.** Whole-cell recording configuration allows to record from neurons and modify the intracellular medium by filling the cell with a solution of a known composition through the patch pipette. To achieve the whole cell configuration a patch pipette is filled with an appropriate solution containing low calcium concentration, comparable to the one inside the cell. On entering the bath the liquid junction potential of the pipette is compensated and measurement of pipette resistance is performed constantly to monitor the quality of the pipette and for subsequent compensation of the series resistance during current-clamp recording. The pipette is driven to the surface of the cell and the positive pressure is applied to keep the tip clean. When touching the cell's surface the positive pressure is released and changed to negative one, which leads to „gigaseal“ formation. After the gigaseal formation the
pipette potential is put to negative value (-70 mV below the bath potential) and repetitive voltage steps of 3 mV are given and fast capacitance compensation is applied to cancel out capacitive artifact caused by capacitance of the pipette's wall and pipette holder. After compensation of capacitive artifact a negative pressure is applied to the pipette until a sudden increase of the capacitive transients occurs. This increase in recorded current is a result of contribution of cell's membrane capacitance to the circuit after the patched membrane has been broken (Fig. 4.1.1).

**Electrical properties of a cell.** The electrical circuit model of a small round cell is shown in figure 4.1.2. It is assumed that the recorded cell has an access (or series) resistance $R_s$ during the recording. When the potential of the pipette $V_{pip}$ follows an imposed square step $dV$, the current flowing through this circuit can be described by a single exponential function of time. Normally series resistance is negligible compared to the resistance of cell's plasma membrane (R) that is clamped, therefore:

\[ I_{in} = \frac{dV}{R_s}, \]
\[ I_{ss} = \frac{dV}{R}, \]
\[ \tau = R_s C, \]

where $I_{in}$ is the instantaneous current, measured immediately after voltage jump, $\tau$ is the time constant of current decay and $I_{ss}$ is a steady-state current. All those parameters can be obtained from the current record, and the equations can be used to calculate the rest of cell's electrical parameters, except the resting potential of the cell. Series resistance is generally 2-3 times higher than the pipette resistance measured before the formation of the seal.

Neurons, however, including visual cortical pyramidal cells can not be described precisely by this simple circuit, because they possess extended dendritic arborizations, that can not be instantly charged when square voltage step is applied to the soma. For instance, in cerebellar Purkinje cells the capacitive current response to hyperpolarising voltage step is best described by the sum of two exponentials, while the pyramidal neurons in the hippocampus display capacitive currents that follow a three-exponential decay. Generally, because of those geometrical considerations neocortical neurons can be faithfully space-clamped in their soma and proximal dendrites, while more spatially
distant signals are subject to dendritic filtering (Sakmann & Neher, 1995).

4.1.5. Experimental set-up

The recording chamber and manipulators were installed on the upright infrared phase contrast microscope (Olympus BX50WI), used to visualize neurons within a brain slice during establishment of the recording (Dodt and Ziegglansberger, 1990). The microscope was equipped with a 2.5x and a 40x objective; the intermediate magnifications were achieved by usage of 10x ocular.

Image was recorded and constantly presented during the experiment with infrared-sensitive CCD camera (C5405, Hamamatsu) on a monitor (WV-BM1410, Panasonic). The microscope was installed on a vibration resistant table (Luigs and Neumann) and surrounded by a Faraday cage. The brain slices were placed in a submerged recording chamber. Throughout the experiment, the brain slices were kept on a nylon net in a fixed position using short silver wire weights. ACSF was continuously bubbled with 95% oxygen and 5% carbon dioxide and kept at 36 ºC with help of the water bath, located outside of the Faraday cage. To provide constant perfusion of slices with fresh ACSF we used a tube pump (mini pulse 3, Gilson, France). The perfusion rate was adjusted to 14 ml/min. The patching was done under visual guidance. To keep the tip of the pipette clean, a constant positive pressure was applied to the pipette during cell approach. When the tip touched the surface of the cell, the pressure was released and slight negative pressure was applied to achieve a gigaseal. Repetitive application of the 3mV step the pipette resistance was monitored throughout the experiment. To establish whole-cell configuration, a negative pressure pulse was applied, until the intracellular space was accessed as evidenced by sudden increase of capacitive artifacts (Fig. 4.1.1).

The recordings of the ionic currents were made using an Axopatch 200B patch-clamp amplifier (Axon Instruments). After digitization of the amplified and filtered signals by an A/D D/A transducer (Digidata 1440, Axon Instruments) the recordings were stored on a hard disk. pClamp10/Clampfit software (Axon Instruments) was used to visualize and analyze recordings. Signals received by the amplifier were represented independently on the oscilloscope (TC 305, Hameg) for independent control. The extracellular stimulation of fibers was done via stimulation unit with adjustable stimulation intensity, controlled by the A/D D/A converter. The rectangle stimuli had a duration of 50μs and were applied via low-
resistance glass pipettes. The stimulation electrode was always located in at least 300 μm away the cell soma, and for stimulation of horizontal connections ~ 800 μm away laterally.

### 4.1.6. Production of the pipettes

Patch pipettes were pulled from 100 mm long borosilicate glass capillaries (GB 150F-8P, Science Products, Hofheim, Germany). These capillaries had an outer diameter of 1.5 mm and the inner diameter of 0.86 mm. Inner wall of the tubes contained a filament to facilitate the filling of the pipettes and prevent occurrence of air bubbles. The pipettes were produced with horizontal electrode puller (DMZ Universalpuller, Zeitz). The electrode resistance amounted to 5-6 MΩ after filling with intracellular solution.

### 4.1.7. Recording and analysis of electrophysiological data

Electrophysiological recordings were obtained using an Axopatch 200 B amplifier (Axon Instruments, Union City, CA, USA). Data were filtered at 5 kHz and digitized using a Digidata-1440 system with PClamp10 software (Molecular Devices, Sunnyvale, CA, USA). During voltage clamp recordings liquid junction potential was corrected before establishment of a gigaseal. No series resistance compensation was applied in voltage-clamp mode. Input and series resistance was monitored throughout the recordings and cells were discarded if those parameters changed more than 20% from baseline value. In current-clamp mode both liquid junction potential and series resistance compensation were applied. Layer II/III cells were visually identified in each slice by their morphology and location relative to the pia. Pyramidal neurons were also identified by their firing pattern and in some cases further verification of cell class was made by injection of 3-4% biocytin (Sigma) or Lucifer Yellow (0.15%, Sigma) through the patch-clamp pipette. After fixation in 4% paraformaldehyde the slices were immunohistochemically processed, and the biocytin/Lucifer Yellow staining revealed recordings exclusively made from layer 2/3.
Figure 4.1.1. Whole-cell recording. A. On approach to the cell and release of the negative pressure a gigaseal is formed, which can be monitored by appearance of the capacitive artifacts and reduction in the steady-state current magnitude. B. Application of the positive pressure leads to the rupture of the membrane under the pipette tip, leading to establishment of the whole-cell configuration, evidenced by sudden increase in amplitude of capacitive artifacts (lower panel).

Figure 4.1.3. Scheme of the patch-clamp setup used for electrophysiological recordings. a – ACSF perfusion input to the slice chamber, b – stimulation electrode, c – visual cortical slice, d – patch electrode, e – ACSF perfusion output, f – Faraday cage.

Pyramidal shaped cells. All neurons perfused with potassium-based solutions responded to depolarizing current injection with regular, frequency-adapting spikes. The cell position in regard to the lesion projection zone was also verified in some cases by double staining of slices for zif268 and Lucifer Yellow or biocytin (Fig. 4.2.1.A).

4.1.8. Intrinsic excitability and passive membrane properties

Passive membrane properties, firing patterns of neurons and action potential thresholds were studied in whole-cell current clamp mode. A DC current was injected to bring the membrane potential to −70 mV. Square current steps of 600 msec duration were then injected, consequently increasing the step's amplitude. The instantaneous firing frequency for the first and subsequent steps was calculated for each neuron. Input
resistance was measured from the steady-state voltage deflection produced by current steps of ~20 pA. The intracellular solution contained (in mM): 140 K-Gluconate, 8 KCl, 2 MgCl, 10 Hapes, 4 Na2-ATP, 0.3 Na2GTP, 10 Na-Phosphocreatine. The pH was adjusted to 7.3 with KOH. Throughout the experiments all major synaptic input was blocked by synaptic blockers: 20μM DNQX, 50 μM PTX and 50μM D-AP5. To measure spontaneous firing rates of the cells the slice was placed in modified ACSF, containing in mM: 126 NaCl, 5 mM KCl, 0.5 mM MgCl₂, 1.25 NaHPO₄, 25 NaHCO₃, 1 CaCl₂, and 25 D-Glucose, bubbled with 95 % O₂ and 5 % CO₂ to pH 7.4

4.1.9. Miniature postsynaptic currents

The recordings of miniature postsynaptic miniature currents were obtained in whole cell voltage clamp mode. The mEPSCs were recorded at a holding potential of -80 mV. The ACSF contained 0.5 μM TTX, in order to exclude action potential-related transmission. For isolation of AMPA receptor mediated excitatory transmission the ACSF contained the GABAₐ receptor antagonist picrotoxin (PTX) (100 μM) and D-AP5 (50 μM) to block NMDA-receptor mediated currents. Intracellular solution contained in mM: 140 K-Gluconate, 8 KCl, 2 MgCl, 10 Hapes, 4 Na2-ATP, 0.3 Na2GTP, 10 Na-Phosphocreatine. The pH was adjusted to 7.3 with KOH.

For pharmacological isolation of mIPSCs mediated by GABAₐ - receptor operated channels 20 μM DNQX (the AMPA receptor-operated channels' locker) was added to the ACSF in place of PTX. The recordings were made at +20 mV with cesium-based intracellular solution containing (in mM) 135 CsCl, 10 HEPES, 2 MgCl, 20 TEACl, 10 EGTA. The pH was adjusted to 7.3 with CsOH. For each cell, at least 5 minutes of recording were obtained. For the detection of spontaneous events, the “threshold search” protocol was used and each event was manually checked. Events were accepted on the basis of current amplitude (more than 5 pA); events with unusually long or short decay times were discarded, unless they belonged to the „burst“.
4.1.10. Evoked postsynaptic currents

Evoked excitatory and inhibitory postsynaptic currents were recorded in layer 2/3 from the same pyramidal neuron to explore the ratio of excitatory and inhibitory input for each individual cell. For this we used a cesium-gluconate based solution of the following content (in mM): 125 Cs-Gluconate, 5 CsCl, 10 EGTA, 2 Na$_2$-ATP, 2 MgCl$_2$, 0.4 Na$_2$ – GTP, 10 HEPES. pH was adjusted to 7.3 with CsOH. With this solution the reversal potential of eEPSC was found to be at -50 mV, while the reversal potential of eIPSC was at +10 mV. The recording of eIPSCs was made at reversal potential of eEPSC and the recording of eEPSC was made at the reversal potential for the eIPSC (Fig. 4.2.9B & 4.2.12B). For accessing vertical inputs the stimulation electrode was placed in layer 4 and for horizontal stimulation the electrode was placed in layer 2/3 600 – 800 micrometers lateral to the recording site.

4.1.11. Long-term potentiation of synapses at horizontal inputs.

For LTP measurements the following potassium gluconate-based solution was used: 140 K-Gluconate, 8 KCl, 2 MgCl, 10 Hpes, 4 Na$_2$-ATP, 0.3 Na$_2$GTP, 10 Na-Phosphocreatine. The pH was adjusted to 7.3 with KOH. The positioning of the stimulation electrode in layer 2/3 was the same as for recordings of eEPSCs. The amplitude of baseline eEPSP was adjusted to remain below 4 mV to prevent unspecific suppression of inhibitory inputs in the vicinity of the recorded cell. We used a stimulation protocol to pair the theta-burst stimulation via stimulation electrode with action-potentials evoked by current-injection via the patch pipette. Generally, each eEPSP in a burst was leading an AP by 2 – 15 ms (Fig. 4.1.4). An input resistance of the neuron was monitored through the experiment, and neurons displaying input resistance change more than 20% of baseline were discarded.
Figure 4.1.4. LTP induction procedure. A. Protocol used to induce LTP. Each theta-burst contained 6 stimuli delivered at 100 Hz and was paired with a depolarizing current injection through the patch pipette. One burst was delivered every 250 ms for 2 seconds. A protocol consisted of 6 episodes of such stimulation. B. A 50 ms depolarizing step was paired with each episode of 100 Hz stimulation. 2 – 4 intrinsic action potentials resulted from depolarizing step, depending on cell's intrinsic properties. Asterisks mark the positions of stimulation-evoked EPSPs. Each EPSP preceded the AP by 2 – 15 ms.
4.1.12. Histological procedures

For histological procedures slices of visual cortex, retinae and brains were first fixed in 4% paraformaldehyde.

4.1.12.a. Nissl staining

Cresylviolet dye solution containing 1000 ml deionized water, 1.00 g Cresylviolet (Sigma C5042), 0.25 g sodiumacetate and 3.1 ml acetic acid was used for Nissl staining of frozen coronal sections. The sections were defatted in ascendic alcohols for 3 minutes each, then three times in Xylene for 3 minutes and, finally, in descendic alcohols 3 minutes each. Then the sections were rinsed in deionized water and incubated in cresylviolet dye solution for 3 – 5 minutes. After rinsing off the dye with several changes of deionized water the sections were placed first in 70% ethanol, then twice in 100% ethanol until no dye traces were left. Then the sections were dehydrated in isopropanol for 3 minutes and cleared in xylene. The slices were coverslipped with DePex (Serva).

4.1.13.b. Determination of LPZ's position in V1 by zif268 expression

Protein product of immediate early gene zif268 is known to change its expression depending on the amount of neuronal activity, also in V1 after the lesions of retina (Arckens et al., 2000b, Hu et al., 2009). We made use of those observations to define the location of the LPZ in order to determine the position of the stimulation and recording electrodes in primary visual cortex of lesioned animals. The rats received either binocular or monocular retinal lesion as described above (see Retinal Lesions section 4.1.2.). Animals were intracardially perfused with cold 4% PFA in phosphate-buffered saline 10 days after the lesion was performed at P21. The brains were removed and placed in the perfusion solution for 24 hours and then placed in 40%
**Figure 4.1.5. Determination of the lesion projection zone (LPZ) by the level of zif268 expression.**

A. Expression of zif268 protein in a section of primary visual cortex of rat, that received a monocular retinal lesion at P21 10 days post lesion (lower panel) and age-matched control rat (upper panel). In lesioned rat an area of reduced zif268 staining is evident in contralateral V1, while V1 from control rat displays even staining in both hemispheres. Stippled boxes, denoted with lower case letters show location of panels from B. Bar = 1 mm.

B. Effect of retinal lesion upon expression of zif268 in primary visual cortex at higher magnification. Lower case letters denote location of areas shown in lower magnification picture from A. a – zif 268 expression in primary visual cortex of a normal 31 day old rat; b - zif 268 expression in ipsilateral primary visual cortex of rat 10 days after monocular lesion; c - zif 268 expression at the border of the LPZ in contralateral V1 of rat 10 days after monocular lesion (arrow marks the border of the LPZ); d - zif 268 expression at the center of the LPZ in contralateral V1 of rat 10 days after monocular lesion. Bar = 200 μm.

C. Mean amount of zif268-positive cells was reduced inside the LPZ compared to adjacent primary visual cortex (p < 0.04, t-test).

D. Nissl staining of sections of contralateral visual cortex showed that total number of living cells inside the LPZ was about the same as in nearby cortex (spared). Therefore, reduction in number of zif268-positive cells was not due to cell death inside the LPZ. Bar = 200 μm.
sucrose solution for cold-protection. Slices were made from occipital cortex containing the V1 up to bregma position (-5 mm). The position of lesion-projection zone was defined as the area of reduced zif268 staining. The amount of zif268 positive cells inside the LPZ was reduced in all cortical layers, compared to normal primary visual cortex from non-lesioned age-matched controls, primary visual cortex from ipsilateral hemispheres and visual cortex located next to the LPZ (Fig. 4.1.5. A & B). At the same time Nissl staining of adjacent sections did not reveal any reduction in total number of cells. Therefore, the observed decrease in zif268-positive cells was not due to unspecific cell death. In few cases staining for zif268 was performed after patch-clamp experiment (see below, also Fig. 4.2.1A). For this the recorded cell was filled with Lucifer Yellow or biocytin during the experiment and the slice was placed in 4% PFA after recording was finished.

Immunohistochemistry was performed on free-floating coronal sections. The sections were rinsed for 10 minutes in PBS three times and incubated for 90 min at room temperature in the blocking solution (10 % normal goat serum, 0.01 M PBS containing 0.2 % TritonX100 and 20% avidin). Consecutively, the sections were incubated overnight at room temperature in primary antibody solution, containing anti-Zif268 (Egr-1, C-19, Santa Cruz sc-189, 1:500 dilution). Normal goat serum (1%), 0.2 % TritonX100, and 20 % biotin were used to limit non-specific binding of antibodies and to increase the membrane permeability. Following washing in PBS sections were incubated for 90 min at room temperature in secondary antibody (biotinylated goat anti rabbit IgG, Vector, 1:200). After rinsing in PBS, sections were processed with the ABC reagent (Vector, 1:500) for 90 minutes at room temperature and then rinsed three times in PBS for 10 min. The reaction product was visualized by transferring sections to 0.05 M PBS solution containing 0.05 % diaminobenzidine and 0.01 % H₂O₂. Following washing in PBS, sections were mounted on chrome-alumgelatin-coated slides. The sections were dehydrated through a graded series of alcohols, Xylol and coverslipped with DePex (Serva).

For simultaneous visualization of Lucifer Yellow-labelled neurons and zif268-positive cells in slices after patch-clamp recordings, the detection with ABC reagent was substituted to detection with CY3-conjugated streptavidin (StreptAV-CY3, Dianova), after which the resulting staining could be visualized with 540 nm fluorescent light.
4.1.13. Statistical analysis

Normally distributed data were assessed with parametric Student's t-test for statistical evaluation. For data sets that considerably deviated from normally distributed (LTP experiments) non-parametric Mann-Whitney U-test was used. Results are presented as mean ± SEM, and critical significance level was set to $p < 0.05$ (unless otherwise stated).
4.2 RESULTS

4.2.1. Increased circuit activity in layer 2/3 pyramidal neurons inside the LPZ 8 – 10 days post lesion.

Whole-cell current-clamp recordings were obtained from L2/3 pyramidal cells in the V1 at temperature 36 ± 1°C. Recordings were made in L2/3 neurons because their dendrites represent targets for the long-range horizontal inputs coming from the neurons located outside of the LPZ. Besides, extragranular cortical layers contain most of the horizontal intrinsic connectivity and have been reported to remain plastic from birth into the adulthood (Goel & Lee, 2007), in contrast to layer 4, which is a primary target of a subcortical input. Slices incubated in standard ACSF typically exhibit very low levels of spontaneous activity. However, when placed in modified ACSF (see Methods, Section 4.1.8) containing reduced Mg\(^{2+}\) (one-third, compared with standard ACSF), Ca\(^{2+}\) (one-half) and slightly elevated potassium (Sanchez-Vives & McCormick, 2000), L2/3 pyramidal neurons exhibited spontaneous firing at 0.01-8 Hz. The changes in ACSF ionic composition may enhance NMDA-receptor-dependent synaptic transmission, reduce calcium-dependent potassium currents, and reduce short-term synaptic depression, relative to that observed with standard ACSF. The ionic content of this modified ACSF is more similar to in vivo rodent cerebrospinal fluid than to standard ACSF (Dani et al., 2005). Previously it was demonstrated that experience-dependent changes in circuit excitability can be readily revealed under these “active-slice” conditions (Maffei et al., 2004). Regular spiking pyramidal cells were selected for the recordings. The cell's identity was further proven in several cases via filling of the cell with Lucifer Yellow or biocytin (Fig. 4.2.1.A). Analysis of spontaneous firing of L2/3 pyramidal cells demonstrated an about ≈2-fold increase (p < 0.02) in the mean firing rate of intra-LPZ pyramidal neurons 8-10 days after the lesion, compared with pyramidal neurons recorded in primary visual cortex of normal age-matched rats (frequency of spontaneous firing, controls: 2.4±0.3 Hz (n = 13), lesion: 4.2 ±0.64 Hz (n = 6), Fig. 4.2.1.B & C).
Figure 4.2.1. Spontaneous activity of pyramidal neurons in the LPZ. Spontaneous firing rates of regularly spiking L3/2 pyramidal cells are increased inside the LPZ compared to primary visual cortex of normal age-matched controls. To induce spontaneous firing the slices were perfused with modified ACSF (see Methods, Section 4.1.8) and recorded cell was brought to threshold (about -50 mV in both groups). A. Lucifer Yellow stained pyramidal cell in layer 2/3 of the LPZ (green). Orange – post-hoc staining of the slice for zif268 expression, confirming the intra-LPZ position of the neuron. B. Representative spontaneous firing recordings made at about 36°C from a primary visual cortex of a normal 29 days old rat (upper panel) and from the LPZ of a 29 days old rat 9 days post lesion (lower panel). C. Average spontaneous firing rate of L2/3 pyramidal neurons from normal rats and LPZ of the lesioned rats. The ≈2-fold difference in mean firing rate was statistically significant (Student's t-test, p < 0.01).
4.2.2. Intrinsic membrane properties of the layer 2/3 pyramidal neurons in the LPZ were not affected by removal of vertical inputs.

The increase in frequency of spontaneous firing observed after the lesion may be a result of different processes, including changes in expression of voltage-gated channels, leading to alterations in spike-generation thresholds, input resistance of the cellular membrane and changes in intrinsic excitability of the neurons (Kotak et al., 2005; Maffei & Turrigiano, 2008; Turrigiano, 2008). Therefore, we next addressed the possibility that increase of spontaneous firing rates was due to increased intrinsic excitability. For this the slices were placed in the ACSF medium containing the „blocker cocktail“ - 20µM DNQX, 50µM PTX and 50µM D-AP5 to remove activity coming from synaptic inputs. The cells were held at – 70 mV and 600 ms positive current steps of increasing intensity were delivered to drive the cell to firing threshold. The firing threshold was determined as the voltage at which dV/dt reached 3 S.D. from baseline (Fig. 4.2.2.A & B and Fig. 4.2.3.C). Firing rate at every step was evaluated to build an input-output curve. To measure input resistance of the cell the negative step of 20pA was delivered and the steady-state amplitude of the passive membrane response was measured. Pyramidal neurons both in the LPZ and in control V1 responded to depolarizing somatic current injection with regular and frequency-adapting action potentials, a typical property of cortical pyramidal neurons (Trettel and Levine, 2002; Connors and Gutnick, 1990). No significant changes in resting membrane potential, frequency of evoked firing or input resistance of the cell could be observed (Fig. 4.2.3.). Membrane potential, controls: -81.57±1.64 mV (n = 9), lesion: -82.39±1.5 mV (n = 7); input resistance, controls: 400.4±44.25 MΩ (n = 9), lesion 455.6±34.87 MΩ (n = 7); spike generation thresholds, controls: 38.52±0.22 (n = 9), lesion: 37.98±0.52 mV (n = 7). Input-output curves for evoked firing rates are depicted in Fig. 4.2.2.B.
Figure 4.2.2. Intrinsic properties of the pyramidal neurons in the LPZ – 1.
Intrinsic excitability of L2/3 pyramidal neurons from the LPZ is unchanged compared to primary visual cortex of age-matched normal controls. Slices were continuously perfused in standard ACSF containing APV, DNQX, and picrotoxine. **A.** Sample traces of spikes evoked by a depolarizing current step of 160 pA in a normal (upper, black) and LPZ-located (lower, gray) L2/3 cell. **B.** Mean firing rate vs. injected current amplitude (F-I curve), open circles – control, filled gray circles – LPZ.
Figure 4.2.3. Intrinsic properties of the pyramidal neurons in the LPZ - 2. A. Input resistance of the intra-LPZ displayed a slight increase compared to neurons of V1 from non-lesioned animals but it was non-significant. B. Resting membrane potential of the intra-LPZ neurons was unchanged compared with non-lesioned controls. C. The mean spike firing threshold of LPZ-located cells was unchanged compared to controls.
4.2.3. Partial removal of subcortical input leads to synaptic scaling of glutamatergic synapses onto layer 2/3 pyramidal cells located in the LPZ.

Increased circuit activity together with normal cellular excitability suggest that the balance between total excitatory synaptic drive and total inhibitory synaptic drive onto L2/3 pyramidal cells of the LPZ may be changed as a result of retinal lesion. To test this implication we first looked at the properties of excitatory and inhibitory miniature postsynaptic currents. The mEPSC were isolated by applying TTX to the bath to block action potentials and in presence of picrotoxin and D-AP5 to block inhibitory and NMDA-related currents, respectively. While the frequency of mEPSCs was unchanged after the lesion (Fig. 4.2.4.A & B, controls: 10.6±1.076 Hz (n =8), lesion: 12.31±1.38 (n = 7)), there was a significant increase of the quantal amplitude of glutamatergic AMPA-mediated transmission (Fig. 4.2.5 & Fig. 4.2.8.A, controls: 10.58±0.86 pA (n = 8), lesion 14.06±1.01 pA (n = 7), p < 0.02). Therefore, depriving the neurons of their dominant subcortical inputs lead to compensatory homeostatic increase in quantal amplitude of EPSCs without affecting the frequency. The increase of mEPSC amplitudes is attributable to synaptic scaling, as demonstrated in figure 4.2.8.A: the whole mEPSC amplitude distribution is shifted to larger amplitude values.

4.2.4. Retinal lesion resulted in marked decrease of frequency of miniature inhibitory postsynaptic currents and reduction of mIPSC amplitudes in the intra-LPZ pyramidal cells.

Next, the properties of miniature inhibitory synaptic transmission were evaluated. mIPSCs were recorded in presence of TTX, DNQX and D-AP5 to block glutamatergic transmission. In contrast to mEPSCs an opposite trend was observed for the mIPSCs: 8 – 10 days after the lesion frequency of mIPSCs was decreased nearly 2-fold in the intra-LPZ pyramidal cells compared to normal cells (Fig.4.2.6.A & B: control: 16.73±0.69 Hz (n = 9), lesion: 9.62±1.36 Hz (n = 8), p < 0.0005). A highly significant decrease in amplitude of mIPSCs also occurred in the intra-LPZ neurons (Fig. 4.2.7 & Fig. 4.2.8.B, controls: 21.42±1.35 pA (n = 9), lesion: 13.82±1.2 Hz (n = 8), p<0.0005). This effect of retinal lesion on mIPSC amplitude is a result of synaptic
Figure 4.2.4. No change in frequency of mEPSCs 8 – 10 days post lesion. mEPSCs were recorded from L2/3 pyramidal cells in the presence of D-AP5, PTX, and TTX. **A.** Sample traces from control (upper black traces) and LPZ-located cells (lower gray traces), voltage clamped at -70 mV. Lower panels represent blockade of mEPSCs by addition of DNQX to the bath. **B.** A small, statistically non-significant (p = 0.12, t-test) increase in the mean mEPSC frequency was observed in LPZ-located cells.
Figure 4.2.5. The amplitude of excitatory quantal transmission in L2/3 pyramids inside the LPZ was increased 8 – 10 days post lesion. mEPSCs were recorded from L2/3 pyramidal cells in the presence of D-AP5, PTX, and TTX. A. Average of 500 mEPSC events from random-chosen control (black) and LPZ-located cell (gray). B. Cumulative histograms of 300 to 600 events from each of normal cells and LPZ-located cells showing a significant rightward shift in the mEPSC amplitude for LPZ-located cells. C. Mean mEPSC amplitude was increased by about 20%, and this difference was statistically significant (p < 0.02, t-test).
scaling, as the whole distribution of mIPSC amplitudes was shifted towards smaller amplitude values (Fig.4.2.8.B). Therefore, retinal lesion scaled the amplitudes of mIPSCs and mEPSCs in opposite directions (Fig.4.2.8.A & B), indicative of potential shift of excitatory-inhibitory balance in the pyramidal neurons of the LPZ.

It has been proposed that long-range lateral connectivity targeting the pyramidal neurons in layer 2/3 and layer 5 comprises a background for cortical reorganization seen in vivo after prolonged survival times. However, under normal developmental conditions the flow of information through the plexus of long-range connections is limited by strong inhibitory influences. Therefore, a shift of excitatory-inhibitory balance may play a critical role in a possible unmasking of horizontal inputs to pyramidal neurons. Indeed, the present study of properties of miniature postsynaptic currents demonstrates that a shift in relative strength of inhibition and excitation is present in layer 2/3 pyramidal neurons 8 – 10 days after the lesion. However, those results are not informative of the source of such change nor do they address the excitatory-inhibitory balance in horizontal inputs specifically. Recordings of miniature postsynaptic currents sample from all excitatory and inhibitory inputs onto a neuron, which arise from several sources. One source of input to layer 2/3 pyramidal cells are excitatory inputs from layer 4 regular spiking pyramidal cells and spiny stellate neurons and inhibitory inputs from layer 4 large basket cells. Other sources of inputs are represented by short- and long-range lateral connections coming from layer 2/3 and layer 5 pyramidal cells and large basket cells from outside of the LPZ (Tanifuji et al., 1994; Paxinos, 2004; Hensch, 2005; McDonald & Burkhalter, 1993). Therefore, we performed additional set of experiments to separately study the relative strength of inhibition and excitation in those horizontal inputs, coming from layer 2/3 and inputs from layer 4 (vertical).
Figure 4.2.6. Unitary mIPSC of layer 2/3 pyramidal neurons in the LPZ displayed a decrease in frequency. mIPSCs were recorded from L2/3 pyramidal cells in the presence of D-AP5, DNQX and TTX. A. Sample traces from normal (upper, black) and LPZ-located cells (lower, gray) voltage clamped at +20 mV. Lower panels represent blockade of mIPSCs by addition of PTX to the bath. B. A drastic decrease in mIPSC frequency was observed in LPZ-located neurons (p < 0.0002, t-test).
Figure 4.2.7. Reduction in the amplitude of mIPSCs in the LPZ-located pyramidal cells. A. Mean of 500 events taken from random-chosen LPZ-located pyramidal neuron and pyramidal neuron from non-lesioned animal. B. Cumulative histograms of 300 to 600 events from each of normal cells and LPZ-located cells showing a leftward shift in the amplitude of mIPCS for LPZ-located cells. C. mIPSC amplitude was reduced by about 42 % inside the LPZ, and this difference was highly statistically significant (p<0.0005, t-test).
Figure 4.2.8. Bidirectional synaptic scaling of miniature postsynaptic currents. The shift of the whole amplitude distribution is indicative of global nature of amplitude change, characteristic of homeostatic scaling. In LPZ neurons the amplitudes of mIPSCs were scaled down, while at the same time the amplitudes of mEPSCs were scaled up. A. Distribution of mEPSC amplitudes in control and LPZ neurons. Rightward shift of total amplitude distribution is observed after the lesion (blue arrows). B. Distribution of mIPSC amplitudes after lesion underwent a leftward shift (blue arrows).
4.2.5. Excitatory-Inhibitory balance did not change for vertical inputs.

To record vertically evoked PSCs a stimulation electrode (low-resistance glass pipette) was placed in layer 4 and pyramidal cells in layer 2/3 inside the LPZ were patched (Fig. 4.2.9.A). The slices were washed in normal ACSF without application of blockers of glutamatergic or GABAergic transmission. To block voltage-gated sodium currents, QX-314 was added to intracellular solution. eEPSCs were recorded at measured reversal potential for the eIPSC (-50 mV), while eIPSCs were recorded at measured reversal potential for eEPSC (+10 mV) (see Fig. 4.2.9.B for exemplary traces). Both vertically evoked EPSCs and IPSCs were characterized by a decreased amplitude across the whole range of stimulation intensities and reduced maximally evoked response (maximally evoked EPSC, control: 1320.09±253.8 pA (n = 6), lesion: 596.34±154.02 pA (n = 8); maximally evoked IPSC, control: 1920±238.7 pA (n = 6), lesion: 798.7±106.58 pA (n = 8)). These results are demonstrated in Fig. 4.2.10. However, the excitatory/inhibitory ratio did not change as a result of such drastic decrease of both response types (maximal eEPSC/eIPSC ratio, control: 0.675±0.082 (n = 6), lesion: 0.78±0.13 pA (n = 8), Fig. 4.2.11.B). eEPSC/eIPSC ratio at lower stimulation intensities, as seen in Fig. 4.2.11.A did not differ significantly from non-lesioned controls.

4.2.6. A shift of excitatory-inhibitory balance in favor of excitation was observed for horizontal inputs to layer 2/3 pyramidal neurons.

Horizontally evoked PSCs were recorded under the same conditions as vertically evoked PSCs. The stimulation electrode was placed in layer 2/3 about 800 μm away from the recorded neuron towards the lateral side of the slice (Fig. 4.2.12.A). Horizontally evoked IPSCs displayed a systematic reduction in amplitude across the whole range of stimulation intensities, as depicted in figure 4.2.12.B, figure 4.2.13.B and figure 4.2.13 D. Maximally evoked inhibitory response was about 2-fold reduced
Figure 4.2.9. Vertical inputs: eEPSC & eIPSC recording. Vertically evoked excitatory postsynaptic currents were recorded from the same cell with stimulation electrode placed in layer 4. A. Placement of stimulation electrode (black) and patch pipette (white) was made based on zif268-positive cell distribution in primary visual cortex relative to white matter, so, that the recorded cell was located approximately in the center of the LPZ, which we could locate by the position of the ventricle hem (marked with “+”). B. Exemplary traces of eEPSCs and eIPSCs recorded from LPZ-located (gray traces) and control cell (black traces) at stimulation intensity 100 µA and holding potential -50 mV for eEPSC and +10 mV for eIPSC.
Figure 4.2.10. Vertical inputs: dramatic decrease of both eEPSC & eIPSC amplitudes. A. Input-output curve for vertically evoked EPSC. A significant decrease in vertically evoked eEPSCs' amplitudes was observed 8-10 days after the lesion even at low stimulation intensities. B. Input-output curve for vertically evoked IPSC. A significant decrease in vertically evoked eIPSC amplitude was observed 8-10 days after the lesion even at low stimulation intensities, similarly to eEPSCs. C & D. Maximal stimulation (130 µA) yielded significantly smaller vertical eEPSC and eIPSC in LPZ-located cells than in control cells from non-lesioned animals.
Figure 4.2.11. Vertical inputs: the balance between eIPSC and eEPSC was unchanged despite drastic effect of the subcortical input removal on the amplitudes of eEPSC and eIPSC. A. eEPSC/eIPSC ratio for used stimulation intensity range (20 to 130 µA). The difference seen for some intensities was non-significant (p > 0.05, t-test) B. eEPSC/eIPSC ratio for maximal stimulation (130 µA). No statistically significant difference was found for LPZ-located cells compared to neurons from non-lesioned animals.
Figure 4.2.12. Horizontal inputs: eEPSC and eIPSC recording. Horizontally evoked excitatory and inhibitory postsynaptic currents were recorded from the same cell with stimulation electrode placed in layer 2/3 about 800 microns away from recorded neuron. The position of the recorded cell was chosen in the same way as for vertical input stimulation (see Fig. 4.2.9.A). A. Placement of stimulation electrode (black) and patch pipette (white) was made based on zif268 positive cell distribution in V1 relative to white matter, so that the recorded cell was approximately in the center of the LPZ (“+” marks the hem of a ventricle, corresponding to the center of LPZ). Stimulation electrode was always placed away from the midline. B. Exemplary traces of eEPSCs and eIPSCs recorded from LPZ-located (gray traces) and control cell (black traces) at stimulation intensity 100 µA and holding potential -50 mV for eEPSC and +10 mV for eIPSC.
in the intra-LPZ neurons compared with control cells (control: 2327.72±448.36 pA (n = 7), lesion: 1221.19±129.24 pA (n = 12), p < 0.005, Fig. 4.2.13.D). In contrast, horizontally evoked EPSC showed a different dynamics: at small stimulation intensities no difference in amplitude was seen between lesioned and control groups; however at intermediate and large stimulation intensities intra-LPZ neurons demonstrated a significant increase in eEPSC amplitude compared to control cells. Maximally evoked glutamatergic response in the intra-LPZ neurons showed about 80% increase compared to control cells (control: 536.43±86.4 pA (n = 7), lesion: 1023.3±135.5 pA (n = 12), p < 0.005, Fig. 4.2.13.A & C). Hence, together those observations demonstrate dramatic shift of excitatory-inhibitory balance towards excitation (control: 0.251±0.02 (n = 7), lesion: 0.898±0.177 (n = 12), p<0.001, Fig.4.2.14.B). This shift was most prominent for strong stimulation intensities, but occurred also at lower stimulation intensities (Fig.4.2.14.A, p<0.05).
Figure 4.2.13. A bidirectional change in the amplitudes of horizontally evoked EPSCs and IPSCs occurred 8 – 10 days after lesion. A. Input-output curve for horizontally evoked EPSC. A significant increase in horizontally evoked eEPSC amplitude was observed 8-10 days after the lesion at intermediate and high stimulation intensities, but not at low stimulation intensities. Asterisk marks first intensity (90 µA), where the difference first becomes significant and corresponding p-value range. B. Input-output curve for horizontally evoked IPSC. A significant decrease in horizontally evoked eIPSC amplitude was observed 8-10 days after the lesion even at small stimulation intensities. C & D. Maximal stimulation (130 µA) yielded significantly larger eEPSC and smaller eIPSC in LPZ-located cells than in control cells during horizontal stimulation.
Figure 4.2.14. Horizontal inputs: the balance between eIPSC and eEPSC was shifted in favor of excitation in L2/3 pyramidal neurons inside the LPZ. A. eEPSC/eIPSC ratio across used stimulation intensity range (20 to 130 µA). Difference seen for all intensities was significant (p < 0.05, t-test). B. eEPSC/eIPSC ratio for maximal stimulation intensity (130 µA). A dramatic significant shift in favor of excitation occurs 8 – 10 days after lesion in LPZ-located cells.
4.2.7. LTP of horizontal inputs in layer 2/3 pyramidal cells is impaired 8 – 10 days after the lesion.

Shift of excitatory-inhibitory balance in favor of excitation may result in facilitation of induction of long-term potentiation in the intra-LPZ pyramidal cells, in a fashion similar to the LTP enhancement occurring in the surround of cortical lesions (Mittmann & Eysel, 2001). To induce LTP in horizontal inputs we applied a TBS paired with postsynaptic spikes, induced by current injection step through the patch pipette (Fig.4.1.4). This protocol induced a robust LTP of horizontal inputs in control animals 28 – 30 days of age (Fig.4.2.15). However, the same treatment of intra-LPZ neurons did not lead to any net changes in the slope of EPSP (Fig. 4.2.15). Moreover, several cells from the sample showed rather depression than potentiation or absence of changes in the slope (Fig. 4.2.16.B). Horizontal EPSP slope was significantly increased compared to baseline only in control non-lesioned animals (EPSP slope (mean over last 10 minutes of recording), control: 1.304±0.055 mV/ms (n = 8), lesion: 0.9388±0.104 mV/ms (n = 8), p < 0.005, Mann-Whitney U-test) (Fig.4.2.15C & Fig.4.2.16).
Figure 4.2.15. Absence of long-term potentiation of horizontal inputs in layer 2/3 pyramidal neurons of the LPZ. To induce LTP in layer 2/3 pyramidal neurons at their horizontal excitatory inputs, a presynaptic theta-burst stimulation was paired with postsynaptic depolarization step that induced intrinsic firing (see Methods, Section 4.1.11 & Fig.4.1.4). A. Exemplary recordings made from a neuron in control slice of V1 from 28 day old control rat (upper black) and from LPZ of 29 day old rat 9 days after lesion (lower gray). Solid lines represent baseline EPSP, dotted lines represent post-pairing EPSP. Note the pronounced increase in EPSP amplitude after pairing in the control animal and virtually no change in EPSP amplitude in the LPZ neuron. B. Time course of changes in EPSP slope during the experiment. Upper panel shows the control neuron, lower panel shows the LPZ neuron (same neurons as in A). Red bar – pairing. While in the control neuron the increase in eEPSP slope was persistent for the duration of recording, in LPZ neuron the slope values returned back to baseline after brief initial increase. C. Mean LTP measurements in layer 2/3 cells from the LPZ and from age-matched control primary visual cortex (non-lesioned animals). LTP of horizontal inputs to the intra-LPZ cells could not be induced 8 – 10 days after the lesion. Black – average of control cells (n = 8), show persistent potentiation of eEPSP amplitude resulting from application of pairing protocol. Gray – intra-LPZ statistics (n = 8). No significant change from baseline could be observed in the LPZ-located neurons.
Figure 4.2.16. Summary of LTP experiments: intra-LPZ pyramidal cells could not display LTP resulting from pairing of the TBS with intrinsic APs, like their counterparts from primary visual cortex of non-lesioned animals. A. A statistically significant increase in eEPSP amplitude was observed in control L2/3 pyramidal neurons, but not in intra-LPZ L2/3 pyramidal neurons 30 minutes after pairing ($p < 0.005$, Mann-Whitney U-test). B. Distribution of individual cell values of potentiation percentage during last 10 minutes of the recording in control (black circles) and intra-LPZ pyramidal cells (gray filled circles). While control cells displayed various degree of potentiation, many LPZ neurons did not show any change or even showed moderate depression of the responses.
4.3. DISCUSSION

4.3.1. Hyperexcitability of the pyramidal cells inside the LPZ

Altered excitability and spontaneous firing in the visual cortex can be caused by different mechanisms coming into play after the lesion is introduced in the retina. It is known that different manipulations causing partial or complete deprivation of visual cortical neurons can lead to changes in their intrinsic excitability, amount of synaptic drive they get and level of cortical inhibition they are under. All those factors are known to contribute to changes in spontaneous firing rates. The exact nature of recruited mechanisms may depend on the severity of the deprivation protocol. For example, Maffei and Turrigiano (Maffei & Turrigiano, 2008) have demonstrated that both lid suture and tetrodotoxin injection into one eye lead to increase in spontaneous firing of pyramidal neurons in layer 2/3 of the contralateral primary visual cortex. However, the ways to achieve this effect were different: where tetrodotoxin application led to shifted balance of excitation and inhibition in the deprived cortex without altering intrinsic properties of neurons, lid suturing boosted intrinsic excitability of the pyramidal cells. Since lid suturing is known to cause a stronger LTD at layer 4 to layer 2/3 connections than TTX injection, it was proposed that increasing the strength and reach of excitatory drive alone was not enough to overcome the effect of this LTD onto the firing rate of the neurons, requiring the recruitment of additional homeostatic mechanisms, namely global scaling of intrinsic excitability of the involved cells (Maffei & Turrigiano, 2008). Alterations in intrinsic membrane properties together with accompanying changes in excitatory-inhibitory balance were also observed after sensorineural hearing loss in area A1 layer 2/3 pyramidal neurons (Kotak et al., 2005). Several days after bilateral ablation of cochlea A1 layer 2/3 neurons displayed a depolarized resting membrane potential, an increased input resistance, and a higher incidence of sustained firing. They also exhibited significantly larger thalamocortically and intracortically evoked excitatory synaptic responses, including a greater susceptibility to the NMDA receptor antagonist AP-5 and the NR2B subunit antagonist ifenprodil. In our model we demonstrated, that intrinsic excitability of the intra-LPZ neurons, their resting membrane potentials, spike thresholds and input resistance were not affected by the retinal lesion (Fig. 4.2.2 & Fig. 4.2.3). These results rule out the plastic changes of
intrinsic excitability and membrane properties of the neurons as a possible cause of increased circuit activity. At the same time we observed moderate increase of excitatory drive together with a dramatic disinhibition of pyramidal cells in the LPZ (Figs. 4.2.5 – 4.2.14), leading to shift of balance between excitatory and inhibitory drive in the intra-LPZ neurons in favor of excitation, which may be the cause for increased firing rates.

4.3.2. Disinhibition of the pyramidal cells inside the LPZ

Massive disinhibition has long been proposed to be responsible for expansion of receptive fields of neurons located at the border of the lesion projection zone after the lesion and was thought to be one of the earliest changes in function of cortical circuitry happening instantly after the lesion (Buonomano & Merzenich, 1998, Gilbert & Wiesel, 1992, Gilbert, 2008; Arckens et al., 2000a,b; Massie et al., 2003a). However the underlying synaptic nature and exact meaning of those changes for further reorganization of the visual cortical circuitry is a matter of controversy. Regulation of action potential timing by a certain level of inhibition may play an important role in induction and stabilization of effects, brought about by retinal lesion. 8 – 10 days after the lesion we observed profound changes in inhibitory synaptic transmission, affecting both pre- and postsynaptic sites. First, the decrease of amplitudes of miniature IPSCs was observed (Fig. 4.2.7 & Fig. 4.2.8.B). Second, a significant reduction in frequency of mIPSCs also occurred (Fig. 4.2.6.). Evoked IPSCs also were affected – amplitudes of eIPSCs were dramatically reduced both at layer 4 to layer 2/3 connections (vertical) and layer 2/3 to layer 2/3 (horizontal) connections. Together those changes amount for overall reduction of the level of inhibitory drive inside the LPZ. This situation can have different implications. On one hand, reducing the inhibitory control may affect the propagation of excitation both inside V1 and between V1 and higher visual areas (Contreras & Llinas, 2001; Rozas et al., 2001; Xu et al., 2007; Bosman et al., 2005) and lead to unmasking of subthreshold long-range horizontal connections, thought to be responsible for initial expansion of receptive fields of perilesional neurons (Gilbert, 1998).

On the other hand, decrease of inhibitory control can also profoundly influence the induction of plastic changes in excitatory synapses by correlation-dependent or spike-timing dependent mechanisms. Many circuits become less susceptible to
development of LTP with increase of animal's age, bringing about a developmental increase in the strength of inhibitory drive. For example, in hippocampus it becomes harder to induce LTP by pairing pre- and postsynaptic spikes in the Schaeffer collateral input to CA1 pyramidal cells with age, requiring postsynaptic spike bursts for LTP induction in older animals. Such developmental inhibitory "gating" of LTP induction can be reversed by application of GABA_\text{A} antagonist bicuculine (Meredith et al., 2003). Contrastingly to this, diazepam application, making inhibition stronger, can prevent LTP induction (Trepel & Racine, 2000; del Cerro et al., 1992). Interestingly, application of picrotoxin, also a GABA_\text{A} antagonist, slowed down the development of LTP in freely moving preparation, although did not block it (Trepel & Racine, 2000). It is known that both feedback and feedforward inhibition affects dendritic depolarization induced by excitatory inputs and, therefore, modulates the elevation of postsynaptic calcium concentrations in response to activity. As a result, the presence of correlated GABAergic inputs onto the postsynaptic neuron may influence the possibility of induction of LTP (Wigstrom & Gustafsson, 1986; Dan & Poo, 2006). Regulation of LTP induction by the level of inhibition was also found in subcortical structures, for example, in rat VTA dopaminergic neurons (Liu et al., 2005). Repeated cocaine exposure, causing reduction of inhibition in VTA, facilitated LTP induction in dopaminergic neurons in VTA, while diazepam application in VTA, leading to restoration of inhibition, prevented this effect of cocaine (Liu et al., 2005). Moreover, the level of inhibitory control was shown to change during induction of LTP of excitatory inputs. For example, in CA1 area of hippocampus tetanization leading to LTP of excitatory transmission also induced LTD of IPSPs, resulting in decrease of inhibitory drive onto CA1 pyramidal cells, which raised the ability of their EPSPs to generate action potentials (Lu et al., 2000). In the visual cortex of the rat the possibility of relaying high-frequency firing, coming from activation of subcortical structures, to the supragranular levels was shown to be markedly reduced with maturation of inhibition. The responses of layer 2/3 neurons to repetitive stimulation of white matter become more and more depressed with age and closure of critical period, marked by dramatic increase in inhibitory drive. This depression, however can be prevented by dark rearing (delaying the development of inhibition) and direct application of blockers of inhibitory transmission (Rozas et al., 2001).

However, disinhibition also may disrupt expression of spike-timing dependent changes in synaptic strength (Hensch, 2005). Therefore, an initial disinhibition may
allow LPZ neurons to keep the firing rates close to pre-lesion values, allowing normally restricted lateral excitation to reach farther inside the deprived areas. Together with scaling up of excitatory synapses, increasing the gain of excitatory connections of the intra-LPZ neurons, this may lead to potentiation-like changes in horizontal inputs to intra-LPZ neurons. However, it is unclear how stable those changes would be in the face of increased firing rates together with poor correlation between post- and presynaptic firing (Abbott & Nelson, 2000). For instance, studies in mice with retinal lesions showed that newly formed dendritic spines in the intra-LPZ layer 5 pyramidal cells were highly unstable in the first two weeks post lesion (Keck et al., 2008). Studies in cats with retinal lesions employing in vivo microdialysis showed that after a period of initial disinhibition inside the LPZ the extracellular concentrations of GABA returned to normal levels after 1 – 2 months post lesion together with levels of expression of immediate early gene products zif268 and c-fos (Massie et al., 2003a; Arckens et al., 2000a,b). The timecourse of restoration of the level of inhibition crudely matched the timecourse of appearance of stable changes in cortical visuotopic map (Giannikopoulos & Eysel, 2006; Arckens et al., 2000a; Massie et al., 2003a).

4.3.3. Synaptic scaling of glutamatergic synapses inside the LPZ

The reduction of cortical GABAergic inhibition was accompanied by synaptic scaling of excitatory postsynaptic currents 8 -10 days post lesion. Increase of amplitude of mEPSCs without significant change in their frequency after a period of decreased network activity, known as synaptic scaling, has been observed in several studies both in neuronal cell culture and after manipulating visual experience in vivo (Turrigiano & Nelson, 2004; Turrigiano, 1998; Maffei and Turrigiano, 2008, Ibata et al., 2008; Turrigiano, 2008). Such scaling may explain the observed increase in spontaneous firing rates of pyramidal neurons inside the LPZ and may be triggered by several factors. First, removal of vertical inputs leads to depression of eEPSCs in the L4 to L2/3 synapses (Fig. 4.2.10.A & C). Such depression was also observed after whisker removal in barrel cortex in barrels corresponding to removed whiskers (Allen et al., 2003) and in visual cortex after monocular deprivation (Rittenhouse et al., 1999) and was shown to share mechanisms with homosynaptic LTD. Depression in the vertical pathways
together with horizontal inputs being weak immediately after the lesion leads to drop in firing rates of neurons in the LPZ. A drop of firing was shown to be a trigger for synaptic scaling (Ibata et al., 2008). Another triggering factor may be a global decrease in inhibitory drive in the LPZ, observed in this study. It was shown that reducing the level of inhibition with chronic application of gabazine led to scaling up of AMPA-receptor mediated EPSCs in spinal motoneurons, even though the average firing of neurons returned to pre-application values 12 hours after the start of application (Wilhelm & Wenner, 2008).

It was demonstrated that synaptic scaling depends on insertion of additional AMPA-receptors into the postsynaptic sites in response to decrease of firing brought about by initial removal of input (Ibata et al., 2008). The cellular and molecular changes leading to scaling have a timecourse much slower than that of Hebbian forms of plasticity – from a few hours to several days and weeks (Ibata et al., 2008; Turrigiano, 2008). In the LPZ multiplicative scaling of excitatory synapses combined with disinhibition may lead to increase in excitability of the cortical network and spontaneous firing rates in the LPZ, increase in gain of cortical neurons and eventual unspecific strengthening of spared horizontal inputs to the intra-LPZ pyramidal neurons. The regulation of the firing rates in face of various perturbations was demonstrated by numerous studies in neuronal cell cultures and also predicted by computational studies. Hyperexcitability resulting in increase of firing rates can have only a short lifespan, as the network aims at keeping neuronal firing rates at a certain optimal value (Turrigiano, 2008). Indeed, a short-lived hyperexcitability of cortical network after deprivation was implied after cat matching binocular retinal lesions (Arckens et al., 2000a). Employing binocular retinal lesions in cat the whole LPZ was shown to undergo a hyperexcitability profile that initially appeared at the rim of the LPZ and moved inside the LPZ over a time of a few weeks, comparable with the timecourse needed for functional reorganization in cat (Giannikopoulos & Eysel, 2006).
4.3.4. Excitatory-inhibitory balance inside the LPZ

Our results show that excitatory-inhibitory balance for layer 4-originating "vertical" inputs and horizontal long-range inputs to layer 2/3 pyramidal cells is affected differently by the retinal lesion. Vertical inputs, while displaying a major depression of both eEPSCs and eIPSCs did not show any change in the ratio between excitatory and inhibitory drive (Fig. 4.2.11.). Oh the other hand, ratio of excitation to inhibition significantly increased for the horizontal inputs. This shift of horizontal E/I ratio may have several important consequences. First, an increase in mean firing rate of the neurons in the LPZ may lead to upregulation of the intracellular calcium concentration, making cells prone to expressing certain forms of long-term plasticity (Barmashenko et al., 2003; Barmashenko et al., 2001; Mittmann & Eysel, 2001). This opens an opportunity for further increase of strength of lateral inputs promoting their influence upon pyramidal cell's activity from modulatory subthreshold to driving suprathreshold, as layer 4 inputs after the lesion loose their visual drive. Thus, the E/I ratio shift may provide background for establishment of the new receptive fields, that occurs over the timecourse of reorganization. Single-unit recordings in cat demonstrated, that shifting of receptive fields and their expansion after the lesion are associated with increase in background firing rates and evoked responses of neurons in the LPZ (Giannikopoulos and Eysel, 2006), which can be the result of increased neuronal gain. A possible mechanism, by which such gain changes associated with shifted ratio of excitatory and inhibitory drive is synaptic scaling. Synaptic scaling is triggered in the visual cortex through a decrease of feedforward input (Desai et al., 2002) and can regulate the excitatory-inhibitory balance within a network by exerting opposite effects on amplitudes of mEPSCs and mIPSCs as observed in several preparations (Turrigiano et al., 1998; Kilman et al., 2002; Maffei & Turrigiano, 2008) and also in the present work. Interestingly, the direction of synaptic scaling also can depend on the identity of the neuron. For example, Rutherford et al. have shown that quantal amplitudes of excitatory synapses located in pyramidal cells grown in culture are decreased by chronic BDNF application, but in excitatory synapses of bipolar interneurons in the same culture the same treatment scales down quantal amplitudes (Rutherford et al., 1998). Cooperative gain changes may also contribute to shift of excitatory-inhibitory balance in favor of increase of net firing exhibited by the network.
4.3.5. Absence of LTP of horizontal inputs inside the LPZ.

To induce the LTP of long-range horizontal connections in the primary visual cortex we paired postsynaptic spikes evoked by positive current injection with theta-burst stimulation. Previously, alike pairing of theta-burst with postsynaptic firing was used to explore the properties of LTP induction in the surround of cortical lesions in rat V1 (Mittmann & Eysel, 2001). Surprisingly, we were unable to observe an enhancement of LTP, and in several cells LTP was not induced at all, unlike in a cortical lesion model (Fig. 4.2.15 & Fig. 4.2.16).

Several possibilities could explain this behavior. First, it is tempting to speculate that an occlusion of LTP has taken place. Such occlusion of LTP and LTD by prior LTP-like or LTD-like changes correspondingly was reported before in several preparations. For instance, in hippocampus induction of LTP in CA1 could be diminished by prior avoidance training (Whitlock et al., 2006), in somatosensory barrel cortex of rodents the occlusion of LTD occurred after whisker removal and in visual cortex the LTD of feedforward excitatory inputs to layer 3 was occluded by monocular deprivation (Allen et al., 2003; Heynen et al., 2003). Therefore, it is possible that occlusion takes place also after retinal lesion and is a result of lesion-mediated LTP-like changes in horizontal inputs. Indeed, an amplitude of excitatory postsynaptic currents evoked horizontally was increased after the retinal lesion at high stimulation intensities (Fig. 4.2.13. A & C). Additionally, a change in firing order of the intra-LPZ neurons and neurons from unaffected V1 after the lesion creates ground for engagement of spike-timing dependent plasticity mechanisms. This also could occlude the next attempts to induce LTP with a spike-timing dependent protocol.

However, a number of other explanations may be more acceptable. First, we observed a dramatic depression of stimulus-related inhibitory transmission (Fig. 4.2.10.B,D & 4.2.13.B,D). This disinhibition can have long-standing consequences on expression of plasticity of glutamatergic synapses. Classical correlation-based LTP induction protocols, relying on the mean amount of firing induced by the protocol are known to be blocked by enhancing inhibition with application of benzodiazepine agonists (del Cerro et al., 1992; Trepel and Racine, 2000). However, the effects of reduction of inhibition on LTP induction can vary. For example, picrotoxin administration slowed down the induction of LTP in the neocortex of a freely moving
rat (Trepel & Racine, 2000). Disinhibition together with global increase of the strength of excitatory synapses, leads to increased spontaneous firing inside the LPZ, which may trigger a homeostatic regulation of LTP induction, analogous to the mechanism of homeostatic shutdown of LTP in hippocampus after widespread synaptic strengthening induced by chemical means (Roth-Alpermann et al., 2006).

Another possibility is that this form of LTP depends on molecular machinery that is also used during scaling of excitatory synapses, which we observed in our study (Fig. 4.2.8) and the induction of LTP is occluded by prior scaling. It was demonstrated in several studies that both Hebbian and homeostatic forms of plasticity can simultaneously exist in neocortical excitatory and inhibitory synapses. For example, feed-forward connections onto layer 2/3 pyramidal cells express a form of LTD in visual and barrel cortex, that can be partially occluded by previous sensory deprivation (Kirkwood et al., 1996; Allen et al., 2003; Crozier et al., 2007). Also, in synapses formed by excitatory recurrent connections in layer 5 of rodent visual cortex postsynaptic and presynaptic forms of LTP exist, together with a presynaptic form of LTD. An interplay between those forms of plasticity can be manipulated by different combinations of correlated presynaptic and postsynaptic firing, as well as availability of biochemical machinery, like, for example, endocannabinoids (Markram et al., 1997; Sjostrom et al., 2001, 2003, 2007). Synaptic scaling has been induced previously in layer 4 excitatory synapses early in development and in layer 2/3 after first 3 weeks of mouse development and into the adulthood (Goel & Lee, 2007). Both intraocular TTX injection and dark rearing managed to drive synaptic scaling (Desai et al., 2002; Goel & Lee, 2007), albeit in different layers. Our data show that scaling of both excitatory and inhibitory synapses occur in layer 2/3 pyramidal cells in the LPZ and LTP of horizontal inputs to the intra-LPZ neurons could be induced in intact animals (Fig. 4.2.15.A & Fig. 4.2.16). It is not known to which extent molecular machinery responsible for scaling can interact with molecular machinery that mediates LTP. Both scaling and LTP depend on insertion of new AMPA-receptors into postsynaptic density (Malinow & Malenka, 2002). However, scaling is a slow process, depending on regulation of immediate early gene expression and engagement of transcription machinery (Ibata et al., 2008; Turrigiano, 2008; Turrigiano, 1998), while initial stages of LTP in some cases depend on fast insertion of premade AMPA receptors into the postsynapse, happening on the scale of minutes (Malinow & Malenka, 2002). Apart from glutamate receptors, chronic long-term changes in activity levels have a strong impact on a large array of synaptic
proteins comprising the postsynaptic density (Ehlers et al., 2003). One group of molecules, suggested to have an important role in both LTP and scaling are calcium/calmodulin-dependent kinases (Thiagarajan et al., 2002; Turrigiano, 2008; Malenka & Bear, 2004). While CaMKII was found to have a critical role in many forms of LTP, scaling was shown to rely more on CaMKIV (Ibata et al., 2008), although a role for CaMKII also was implied (Thiagarajan et al., 2002). Interestingly, inside of the LPZ of visual cortex of cats with retinal lesions, an increase in CaMKII autophosphorylation was observed, reaching the peak values only 14 days after the lesion, while shortly after the lesion (3 days) the increase was insignificant (van den Bergh et al., 2003). This CaMKII autophosphorylation increase indicative of increased protein kinase activity and related LTP-like changes in synaptic strength depended on competition of deprived and non-deprived inputs, since it did not occur in animals whose optic tract and cortex callosum were surgically cut (van den Bergh et al., 2003). These data also indicate, that persistent activation of signaling cascades engaged in LTP also did not occur until two weeks post lesion. Therefore, it may be that LTP enhancement may be still observed in our model, although not in the chosen time window. In vivo we observed a specific reorganization, accompanied by reinstallment of receptive fields and enhancement of the amplitude and latency of stimulus-driven lateral activation 1 – 1.5 months after the lesion, but not 4 – 7 days after the lesion, although lateral propagation of excitation wave was evident already 4 days after the lesion (Fig. 3.2.5 – Fig. 3.2.7). Intrinsic optical imaging study in mice also showed significant reorganization and recovery of optical signals inside of the LPZ only after 2 weeks post lesion (Keck et al., 2008). Authors of the this study also performed chronic two-photon imaging of dendritic spine dynamics. A dramatic increase in spine turnover rate immediately after the lesion and several weeks after the lesion was observed compared to unaffected areas of visual cortex, with nearly 90% of spines being replaced over the first week post lesion. Despite such a remarkable increase in spine dynamics, the newly formed spines were also highly unstable, indicating that they could not serve as anatomical basis for formation of new synapses and their strengthening. Persistent stable spines that can serve as anatomical basis of changes in synaptic strength were formed in the LPZ only after 2 weeks post lesion, and the number of newly formed spines after 2 months post lesion was twofold greater than in the visual cortex of control animals and animals with complete ablation of the retina in both eyes. Such instability of newly formed spines may point to impossibility of consolidating the short-term changes in synaptic strengths.
during the first two weeks after the lesion, thus preventing consolidation of LTP-like changes. On the other hand, a recent study in motor cortex showed, that motor learning, leading to transient increase in the width of dendritic spines also transiently occluded induction of LTP in slices of trained primary motor cortex (Harms et al., 2008).

Third, a metaplasticity mechanism may come into play, triggered by removal of subcortical input (Abraham & Bear, 1996; Rioult-Pedotti et al., 2007). Metaplasticity was documented in layer 3 neurons: after average level of evoked neuronal cortical activity was reduced by a period of binocular deprivation LTP could be induced in layer 4 to layer 2/3 synapses by lower stimulation intensities than under control conditions (Kirkwood et al., 1996). This study established that the magnitude of synaptic strength change and its polarity depends on the level of postsynaptic response relative to a modification threshold, the value of which varies with the history of cortical activity. In our study initial loss of visually-driven firing inside the LPZ caused an eventual increase of spontaneous firing rates resulting from bidirectional synaptic scaling of excitatory and inhibitory synapses. This increase may have a profound effect on intracellular calcium concentrations and associated activation levels of different forms of CaMKII, involved in LTP (Turrigiano, 2008; Thiagarajan et al., 2002, 2007). For instance, it was shown, that levels of alpha- and beta-CaMKII and associated sensitivity of holoenzyme to calcium influx induced by electrical stimulation are inversely regulated by changes in neuronal activity (Thiagarajan et al., 2002, 2007). Thus, this ratio may determine the threshold for LTP induction and depends on the global level of activity prior to LTP induction. Prior inactivity or, alternatively, increased activity, also were shown to exert a strong influence on other cellular events and molecular machinery, that underlie LTP, and efficiency of LTP-inducing protocols as was demonstrated in several studies (Thiagarajan et al., 2007; Slutsky et al., 2004; Ehlers, 2003). Hence, it is possible, that persistent increase in spontaneous firing inside the LPZ may lead to shift in modification threshold for LTP via change in expression and activity of LTP-related molecular substrates, thus preventing LTP induction with our protocol.
4.4. CONCLUSIONS – 2.

A number of changes in the visual cortical synaptic transmission resulting from retinal lesions were registered in this study. During second week post lesion the spontaneous firing rates of pyramidal cells inside the lesion projection zone were increased as a result of synaptic scaling of excitatory synapses combined with major decrease in inhibitory drive inside of the LPZ. Those bidirectional changes in inhibitory and excitatory transmission led to pronounced shift of excitatory-inhibitory balance in favor of excitation for horizontal inputs in the intra-LPZ pyramidal neurons. Reduction of inhibitory drive may lead to enhanced and more far-reaching horizontal spread of activity inside the LPZ; and homeostatic scaling of excitatory synapses, increasing the gain of intra-LPZ neurons, may facilitate propagation of activity in the cortex and lead to shortening of latencies of horizontal spread, observed in our VSDI data after prolonged recovery. However, the sharpening of receptive fields and appearance of orientation selectivity of neurons inside the LPZ is more likely to depend on local, directional mechanisms, like spike-timing dependent plasticity (Young et al., 2007; Yao & Dan, 2001), rather than global homeostatic mechanisms. In this work we could not induce LTP in the intra-LPZ neurons with a spike-timing dependent protocol, otherwise effective in normal animals. The reasons for this result may be ambiguous: occlusion by a prior LTP-like change, scaling-related or firing rate-related metaplasticity mechanism or even homeostatic shutdown, resulting from increased circuit activity. Thus, the role of STDP in retinal lesion-induced cortical reorganization remains unclear, and more studies at different time points during reorganization timecourse are needed to elucidate it.
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6. Supplementary materials:

Structure of primary visual cortex

Primary sensory cortices share common anatomical architectures and local circuit wiring. Sensory cortex is generally divided into six layers numbered from 1 to 6. Functionally, primary visual cortex is found in the occipital part of the hemispheres of rat brain extending from bregma position – 9 mm to about – 4.5 mm and laterally from 1.8 mm to 5.5 mm (Paxinos et al., 2004; Paxinos & Watson, 2007). Sensory information reaches the primary visual cortex from the thalamus via the thalamocortical axons arising from the dLGN. The thalamocortical axons terminate primarily in layer 4, innervating the spiny stellate neurons and pyramidal cells; to a lesser extent the thalamocortical afferents end up in layer 6 and lower layer 3 of cat visual cortex (LeVay & Gilbert, 1976; Buonomano & Merzenich, 1998). Based on anatomical studies, latency, and pharmacological data, the principal vertical flow of information through the cortical layers in rodents and cats is layer 4 > layer 2/3 > layer 5 > layer 6 or layer 4 > layer 2/3/5 > layer 6 (Bolz et al., 1989; Armstrong-James et al., 1992; Buonomano & Merzenich, 1998). Besides this dominant pathway, numerous intralaminar horizontal (Burkhalter & Charles, 1990; McDonald & Burkhalter, 1993) and interlaminar (layer 6 > layer 4) projections exist (Buonomano & Merzenich, 1998). Output to other cortical areas and subcortical top-down projections is carried out via pyramidal cells in layers 5 and 6.

Correspondingly to the described information flow pathway, the response properties of neurons in different cortical layers change, as neurons of each layer contribute to extracting visual information from the stimuli in layer-specific manner. Receptive fields become larger when going from layer 4 to extragranular layers. Experiments in rat and monkey somatosensory cortex indicate that the smallest receptive fields are found in layer 4, while supragranular layers exhibit larger receptive fields than those observed in layer 4, and infragranular layers exhibit the largest receptive fields or sizes equivalent to those in the supragranular layers (Sur et al 1985; Buonomano & Merzenich, 1998). In cat visual cortex complex cells are concentrated mostly in extragranular layers and are quite scarce in layer 4; layer 4 contains the largest proportion of simple cells, which are also found in deep layer 3 and layer 6. Layer 4-located cells have the smallest receptive fields; and layer 6 – located cells have the
largest receptive fields, while cells in layers 2, 3 and 5 have receptive fields of intermediate sizes (Gilbert, 1977). Similarly cells in extragranular layers tend to have sharper orientation tuning, than those of layer 4 (Chapman & Stryker, 1993). In addition to the vertical flow of information, profound horizontal connectivity exists integrating inputs from neighboring regions and from distant cortical parts.

Figure SM1. Inputs to pyramidal cells in the supragranular layers of rodent primary visual cortex. Excitatory pyramidal cells (Pyr) provide long-range connectivity between distantly located neurons in V1. The targets of long-range connections (red) are dendritic trees of layer 2/3 and 5 pyramidal cells. Inhibitory neuron subtypes are represented with parvalbumin (PV+) and cholecystokinin-expressing (CCK+) large-basket cells whose axons can span the width of several columns (about 500 µm) and provide perisomatic and proximal dendritic inhibition, thus playing a role in regulation of spike-timing. Other interneurons are chandelier cells, forming axoaxonic contacts (Ch) and double-bouquet cells, forming synapses on distal dendritic sites (DB) (modified from Hensch, 2005).
Horizontal connectivity plays an important role in modulating responses of neurons to dominant vertical inputs and also in communication of distant groups of neurons (Malach et al., 1993; Lamme et al., 1998; Gilbert, 1998; Buzas et al., 2001). Excitatory horizontal connections arise mostly from pyramidal cells and target preferentially cells in supra- and infragranular layers of the cortex (Tanifuji et al., 1994). Inhibitory lateral connectivity is provided mainly by large basket cells, expressing parvalbumin. In cat and monkey horizontal connections can extend as much as 8 mm in the cortex, and more than 1 mm in rat (Tanifuji et al., 1994; McDonald & Burkhalter, 1993). The number of synapses formed by extracolumnar inputs in extragranular layers of V1 is variable and not known precisely. In layer 4 only a minor portion of the synapses are formed by thalamocortical afferents, less than 15 % (Peters & Payne, 1993; Ahmed et al., 1997). Therefore, most synaptic input seems to originate from intra- and interlaminar neurons even in the entry layer of V1. Horizontal connections were implied as a possible substrate of cortical reorganization, based on anatomical and functional properties of newly established receptive fields of deprived neurons and topographic maps (Gilbert, 1998; Calford et al., 2003).
7. APPENDIX

7.1 CURRICULUM VITAE

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7.2. PUBLICATIONS


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