FUNCTIONAL ALTERATIONS OF GLUTAMATERGIC AND GABAERGIC NEUROTRANSMISSION IN THE CORTICAL NETWORKS SURROUNDING A FOCAL LASER LESION IN RAT VISUAL CORTEX

by

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This research was conducted at the Department of Neurophysiology, within the Faculty of Medicine the Ruhr University under the supervision of Prof. Dr. Thomas Mittmann

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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 a similar form at this or any other domestic or foreign institution of higher learning as a dissertation.

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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin biotin complex</td>
</tr>
<tr>
<td>Abs</td>
<td>antibodies</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate</td>
</tr>
<tr>
<td>AMPARs</td>
<td>AMPA receptors</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>CB</td>
<td>calbindin</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
</tr>
<tr>
<td>CHR</td>
<td>chromogen</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSPGs</td>
<td>chondroitin sulphate proteoglycans</td>
</tr>
<tr>
<td>CR</td>
<td>calretinin</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>eEPSCs</td>
<td>evoked excitatory postsynaptic currents</td>
</tr>
<tr>
<td>EPSCs</td>
<td>excitatory postsynaptic currents</td>
</tr>
<tr>
<td>EPSPs</td>
<td>excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>eIPSCs</td>
<td>evoked inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>fEPSPs</td>
<td>field excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GABA(_A)Rs</td>
<td>GABA(_A) receptors</td>
</tr>
<tr>
<td>GABA(_B)Rs</td>
<td>GABA(_B) receptors</td>
</tr>
<tr>
<td>GAD 67</td>
<td>glutamic acid decarboxylase 67</td>
</tr>
<tr>
<td>GAT</td>
<td>GABA transporter</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IPSCs</td>
<td>inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>IPSPs</td>
<td>inhibitory postsynaptic potentials</td>
</tr>
<tr>
<td>ir</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>ISI</td>
<td>inter-stimulus interval</td>
</tr>
<tr>
<td>L2/3-L2/3</td>
<td>layer 2/3-layer 2/3</td>
</tr>
<tr>
<td>LFS</td>
<td>low frequency stimulation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>LGN</td>
<td>lateral geniculate nucleus</td>
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<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NMDARs</td>
<td>NMDA receptors</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PNs</td>
<td>perineuronal nets</td>
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<tr>
<td>POD</td>
<td>peroxidases</td>
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<tr>
<td>PPD</td>
<td>paired-pulse depression</td>
</tr>
<tr>
<td>PPF</td>
<td>paired-pulse facilitation</td>
</tr>
<tr>
<td>PPR</td>
<td>paired-pulse ratio</td>
</tr>
<tr>
<td>Pr</td>
<td>initial probability of release</td>
</tr>
<tr>
<td>PrRRP</td>
<td>initial probability of release of the RRP (estimation of Pr)</td>
</tr>
<tr>
<td>PV</td>
<td>parvalbumin</td>
</tr>
<tr>
<td>Q</td>
<td>charge transfer</td>
</tr>
<tr>
<td>Ra</td>
<td>access resistance</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RRP</td>
<td>readily releasable pool</td>
</tr>
<tr>
<td>SD</td>
<td>spreading depression</td>
</tr>
<tr>
<td>sEPSCs</td>
<td>spontaneous excitatory postsynaptic currents</td>
</tr>
<tr>
<td>sIPSCs</td>
<td>spontaneous inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TBS</td>
<td>theta burst stimulation</td>
</tr>
<tr>
<td>VGAT</td>
<td>vesicular GABA transporter</td>
</tr>
<tr>
<td>WFA</td>
<td>wisteria floribunda Agglutinin</td>
</tr>
</tbody>
</table>
### List of Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula / Description</th>
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<tbody>
<tr>
<td>CGP 55845</td>
<td>(2S)-3-[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid hydrochloride</td>
</tr>
<tr>
<td>D-AP5</td>
<td>D-(-)-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>MK-801</td>
<td>5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate</td>
</tr>
<tr>
<td>PTX</td>
<td>picrotoxin</td>
</tr>
<tr>
<td>QX-314</td>
<td>N-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium bromide</td>
</tr>
</tbody>
</table>
Abstract

Focal brain injuries can lead to severe neurological deficits. Nonetheless, a gradual functional recovery is often observed with the course of time. This restoration of function has been primarily attributed to a functional reorganization of the neuronal networks in the surround of the injured tissue.

The present study focused on the changes in synaptic plasticity induction and in the GABAergic transmission taking place in the early phase of this functional reorganization by using a well established ex vivo- in vitro model of focal laser lesion in rat visual cortex.

The physiological changes occurring in the cortical tissue surrounding the lesion were evaluated by combing immunohistochemical analyses and in vitro whole cell patch clamp recordings after a survival time of three to six days post-lesion.

An enhanced long-term potentiation (LTP) and a reduced long-term depression (LTD) were observed at synapses formed by horizontal fibers in the cortical layers 2/3, at 2–4 mm lateral from the border of the lesion. This metaplastic shift toward the strengthening of these synapses could be essential to guarantee a proper cortical rewiring post-lesion throughout a widespread area.

Recordings of spontaneous and evoked GABAergic synaptic currents disclosed an impaired GABAergic transmission in a large part of the visual cortex ipsilateral to the lesion as well as in the contralateral untreated hemisphere. This impaired inhibitory transmission did not seem to be accompanied by a degeneration of vulnerable interneurons or by a reduced GABAergic innervation but was most likely due to a reduction in the probability of release for GABA. A lesion-induced tonic activation of presynaptic GABABRs seems to mediate this reduced GABA release.

The impaired GABAergic transmission was coupled with a moderate, spatially restricted, reduction in glutamatergic transmission. These circumstances may lead to a shift in the excitation/inhibition balance in favor of excitation in a large cortical tissue surrounding the lesion.

Surprisingly, recordings from the hemisphere contralateral to the lesion revealed a higher excitability, in term of neuronal firing rates, compared to the ipsilateral cortex. This might implicate an important participation of the cortex contralateral to the lesion during the processes of functional reorganization in the visual cortex following focal injuries.
1. Introduction

1.1 Focal brain injuries: from the clinic to the experimental models
A focal brain injury is a pathological condition resulting in the damage of a localized brain area with a consequent functional neurological deficit which normally reflexes the loss of function of the injured region.

The common causes of focal brain injuries are traumatic events and cerebrovascular diseases. Other causes of brain injuries are surgery, some neurological illnesses, tumoral mass which growing can compress and damage other brain areas and occasionally infections and metabolic factors.

A brain damage induces normally functional and structural alterations which evolve in time and space and are not limited to the lesion itself but can be observed in perilesional as well as in remote brain areas (Andrews 1991). These lesion-induced disturbances may also contribute to the clinical deficits altering neuronal network functions, causing an enlargement of the initial damage and eventually leading to the generation of epileptic events which occur in 5-15% of stroke patients (Kotila and Waltimo 1992) and even more frequently after a traumatic brain injury (Salazar et al. 1985; Dinner 1993).

However, these alterations spreading in the surrounding uninjured tissue and in remote areas are not always maladaptive processes but they seem also crucially important to guarantee the recovery of function (at least in part) in patients suffering from this pathological condition.

It should be emphasized that the pathophysiologic events triggered by the lesion can notably differ depending on many factors, such as the nature of the insult (cerebrovascular, traumatic, etc), the extent of the damaged area as well as the brain structures affected.

The nature of these processes will contribute to define the boundaries of the lesion core as well as the typical features of the functional and metabolic disturbances spreading into uninjured areas (Witte et al. 2000; Park et al. 2008).

This large variability led many researchers to develop several animal models of focal brain injury in the attempt to reproduce different aspects of this pathological condition. It is mostly thanks to these various experimental animal models that today...
the cellular mechanisms of the lesion-induced alterations and their functional consequences are becoming clear.

1.2 Spontaneous recovery of function post-lesion

Despite the wide range of variability in the etiology, severity and location of brain lesions, a gradual recovery from the functional disability has often been observed with the course of time in many patients suffering from stroke or severe traumatic brain injuries (Zihl and von Cramon 1985; Sbordone et al. 1995; Naeser et al. 1998) as well as in experimental animal models of focal brain lesion (Jenkins and Merzenich 1987; Eysel and Schweigart 1999).

Thus, although we can not compare under many aspects a stroke with a traumatic brain injury or with a particular experimental model of focal brain lesion, this partial restoration of function, with some degree of variability, seems to be a general phenomenon following a lesion in the central nervous system (CNS) and might be therefore based on a process commonly triggered by a brain damage.

1.3 Reorganization of neuronal networks surrounding the injured area

Since neuronal regeneration is absent in the most part of the CNS, the observed functional recovery is probably based on a functional and structural reorganization of the neuronal networks surrounding the injured area.

Different studies support this hypothesis. Jenkins and Merzenich could appreciate that, weeks to months after removing the somatosensory cortex representing some part of the hand in owl monkeys, neurons in the surround of the damaged area became responsive to sensory inputs previously represented by the injured tissue (Jenkins and Merzenich 1987). This finding implicated that a change in the cortical representation of sensory inputs occurred and was responsible for the partial functional recovery.

Subsequently, Eysel and Schweigart showed an increase in the receptive field size of neurons located at the border of a focal ibotenic acid lesion in adult cat visual cortex 2 months after the insult. These neurons with enlarged receptive fields began to represent previously unresponsive regions of the visual field suggesting that they can, at least partially, take over the function previously belonging to the lost brain area (Eysel and Schweigart 1999).
Multiple studies have shown that brain injuries often increase the neurogenesis in the subventricular zone (SVZ) and promote the emigration of neuroblasts from this region toward the injured area (and not only into the olfactory bulbs) (Goings et al. 2004; Romanko et al. 2004; Sundholm-Peters et al. 2005; Kernie and Parent 2010). However because of their limited number, the unclear differentiation fate – the morphology and the expression of glial markers of many cells suggested that they will differentiate into glial cells (Holmin et al. 1997; Blizzard et al. 2010) – and the difficult integration into the pre-existing neuronal networks, it is very unlikely that they could play a role in the functional recovery after a brain lesion by functionally substituting dead neurons.

Finally, the reactivation of reversibly damaged, temporary functionally suppressed neurons and the resolution of edema normally occurring in the surround of the lesion, could also contribute to the restoration of function but mostly in the first few days post-lesion.

The functional reorganization of the surrounding neuronal circuits seems therefore the most relevant process mediating the restitution of function post-lesion.

Several neurobiological mechanisms have been proposed to mediate this remodeling of neuronal connections and most probably many of these mechanisms work together, with some being more or less relevant depending on the characteristics of the lesion and the considered time window post-lesion.

1.4 Neurobiological mechanisms proposed as responsible for the reorganization of the neuronal networks post-lesion

The modulation in the efficacy of pre-existing connections has been attributed as one mechanism which can account, at least in part, for the functional modification of neuronal circuits post-lesion.

1.4.1 Synaptic plasticity: an overview

Synaptic plasticity refers to the capability of a synapse to modify its efficacy in response to neuronal activity and it is considered the cellular substrate for processes of learning and memory.

Donald Hebb postulated, already in the 1949, a theory to explain the neuronal basis of learning and memory. The Hebbian Theory states that when two neurons are
repetitively active at the same time they will tend to become “associated” or their connections will be more efficient. The paradigm is better summarized with the statement “cells that fire together, wire together” (Hebb 1949).

The first experimental data validating the Hebbian mechanisms of learning was provided, in the early '60, by Eric Kandel, who performed recordings from a single cell in a ganglion of the marine gastropod *Aplysia californica*. With this very simple animal model he demonstrated for the first time a stimulus dependent modification of synaptic efficacy. He therefore characterized the cellular structures involved in the reinforcement or weakening of the functional connections between neurons.

In the following years, many others follow-up studies confirmed and extended the knowledge on synaptic plasticity. The induction of synaptic plasticity has been described in a large number of brain structures and in different types of synapses, in innumerable *in vitro* studies. Generally long-term potentiation (LTP) refers to the potentiation of the synaptic strength and long-term depression (LTD) to the depression of synaptic transmission, which can be recorded for a relative long period of time (hours to days).

Many forms of synaptic plasticity have been found to be associative, indicating that the LTP induction of a “weak” input could be achieved if this input was simultaneously active with a strong stimulus onto a convergent input (for review see Bi and Poo 2001).

NMDA receptors are considered to play a crucial role in this form of synaptic plasticity.

The peculiarity conferring this important role is a binding site for Mg$^{2+}$ within the receptor channel. Mg$^{2+}$ normally blocks the conductance of the channel and can be removed only with a prolonged membrane depolarization. A presynaptic glutamate release will therefore activate the receptors only when a timely simultaneous postsynaptic membrane depolarization takes place. In this way NMDARs can work as a coincidence detector coupling separated neuronal events which occur simultaneously (for review see Bourne and Nicoll 1993). Because NMDARs are permeable to Ca$^{2+}$, the activation of these receptors will promote Ca$^{2+}$ influx into the postsynaptic neuron. The Ca$^{2+}$ influx activates different intracellular signal transductions, which eventually can lead to a modulation of the synaptic strength. The Ca$^{2+}$ influx into the postsynaptic neuron is believed to be important both for LTP and LTD induction. A transient and robust increase of Ca$^{2+}$ does normally lead to LTP,
while a sustained but low increase of intracellular of Ca$^{2+}$ to LTD. The different kinetics of Ca$^{2+}$ transient can activate either protein kinases (inducing LTP) or phosphatases (inducing LTD) (Lisman 1989; Malenka et al. 1989). The activity of protein kinases and phosphatases can dynamically regulate the insertion or removal of AMPARs from the postsynaptic membrane (Shi et al. 1999) and the phosphorylation state of these receptors (Lee et al. 2000) thereby modulating the strength of the postsynaptic response.

Different forms of synaptic plasticity independent of NMDARs activation have been also described. Some forms of LTP/LTD require the activation of metabotropic glutamate receptors (for review see Bortolotto et al. 1999). Other forms of NMDARs-independent synaptic plasticity have been reported to be induced at the presynaptic site through a modulation of transmitter release (Castro-Alamancos and Calcagnotto 1999). This presynaptic form of plasticity is believed to be predominately based on retrograde signaling from the post- to the presynaptic cell (i.e. molecules produced at the postsynaptic site are secreted and act on the presynaptic site activating specific receptors and modulating transmitter release). Arachidonic acid, platelet-activating factor, nitric monoxide (NO), carbon monoxide (CO) and endocannabinoids are considered the most important factors mediating retrograde signaling (Tao and Poo 2001).

In many laboratories the protocols used to induce synaptic plasticity consist of different patterns of presynaptic stimulations paired with the induction of spikes in the postsynaptic cell. In this regard, an interesting finding was the importance of the temporal order between the pre- and postsynaptic stimulation for the potentiation versus depression of the synaptic response. In particular, it was observed that when the postsynaptic spike was triggered after the onset of an excitatory postsynaptic potential (EPSP) (within a narrow temporal windows of few tens of ms) LTP followed, while when the temporal order was reversed (postsynaptic spike first, followed by EPSP) LTD was induced (Markram et al. 1997). This phenomenon is known as “spike-timing-dependent plasticity”. The kinetic and the amount of Ca$^{2+}$ influx seem to play a role in this process. Action potentials (APs) following an EPSP within few tens of milliseconds were accompanied by a supralinear summation of the postsynaptic Ca$^{2+}$ signal which was not observed when the order of APs and EPSP was reversed (Nevian and Sakmann 2004).
1.4.2 Increased synaptic plasticity

Some *ex vivo- in vitro* studies reported an increased synaptic plasticity post-lesion. In particular, an increased long-term potentiation (LTP) induction was found in the surround of an experimentally induced focal cortical infarction in the somatosensory cortex of rats (Hagemann et al. 1998) as well as in a focal laser lesion model in the rat visual cortex (Mittmann and Eysel 2001).

This enhanced synaptic plasticity, was observed in layers 2/3 pyramidal cells after tetanisation of the ascending inputs. The strengthening of these fibers could promote the emergence of previously silent, functional connections and facilitate the reinforcement of others. However, because this phenomenon was found to be more strongly expressed within a defined temporal window after the lesion induction (1 week survival time) (Mittmann and Eysel 2001) it could contribute in the early phase of a functional reorganization post-lesion, but subsequently structural changes might be necessary to guarantee a stable and long-term rewiring of cortical networks.

1.4.3 Structural changes

Beyond the modulation of pre-existing connections, structural changes have been also imputed as important processes implicated in the neuronal circuits reorganization post-lesion.

The formation of new and the removal of old synaptic contacts can mainly take place due to the dynamic nature of the dendritic spines. Dendritic spines stick out from the dendrites of most principal neurons in the brain and constitute the site where axonal terminals (varicosities) end forming usually excitatory synapses (Harris 1999). Although they are described as relatively stable structures in the adult brain (Grutzendler et al. 2002) they also manifest plastic properties, particularly in response to a modified level of synaptic activity or alteration of sensory experience, (Trachtenberg et al. 2002; Zuo et al. 2005; Holtmaat et al. 2006; Keck et al. 2008) and could therefore constitute an optimal anatomical substrate for a structural plasticity after a brain injury. In support to this hypothesis a recent in-vivo two-photon imaging study, conducted in a phototrombotic model of injury in the sensorimotor cortex of mice, reported an extensive turnover of dendritic spines in the peri-infarcted cortex 1, 2 or 6 weeks after the lesion induction (Brown et al. 2007). Similarly, an enhanced turnover of mushroom spines was observed in the contralateral (left) non-injured
hemisphere after a cerebral infarction in the right somatosensory cortex of mice (Takatsuru et al. 2009).

Finally, a more stable rewiring of neuronal networks could be achieved through robust structural changes like axonal sprouting and growing of new dendritic arborizations.

Evidence for such a structural plasticity has been reported following different models of focal brain injuries.

In a model of partially isolated somatosensory cortex layer 5 pyramidal cells, located in the surrounding cortical areas, presented an increase in total axonal length, in number of collateral axonal branches and in number of axonal boutons (Salin et al. 1995). In addition, in an electrolytic model of lesion, in the sensorimotor cortex of rats, an extensive increase in dendritic arborization of layer 5 pyramidal neurons was observed in the contralateral homotopic cortical area with a peak between 14 to 18 days post-lesion.

Lesion induced, probably activity dependent, upregulation of trophic growth factors (Kawamata et al. 1997; Sulejczak et al. 2007) are most likely responsible for these structural rearrangements.

Although these anatomical changes, with their specific spatio-temporal profiles, can vary depending on the lesion etiology and the affected brain structures, an enhanced structural plasticity of the tissue seems to be a general phenomenon following a focal brain lesion (Macias 2008).

This increased structural plasticity could facilitate the reorganization of the neuronal networks post-lesion thereby allowing a functional recovery to take place. Nevertheless a correlation between these structural modifications and the restitution of function post-lesion is still controversial. While some groups were able to show a relation between reorganization in dendrite morphology, axonal sprouting and behavioral improvement (Jones and Schallert 1992; Stroemer et al. 1995), other failed to find this link (Gonzales and Kolb 2003). Furthermore these structural changes, increasing the number and complexity of synaptic connections among neurons, could generate excessive recurrent excitatory circuits, which might cause an abnormal neuronal activity and promote the generation of epileptiform events, not rarely observed after a brain injury (Salin et al. 1995; McKinney et al. 1997, Prince et al. 2009).
1.4.4 Functional suppression and neuronal hyperexcitability

Many experimental animal models of focal brain lesion reported functional alterations of neuronal activity in the tissue surrounding the injury. These functional alterations seem even to share, to some extent, a similar spatio-temporal distribution. The immediate surround of a cortical lesion is normally functionally suppressed, especially in the first days after the primary insult.

An area of reduced activity has been observed in models of focal ischemic brain injury which normally show an extended penumbra (a brain region that suffers from ischemia but in which the ischemic damage is potentially or at least partially reversible) (for review see Witte et al. 2000).

However a study of microcirculation in the surround of a focal laser-induced lesion also reported a reduced perfusion and edema at 1 mm from the primary lesion. These hemodynamic changes were probably accompanied by a reduced neuronal activity (Lindsberg et al. 1991).

Furthermore a functionally suppressed area, with a reduced spontaneous neuronal activity as well as decreased stimulus driven responses, has been also described in the immediate vicinity of a heat model of lesion in cat visual cortex (Eysel and Schmidt-Kastner 1991).

In parallel, an abnormal neuronal excitability has been also reported following different experimental models of focal brain injury. This hyperexcitable area seems to further surround the functionally depressed region.

Hyperexcitability has been described in terms of increased neuronal firing in different in vivo studies of brain injury. In a heat model of lesion in cat visual cortex, neurons located between 1 to 2.5 mm from the border of the injury were overresponsive to visual stimuli and exhibited an increased spontaneous activity. The effects were more prominent at the post-lesion days 1 and 2. However a trend remained up to 30 days post-lesion (Eysel and Schmidt-Kastner 1991).

An increased spontaneous activity of neurons was also observed in the vicinity of photothrombotic infarcts with a peak, this time, between 3 to 7 days after lesion induction (and it was visible up to 4 months) (Schiene et al. 1996). Furthermore several in vivo and in vitro studies conducted in lesion models of partially isolated cortex, “undercut cortex”, reported a progressive increased susceptibility to epileptiform discharges and the generation of paroxysmal activity over a period of
several weeks after the lesion induction (Prince and Tseng 1993; Topolnik et al. 2003; Nita et al. 2006). This lesion-induced epileptogenesis is a clear indication of an abnormal excitability of the brain area surrounding the damaged tissue. Finally acute seizures and epilepsy have been often reported in patients suffering from brain injury (Kotila and Waltimo 1992; Dinner 1993) attributing a clinical relevance to the experimental data. All together these studies indicate that focal cortical injury, independently of their etiology, can lead to similar functional changes in regard to the activity of the surrounding neuronal networks.

1.4.4.1 Modest alteration of neuronal intrinsic properties
The lesion-induced alterations in neuronal activity could arise from changes in the intrinsic excitability and/or in the synaptic properties of neurons. Only moderate changes in the intrinsic membrane properties of neurons were observed in a photochemically induced ischemic lesion model in the rat neocortex. In this study the resting membrane potential of neurons located in the vicinity of the injury was significantly shifted to more positive values, while the input resistance remained unaltered (Neumann-Haefelin et al. 1995). Other authors reported a positive shift in the resting membrane potential as well as an increased input resistance in response to a local thermo-lesion in the rat neocortex (Mittmann et al. 1994). Both changes increase the probability of neurons to generate APs in response to excitatory inputs. More profound lesion-induced alterations were however observed in the synaptic properties of neurons.

1.4.4.2 Functional impairment of intracortical GABAergic transmission
A decreased GABAergic intracortical inhibition was observed by numerous groups in many different cortical lesion models. In a photochemically induced cortical infarct (Domann et al. 1993, Buchkremer-Ratzmann et al. 1996) as well as in a model of middle cerebral artery occlusion in Wistar rats (Reinecke et al. 1999; Neumann-Haefelin and Witte 2000), field potential recordings performed from neocortical slices in lesion-treated animals revealed an impaired paired-pulse inhibition extending to relative distal areas from the lesion core in the ipsilateral as well as in the intact contralateral hemisphere. According to the
authors this paired-pulse inhibition is based on the activation of recurrent local interneurons with the first pulse. The activated GABAergic neurons projecting back to the principal cells will inhibit their responses to a second pulse if it arrives within short time interval (20-30 ms). Thus, the lack of paired-pulse inhibition post-lesion was interpreted as a reduced strength of GABAergic transmission. Intracellular recordings from layers 2/3 pyramidal cells in the vicinity of a local thermo-lesion (Mittmann et al. 1994) as well as a phototrombotic cortical lesion (Neumann-Haefelin et al. 1995) strongly supported these results. The conductances of both early and late inhibitory postsynaptic potentials (IPSPs), induced by activation of GABA\textsubscript{A} and GABA\textsubscript{B} receptors, respectively, were found to be reduced. In addition these intracellular recordings disclosed a shift in the reverse potential of the early IPSPs to more positive values conferring to GABA\textsubscript{A} receptors a potential depolarizing action. Finally, whole-cell patch clamp recordings from layer 5 pyramidal neurons, in slices from animals which underwent a partial cortical isolation, revealed a decreased frequency of spontaneous and miniature inhibitory postsynaptic currents (Li and Prince 2002). In addition to these electrophysiological data, quantitative receptor autoradiography studies reported a down-regulation of \textsuperscript{[3H]}muscimol binding to GABA\textsubscript{A} receptors in the surround of a cerebral phototrombosis (Schiene et al. 1996) as well as in the contralateral intact hemisphere (Que et al. 1998a). The same result was observed also after unilateral permanent focal cerebral ischemia in the rat brain (Que et al. 1998b). This decreased binding of radiolabeled muscimol was interpreted as a reduced density of GABA\textsubscript{A} receptors or a reduced affinity of the receptor for its agonist. A decreased axonal length of layer 5 fast-spiking (FS) interneurons was also detected in an “undercut” model of lesion (Prince et al. 2009). Finally an immunohistochemical study revealed a down-regulation of the \(\alpha_1\) subunit (the most prominent \(\alpha\) subunit in adult rat neocortex) of GABA\textsubscript{A} receptors and signs of degeneration of parvalbumin positive interneurons (which constitute the largest sub-population of interneurons) in the surround of a photochemically induced cortical lesion in rats (Neumann-Haefelin et al. 1998). In the visual cortex, a mature intracortical GABAergic inhibition has been reported to be a potential limiting factor in the induction of adult cortical plasticity (Kato et al.
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In accordance with this, an increased synaptic plasticity (Kirkwood and Bear 1994) and the reactivation of ocular dominance plasticity (Harauzov et al. 2010) have been observed by pharmacological reduction of GABAergic inhibition in the visual cortex of adult rats.

It is therefore probable that the impaired GABAergic inhibition observed after a cortical injury could have a dual role: on the one hand it can cause abnormal excitability, probably responsible for the development of seizures, but on the other hand it might favor the functional reorganization of the surrounding neuronal networks by conferring enhanced plastic properties to the tissue.

1.4.4.3 Enhanced efficacy of excitatory transmission

Excitatory synaptic transmission has also been found to be affected in some models of focal brain injury.

In an “undercut” lesion model, in vitro synaptic activity measurements of layer 5 pyramidal cells from chronically injured, epileptogenic sensory-motor neocortex of rats revealed a higher frequency of both spontaneous and miniature excitatory postsynaptic currents (EPSCs) and an increase of evoked, non-NMDARs mediated EPSCs (Li and Prince 2002), indicating a higher number of excitatory synaptic contacts and/or an enhanced probability of glutamate release at synaptic terminals post-lesion.

Further investigations in the same lesion model disclosed a decreased paired-pulse ratio (PPR) of EPSCs elicited by repetitive presynaptic stimulations at high frequencies (40-66.7 Hz), pointing towards an enhanced initial probability of glutamate release from the presynaptic terminals (Li et al. 2005).

However, an increased complexity of synaptic connections post-lesion is also likely contributing to this higher excitatory transmission especially when longer time windows (weeks post-lesion) are considered.

This was firstly supported by different histological studies (Salin et al. 1995; Stroemer et al. 1995). Furthermore in vivo recordings, conducted in the cat’s supra-sylvian gyrus which underwent a partial deafferentation, gave a physiological relevance to these anatomical evidence. Here an enhanced excitatory coupling was revealed by an increased probability of finding synaptically connected pairs of cortical pyramidal neurons 2, 4 and 6 weeks after the lesion induction (Avramescu and Timofeev 2008).
1. INTRODUCTION

The controversial role of the NMDARs

Some of the functional alterations observed in many focal cortical lesion models seem to be mediated by changes in NMDARs expression and/or functionality.

The brain tissue which surrounds ischemic and traumatic brain lesions is often the site of origin of massive neuronal depolarization (accompanied by a transient suppression of neuronal activity) which propagates as a wave across the gray matter (Hossmann 1996; Mayevsky et al. 1996; Strong et al. 2002). This phenomenon, known as spreading depression (SD) (for reviews see Martins-Ferreira et al. 2000; Somjen 2001), causes a large metabolic stress on the tissue and progressively recruits part of the partially damaged tissue into the irreversible lesion (Church and Andrew 2005).

MK-801, a competitive antagonist of NMDARs, was found to prevent SD (Lauritzen and Hansen 1992; Dietrich et al. 1994; Church and Andrew 2005).

NMDARs were also found to mediate epileptiform neuronal activity in different experimental lesion models. Long-lasting multiphasic NMDARs-mediated postsynaptic responses were recorded in vitro from pyramidal neurons in the vicinity of thermo-lesions (Mittmann et al. 1994) as well as ischemic lesions (Neumann-Haefelin et al. 1995) in the somatosensory cortex of rats.

On top of these functional alterations an increased binding density of NMDARs was found bilaterally in a quantitative autoradiography study following a photothrombotic lesion in rat brain (Que et al. 1999b).

The hyperactivity of NMDARs probably due to a pathological increase in glutamate release and/or to a partial postsynaptic membrane depolarization can cause irreversible cellular damage allowing a massive Ca$^{2+}$ influx into the postsynaptic neurons.

This evidence implicates a relevant participation of NMDARs in the pathophysiological processes responsible for the enlargement of the primary insult.

The blockage of these receptors was therefore considered a potential pharmacological approach to rescue the reversible damaged tissue. However, administration of NMDARs antagonists in rodent models of stroke and traumatic brain injury produced only a temporally restricted neuroprotective effect (Simon et al. 1984; Meldrum 1990; Lee et al. 1999) and several clinical trials failed to show any efficacy (Morris et al. 1999; Davis et al. 2000).

This negative result has been partially attributed to the fact that the Ca$^{2+}$ influx through NMDARs, within certain physiological ranges, can also promote neuronal
survival by activating Ca\(^{2+}\) dependent signal transductions which culminate in the expression of different pro-survival genes (Ikonomidou and Turski 2002). The blockade of the receptors could therefore prevent this beneficial effect.

In addition, NMDARs represent an essential molecule for the induction of many forms of synaptic plasticity and they could therefore play an important role in mediating the functional reorganization post-lesion.

In this regard a study from our laboratory showed that the enhanced LTP, observed in the surround of a focal lesion in the visual cortex of rats, was mediated by NMDARs containing the NR2B subunit. The application of ifenprodil, a specific blocker of this subunit, reduced the LTP in lesion-treated animals to the level of the control ones (Huemmke et al. 2004).

1.5 The role of glial cells in focal brain injury

Although much attention has been devoted to the neuronal alterations induced by focal brain lesions, one should not forget that glial cells undergo a series of modifications as a consequence of an injury in the central nervous system as well.

The first few hours after a lesion are normally characterized by a reactive astrogliosis, in which astrocytes increase in cell volume, in the number and extension of their cell processes, and upregulate dramatically the expression of the glial fibrillary acidic protein (GFAP). These reactive glia divide, migrate, surround the lesion, and contribute to the formation of a glial scar (Fawcett and Asher 1999).

However astrocytes perform numerous functions which go beyond the formation of a scar tissue. In particular by guaranteeing glutamate uptake, buffering the extracellular K\(^+\) and H\(^+\) and releasing neurotrophic factors they can critically influence the neuronal survival during a brain injury. Furthermore, controlling neuronal activity, neurite outgrowth and synaptic plasticity they could play a role in the long-term recovery post-lesion (Chen and Swanson 2003).
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1.6 The visual system in rats

This study has been entirely carried out in a well-established model of focal laser-lesion induced in vivo in rat visual cortex.

Rats have a relatively low visual acuity and rely mostly on other sensory modalities like olfaction and somatosensory system. However, the visual system of rats is highly developed and contains most of the elements seen in primates.

Similarly, in rats and primates, photons can activate particular molecules (opsins) on the photoreceptor cells in the retina. The activation of these opsin results in the transduction of light into an electrical signal, which contains the visual information. The visual information from ganglion cells in the retina is sent to the lateral geniculate nucleus (LGN) in the thalamus and finally from the LGN to the visual cortex. However, some ganglion cells in the retina do not send axons to the LGN but to the superior colliculus, a structure in the midbrain primarily involved in the control of eye movements, to the pretectum, for the pupillary reflex and to other brain structures implicated in the control of circadian rhythms and sleep.

The rat visual cortex is located in the occipital lobe of the cerebral cortex and it is specialized in processing visual stimuli. It can be subdivided in primary visual cortex (or V1), which receives the main thalamic input from the LGN, and in higher order cortical areas which further integrate the information coming from V1 and contribute to the final perception of the visual scene. Similar to the organization of the visual cortex in cat and monkeys, multiple distinct higher order visual areas, organized in a multilevel hierarchy, have been identified in the surround of the rat primary visual cortex (Coogan and Burkhalter 1993).

The rat visual cortex is divided into 6 cortical layers functionally and structurally similarly organized as in higher species. Layer 4 receives the main projections from the LGN. From layer 4 topographically precise afferents project to layers 2/3. The main output of layers 2/3 is layer 5. Layer 5, in turn, projects to layer 6 and also back to layers 2/3 and 1. Visual inputs undergo different levels of processing through these interlaminar vertical connections (Burkhalter 1989). Beyond forward projections intracortical fibers in rat visual cortex share also many features with those found in primates (Coogan and Burkhalter 1993).

The receptive field properties of rat V1 neurons are also, in many aspects, as specialized as those of cats and primates (Girman et al. 1999).
An important difference between rodents and primates is the field of vision. Rats have laterally-placed eyes (i.e. the eyes are located at either side of the head). This implicates that rats have a larger field of vision but a reduced binocular vision compared to animals having the eyes placed on the front of the head. Nevertheless the binocular part of the rat visual cortex is relatively large (Zilles et al. 1984) and contains a relative high percentage of binocular cells compared to other mammals (Fagiolini et al. 1994). Furthermore the expression of ocular dominance plasticity in the rat visual cortex has been reported by many authors when the visual inputs from one eye are manipulated during development (and in particular circumstances even in adult animals) (Maffei et al. 1992; Fagiolini et al. 1994; Di Cristo et al. 2001; Pizzorusso et al. 2002; He et al. 2006). Because of this plastic property and the relative rapidity of collecting anatomical and electrophysiological data from these animals, the rat visual cortex has therefore emerged as one of the favorite models to study the cellular mechanisms responsible for the experience-dependent modification of cortical networks.
1.7 Goals of the study

An increased LTP induction at synapses formed by ascending fibers, projecting to the cortical layers 2/3, has been already reported following different experimental animal models of lesion (Hagemann et al. 1998; Mittmann and Eysel 2001).

The first goal of the present study was to investigate whether potential changes in the induction of synaptic plasticity could be also expressed at intracortical horizontal fibers in the vicinity of a focal laser lesion in rat visual cortex. Horizontal connections run tangentially to the cortical surface forming a dense network in the superficial cortical layers (Burkhalter and Charles 1990). On the basis of their anatomical structure they seem to constitute an optimal substrate to guarantee a proper cortical rewiring extending laterally across the cortical surface. The plasticity of these connections might be therefore essential to achieve a proper cortical reorganization, post-lesion. Modelling of neuronal networks also predicted a fundamental role of adapting lateral interactions for the cortical reorganization post-lesion (Sirosh and Miikkulainen 1994).

However, experimental data on animal models are still missing.

A severe impairment of the inhibitory transmission is one of the most prominent lesion-induced functional alterations as shown in several lesion models. However, the cellular mechanisms responsible for these changes remain to be elucidated.

Thus, the second aim of this study was the characterization of the GABAergic system after the lesion induction. Furthermore, to characterize the spreading of the effects of the lesion in the surrounding neuronal networks, cortical regions at different distances from the border of the lesion (in the ipsilateral hemisphere) as well as the intact contralateral hemisphere were investigated.

The goal of the last part of the study was to investigate whether the potentially altered balance between excitatory and inhibitory synaptic transmission, in the cortical areas surrounding the focal lesion could influence the spontaneous neuronal firing rate.
2. Materials and Methods

2.1 In-vivo lesion induction in the visual cortex

Infrared laser lesions were performed \textit{in vivo} in the right visual cortex of juvenile Long-Evans rats. Animals at the age of 20-21 days were anaesthetized by intraperitoneal injection of chloral hydrate (4%; 0.1ml/10g) and by a subcutaneous injection of lidocain (0.08%, 2mg/kg) into the area above the visual cortex. Subsequently the animals were fixated in a stereotaxic apparatus, the skull was exposed and cautiously drilled above the right visual cortex parallel to the midline in a rectangular area 1 mm width beginning right anterior at the lambda suture and extending 3 mm towards the bregma without penetrating the dura mater. Cortical lesions were made under visual control with an 810nm infrared diode laser (OcuLight SLx, Iris Medical, USA) attached to a binocular operating microscope. Multiple, partially overlapping round lesions were performed about 2 to 2.5 mm lateral from the midline in order to form an elongated lesion of 1 mm mediolateral width and 3 mm anteroposterior length starting anterior to the lambda suture in the visual cortex (areas V1M, V2ML V2MM). Sham-operated animals of the same age were treated similarly. However, after opening of the skull no laser lesions were induced.

2.2 Electrophysiology

2.2.1 Acute slices preparation

After a survival time of 3-6 days the animals were deeply anaesthetized with ether and decapitated. Coronal slices containing the visual cortex (thickness: 350 \( \mu \text{m} \)) were prepared from the lesioned and the contralateral untreated hemisphere by use of a vibratome (LEICA, VT-1000-S, Germany). The tissue was incubated for at least 1 hour at room temperature in a standard artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 25 NaHCO\(_3\), 2.5 KCl, 1.5 MgCl\(_2\), 2 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), and 25 D-glucose (pH 7.4) saturated with 95% O\(_2\) and 5% CO\(_2\).

For electrophysiological investigations single slices were transferred onto a nylon net, located in a submerged recording chamber, mounted on an upright microscope, and superfused with standard ACSF bubbled with 95% O\(_2\) and 5% CO\(_2\).
To measure spontaneous firing of neurons the standard ACSF was substituted with a modified ACSF containing (in mM) 126 NaCl, 25 NaHCO₃, 5 KCl, 0.75 MgCl₂, 1 CaCl₂, 1.25 NaH₂PO₄, and 25 D-glucose (pH 7.4).

During all the experiments the ACSF temperature was kept at 32±2°C with the help of a water bath and a constant perfusion of the slices, with fresh oxygenated ACSF, was provided by a tube pump system (Mini Pulse 3, Gilson, France).

The microscope and micromanipulators were placed on an isolation table electrically grounded to a faraday cage.

**2.2.2 Experimental patch clamp set up**

*Visualization of the tissue and neurons identification*

The microscope (Olympus-BX50WI, Olympus, Japan) was equipped with a combination of infrared illumination and differential interference contrast (Dodt and Ziegglansberger 1990) and with 2.5X dry- and 40X water immersion type objectives to permit the visualization of brain slices.

Images of samples were captured by an infrared-sensitive CCD camera (C2400, Hamamatsu) and visualized on a connected monitor (WV-BM1400, Panasonic).

The 40X water immersion objective allowed the identification of neurons in the tissue and the pyramidal shaped-soma, characteristic of pyramidal neurons, could be easily recognized.

*Patch clamp technique*

The patch clamp technique was developed as a refinement of the voltage clamp technique by Erwin Neher and Bert Sakmann who were honoured, in 1991, with a Nobel Prize in Physiology or Medicine for this invention. This technique allowed for the first time to record ion currents through single ion channels.

The experimental setup that distinguishes patch clamp recordings is the use of a glass micropipette, with a smooth surface tip of around 1 μm in diameter, as recording electrode. If the tip of the micropipette touches the surface of a cell, a gentle suction through the pipette will pull a small portion of the cell membrane (patch) into the pipette’s tip. This results in a high resistance seal in the order of gigaohm (so called gigaohm seal) which is formed between the surface of the cell membrane and the
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pipette. This configuration, known as cell attached configuration, allows the study of ion currents across one or few ion channels in the membrane area enclosed under the pipette tip (Fig. 2.1B).

Patch clamp recordings exist in different types of configurations; the most extensively used is the whole cell configuration, which can be obtained by application of an additional stronger suction through the pipette in the cell attached configuration. This negative pressure causes the rupture of the cell membrane under the pipette’s tip thereby allowing recordings of ion currents across the entire cell membrane (Fig. 2.1C).

All the patch clamp recordings in the present study were conducted in the whole cell configuration which allowed the isolated recordings of ion currents through ligand-gated and voltage-gated transmembrane ion channels indispensable for the investigation of synaptic and intrinsic neuronal properties.

Glass micropipettes production
Micropipettes were pulled from glass tubes (100 mm long borosilicate glass capillaries) (GB 150F-8P, Science Products, Hofheim, Germany) using a horizontal micropipette puller (DMZ-Universal puller, Zeitz-Instr., Germany). The tips of the pipettes were polished, with an additional step, to improve the seal formation. The resistance of the pipettes for patch clamp recordings was 4-6 MΩ when filled. The same procedure was used to produce glass electrodes for extracellular stimulations.

Intracellular solutions
Pipettes were filled with a chemical solution which resembles the intracellular composition. This is important because after the opening of the cell membrane the solution diffuses throughout the cytosol of the cell and dilutes all intracellular contents. However the choice of the intracellular solution can vary depending on the experimental purpose.

For the experiments performed in current clamp mode the intracellular solution contained (in mM): 140 K-gluconate, 8 KCl, 2 MgCl₂, 4 Na₂-ATP, 0.3 Na₂-GTP, 10 Na-phosphocreatin and 10 HEPES, while for experiments conducted in voltage clamp mode contained (in mM): 125 Cs-gluconate, 5 CsCl, 10 EGTA, 2 MgCl₂, 2 Na₂-ATP, 0.4 Na₂-GTP, 10 HEPES. QX-314 (5mM) was added in the experiments in which synaptic properties were investigated.
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The pH was set to 7.3 with either KOH or CsOH. Cs-gluconate based solutions are normally used to study synaptic cell properties. Cesium blocks K\(^+\) permeable channels thereby allowing the isolation of synaptic mediated currents, without the interference of K\(^+\) currents. QX-314 blocks voltage-activated Na\(^+\) channels and has therefore a similar function.

The intracellular solution to study tonic inhibition contained (in mM): 120 CsCl, 2 MgCl\(_2\), 2 CaCl\(_2\), 2 Mg-ATP, 0.3 Na-GTP, 10 Hepes, 10 EGTA and 5 QX-314.

*Performing a patch clamp recording*

The glass pipette electrode, filled with the intracellular solution, was fixed on a headstage holder. A chlorided silver wire, plunged in the intracellular solution, transmitted electrical signals from and to the patch clamp amplifier (Axopatch 200B, Axon Instruments). This electrode was used as a voltage sensor as well as current-passing electrode. Another chlorided silver wire, immerged in the solution into the recording chamber, was connected to the headstage and served as a ground electrode.

The pipette electrode and the headstage were mounted on an electrical micromanipulator which could be moved into the 3 axes with a keyboard controller (Luigs & Neumann, SM I, Germany).

When the pipette entered the bath solution the pipette offset was set to zero to eliminate liquid junction potentials between the bath solution and solution inside the pipette. The resistance of the pipette was monitored continuously by applying repetitive 5 mV pulses (*Fig. 2.1A*).

During the penetration of the brain tissue a positive pressure was applied into the pipette.

Once the pipette tip contacted the cell surface, the positive pressure was removed and an additional negative pressure was applied to enable the gigaohm seal formation, as already described above. The pipette resistance reached here values > 1 G\(\Omega\).

In the cell attached configuration, pipette capacitance transient was canceled or reduced using the fast compensation adjustment at the amplifier (*Fig. 2.1B*). Subsequently the potential of the pipette interior was set to a voltage similar to the expected membrane resting potential and the rupture of the “patch” membrane was obtained by application of a relative strong and brief negative pressure into the pipette. The appearance of large capacity transients at the edges of the 5 mV pulse indicated the achievement of the whole cell configuration (*Fig. 2.1C*).
The access resistance (Ra) (Ra = resistance of the pipette after achieving the whole cell configuration) was not compensated and only cells with Ra < 20 MΩ were used for recordings. The input resistance was monitored in current clamp mode. Furthermore input resistance and Ra were controlled before and after each recording, and cells were discarded, if these parameters changed more than 20 %.

**Fig. 2.1 Schematic illustration of the important steps to perform a whole-cell patch clamp recording.** A) The upper draw shows the tip of a pipette immersed in the bath solution; the lower trace represents the current passing through the tip of the pipette upon application of a voltage step. B) The upper draw symbolizes the achievement of the cell-attached patch clamp. This configuration can be achieved by application of a gentle suction through the pipette when the pipette tip contacts the surface of the cell. The sudden drop in the current through the pipette (trace in the middle) indicates the achievement of the cell-attached configuration. The lower trace shows the reduction of the pipette capacitance transient obtained using the fast compensation adjustment at the amplifier. C) The application of an additional suction into the pipette eventually causes the rupture of the membrane under the pipette tip (upper draw). The appearance of large capacity transients at the edges of the voltage step indicated the achievement of the whole cell configuration (lower trace).
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Current and Voltage clamp

Intrinsic cellular properties, spontaneous firing and synaptic plasticity were investigated in current clamp mode. All the other electrophysiological recordings were conducted in voltage clamp mode.

The voltage clamp allows measurements of ion currents across the cell membrane by maintaining the membrane potential of the cell constantly “clamped” to a command (or holding) potential set by the experimenter.

The voltage clamp operates by negative feedback. A feedback amplifier monitors if the cell membrane potential (Vm) detected by the recording electrode (relative to a ground electrode) is equal to the command potential (Vc), set by the experimenter.

For any shift of Vm from Vc, due to ionic currents across the cell membrane, the amplifier sends an output current through the same electrode (which now works as a current source), into the cell, with a polarity and magnitude necessary to bring the voltage of the cell back to the desired voltage Vc (Fig. 2.2).

The clamp circuit produces therefore a current equal in magnitude and opposite in polarity to the ionic currents responsible for the shift in membrane potential. Since the feedback amplifier operates fast it can be assumed that for all practical purposes Vm = Vc.

Fig. 2.2 Schematic drawing of the patch clamp method in the voltage clamp mode. Briefly, a feedback amplifier monitors the membrane potential (Vm) of the recorded cell. For any change of Vm, from the command voltage (Vc), the amplifier sends into the cell the current necessary to restore Vm = Vc.
2.MATERIALS AND METHODS

Data acquisition
Electrical signals were recorded with an Axoclamp-2B amplifier (Axon Instruments) and visualized either with an oscilloscope (HM407, HAMEG) or digitized at 5 KHz and filtered at 2 KHz using an A/D D/A transducer (Digidata-1400 system, Axon Instruments). The Digidata was connected to a personal computer allowing the visualization of the traces on a computer screen with the PClamp 10 software (Molecular Devices, Sunnyvale, CA, USA).
Intracellular current injections and extracellular stimulations of the tissue were controlled by the software (P Clamp 10) as well. The digital signals were inversely converted into an analog signal by the Digidata and then sent to either the patch electrode for intracellular stimulation or to a stimulation unit finally connected to an extracellular stimulating electrode placed in a region of interest on the brain tissue.

2.2.3 Experimental designs

Synaptic plasticity at horizontal connections
To study synaptic plasticity at horizontal connections, extracellular presynaptic stimulations were performed by placing a glass electrode at around 500 μm adjacent to the patched neuron within layers 2/3. The presynaptic stimulation induced a monosynaptic response, with a stimulus to peak-latency of approximately 5 ms following the stimulus artifact. To prevent polysynaptic activity we used a low stimulation intensity (80-120 μA), which evoked a relatively small postsynaptic responses. Thus, we excluded any parallel activation of adjacent ascending fibers in the synaptic signals.
Only neurons with a resting membrane potential of at least -65 mV were used for further investigations. The input resistance was continuously monitored during the recordings by injection of a hyperpolarizing current pulse (intensity: 50 pA; duration: 250 ms) following each test stimulus.
Baseline EPSPs were recorded every 20 sec for at least 5 min before LTP or LTD induction. The signals had a mean amplitude of 2.5 ± 0.5 mV and 3.5 ± 0.5 mV, respectively, and were similar in both experimental groups.
LTP and LTD was induced by applying a theta-burst stimulation (TBS)- or low-frequency stimulation (LFS) protocol, respectively.
The TBS protocol consisted of five synaptic trains (at 20 s intervals) of five bursts (at 5 Hz) each providing four stimuli at 100 Hz. Bursts were paired with intracellular depolarization delayed by 10 ms (900 ± 200 pA, 45 ms duration) through the recording electrode. Similar protocols have been used to induce NMDAR-dependent LTP in the visual cortex (Kirkwood and Bear 1994, Yoshimura et al. 2003; Huemmeke et al. 2004) (Fig. 2.3A). The LFS protocol consisted of 10 min of synaptic stimulation at a frequency of 1 Hz paired with intracellular depolarization delayed by 10 ms (500 ± 100 pA, 45 ms duration) (Fig. 2.3B). The LTD induction with this protocol did not follow the rule of spike-timing-dependent plasticity. Both type of stimulations induced spikes in all the recorded neurons.

For statistical evaluation the EPSP amplitudes the last 30 EPSPs were averaged in each recorded cell.

Fig. 2.3 Schematic representation of the protocols used for LTP and LTD induction. A) The TBS protocol, used for induction of LTP, consisted of five synaptic trains (at 20 s intervals) of five bursts (at 5 Hz) each providing four stimuli at 100 Hz. The bursts were paired with intracellular depolarization delayed by 10 ms (yellow lines). B) The LFS protocol, used for LTD induction, consisted of 10 min of synaptic stimulation at a frequency of 1 Hz paired with intracellular depolarization delayed by 10 ms.
Isolation of excitatory and inhibitory postsynaptic currents

AMPA-mediated currents were pharmacologically isolated by bath application of the GABA<sub>A</sub> receptor antagonist picrotoxin (PTX) (50 µM) (Biozol, Germany), the NMDA receptor blocker D-AP5 (25 µM) (Biozol, Germany) and acquired at a holding potential of -80 mV. NMDA-mediated currents were isolated by bath application of PTX (50 µM), of the AMPA receptor antagonist DNQX (20 µM) (Biozol, Germany) and acquired at +40 mV. GABA<sub>A</sub> mediated currents were recorded in presence of DNQX and D-AP5 at the above described concentrations and acquired at -80 mV. It is important to mention that in the 4<sup>th</sup> postnatal week (time when the experiments were performed) the GABAergic system is still undergoing developmental changes. Thus, all experiments on GABAergic inhibition were performed in a very narrow temporal window (P24-P28) and care was taken that animals belonging to the two experimental groups were strictly age-matched. Although the Cs-gluconate-based internal pipette solution contained a relative low chloride concentration the reversal potential for GABA<sub>A</sub>R-mediated currents was found to be -45 ± 5 mV. As a result, IPSCs were recorded as inward currents. To block GABA<sub>B</sub> receptors CGP55845 (2 µM) (Biozol, Germany) was added in the bath.

Estimation of initial release probability

Pairs of synaptic stimulations with interstimulus intervals (ISIs) ranging from 20 to 200 ms were used to analyze the paired-pulse ratio (PPR). The stimulus intensity was always set to evoke a first postsynaptic response of ~ 60 pA.

High frequency presynaptic stimulation was also performed to further characterize synaptic function. Forty pulses at 33 Hz were delivered. The stimulus intensity was always set to evoke a first postsynaptic response of ~ 60 pA.

Investigation of tonic inhibition

To study the influence of tonic inhibition on the postsynaptic cell conductance GABA<sub>A</sub>R-mediated currents were recorded before and after the application of bicuculline (20 µM) (Sigma, Germany).

2.2.4 Analysis of electrophysiological recordings

PClamp 10.1 software was used for off-line analysis.
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Spontaneous IPSCs and EPSCs (sIPSCs, sEPSCs) were visually identified and the frequency and amplitude was calculated as the median of 300 events for each cell.

Paired-pulse ratio. The PPR was calculated as the ratio between the peak amplitude of the second evoked IPSC or EPSC (eIPSC, eEPSC) to the first one. The current traces used for the analysis were averages of six to eight consecutive responses repeated every 10 s.

Estimation of initial release probability of readily releasable pool from cumulative amplitude analysis

To estimate the readily releasable pool (RRP) at GABAergic synapses, cumulative eIPSC amplitudes, evoked by a train of presynaptic stimulation at high frequency (40 pulses at 33 Hz), were plotted versus the stimulus number. After 30 pulses, the cumulative plot reached a steady-state where it increased linearly with subsequent pulses.

In the steady-state phase the RRP is assumed to be depleted and the postsynaptic responses generated by an ongoing replenishment of vesicles (Baldelli et al. 2005).

To estimate the RRP the linear component of the cumulative eIPSCs plot (the last 10 data points) was fitted with a linear regression and backextrapolated to the time 0 (Baldelli et al. 2005). Because the cumulative eIPSCs plot was calculated with normalized values, the so-determined RRP was expressed as n-fold the size of the first eIPSC.

It is worth noting that since the presynaptic stimulation intensity was always adjusted to evoke a first eIPSC, with constant amplitude of ~ 60 pA. Assuming that the studied synaptic transmission could be approximated to the binomial model of synaptic transmission first described by Katz (Katz 1969), the size of the first evoked postsynaptic current depends on the number of functional synaptic release sites (N), the quantal size of a single vesicle (q) and the initial probability of release (Pr):

\[
\text{IPSC amplitude} = N \times q \times Pr
\]

Since the number of release sites is the only parameter which can be controlled, being proportional to the number of the stimulated fibers (dependent on the intensity of the presynaptic stimulation), differences in the quantal size and/or in the initial
probability of release had to be compensated by modulating the number of stimulated fibers (increasing or decreasing the presynaptic stimulation intensity) to evoke a constant first eIPSC amplitude in each recorded neuron (60 pA). This approach implies that absolute values of RRP can not be compared between different neurons because they depend on the number of stimulated fibers which differs for each cell (and especially among different experimental groups). However, the probability of any readily releasable vesicle to be released with the first pulse (PrRRP) can be estimated from the ratio between the first eIPSC amplitude and the RRP for each cell (Baldelli et al. 2005):

\[ Pr_{RRP} = \frac{\text{first eIPSC}}{\text{RRP}} \]

Because potential lesion-induced alterations in RRP and in the ongoing vesicles replenishment could not be analyzed and could potentially influence PrRRP, differences in this value were interpreted as indicative but not conclusive of changes in the initial probability of transmitter release.

**Kinetics of postsynaptic currents**

The rise time of evoked postsynaptic currents was calculated between 10% and 90% of the peak amplitude onset, while the decay-time constant was calculated by fitting the decaying current to the following monoexponential function:

\[ f(t) = A_i e^{-t/\tau_i} + C \]

**Spikes**

To measure spike threshold the maximum rate of change of Vm \((dV/dt)_{MAX}\) was computed during the upstroke of each spike. The membrane potential threshold was defined as the voltage at the onset of each spike at which 5% of \((dV/dt)_{MAX}\) was reached. This fraction (5%) of \((dV/dt)_{MAX}\) was chosen because it best matched the threshold assigned by careful visual inspection of the raw data (Azouz and Gray 2000).
For all electrophysiological investigations the value \( n \) corresponds to the number of the analysed neurons for each group.

### 2.3 Histology

For histological and immunohistochemical analyses animals were deeply anaesthetized by intraperitoneal injection of Pentobarbital (0.33 g/kg body weight, Pentobarbital-Sodium, KG Narcoren®, Rhone Merieux GmbH, Germany) and perfused through the left ventricle (perfusion rate: 30\( \mu \)l/sec) for 1 minute with cold Ringer solution containing 1% Heparin (Liquemin®, Roche, Switzerland), followed by 15 minutes with 4% paraformaldehyde diluted in 0.1 M phosphate buffer saline solution (PBS, pH 7.4).

The brains were removed from the skull and postfixed in the same fixative for 24 hours at 4ºC. For cryoprotection the brains were subsequently immersed in 30% sucrose (in 0.1 M PBS) for at least 72 hours at 4ºC.

Coronal sections containing the visual cortex (30 \( \mu \)m thick) were cut on a freezing microtome and collected in PBS.

Nissl stainings were performed in some slices for the morphological characterization of the lesion and to control the quality of the tissue.

Nissl stainings were performed using 0.1% Cresyl Violet solution on slide-mounted and defattened sections.

#### 2.3.1 Immunohistochemistry

Immunohistochemistry (IHC) is a staining procedure which detects specific proteins in biological tissue by an antigen-antibody reaction.

The production of antibodies (Abs) specific for a protein intended to be detected is achieved by infecting an animal (normally mouse or rabbit) with this protein (antigen). The immune system of the animal will program B-cells to produce the specific Abs. Each B-cell synthesizes one antibody for one epitope (a small fraction of amino acids where the Abs bind). Antibody-solutions which contain only the binding site for one epitope are called monoclonal antibodies. They are generated in mouse-hybridoma cells. Since most antigens possess several potential epitopes, a population of B-cells will produce various antibodies (polyclonal Abs) binding different epitopes of the antigen. These polyclonal Abs can be simply purified from the blood plasma.
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Both polyclonal and monoclonal Abs are commercially available and are often used in IHC. The advantage of polyclonal Abs is the lower price (compared to the monoclonal) and the high sensitivity. However monoclonal Abs can offer a much higher specificity and sometimes are necessary to increase the signal-to-noise ratio.

**Protein detection**

The visualization of the antibody-antigen interaction can be achieved with different procedures. Most commonly the antibody is conjugated to an enzyme which catalyses a colour-producing reaction (f.i. peroxidase) or to a fluorophore.

In order to intensify the sensitivity of the detection some additional steps are normally used, which include labeled secondary antibodies specifically binding the primary ones and a complex made of avidin and biotin which on the one hand can bind biotinylated antibodies and on the other hand is coupled with a proper enzyme.

**Detection with peroxidase reaction**

Many stainings in this study utilized a peroxidase reaction to localize the site of antibody-antigen interaction.

Peroxidases (POD) are a specific class of oxidoreductases which act as a catalyzing factor to oxidize a chromogen (CHR) by reducing $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$. The resulting oxidized chromogen changes to dark colour and precipitates.

\[
\text{(CHR)}\text{H}_2 + \text{H}_2\text{O}_2 \xrightarrow{\text{POD}} \text{H}_2\text{O} + \text{(CHR)↓}
\]

The chromogen used in the present study is Diaminobenzidine (DAB) which turns into a brownish product in presence of $\text{H}_2\text{O}_2$ and POD.

To localize the antibody-antigen interaction POD was purchased conjugated with a biotin-avidin complex (ABC) (Vector, USA) (Fig. 2.4A).

**Detection with fluorescent dyes**

Immunofluorescence was performed using Abs conjugated with a fluorescent dye (Fig. 2.4B). In alternative biotinylated Abs were recognized by streptavidin coupled with a fluorophore (Fig. 2.4C).
The principle of using fluorescent dyes is that they can emit light after having absorbed light (or other electromagnetic radiation) with a different wavelength. The emitted light has, in most cases, a longer wavelength and therefore less energy. Several fluorophores emitting light at different wavelengths are commercially available. Using the appropriate illumination, at fluorescence or confocal microscope, one can therefore distinguish clearly between different fluorophores. This peculiarity makes fluorescent dyes the first choice for double (or multiple) staining experiments.

All immunohistochemical stainings in this study were performed on free-floating sections. Briefly, the immunohistochemical procedure for stainings detected with peroxidase reaction consists of the following steps:

- washing 3 X 10 min in PBS;
- quenching endogenous peroxidase activity with 0.3% hydrogen peroxide (H₂O₂) in PBS for 20 min;
- washing 3 X 10 min in PBS;
2. MATERIALS AND METHODS

- blocking endogenous biotin with 20% avidin (block A, blocking kit, Vector, USA), 10% normal serum in PBS/triton (0.2%) for 90 min;
- incubation with primary antibody with 20% biotin (block B, blocking kit, Vector, USA), 1% normal serum in PBS/triton (0.2%) over night;
- washing 3 X 10 min in PBS;
- incubation with biotinylated secondary antibody (1:500) with 1% normal serum in PBS/triton (0.1%) for 90 min;
- washing 3 X 10 min in PBS;
- detecting system with 1% normal serum, ABC (1:500) in PBS/triton (0.1%) for 90 min;
- washing 3 X 10 min in PBS;
- visualization with DAB (0.5mg/ml), 0.01% H$_2$O$_2$ in PBS for some minutes (the incubation time depends on the antibody and normally vary between 4-5 and 10 min);
- washing 3 X 5 min in PBS.

All steps were performed at room temperature. Triton was used to increase the permeability of the tissue for the antibodies. Omission of the primary antibody resulted in a complete lack of immunoreactivity confirming the specificity of the antibody.

For primary polyclonal antibody normal goat serum and biotinylated goat-anti-rabbit secondary antibody (Vector, USA) were used. For primary monoclonal antibody normal horse serum and biotinylated horse-anti-mouse secondary antibody (Vector, USA) were used.

The biotinylated lectin Wisteria floribunda agglutinin (WFA) could be directly detected with either ABC or streptavidin conjugated with a fluorophore.

For immunofluorescence the biotinylated secondary antibodies were finally incubated with streptavidin conjugated with a fluorophore for 90 minutes in the dark. When antibodies were conjugated with a fluorophore this last incubation as well as the block of endogenous biotin were not necessary. Both reactions were done without endogenous peroxidase-blocking.

Finally sections were mounted on gelatinized slides, dehydrated and coverslipped with DePex (Serva, Germany).
The following Abs were used: anti-glial fibrillary acidic protein (GFAP), anti-glutamate decarboxylase (GAD67), anti-parvalbumin (PV), anti-calbindin (CB), anti-calretinin (CR), anti-neuronal nuclei (NeuN) and anti-vesicular GABA transporter (VGAT). Some details about the Abs are resumed in the table 1.

<table>
<thead>
<tr>
<th>Antibody/lectin</th>
<th>Company</th>
<th>Mono- Polyclonal</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>DAKO</td>
<td>Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>GAD 67</td>
<td>Chemicon</td>
<td>Monoclonal</td>
<td>1:2000</td>
</tr>
<tr>
<td>PV</td>
<td>Swant</td>
<td>Monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>WFA (lectin)</td>
<td>Sigma</td>
<td>-</td>
<td>1:3000</td>
</tr>
<tr>
<td>CB</td>
<td>Swant</td>
<td>Monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>CR</td>
<td>Swant</td>
<td>Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>NeuN</td>
<td>Chemicon</td>
<td>Monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>VGAT</td>
<td>SySy</td>
<td>Polyclonal</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 1. Generalities of the used Abs.

### 2.3.2 Analysis of immunohistochemical stainings

Quantification of immunostainings was performed in 2 non-adjacent sections for each animal. The number of animals used for each staining is represented by the value n, if not otherwise indicated.

Images of the stained material, detected with peroxidase reaction, were acquired using Leitz Wetzlar Dialux 20 microscope (Leica, Germany) equipped with a digital colour video camera (DEI-750, Visitron Systems, Puchheim, Germany).

Visually identified GAD67, CB, CR, PV and WFA positive cells were carefully counted from a region of interest (ROI) extending from the pial surface throughout all the cortical layers.

The number of positive cells for these markers was normalized to the number of NeuN positive cells in the same ROI in an adjacent section. The anti-NeuN antibody labeled a nuclear protein expressed in most neuronal cell types throughout the nervous system (Mullen et al. 1992) and it is therefore commonly used as a neuronal marker.

The number of NeuN positive cells did not change post-lesion (Fig. 2.5).
NeuN positive cells were counted using home-made software written in Interactive Data Language (ACCM, automatic cell counting method) (Benali et al. 2003). Briefly, the images were binarised setting a threshold and then, according to the different possible sizes of neurons, a range area was selected. Under this condition the software counted the cell number automatically. A visual inspection was finally carried out to exclude obvious artifacts.

![Image](image_url)

**Fig. 2.5 The number of NeuN positive cells remained unaltered post-lesion.**

Immunohistochemical stainings for NeuN taken from the visual cortex of a lesion-treated animal in the proximity of the lesion and in the homotopic area in a sham-operated animal.

Pictures from immunofluorescence material were acquired using either a confocal microscope (SP5MP, Leica) or an ApoTome microscope (Axiovert Z1, Zeiss). To quantify VGAT expression, optical density (OD) measurements were conducted on converted grey-scaled images with the software Image J (National Institutes of Health, USA). In order to achieve reliable results care was taken in processing all sections under the same conditions. In sections stained with peroxidase reaction, the background from the underlying immunonegative white matter was measured in each slice. The final OD values were obtained by subtracting the background value from the value of the immunopositive neuropil in the grey matter. The quantification of the CR immunopositive neuropil in layer 1 followed the same procedure.
To study perisomatic GABAergic innervation a double immunofluorescence labeling with NeuN and VGAT was performed. Staining for NeuN allowed the visualization of neuronal bodies. Only cells with NeuNir pyramidal shaped body were used for analysis. Because single positive axonal boutons were not clearly distinguishable, to obtain a quantitative estimation of perisomatic GABAergic innervation a ROI was selected around the soma of each cell. The VGAT immunoreactivity was then evaluated after binarizing each section by setting a proper threshold. In each section the threshold value corresponds to the last 3% of the histogram distribution of the grey scale values in each picture. The threshold of 3% was selected after a careful visual inspection. The % of the VGAT positive area was calculated over the total area of each ROI.

2.4 Statistical analysis
Statistical significance was tested with independent sample t-test. Data are presented as mean ± SEM. Pearson’s correlation coefficients were also used when computing correlations between two variables. p values < 0.05 were considered as significant.
3. Results

3.1 Histological characterization of the lesion and investigation of synaptic plasticity in the surround of the lesion

To study the morphological alterations of the tissue at the border of the lesion, Nissl and immunohistochemical stainings were performed between 3 and 6 days post-lesion.

Nissl stainings revealed that the lesion was primarily located in the visual cortical areas V1M, V2MM, V2ML in cortical slices selected from the region Bregma -6 mm to Bregma -7.6 mm (Paxinos and Watson 1998). Slices within this region were therefore chosen for all experimental investigations.

The necrotic tissue damage had a diameter of 1 ± 0.2 mm in mediolateral extent and reached down deep cortical layers (Fig. 3.1A).

Immunohistochemical staining for GFAP showed a clear astrogliosis which was mostly restricted to distances of 300–400 μm from the border of the lesion (Fig. 3.1C-D).

The non-injured contralateral hemisphere as well as the visual cortex of sham-operated rats showed neither histological tissue damage nor sign of astrogliosis (data not shown).

All electrophysiological recordings were performed from visually-identified pyramidal neurons in the cortical layers 2/3.

In the first part of the study the recordings were performed at distances of 2 to 4 mm away from the border of the lesion. This specific region has been previously characterized by changes in synaptic plasticity of vertically ascending synaptic inputs (Mittmann and Eysel 2001) and by an increase in resting and stimulus evoked [Ca^{2+}]i (Barmashenko et al. 2001; Barmashenko et al. 2003). All neurons labeled with biocytin could be identified as characteristic pyramidal shaped-body neurons (Fig. 3.1B).
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Figure 3.1 Histochemistry of the focal laser lesion in rat visual cortex. A) Nissl stained coronal section containing the visual cortex of a lesion-treated animal. Note the laser-induced lesion, located in the medial part of the visual cortex and the position of the recording patch clamp electrode (r) in layers 2/3 and the stimulation electrode (s) lateral in the same layers, to stimulate horizontal fibres. B) Intracellularly biocytin-labeled pyramidal neuron in layers 2/3 of rat primary visual cortex. C) Another slice containing the lesion labelled with the glial marker GFAP disclosed an astrogliosis reaction, mostly restricted to 300–400 µm from the border of the lesion. D) The same slice as shown in C revealed at higher magnification the spatially-limited astrogliosis.

3.1.1 Unaltered intrinsic properties of pyramidal neurons in layers 2/3 post-lesion

Firstly, changes in the intrinsic properties of the neurons surrounding the lesion were investigated. The resting membrane potential was not significantly altered, although it was slightly shifted to more positive values post-lesion (data not shown).

To study the firing properties of neurons, square pulses of hyperpolarizing and depolarizing current were delivered into the patch clamped neurons (at 0.2 Hz, for 400 ms). Neurons from both sham-operated and lesion-treated animals started firing
3.RESULTS

action potentials normally with 300–400 pA of depolarizing current injection and all
of the recorded neurons showed regular frequency-adapting spikes which are
characteristic for cortical pyramidal neurons (Fig. 3.2A) (McCormick et al. 1985). No
significant differences were found in the spike frequency at different current
amplitudes between the 2 animal groups even though, at 800 pA current injection, the
spike frequency of the lesion group was slightly higher (Fig. 3.2B). The input
resistance was also not significantly altered by the lesion (sham-op: 94.22 ± 7.38 MΩ
n= 9, lesion: 109.90 ±11.86 MΩ n= 10) (Fig. 3.2C).

Fig. 3.2 Unchanged intrinsic properties of pyramidal neurons post-lesion. A) Examples
traces of membrane potential shift and firing patterns of two recorded neurons (one from
lesion-treated and one from sham-operated animal) in response to hyperpolarizing and
depolarizing steps of current injections (the diagram on the right side showed the steps of
current injection). For each neuron, the responses to the same three current injection
amplitudes are shown. B) Summary plot of the mean spike frequency in response to
increasing current injection amplitudes into the recorded neurons. C) Mean input resistance of
the recorded neurons.
3. RESULTS

3.1.2 Metaplasticity at horizontal connections in the surround of the lesion

So far it is not known whether horizontal fibers at the border of a focal brain lesion express any changes in synaptic plasticity. Thus, potential changes of LTP and LTD at synapses formed by horizontal connections in layers 2/3 located lateral to the border of the lesion were evaluated.

The TBS protocol, when applied in sham-operated animals, induced a relative small LTP of the signal amplitude in 8 out of 11 neurons tested, 1 neuron expressed only a moderate short-term potentiation which decayed within the first 20 min, and two other neurons did not show any potentiation but rather a slight depression (Fig. 3.3E). The average of relative EPSP amplitude recorded during the last 10 min reached $125.3 \pm 15.3\%$ (n= 11). In contrast, the same protocol led to a significantly higher level of LTP ($p<0.05$) in neurons from lesion treated animals ($174.5 \pm 20.4\%$ n= 16) (Fig. 3.3A-B). In this group only 1 neuron out of 16 failed to show any plastic change and 3 others expressed only short term potentiation, while a relative stable LTP was observed in all remaining cells (Fig. 3.3E).

Next the strength of LTD induction post-lesion was tested. The LFS protocol reliably induced LTD in all neurons from sham-operated animals. In contrast, it induced two types of alterations in the EPSPs amplitude in neurons from lesion-treated animals: half of the neuronal population revealed a LFS-induced LTP while the other half gave rise to LTD (Fig. 3.3F).

While the strength of synaptic depression in the population of LTD-expressing neurons was not different between lesion and sham, the summary diagram of all cells treated with a LFS protocol revealed a significant difference ($sham-op: 60.8 \pm 6.7\%$ n= 8, $lesion: 100.4 \pm 15.4\%$ n= 11, $p<0.05$) (Fig. 3.3C-D). Overall, the synapses at horizontal connections at the border of the lesion show a metaplastic shift towards a strengthening of the glutamatergic synaptic transmission.
3. RESULTS

Fig. 3.3 Lesion-induced metaplasticity at L2/3-L2/3 synapses. A) Representative traces of EPSPs recorded 2 minutes before and 40 minutes after LTP induction. B) Time course of the changes in the relative EPSP amplitudes induced by the TBS protocol. Note the enhanced potentiation of EPSPs post-lesion. C) Representative traces of EPSPs recorded 2 minutes before and 40 minutes after LTD induction. D) Time course of the changes in the relative EPSP amplitudes induced by the LFS protocol. Note the missing induction of LTD post-lesion. E), F) Graph showing the mean of the relative EPSP-amplitudes for each individual neuron recorded between 20-30 min following (E) TBS or (F) LFS.
3. RESULTS

In order to elucidate the mechanisms underlying the observed lesion-induced metaplasticity, possible alterations at glutamatergic and GABAergic L2/3-L2/3 synapses were studied.

3.1.3 Unaltered glutamate release at L2/3-L2/3 synapses post-lesion
To test a potential change in glutamate release the PPR of AMPA-mediated currents evoked by pairs of synaptic stimulations with different ISIs (20-200 ms) was calculated. Most of the recorded neurons showed a paired-pulse facilitation up to 120 ms ISI which was not different between the two experimental groups (Fig. 3.4A-B).

**Fig. 3.4 Unchanged PPR of AMPAR-mediated currents at L2/3-L2/3 synapses post-lesion.** A) Representative traces of AMPAR-mediated EPSCs, recorded at -80 mV, evoked by pairs of synaptic stimulations with an ISI of 30 ms. B) Summary diagram of the paired-pulse ratio (PPR) of AMPAR-mediated EPSCs evoked by two synaptic stimulations with ISIs ranging from 20 to 200 ms. The unaltered PPR indicated that the release probability of glutamate is not affected by the lesion.

3.1.4 The cortical lesion prolonged the decay-time constant of synaptic NMDAR-mediated currents at L2/3-L2/3 synapses
Next, the functional properties of postsynaptic glutamate receptors at L2/3-L2/3 excitatory synapses were investigated. The current-voltage relationship of AMPAR-mediated currents did not change post-lesion (Fig. 3.5A-B). Similarly, the current-voltage relationship of NMDAR-mediated currents was not significantly altered (Fig. 3.5C-D).
3. RESULTS

Fig. 3.5 Unchanged current-voltage curve of AMPAR- and NMDAR-mediated currents at L2/3-L2/3 synapses post-lesion. A) Representative traces of evoked AMPAR-mediated currents recorded at different holding potentials in a neuron from a lesion-treated animal. B) Current-voltage curve of AMPAR-mediated EPSCs in lesion-treated and sham-operated animals. C) Representative traces of evoked NMDAR-mediated currents recorded at different holding potentials in a neuron from a lesion-treated animal. D) Current-voltage curve of NMDAR-mediated EPSCs in lesion-treated and sham-operated animals.

However a significantly prolonged decay-time constant of NMDAR-mediated currents was observed post-lesion (sham-op: 148.9 ± 12.8 ms n= 10, lesion: 185.7 ± 10.8 ms n= 11) (p< 0.05) (Fig. 3.6A-B). In accordance, upon high-frequency synaptic stimulation (40 pulses, 33Hz) the normalized NMDAR-mediated current amplitude was significantly higher in lesion-treated rats (Fig. 3.6 C-D).

This effect is very unlikely to be mediated by alterations of the presynaptic function, such as changes in glutamate release or increased glutamate spillover, because (1) the
PPR of AMPA-receptor mediated EPSCs did not show any differences for all the tested inter-stimulus intervals (20-200ms) ([Fig. 3.4A-B](#)), (2) the temporal dynamics of AMPAR-mediated currents induced by the same protocol (40 pulses at 33 Hz) showed no changes post-lesion (data not shown). NMDARs are permeable to calcium. Thus, the prolonged decay-time constant of NMDARs might enhance the influx of Ca^{2+} into the postsynaptic neuron. The increased Ca^{2+} influx may be responsible, at least partially, for the facilitated induction of LTP post-lesion (see [Fig. 3.3](#)).

![Fig. 3.6 Prolonged decay-time constant of NMDA-receptor mediated currents at L2/3-L2/3 synapses post-lesion.](#)

A) Representative traces of NMDAR-mediated EPSCs recorded at a holding potential of +40 mV from lesion-treated (black trace) and sham-operated (grey trace) animals. B) Diagram showing the decay-time constant of these EPSCs for each recorded neuron and the mean decay-time constant in the two experimental groups. C) Representative traces of NMDA-mediated EPSCs in response to high frequency presynaptic stimulation protocol (40 pulses at 33Hz). D) The normalized amplitudes of NMDA-receptor mediated EPSCs, evoked by the same protocol as in C, were higher in the lesion group.
3.1.5 Unchanged GABA release at L2/3-L2/3 synapses after lesion

Potential lesion-induced alterations of the inhibitory transmission at L2/3-L2/3 synapses were investigated by pharmacological isolation of GABA$_A$R-mediated currents. Extracellular presynaptic paired stimulations revealed a PPR close to 1 for short ISIs (20-50 ms) and slightly $< 1$ for longer ISIs with no difference between the two experimental groups (Fig. 3.7A-B). Repetitive presynaptic stimulations at high frequency (40 pulses, at 33 Hz) were also delivered but no change was observed in the rate of depression of eIPSC amplitudes with subsequent stimuli (Fig. 3.7C-D).

Fig. 3.7 Unchanged properties of L2/3-L2/3 GABAergic synapses post-lesion. A) Representative traces of GABA$_A$R-mediated currents recorded at -80 mV evoked by pairs of synaptic stimulations with an ISI of 30ms. B) Summary diagram of the paired-pulse ratio (PPR) of GABA$_A$R-mediated currents evoked by two synaptic stimulations with ISIs ranging from 20 to 200 ms. The PPR was not significantly different between the 2 groups. C) Representative traces of GABA$_A$-mediated currents in response to high frequency presynaptic stimulation protocol (40 pulses at 33Hz). D) Normalized amplitude of GABA$_A$-mediated responses evoked by the same protocol as in C. No changes can be observed post-lesion.
3.2 Spatial profile of lesion-induced neuronal alterations

Next, the spatial profile of the lesion-induced alterations was investigated in surrounding cortical networks. Immunohistochemical stainings were combined with electrophysiological recordings to study the relation between structural modifications or changes in protein expression and alterations of neuronal functions.

**Immunohistochemistry**

Images for analysis were acquired at 1 mm, 2.5 mm and 4 mm lateral from the border of the lesion, in coronal sections, and in the homotopic areas from the non-injured contralateral hemisphere and from sham-operated animals (Fig. 3.8A).

**Electrophysiology**

Layers 2/3 pyramidal neurons selected for recordings were divided into four groups depending on the location with respect to the border of lesion: **ipsi 1mm** (which included recordings of neurons located in the cortex ipsilateral to the lesion at around 1 mm distance from the border of the lesion), **ipsi 2.5mm** (again in the ipsilateral cortex at around 2.5 mm from the border of the injury), **contra and sham-op** (which included neurons from the untreated contralateral hemisphere of lesion-treated rats and from the cortex of sham-operated rats, respectively, in a homotopic area to the region ipsi 1mm). Synaptically evoked responses were induced by electrical stimulation of ascending fibers in cortical layer 4 below the recorded cell (Fig. 3.8B).
3. RESULTS

Fig. 3.8 Definition of the investigated cortical areas with respect to the location of the lesion. A) Nissl stained coronal section containing the lesion in the visual cortex. The picture illustrates the regions where the images for immunohistochemical analysis were taken at defined distances from the border of the lesion in both hemispheres (1, 2.5 and 4 mm). The homotopic cortical areas were used in sham-operated animals. B) The same Nissl picture as in A shows here the cortical regions where patch clamp recordings were performed. The schematic drawings represent the recording and stimulating electrodes at 1 mm and 2.5 mm from the border of the lesion and in the contralateral hemisphere.
3. RESULTS

3.2.1 Reduced number of GAD67 and PV positive cells in both visual cortical hemispheres post-lesion

Functional alterations of the GABAergic transmission can be reflected by an altered expression of specific markers of inhibitory interneurons. The expression of the major isoform of glutamate decarboxylase (GAD67) was first examined. GAD67 is responsible for the basal GABA production in the brain.

The number of GAD67 positive neurons was found to be reduced in a large part of the visual cortex ipsilateral to the lesion and surprisingly also to the same extent of the contralateral non-lesioned hemisphere (Fig. 3.9A-B). The reduction was significant at 1 and 2.5 mm distance from the border of the lesion as well as in the homotopic regions of the contralateral hemisphere (1 mm = sham-op: 7.97 ± 0.23 n= 6, ipsi: 6.94 ± 0.20 n= 6, p= 0.011, contra: 6.70 ± 0.36 n= 6, p= 0.022, 2.5 mm = sham-op: 8.59 ± 0.28, ipsi: 7.10 ± 0.40, p= 0.019, contra: 6.70 ± 0.54, p= 0.018). At 4 mm distance from the border of the lesion the number of GAD67 immunoreactive (GAD67ir) cells reached the level of control animals (sham-op: 7.68 ± 0.38 n= 5, ipsi: 7.28 ± 0.17 n= 5, contra: 7.67 ± 0.30 n= 4) (Fig. 3.9D).

GAD67 is however expressed in all heterogeneous subpopulations of cortical GABAergic neurons. To study the effect of the lesion on specific classes of interneurons, the expression of the calcium-binding proteins PV, CB and CR were investigated. Together, they labeled a large subpopulation of almost non-overlapping interneurons in rodent visual cortex (Gonchar and Burkhalter 1997).

A reduction in the number of PV immunoreactive (PVir) cells was observed in both the lesioned and the contralateral hemisphere (Fig. 3.9C) but it was mostly restricted to 1 mm distance from the border of the lesion (sham-op: 9.64 ± 0.37 n= 6, ipsi: 7.93 ± 0.62 n= 6, p= 0.057, contra: 6.15 ± 0.66 n= 6, p= 0.002). A slight but not significant reduction was still present at 2.5 mm distance (sham-op: 9.72 ± 0.52, ipsi: 9.39 ± 0.50, contra: 8.10 ± 0.63) which completely disappeared at 4 mm distance (sham-op: 9.61 ± 0.36 n= 4, ipsi: 10.02 ± 0.69 n= 5, contra: 9.40 ± 0.65 n= 5) (Fig. 3.9E).

Surprisingly the intact contralateral hemisphere was stronger affected than the ipsilateral one.
3. RESULTS

Fig. 3.9 Reduction of the number of GAD67- and PV positive cells post-lesion. A) Immunohistochemical stainings for GAD67. B) The same pictures as in A showing the layers 2/3 at higher magnification. C) Immunohistochemical stainings for PV. The pictures were taken from the visual cortex of a lesion-treated animal at 1 mm from the border of the lesion (ipsi) and from the homotopic area in the hemisphere contralateral to the lesion (contra) and in a sham-operated animal (sham). Graphs showing the number of D) GAD67 and E) PV
positive cells normalized to the total number of neurons (NeuN positive) per region of interest (ROI). Analyses were performed from pictures taken at different distances from the border of the lesion (see Fig. 3.8A).

The reduced number of PV positive cells could either be due to a degeneration of this interneuron subpopulation or it might indicate a reduced expression of this calcium-binding protein from intact cells. Beyond the expression of PV, "perineuronal nets" (PNs) – specialized regions of extracellular matrix containing chondroitin sulphate proteoglycans (CSPGs) – highly concentrate around the soma and the proximal dendrites of this subclass of interneurons (Kosaka and Heizmann 1989).

If the cells survive but stop or reduce the production of PV intact PNs should be visible in the cortical regions surrounding the lesion (where PV was found to be reduced). Stainings performed using the lectin WFA, which binds an amino-sugar residue of CSPGs, revealed indeed the presence of intact PNs post-lesion (Fig. 3.10A). The number of neurons surrounded by WFA positive nets did not change after lesion in any of the tested cortical locations (Fig. 3.10B).
3. RESULTS

Fig. 3.10 No sign of PNs degeneration post-lesion. A) Immunohistochemical stainings for WFA. The pictures were taken from the same cortical regions as in Fig. 3.9. B) Graph showing the number of WFA positive cells normalized to the total number of neurons (NeuN positive) per region of interest (ROI).
3. RESULTS

Double stainings with anti-PV antibodies and the lectin WFA revealed that in sham-operated animals the vast majority of perineuronal WFA immunoreactivity was found in the surround of PVir cells (% of WFA positive, PV negative cells: 4.78 ± 1.03 % n= 5). In contrast, the cortical area in the vicinity of the lesion (around 1 mm distance from the border of the injury and in the homotopic area of the contralateral hemisphere) was characterized by the presence of diverse WFA positive PNs, which encircled PV negative neurons (% of WFA positive, PV negative cells, ipsilateral: 22.16 ± 5.81 % n= 6, contralateral: 22.03 ± 6.56 % n= 6) (Fig. 3.11). This result strongly suggested that interneurons survived, but stopped or reduced the expression of PV.

Fig. 3.11 Double immunofluorescence stainings for PV and WFA. Confocal images of the visual cortex (layers 2/3), double labeled for PV (green) and WFA (red), taken from a sham-operated (sham) and a lesion-treated animal (ipsi and contra) in a region where PV expression was found to be reduced (see Fig. 3.9). Note the presence of diverse WFA positive PNs surrounding PV negative neurons only in the lesion-treated animal (white arrows).
3. RESULTS

Because PVir cells represent the largest sub-class of interneurons in the rat cerebral cortex (Celio 1986) it is likely that the lost GAD67ir neurons largely corresponded to the lost PVir cells.

The positive correlation between the two variables points toward this hypothesis ($r=0.459$ $n=36$, $p<0.005$) (Fig. 3.12A). Importantly, no correlation was found between the expression of GAD67 and WFA ($r=-0.064$ $n=24$) excluding that the positive correlation with PV was due to the variability of the stainings among different animals (Fig. 3.12B).

![Fig. 3.12 Positive correlation between GAD67 and PV expression. A) Graph representing the positive correlation between PV and GAD67 positive cells. Each pair of variables represents the normalized number of PV and GAD67 positive cells in a homotopic ROI in serial coronal sections from the same animal. The positive correlation indicate that the same (or at least an overlapping) population of interneurons reduced both the expression of GAD67 and PV. B) The absence of correlation between WFA and GAD67 positive cells excluded that the positive correlation found in A was due to the variability of the stainings between different animals.

The number of CBir and CRir neurons remained unaltered in all the tested cortical regions (Fig. 3.13A-D). CBir cells could be separated into two distinct neuronal populations, one constituted by weakly labeled cells, particularly numerous in layers 2/3, and a second by relatively rare intensely labeled cells. Analyses were performed focusing on this latter neuronal population since many weakly stained cells were found to be immunonegative for GABA (Gonchar and Burkhalter 1997).
3. RESULTS

**Fig. 3.13 Unaltered number of CB- and CR positive cells post-lesion.**

Immunohistochemical stainings for A) CB and B) CR. The pictures were taken from the same cortical regions as in Fig. 3.9. The graphs show no alteration in the number of C) CB and D) CR positive cells normalized to the total number of neurons (NeuN positive) per region of interest (ROI) post-lesion.

However, an interesting observation was a lesion-induced increase in the intensity of the CR stained neuropil particularly expressed in the cortical layer 1, where many ascending dendritic arborizations of CRir neurons branches off (Gonchar and Burkhalter 1999).
At higher magnification a dense network of CR-immunopositive arborizations was appreciable in layer 1 in coronal sections from the visual cortex ipsilateral and contralateral to the lesion. These layer 1 CR positive arborizations were clearly less visible in the homotopic cortical areas from sham-operated animals (Fig. 3.14A). Densitometric analysis revealed a significant enhancement in the CR staining of the neuropil in layer 1 at 1 mm distance from the border of the lesion as well as in the homotopic region in the contralateral cortex (sham-op: 15.49 ± 1.41 n= 6, ipsi: 22.87 ± 1.48 n= 7, p= 0.007, contra: 22.98 ± 1.28 n= 6, p= 0.005) (Fig. 3.14B).

Fig. 3.14 Enhanced intensity of CR-immunolabeled neuropil post-lesion. A) High magnification of the visual cortex (layer 1), immunolabeled for CR, in a sham-operated and in a lesion-treated animal. The pictures were taken at 1 mm distance from the lesion (ipsi) and in the homotopic region in the contralateral cortex (contra) and in control animal (sham). Note the increased intensity of the stainings and the CR immunoreactive arborizations visible post-lesion. B) Optical density (OD) analysis of the stained neuropil in layer 1 showing a significantly stronger immunoreaction for CR in lesion-treated animals, both ipsilateral at 1 mm from the border of the lesion and in the homotopic area in the contralateral cortex. The final OD values were always obtained by subtracting the background value of the immunonegative white matter from the value of the immunopositive neuropil in the grey matter.
3.2.2 Reduction in the frequency of spontaneous IPSCs post-lesion

In search of the functional correlates of the post-lesion reduced expression of PV and GAD67, spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded at a holding potential of -80 mV (Fig. 3.15A).

The frequency of sIPSCs was found to be dramatically reduced post-lesion at all the recording sites (sham-op: 6.45 ± 0.64 Hz n= 14, ipsi 1mm: 3.86 ± 0.42 Hz n= 10, ipsi 2.5mm: 3.96 ± 0.33 Hz n= 12, contra: 4.18 ± 0.24 Hz n= 12, for all p< 0.01) (Fig. 3.15B-C). The sIPSCs amplitude was only slightly reduced close to the lesion (sham-op: 10.86 ± 0.80 pA, ipsi 1mm: 8.66 ± 0.62 pA, p> 0.05) and remained unaltered at more distal areas (ipsi 2.5mm: 10.38 ± 0.68 pA, contra: 10.11 ± 0.70 pA, p> 0.05) (Fig. 3.15D-E). These spontaneous postsynaptic events were blocked by PTX (50 μM) confirming that they were due to the activation of GABA_A receptors (Fig. 3.15A).
3. RESULTS

**Fig. 3.15** Lesion-induced reduction of sIPSCs frequency. A) Representative traces of sIPSCs recorded at -80 mV from neurons located at different distances from the border of the lesion (see Fig. 3.8B). Homotopic regions were selected to record neurons from sham-operated rats. Bath application of 50 µM picrotoxin (PTX) abolished all signals. B) Cumulative probability plot showing a right shift in the interevent interval of sIPSCs in neurons from lesion-treated animals. C) Summary diagram of the mean sIPSCs frequency. D) Cumulative probability plot showing no significant alteration in the amplitude of sIPSCs, post-lesion. E) Summary diagram of the mean sIPSCs amplitude.
3.2.3 Unaltered GABAergic terminals post-lesion

The decreased frequency of sIPSCs could either reflect a decrease in the number of GABAergic synapses onto pyramidal cells or a reduction in the release probability of GABA at the presynaptic terminals.

The former hypothesis was first tested by evaluating the immunoreactivity for VGAT, used by others as a marker of GABAergic terminals (Minelli A et al. 2003; Merchan-Perez et al. 2009) (Fig. 3.16A). Optical density analysis revealed no significant differences in VGAT expression post-lesion (Fig. 3.16B).

Fig. 3.16 Unaltered VGAT immunoreactivity post-lesion. A) Immunohistochemical stainings for VGAT. The pictures were taken from the same cortical regions as in Fig. 3.9. B) Optical density analysis of the stained-neuropil showing no significant differences in VGAT immunoreactivity in all the tested positions, indicating an unchanged number of GABAergic terminals post-lesion.
In addition, because PV positive basket cells preferentially innervate the soma of pyramidal neurons, the perisomatic GABAergic innervation was also evaluated (Fig. 3.17A-B). This analysis was performed selectively in the cortical area where a reduced PV expression was observed (1mm from the border of the lesion and in the homotopic contralateral hemisphere). The double immunofluorescence labeling with NeuN and VGAT revealed an unchanged perisomatic innervation post-lesion. Quantitative estimations of VGATir on the soma of pyramidal cells confirmed this observation (sham-op: 4.17 ± 0.25% n= 26, ipsi: 4.01 ± 0.32% n= 36, contra: 4.12 ± 0.37% n= 23) (Fig. 3.17C). Here, the value n indicates the number of neuronal soma analysed for each experimental group.
Fig. 3.17 Unaltered perisomatic GABAergic innervation post-lesion. A) Images of double immunofluorescence stainings for VGAT (green) and NeuN (red) in layers 2/3 of the visual cortex taken from a sham-operated and a lesion-treated animal in a region where PV expression was found to be reduced (see Fig.3.9). B) Pictures from the same sections as in A at higher magnification. C) Quantitative estimation of VGAT immunoreactivity on the soma of pyramidal neurons normalized to the size of the body of each neuron.
3.2.4 Decreased initial release probability at GABAergic synaptic terminals post-lesion

Next, the functional properties of GABAergic synapses were studied eliciting inhibitory postsynaptic responses (eIPSCs) by paired-pulses of synaptic stimulations in layer 4.

Neurons located in both the ipsilateral and contralateral hemisphere to the lesion responded to pairs of synaptic stimulations with a large increase in PPR compared to neurons from sham-operated animals (Fig. 3.18A). The increase was highly significant for short ISIs (20-50 ms) (20 ms= sham-op: 0.71 ± 0.05 n= 19, ipsi 1mm: 1.19 ± 0.13 n=17, ipsi 2.5mm: 1.05 ± 0.07 n= 18, contra: 1.11 ± 0.08, for all p< 0.001, 30 ms= sham-op: 0.70 ± 0.05, ipsi 1mm: 1.00 ± 0.09 p= 0.005, ipsi 2.5mm: 1.02 ± 0.07 p= 0.001, contra: 1.14 ± 0.17 p= 0.01, 40 ms= sham-op: 0.68 ± 0.04, ipsi 1mm: 1.14 ± 0.14 p= 0.004, ipsi 2.5mm: 0.98 ± 0.07 p= 0.001, contra: 1.17 ± 0.16, p= 0.003, 50 ms= sham-op: 0.67 ± 0.05, ipsi 1mm: 0.91 ± 0.10 p= 0.033, ipsi 2.5mm: 0.98 ± 0.07 p= 0.001, contra: 0.96 ± 0.08, p= 0.001) and present still up to 100 ms ISI (sham-op: 0.65 ± 0.03, ipsi 1mm: 0.82 ± 0.06, p= 0.016 ipsi 2.5mm: 0.91 ± 0.11, p= 0.023, contra: 0.85 ± 0.06, p= 0.006) (Fig. 3.18B).

![Fig. 3.18 Increased PPR of GABA<sub>A</sub>R-mediated currents evoked by synaptic stimulation in layer 4 post-lesion. A) Representative traces of GABA<sub>A</sub>R-mediated currents recorded at -80 mV evoked by pairs of synaptic stimulations with an ISI of 30 ms. B) Summary diagram of the paired-pulse ratio (PPR) for different ISIs (20 to 200 ms). The higher PPR post-lesion indicated a reduced release probability for GABA.](image-url)
To further investigate the effect of the lesion on the initial release probability, synapses were challenged with a train of 40 pulses at 33 Hz and GABA<sub>A</sub>R-mediated postsynaptic responses were evaluated.

The train of eIPSCs in neurons from sham-operated animals started to decay with the second eIPSC while in neurons from lesion-treated animals the first few eIPSCs (3–5) showed similar amplitude and no clear sign of depression, as expected from synapses with a lower initial release probability (Fig. 3.19A).

Because neurons recorded from different locations post-lesion generated very similar postsynaptic responses, in this experiment all neurons recorded from lesioned-treated animals were pooled together.

The time course of the normalized eIPSCs during the train of 40 pulses generated a curve with a slower rate of depression post-lesion as compared to the control animals. The ratio of the second and third IPSC amplitude to the first one was significantly higher in lesion-treated rats (2<sup>nd</sup>/1<sup>st</sup> IPSC: sham-op: 0.71 ± 0.03 n= 8, all lesion: 0.97 ± 0.06 n= 27, p= 0.001, 3<sup>rd</sup>/1<sup>st</sup> IPSC: sham-op: 0.61 ± 0.05, all lesion: 0.86 ± 0.06, p= 0.008, 4<sup>th</sup>/1<sup>st</sup> IPSC: sham-op: 0.50 ± 0.06, all lesion: 0.74 ± 0.06, p= 0.073) (Fig 3.19B-C).

The RRP was estimated from the linear component of the cumulative IPSCs plot from each cell (Fig. 3.20A). From this value, the probability of any readily releasable vesicles to be released with the first pulse (Pr<sub>RRP</sub>) could be calculated (see Materials and Methods). In line with the previous findings the Pr<sub>RRP</sub> was reduced in lesion-treated animals although not significantly (sham-op: 17.96 ± 1.95 %, all lesion: 13.43 ± 1.42 %, p> 0.05) (Fig. 3.20B).

In addition to changes in the release probability this high frequency repetitive stimulation protocol revealed an increase in the total charge transfer (Q) calculated from the area above the curve defined by the eIPSCs (sham-op: 27.74 ± 4.44 pC n= 7, all lesion: 54.65 ± 5.47 pC n= 26, p= 0.022) (Fig. 3.20C-D). This increase was mainly due to a negative shift in the holding current during the presynaptic stimulation.
3. RESULTS

Fig. 3.19 eIPSCs in response to high frequency presynaptic stimulation. 1. A) Representative traces of GABA\(_A\)R-mediated currents in response to a high frequency repetitive synaptic stimulation protocol (40 pulses at 33Hz). B) Normalized amplitude of GABA\(_A\)R-mediated currents in response to the protocol in A. C) Same graph shown in B highlighting the postsynaptic responses to the first 10 pulses. In this experiment all neurons recorded from lesion-treated rats were pooled together.
**3. RESULTS**

**Fig. 3.20 eIPSCs in response to high frequency presynaptic stimulation, 2.** A) Normalized cumulative amplitude of GABA$_A$R-mediated currents in response to the same protocol as in Fig.3.19. The linear part of the function (derived from the last 10 stimuli) was back extrapolated to the y-axis to obtain an estimation of the size of the readily releasable pool (RRP) (normalized to the amplitude of the first response). B) Estimation of the initial probability of release obtained by dividing the amplitude of the evoked GABA$_A$R-mediated current in response to the first stimulus to the size of the RRP in each neuron. Here, once again, all neurons recorded from lesion-treated rats were pooled together. C) Same traces shown in Fig.3.19A. Here, the total charge transfer is represented by the dashed area above the curve defined by the eIPSCs. D) Diagram showing the total charge transfer of GABA$_A$R-mediated currents during the high frequency presynaptic stimulation. Note the net increase post-lesion.
**3. RESULTS**

**3.2.5 GABA<sub>B</sub> receptors modulated the spontaneous and evoked GABA release in lesion-treated animals**

Potential cellular mechanisms responsible for the lesion-induced decrease in spontaneous and evoked transmitter release at GABAergic terminals could be a reduced presynaptic voltage-dependent Ca<sup>2+</sup> influx and/or an increased K<sup>+</sup> conductance. Both could be mediated by the activation of presynaptic G protein-coupled GABA<sub>B</sub>Rs which have been shown to be present at axonal terminals of both GABAergic and glutamatergic synapses in the rat visual cortex (Gonchar et al. 2001). To disclose whether a tonic activation of presynaptically located GABA<sub>B</sub>Rs could be responsible for the reduced spontaneous GABA release post-lesion, sIPSCs were recorded in presence of CGP55845 (2 μM), a specific GABA<sub>B</sub>Rs blocker.

The mean frequency of sIPSCs, in presence of this blocker, did not change in neurons from sham-operated animals (*sham-op with CGP* = 6.27 ± 0.42 Hz n= 18, *sham-op without CGP* = 6.45 ± 0.64 Hz n= 14). In contrast, CGP55845 dramatically increased the sIPSCs frequency in lesion-treated animals (*p*< 0.001, in all the three locations post-lesion) (**Fig. 3.21A-B**). The mean sIPSCs amplitude was found to be slightly increased in presence of CGP55845 in all the experimental groups (the increase in sIPSCs amplitude was significant at 1 mm ipsilateral to the lesion, *p* = 0.014) (**Fig. 3.21A,C**).

Surprisingly, in presence of CGP55845, neurons located at 1 mm distance from the border of the lesion and in the contralateral intact hemisphere showed a mean sIPSCs frequency significantly higher than neurons from sham-operated animals (*sham-op: 6.27 ± 0.42 Hz n= 18, ipsi 1mm: 8.04 ± 0.66 Hz n= 13, ipsi 2.5mm: 6.49 ± 0.46 Hz n= 12, *p* > 0.05, contra: 8.40 ± 0.84 Hz n= 14, *p* = 0.027) (**Fig. 3.22 A-C**).

In presence of the blocker, the sIPSCs amplitude was similar in all the groups (*sham-op: 12.62 ± 0.84 pA n= 17, ipsi 1mm: 12.20 ± 1.00 pA n= 13, ipsi 2.5mm: 10.72 ± 0.45 pA n= 12, contra: 11.77 ± 0.43 pA n= 14) (**Fig. 3.22 A,D-E**).
3. RESULTS

Fig. 3.21 Increased frequency of sIPSCs in presence of the GABA$_{	ext{B}}$R specific blocker CGP55845 post-lesion. A) Representative traces of sIPSCs recorded in absence (-) (from Fig. 3.15) and in presence (+) of the GABA$_{	ext{B}}$R specific blocker, CGP55845. Traces for each experimental group, are represented next to each other for a clear observation of the effect of the blocker. Summary diagram of sIPSCs B) mean frequency and C) mean amplitude in absence (-) and presence (+) of CGP55845. Note the dramatic increase in the frequency of sIPSCs only in lesion-treated animals.
3. RESULTS

Fig. 3.22 The frequency of sIPSCs in lesion-treated rats is higher than control in presence of CGP55845. A) Representative traces of sIPSCs in presence of CGP55845 (from Fig. 3.21). Bath application of 50 µM picrotoxin (PTX) abolished all signals. B) Cumulative probability plot showing the distribution of the interevent intervals of sIPSCs for the different experimental groups. C) Summary diagram of the mean sIPSCs frequency. D) Cumulative probability plot showing the distribution of the amplitude of sIPSCs. E) Summary diagram of the mean sIPSCs amplitude.
The paired-pulse stimulation protocol was also repeated in presence of CGP55845. The blocker reduced the PPR in neurons from lesion-treated animals to the level of control animals (Fig. 3.23A-B). No change in PPR was observed in neurons from sham-operated rats by application of CGP55845. These findings strongly suggested that tonically active GABA_{B}Rs are responsible for the reduced spontaneous and evoked GABA release post-lesion.

**Fig. 3.23** CGP55845 reduced the PPR of GABA_{A}R-mediated currents in lesion-treated animals to the level of control. A) Representative traces of GABA_{A}R-mediated currents recorded at -80 mV in presence of CGP55845 evoked by pairs of synaptic stimulations with an ISI of 30 ms. B) Summary diagram of the paired-pulse ratio (PPR) for different ISIs (20 to 200 ms). The application of the blocker reduced the PPR of neurons from lesion-treated rats to the level of sham-operated animals.
3. RESULTS

3.2.6 No sign of tonic inhibition post-lesion

A tonic activation of GABA$_B$Rs could be mediated by an increased ambient GABA. A potentially higher concentration of GABA in the extracellular space might be sufficient to cause a persistent activation of extrasynaptic GABA$_A$Rs thereby generating a tonic inhibition expressed as increase in the input conductance of the postsynaptic cell (Farrant and Nusser 2005).

A GABA$_A$R-mediated tonic conductance can be distinguished because it produces a shift in the holding current of a voltage clamped cell. Neurons from lesion-treated animals were voltage clamped at -80 mV and the mean current, together with sIPSCs, was measured in presence of glutamatergic receptor blockers. The use of a high Cl$^-$ concentration in the intracellular solution strongly enhanced GABA$_A$R-mediated currents.

The application of bicuculline (20 μM), an antagonist of GABA$_A$Rs, was followed by the abolishment of sIPSCs. However, this was not accompanied by an outward shift in the holding current in none of the neurons tested from lesion-treated animals (n= 6) as expected if the level of ambient GABA would be sufficient to activate extrasynaptic GABA$_A$Rs (Fig. 3.24).

![Bicuculline](image)

**Fig. 3.24 No sign of GABA$_A$R-mediated tonic conductance post-lesion.** Example trace of GABA$_A$R-mediated currents in a neuron clamped at -80 mV, from a lesion-treated animal. Note that Bicuculline application is followed by a rapid disappearance of sIPSCs which is however not accompanied by a shift in the holding current.
3.3 Lesion-induced shift in the excitation/inhibition balance

The above described findings demonstrated a clear functional impairment in the GABAergic transmission post-lesion. To disclose whether these changes might produce a shift in the excitation/inhibition balance, sEPSCs were recorded at -80mV in presence of PTX (Fig. 3.25A).

Bath application of DNQX (20 µM) abolished all signals indicating that they were mediated by the activity of non-NMDA glutamate receptors. The frequency of these spontaneous events was reduced only in cells recorded at 1 mm distance from the border of the lesion (sham-op: 11.38 ± 1.04 Hz n= 13, ipsi 1mm: 8.14 ± 0.93 Hz n= 16 p< 0.05) possibly due to the degenerated intracortical connections projecting from the injured area, while it was unchanged at more distant locations (ipsi 2.5mm: 12.48 ± 0.95 Hz n= 14, contra: 10.43 ± 0.92 Hz n= 12) (Fig. 3.25B-C). No significant alteration in the sEPSCs amplitude was observed (sham-op: 11.27 ± 0.79 pA, ipsi 1mm: 9.69 ± 0.59 pA, ipsi 2.5mm: 11.33 ± 0.93 pA, contra: 10.22 ± 0.74 pA) (Fig. 3.25D-E).
Fig. 3.25 Moderate and spatially restricted reduction in the frequency of sEPSCs. A) Representative traces of pharmacologically isolated sEPSCs recorded at -80 mV. Bath application of 20 µM DNQX abolished all signals. B) Cumulative probability plot showing the distribution of the interevent intervals of sEPSCs for the different experimental groups. C) Summary diagram of the mean sEPSCs frequency. D) Cumulative probability plot showing the distribution of the amplitude of sEPSCs. E) Summary diagram of the mean sEPSCs amplitude.
3.3.1 Modulation of the spontaneous firing rate post-lesion

The moderate decrease in the spontaneous excitatory synaptic transmission, spatially restricted to the vicinity of the injury, coupled with the extensive and prominent reduction in the spontaneous inhibitory transmission indicated a shift in the excitation/inhibition balance in favor of excitation in a large cortical area surrounding the lesion and in the contralateral hemisphere.

To measure if this lesion-induced shifted balance could result in an increased spontaneous firing rate, neurons were current clamped and spontaneous action potentials were generated by injecting a constant positive current to set the membrane potential of the cells to -50 mV (Fig. 3.26A).

Surprisingly, an increased spontaneous spike frequency was observed only in the hemisphere contralateral to the lesion (sham-op: 3.45 ± 0.36 Hz n= 19, contra: 4.63 ± 0.43 Hz n= 18 p < 0.049) while ipsilaterally, at 1 mm distance, the firing rate was slightly but not significantly reduced and remained unchanged at 2.5 mm distance from the border of the lesion (ipsi 1mm: 2.81 ± 0.51 Hz n= 14, ipsi 2.5mm: 3.54 ± 0.46 n= 18, p > 0.05) (Fig. 3.26B). The histogram in Fig. 3.26C shows the distribution of inter-spike-intervals. A clear peak, ranging between 170 and 200 ms, was distinguishable only in neurons recorded from the contralateral hemisphere.
3.3.2 The lesion prolonged the kinetics of IPSCs selectively in the 
ipsilaterial hemisphere

When looking for the cellular mechanisms responsible for the discrepancy in firing frequency between the two hemispheres (ipsilateral and contralateral to the lesion) one should consider that not only the frequency and amplitude of sEPSCs and sIPSCs, but also the kinetic properties of these postsynaptic events can influence the strength of synaptic transmission by controlling the amount of ionic charge passing through the cell membrane.

The kinetics of AMPA-mediated currents remained unaltered post-lesion (data not shown). However, a prolonged rise and decay time of eIPSCs was observed in neurons located in the cortex ipsilateral to the lesion. The most prominent effect was
expressed at 1 mm distance from the border of the lesion (Fig. 3.27A) but it was still evident at 2.5 mm while a non-significant but still mild effect was observed in the contralateral hemisphere (rise time= sham-op: 2.38 ± 0.25 ms, ipsi 1mm: 3.13 ± 0.29 ms, p= 0.03, ipsi 2.5mm: 3.21 ± 0.27 ms, p= 0.043, contra: 2.88 ± 0.34, p> 0.05, decay time= sham-op: 15.87 ± 1.30 ms, ipsi 1mm: 24.92 ± 2.24 ms, p= 0.02, ipsi 2.5mm: 21.69 ± 2.20 ms, p= 0.055, contra: 18.95 ± 1.76 ms p> 0.05) (Fig. 3.27B-C).

The alteration in the temporal dynamics of eIPSCs, almost exclusively expressed in neurons from the hemisphere ipsilateral to the lesion, correlated well with the lack of increase in the spontaneous firing rate. The prolonged duration of eIPSCs could enhance the inhibitory strength on the postsynaptic cell thereby compensating, at least partially, for the reduced presynaptic GABA release.

**Fig. 3.27 Prolonged kinetics of eIPSCs in the hemisphere ipsilateral to the lesion.** A) Representative traces of GABA$_A$R-mediated currents, recorded at -80 mV, evoked by a synaptic stimulation in layer 4. Note the prolonged rise- and decay-time in the neuron recorded 1 mm lateral to the lesion (dashed trace) compared to sham-operated rats (grey trace). Diagram showing B) the rise time and C) the decay-time constant of these eIPSCs for each recorded neuron and the mean values for each experimental group.
3. RESULTS

To evaluate the impact of the lesion-induced prolonged kinetics of eIPSCs on the strength of the spontaneous inhibitory transmission the ionic charge of sIPSCs was measured.

The charge transfer of sIPSCs (Q) was defined as the area above the curve defined by each sIPSC and the charge transfer was calculated as the median of 30 events for each cell (Fig. 3.28A).

Ipsilaterally close to the lesion Q was very similar to control values indicating that the prolonged IPSC kinetics fairly compensated the reduced events amplitude (sham-op: $6.40 \times 10^{-2} \pm 0.36 \times 10^{-2}$ pC n= 13, ipsi 1mm: $6.57 \times 10^{-2} \pm 0.57 \times 10^{-2}$ pC n= 10). At a more distant location from the border of the lesion, where the amplitude of sIPSCs was not different from control animals, Q was slightly increased (ipsi 2.5mm: $7.47 \times 10^{-2} \pm 0.46 \times 10^{-2}$ pC n= 12, p= 0.09). Finally, in the contralateral hemisphere it was unaltered, as expected from the unchanged sIPSC amplitude and eIPSC kinetics (contra: $5.96 \times 10^{-2} \pm 0.42 \times 10^{-2}$ pC n= 11) (Fig. 3.28B).

These findings could, at least partially, explain the lack of any increase (and even a mild decrease at ipsi 1mm) in the frequency of APs in a neuronal network characterized by an impaired GABA release accompanied by only a mild and localized (ipsi 1mm) reduction of glutamatergic synaptic transmission.

**Fig. 3.28 Charge transfer of sIPSCs.** A) Representative traces of sIPSCs recorded in a neuron from a lesion-treated and a sham-operated rat. The dashed area represents the charge transfer for these spontaneous events. B) Summary plot of the mean sIPSCs charge transfer for each experimental group.
3.3.3 Altered spike threshold and amplitude ipsilaterally to the lesion

The mean membrane potential threshold for spike induction (spike threshold) was found to be significantly shifted to more positive values selectively in the hemisphere ipsilateral to the lesion (sham-op: -42.27 ± 0.40 mV n= 19, ipsi 1mm: -40.13 ± 0.66 mV n= 14, p= 0.008, ipsi 2.5mm: -40.38 ± 0.55 mV n= 18, p= 0.01, contra: -42.00 ± 0.46 mV n= 18, p> 0.05) (Fig. 3.29A-C). This shift in the spike threshold to more positive membrane potentials could lead to a lower frequency of APs because neurons need stronger membrane depolarizations to generate a spike. Indeed, when the spike threshold was plotted against the spike frequency for each recorded cell (from all the experimental groups), the two variables showed a clear negative correlation (r= -0.606 p< 0.000, n= 68) (Fig. 3.29D).
3. RESULTS

Fig. 3.29 Positive shift of the spike threshold in the hemisphere ipsilateral to the lesion. A) Voltage traces representing a single AP in a neuron from a lesion-treated (dashed trace) and a sham-operated rat (grey trace). Note the positive shift in the spike threshold and the reduced spike amplitude post-lesion. B) The picture illustrates the method for calculating spike threshold. In black is represented a single AP and in grey its derivative \((dV/dt)\). After calculating the \((dV/dt)_{\text{MAX}}\) (the peak slope of the rising phase), the membrane potential threshold was defined as the voltage at which 5\% of \((dV/dt)_{\text{MAX}}\) was reached. C) Summary plot of the mean spike threshold for each experimental group. D) Graph showing the correlation between the spike frequency and the spike threshold in each recorded neuron (from all experimental groups).

In parallel, the resting membrane potential was unchanged indicating that in vivo larger depolarizing postsynaptic potentials might be necessary to reach the potential threshold for spike induction (sham-op: -71.12 ± 1.45 mV, ipsi 1mm: -70.53 ± 1.07 mV, ipsi 2.5mm: -70.43 ± 0.70 mV, contra: -70.61 ± 0.64 mV, p> 0.05) (Fig. 3.30D). The mean spike amplitude was also significantly reduced ipsilateral to the lesion (sham-op: 100.28 ± 1.79 mV, ipsi 1mm: 94.47 ± 1.64 mV, p= 0.031, ipsi 2.5mm: 93.69 ± 1.88 mV, p= 0.02, contra: 98.68 ± 1.99) (Fig. 3.30B) and the mean spike...
half-width was slightly larger (sham-op: 1.39 ± 0.04 ms, ipsi 1mm: 1.48 ± 0.07 ms, ipsi 2.5mm: 1.57 ± 0.06 ms, contra: 1.35 ± 0.05 ms) (Fig. 3.30C).

Fig. 3.30 Decreased spike amplitude in the hemisphere ipsilateral to the lesion. A) The trace represents a single AP and illustrates how the spike amplitude and the spike half-width were measured. Summary plot of B) the mean spike amplitude, C) the mean spike half-width and D) the mean resting membrane potential for each experimental group.
### Table 2. Summary table of the lesion-induced electrophysiological changes.

The table resumes the most important functional alterations observed in the different groups of lesion-treated animals compared to sham-operated animals. A dash line (–) indicates no change in the measured parameter (left column), upward and downward arrows indicate an increase or a decrease, respectively. Two arrows indicate highly significant changes (p < 0.01).

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<th>exp.</th>
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4. Discussion

4.1 Lesion-induced metaplasticity at horizontal connections in layers 2/3

In the first part of this study Hebbian forms of synaptic plasticity in the cortex ipsilateral to a focal laser lesion were investigated. The modification in the efficacy of vertical synaptic connections projecting onto layers 2/3 has been already described in different cortical lesion models (Hagemann et al. 1998; Mittmann and Eysel 2001) and it seems to be an important mechanism for the functional reorganization of surviving cortical areas in the surround of a focal brain injury. However, whether lesion-induced plastic changes occur at intracortical horizontal connections, was not yet investigated.

Here, an enhanced LTP and reduced LTD of L2/3-L2/3 synapses was observed at horizontal connections in the vicinity of the lesion. This result indicated a lesion-induced shift toward strengthening of these synapses disclosing for the first time the metaplastic capacity of these intracortical horizontal fibers at the border of a focal injury (Fig. 4.1).

Fig. 4.1 Hypothetical model of lesion-induced metaplasticity at horizontal connections.

The hypothetical curve, of which only two stimulation frequencies have been tested (TBS, LFS), represents the variation of synaptic strength in relation to the applied stimulation frequency. The curve might be shifted to the left post-lesion (modified from Bienenstock et al. 1982).
In general LTP and LTD have been well studied at synapses of ascending fibers in layers 2/3 of the visual cortex (Artola and Singer 1987; Kirkwood and Bear 1994; Mittmann and Eysel 2001; Barmashenko et al. 2003). A few studies reported that synaptic plasticity can also be induced at horizontal connections in superficial cortical layers in the rat motor (Hess et al. 1996) and sensory cortex (Marik and Hickmoon 2009).

This study provided evidence that these intracortical fibers in rat visual cortex are also able to undergo synaptic metaplastic changes in the context of a focal brain injury. Normally, the strength of the postsynaptic response determines whether a synapse undergoes depression or facilitation. Furthermore, the threshold defining whether LTP or LTD is evoked has been reported to vary as a function of the history of a synapse (Bienenstock et al. 1982). For example, in the visual cortex it has been shown that a reduced visual input (e.g. under conditions of light deprivation) shifted this threshold in favour of LTP induction (Kirkwood et al. 1996).

The present data suggest that the threshold for LTP induction at intracortical fibers in layers 2/3 might be also shifted post-lesion. This could explain why the relatively weak LFS protocol reliably induced a depression of EPSPs in sham-operated animals while it led to a bidirectional change in EPSPs (with some neurons responding with LTP and other with LTD) in lesion-treated animals, and why the high-frequency stimulation (TBS) gave rise to an increased level of LTP post-lesion.

Looking for pre and postsynaptic changes which might account for these results, a moderate prolonged decay time constant of NMDAR-mediated currents was found to accompany these metaplastic changes.

The slower decay-time constant of NMDARs could be mediated by changes in the subunit-composition of NMDARs, especially through a relative increase of NMDARs containing the NR2B subunit (Monyer et al. 1994; Flint et al. 1997). This resembles the developmental status of a more juvenile brain, where neuronal networks show relatively high plastic properties. In line with this, a previous study from our laboratory showed a lesion-induced enhancement of LTP at synapses of ascending fibers projecting from layer 4 to layers 2/3. Here the increased LTP could be reduced to the level of control animals by application of ifenprodil, a specific blocker of NMDARs containing the NR2B subunit (Huemmeke et al. 2004).

However, other mechanisms might also contribute to the lesion-induced metaplastic shift.
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Although no functional changes of eIPSCs were observed during paired or repetitive presynaptic stimulations at the horizontal connections, an impaired GABAergic transmission expressed at ascending fibers could still indirectly influence the induction of LTP/LTD at horizontal connections. Local interneurons mediating recurrent inhibition in layers 2/3 are most likely activated during the TBS/LFS-induced postsynaptic firing. These GABAergic cells, projecting back to the recorded neuron, could modulate the postsynaptic response during TBS/LFS, thereby potentially influencing synaptic plasticity induction. Future challenging studies are necessary to investigate this hypothesis.

It should be pointed out that most of the long-range horizontal connections do not follow the coronal plane of brain slices and therefore they were truncated during the slice preparation. This limitation however did not prevent to study the synaptic strength of the remaining lateral fibers in a cortical area which has been shown to be functionally altered up to 4 mm from the border of the injury (Barmashenko et al. 2001).

Taken together, the facilitated synaptic plasticity, which was previously observed at ascending and presently at lateral projections could be both essential to guarantee a proper cortical rewiring post-lesion.

Anatomical studies conducted in cat and tree shrew visual cortex emphasized the exuberance of horizontal connections leading to the conclusion that not all, but only a defined subset of inputs activate a target cell in response to a specific visual stimulation (Gilbert and Wiesel 1979; Rockland and Lund 1982). Therefore, horizontal intracortical fibers seem to constitute a very flexible framework offering numerous connections with variable weights, the modification of which could have a strong impact on the processing of visual inputs. Assuming a similar distribution of intracortical connections in rat visual cortex, some subthreshold horizontal inputs could be unmasked, upon visual stimulation, by the described facilitated synaptic potentiation.

4.2 Functional changes of GABAergic transmission post-lesion and the underlying cellular mechanisms

Focal cortical lesions have been shown to cause functional alterations extending in a widespread brain area and even in the contralateral undamaged hemisphere. These
lesion-induced functional changes were attributed, in many studies, to an impaired GABAergic inhibition (Buchkremer-Ratzmann et al. 1996; Reinecke et al. 1999). This might have a strong implication for brain functions, since the GABAergic system provides the main source of inhibition in the mammalian brain.

The lesion-induced deficit in GABAergic transmission could cause serious consequences in the cortical information processing and it has been proposed to be implicated both in the long-term functional recovery post-lesion, by offering higher plastic properties to the surrounding uninjured tissue, but also in the generation of hyperexcitable networks potentially leading to the generation of epileptic activity (Neumann-Haefelin et al. 1995).

The GABAergic system is however extremely heterogeneous, being composed by diverse interneuron cell types that display distinct morphological, electrophysiological and biochemical features which most likely attribute class-specific functional properties (Petilla Interneuron Nomenclature Group 2008).

Therefore, it is important to understand whether the lesion affected differentially distinct subpopulations of GABAergic cells and to define the cellular mechanisms responsible for the reduced efficacy of GABAergic transmission.

4.2.1 Immunohistochemical characterization of the GABAergic system in the cortical regions surrounding the lesion

In order to answer these questions immunohistochemical analyses were performed to evaluate the spatial profile of the expression of different interneuronal markers post-lesion.

Distinct subpopulations of GABAergic cells can be characterized by the expression of distinct biochemical markers, such as the calcium-binding proteins parvalbumin, calbindin and calretinin and the neuropeptides neuropeptide Y, vasoactive intestinal peptide, somatostatin and cholecystokinin (for review see Markram et al. 2004).

Potential lesion-induced alterations in the expression of these markers could give an indication of the effect of the lesion on distinct interneuronal populations.

The major outcome was a net decreased of GAD67 positive neurons affecting a large cortical area lateral to the lesion and extending into the contralateral hemisphere.

GAD67 is the major isoform of the GABA-synthesizing enzyme, it is constitutively active and responsible for over 90% of the basal GABA synthesis (Asada et al. 1997).
Interestingly, the expression of GAD67 is the limiting factor for the production of GABA and its transcription is activity dependent (Huang 2009). The observed decrease in the number of GAD67ir neurons could therefore be due to a reduced activity of this cell population. Under this potential circumstance the expression of the enzyme might fall under the threshold for being detected with the used staining procedure thereby diminishing the number of cells identified as positive. As alternative hypothesis, the degeneration of a particularly vulnerable subpopulation of interneurons can not be excluded. However, since the effect was observed across a large visual cortical area and even in regions remote to the primary lesion (in the contralateral hemisphere), it was considered very unlikely.

The expression of PV, CB and CR was also evaluated. These three calcium-binding proteins were chosen for two reasons. First, because they label a largely nonoverlapping interneuronal population (Gonchar and Burkhalter 1997) and second because by buffering intracellular calcium they could strongly influence important neuronal functions.

Since in rat visual cortex, as in many other cortical areas, PV positive cells constitute about half of the GABAergic neurons, the higher number of PVir cells, compared to the number of GAD67ir cells (which should be expressed in all GABAergic neurons), for the same ROI could be at first glance a paradoxical outcome. However this might be due to the fact that many interneurons express GAD67 below the threshold level for detection with the used staining procedure.

Among the three calcium-binding proteins tested only the number of PV positive cells was found to be decreased post-lesion. Once again, this effect was observed in both visual cortical hemispheres although in a more restricted region compared to GAD67. A previous histological study, on a photochemically-induced infarct model in the somatosensory cortex of rats, already revealed a reduced number of PVir neurons in the immediate vicinity of the lesion. The shrunken soma and the greatly reduced number and length of labeled dendrites and axons of the few PV stained cells led to the conclusion that this interneuronal subpopulation was degenerating as a consequence of the lesion (Neumann-Haefelin et al. 1998). In this study however the presence of intact PNs post-lesion argues against a neuronal degeneration hypothesis. PNs are, in fact, known to envelope almost exclusively this type of GABAergic cells (Kosaka and Heizmann 1989). Thus, it is very likely that the observed phenotype was due to a reduced expression of PV from intact cells.
Because PV has an important role in buffering intracellular Ca\textsuperscript{2+}, a reduced level of this protein might lead to an impaired Ca\textsuperscript{2+} homeostasis with consequent disturbances in different neuronal functions. However, a reduced PV level could just reflect a functional suppression of this interneuronal population which would then require less Ca\textsuperscript{2+} buffering due to the diminished neuronal activity.

Neuronal activity indeed was found to modulate PV expression in different studies. In rat visual cortex the expression of PV is developmentally regulated, and it has been shown to be influenced by visual input (Cellerino et al. 1992). Furthermore, in organotypic culture prepared from newborn visual cortex, cortical activity and TrkB ligands were found to be essential to trigger PV expression (Patz et al. 2004). A potential modified cortical activity, as a consequence of the lesion, might be therefore responsible for the PV downregulation.

The lesion effect on PV expression was found to be relatively variable among different animals without any clear correlation with the lesion size. In some sections the reduction in PV expression was more strongly expressed in layer 4 and in the supragranular layers 2/3 (more accentuated in layer 3) but in others it seemed to be homogeneously distributed throughout all cortical layers. The reduction in GAD67ir cells followed a similar distribution. Indeed, a positive correlation between the number of PVir and GAD67ir neurons in adjacent sections was found, indicating a probable relationship in the expression of the two markers. This finding, however, does not exclude that the GAD67 expression could be altered in other interneuronal subclasses as well.

Which are the consequences of a reduced PV expression on the functions of cortical neuronal networks is still an open question. However, based on the electrophysiological properties, the morphology and the preferential innervation on selective membrane subdomains of the target cells, some considerations can be done.

PV expressing cells are morphologically classified in basket cells, which mainly send axon endings onto the soma and the proximal dendrites of principal neurons, and in the axon-targeting chandelier cells, which form characteristic short vertical rows of boutons, resembling a chandelier (also known as cartridges), on the axons of the target cells (for review see Markram et al. 2004). The innervation of these interneurons is therefore strategically positioned to control the output of the target cells. In particular, axon endings of chandelier cells are ideally suited to control the generation and timing of action potentials allowing the synchronization of firing of
4.DISCUSSION

large groups of principal cells (Freund 2003). This salient feature, together with their characteristic fast-spiking behavior, has suggested this cell type to be involved in the generation of gamma oscillations (30 to 80 Hz), hypothesis recently proven by an optogenetic study (Sohal et al. 2009). Since gamma oscillations seem to be critically important in the cortical information processing, a dysfunction or reduced function of PVir cells might have severe consequences on the cortical activity.

Another distinctive feature of PVir cells is the envelope of PNs. Beyond being involved in structural functions, like synapses stabilization and neuroprotection (Karetko and Skangiel-Kramska 2009), the PNs might serve to locally buffer cations accumulating in the extracellular microenvironment, close to the cell membrane, thereby allowing PV expressing cells to establish their fast firing phenotype (Haertig et al. 1999; Hensch 2005).

The lack of any observable degradation of the PNs post-lesion could guarantee on the one side the maintenance of the firing properties of these cells, but, on the other side, only recordings from these neurons could provide direct evidence of potential alterations in their physiological properties.

Finally PVir cells seem to be critical to define the opening and closure of the critical period for ocular dominance plasticity (Fagiolini et al. 2004). An altered function of this subset of neurons might therefore greatly influence cortical plasticity.

In contrast to the lesion-induced PV reduction, the number of CB and CRir neurons did not show any alteration post-lesion.

Although no difference was found in the number of positive cells, CR labeled sections showed a significant more intensively stained neuropil in layer 1 both in the hemisphere ipsilateral and contralateral to the lesion.

Anatomical studies showed that CR positive neurons, located in layers 2/3 of rat visual cortex, send vertical oriented ascending dendrites. Many ascending dendritic branches extend in layer 1 where they can run for long distances (> 1 mm in adult rats) parallel to the pial surface (Gonchar and Burkhalter 1999). The increased staining of the neuropil in layer 1 could therefore indicate either an increase CR expression in the dendrites of these interneurons or the growth of new dendritic arborizations post-lesion. The latter hypothesis may imply an increased synaptic input onto this subpopulation of interneurons.
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In contrast to other type of interneurons, the majority (68%) of CR positive axonal terminals in layers 2/3 form synapses with other GABAergic neurons (Gonchar and Burkhalter 1999), regulating and coordinating their activity (Caputi et al. 2009) and finally exerting a disinhibitory action on principal cells. Potential functional alterations of CRir cells, which might accompany these structural changes, could therefore lead to complex alteration in the function of neuronal circuits.

The expression of somatostatin positive cells, which constitute another relative large subpopulation of interneurons, accounting for roughly 17% of all GABA expressing cells in rat visual cortex, was not investigated. However a large fraction of this biochemical-distinct cell population has been reported to co-express CB (Gonchar and Burkhalter 1997).

4.2.2 Lesion-induced drop in the frequency of sIPSCs

The spontaneous GABAergic transmission was found to be drastically reduced post-lesion, as reflected by the drop in the frequency of sIPSCs. The effect was visible at all recording positions post-lesion and matched the reduced GAD67 expression revealed by immunohistochemistry. The unchanged immunoreactivity for VGAT, used as marker of GABAergic synaptic terminals argued against a decreased GABAergic innervation as responsible for the decreased frequency of these spontaneous events. Because the maturation of perisomatic GABAergic innervation, during development, has been shown to depend on neuronal activity and visual inputs (Chattopadhyaya et al. 2004), most probably through a modulation of GAD67-mediated GABA synthesis (Chattopadhyaya et al. 2007), the lesion could have caused a selective reduction of perisomatic innervation. However, the level of perisomatic VGAT immunoreactivity, in sections from lesion-treated animals, was comparable to sham-operated controls ruling out this hypothesis.

4.2.3 Reduced initial release probability of GABA post-lesion

Another possible explanation for the low level of spontaneous GABA release could be a lower initial release probability of GABA (Pr).

To assess changes in Pr, pairs of presynaptic stimulations were applied at different ISIs and isolated postsynaptic GABAergic currents were evaluated. Neurons from
sham-operated animals responded with a paired-pulse depression (PPD) (PPR< 1) to all the tested ISIs. This form of short-term synaptic depression at GABAergic synapses of layers 2/3 pyramidal cells has been already described by others (Morales et al. 2002) in the visual cortex of juvenile animals. PPD is normally observed at synapses with high release probability and it is commonly attributed to the depletion of the readily releasable pool with a consequent decreased transmitter release upon arrival of the second pulse (Thomson 2000). Thus, the reduced PPD was interpreted as a reduced Pr for GABA.

Nevertheless it is worth pointing out that although PPD is commonly attributed to a presynaptic mechanism, the saturation or desensitization of postsynaptic receptors could also contribute (Jones and Westbrook 1995). It is however unlikely that the drastic increase in PPR post-lesion could be attributed to a reduced desensitization-saturation of postsynaptic receptors; if these postsynaptic mechanisms would account for the observed PPR, increasing the ISI duration (giving more time to GABAARs to recovery from desensitized state) should rather result in a progressively higher PPR instead of a lower, as reported here.

Finally PPR is strongly influenced by Ca\(^{2+}\) ions entering the axonal terminal with the first stimulus which might still be present upon arrival of the second AP. This residual Ca\(^{2+}\) will then increase transmitter release in response to the second stimulus. Remarkably, differences in local Ca\(^{2+}\) buffering might modify the residual Ca\(^{2+}\) concentration thereby leading to a variable PPR. An interesting study showed that in PV knockout mice the PPD, normally observed at GABAergic synapses of Purkinje cells in the cerebellum, was converted into paired-pulse facilitation (PPF). The slow buffering properties of PV indicate that this calcium-binding protein will be inefficient in reducing the fast peak Ca\(^{2+}\) transient following the invasion of an AP in the axonal terminal but it might accelerate the rate of decay of this transient diminishing the residual Ca\(^{2+}\) when a second AP comes. The observed PPF in PV knockout animals was therefore attributed to an increase residual Ca\(^{2+}\) due to the lost Ca\(^{2+}\) buffer (Caillard et al. 2000).

The observed lesion-induced reduction in PV expression could be therefore potentially responsible for the increased PPR. Although this assumption can not be excluded it would not easily explain the reduced frequency of sIPSCs.
4. DISCUSSION

The initial probability of release of any given vesicles in the RRP \( (Pr_{RRP}) \), which is independent of residual \( \text{Ca}^{2+} \), was also found to be reduced post-lesion, further arguing for a lower initial probability of GABA release.

A similar decrease in release probability for GABA is normally observed in the first postnatal weeks in rodents. However, this developmental change is coupled with a progressive increase in the number of synaptic contacts (Morales et al. 2002).

The decreased \( Pr \) for GABA post-lesion was instead coupled with an unaltered number of synaptic contacts leading to a general reduction in the GABAergic strength.

GABA\(_A\)-mediated currents evoked by high frequency synaptic stimulation showed a reduced rate of depression post-lesion. However, this result can not be directly interpreted as an increase GABAergic transmission. The IPSCs evoked by subsequent stimuli are normalized to the size of the first eIPSC amplitude (see Fig. 3.19B-C).

Therefore, the increased ratio between the amplitude of subsequent IPSCs to the first IPSC is most probably due to the reduced initial release probability post-lesion and not to an enhanced GABAergic transmission.

Finally, in the rat neocortex synaptic connections formed by anatomically and electrophysiologically distinct interneuronal subpopulations onto pyramidal cells have been shown to give rise to highly heterogeneous postsynaptic responses. Indeed, GABAergic synapses can show different degrees of synaptic depression or facilitation depending on the anatomical and physiological properties of both the pre- and postsynaptic cell (Gupta et al. 2000). Therefore, changes in the temporal dynamics of GABA\(_A\)-mediated currents upon repetitive stimulations might also result from a differential contribution of specific interneuronal subpopulations in generating these postsynaptic responses.

In summary, the focal lesion led to a functional impairment of spontaneous GABAergic transmission in a large cortical area around the lesion as well as in the contralateral hemisphere, which does not seem to be related to a reduction in the number of GABAergic synapses onto principal neurons but rather to a reduced probability of transmitter release from the presynaptic terminals. Whether this was triggered by a functional suppression of GAD67 and PV positive neurons remains an open question.
4.2.4 A tonic activation of presynaptic GABA\textsubscript{B}Rs is likely responsible for the reduced spontaneous and evoked GABA release

Presynaptic G protein-coupled GABA\textsubscript{B}Rs have been suggested to dynamically regulate GABAergic and glutamatergic transmission. They mainly act by decreasing Ca\textsuperscript{2+} currents, thereby reducing transmitter release (GABA\textsubscript{B}Rs are also known to increase K\textsuperscript{+} conductance, however this mechanism is predominantly used at the postsynaptic site) (Misgeld et al. 1995).

The function of presynaptic GABA\textsubscript{B}Rs might be to establish a local negative feedback by reducing GABA release when the local concentration of GABA is high. Nevertheless, a tonic inhibition of constitutive GABA release by persistent activation of GABA\textsubscript{B}Rs is still controversial and might depend on the investigated brain area (Rohrbacher et al. 1997).

In the present study, bath application of CGP55845 (2 \(\mu\)M), a specific blocker of GABA\textsubscript{B}Rs, did not alter the frequency and increased only slightly the amplitude of sIPSCs in sham-operated animals. The PPR of eIPSCs was also unchanged indicating that under physiological conditions GABA\textsubscript{B}Rs are not constitutively active in the rat visual cortex.

In contrast, a drastic increase in the frequency of sIPSCs together with the reduced PPR of eIPSCs was observed in lesion-treated animals in presence of the GABA\textsubscript{B}Rs blocker. This result suggested a lesion-induced tonic activation of presynaptic GABA\textsubscript{B}Rs.

The tonic activation of presynaptic GABA\textsubscript{B}Rs depends on the level of ambient GABA (Laviv et al. 2010). It is therefore likely that an increase in the extracellular concentration of GABA post-lesion might be responsible for this effect.

Commonly, an increased ambient GABA is attributed to a higher ongoing neuronal activity (through synaptically released GABA) or to the activity of GABA transporters operating in the reverse mode and thereby releasing, instead of uptaking GABA. In this regard, a recent work reported that, even under physiological conditions, the reversal potential of GAT-1 (the predominant neuronal GABA transporter localized at GABAergic axons and terminals) is very close to the normal resting potential of neurons (Wu et al. 2007).

Furthermore, because the translocation of GABA is coupled to 2 Na\textsuperscript{+} and 1 Cl\textsuperscript{−} (Lu and Hilgemann 1999), alteration in the intracellular (or extracellular) concentration of
these two ions will also shift the reversal potential of GAT-1 to more depolarized or hyperpolarized potentials. A lesion-dependent depolarization of the resting membrane potential or an increase in the intracellular concentration of these ions at axonal terminals might therefore promote the transporter to operate in the reverse mode. The post-lesion prolonged decay time constant of GABA\textsubscript{A}Rs mediated currents (although spatially confined to the ipsilateral hemisphere) as well as the increased total charge transfer of eIPSCs during high frequency stimulations could also be due to the same phenomenon. The glial GABA transporter subtypes, GAT-2 and GAT-3, could also contribute to the elevation of extracellular GABA by working in the reverse mode (Heja et al. 2009). Interestingly, the application of CGP55845 (2 $\mu$m) raised the mean frequency of sIPSCs not only up to the level of control animals but even to a significantly higher value at 1 mm distance from the lesion (\textit{ipsi 1mm}) and also in the homotopic area in the contralateral cortex (\textit{contra}). This surprising result can not be explained by an increased number of GABAergic synapses (previously masked by the reduced probability of transmitter release) because immunohistochemical stainings showed no alteration in VGAT expression. An even higher release probability of GABA could be therefore unmasked by blocking GABA\textsubscript{B}Rs post-lesion.

### 4.2.5 Absence of tonic inhibition post-lesion

A tonic elevation of ambient GABA can also cause a persistent activation of extrasynaptic GABA\textsubscript{A}Rs leading to a tonic inhibitory conductance in the postsynaptic neurons (Farrant and Nusser 2005). However, the present study failed to show any tonic GABA\textsubscript{A}Rs-mediated currents post-lesion. Nevertheless, an increased ambient GABA could still take place but it might be limited to a spatially restricted domain enough to activate presynaptic GABA\textsubscript{B}Rs but not extrasynaptic GABA\textsubscript{A}Rs (\textbf{Fig. 4.2}).
In conclusion, these results strongly suggest a tonic activation of presynaptic GABABRs, which functionally impaired the spontaneous GABAergic transmission post-lesion.

The constitutive activation of these receptors might be due to an increased extracellular concentration of GABA. Future experiments should explore potential alterations in the function of the GABA transporters (GAT-1 and GAT-2/3) as potential source of extracellular GABA.

Nevertheless, one can not exclude a higher affinity of GABABRs for GABA or a higher expression of presynaptic GABABRs as responsible for these results. An increase in the binding density of [3H]baclofen (a GABABRs agonist) observed in a previous autoradiography study in a model of cerebral ischemia (Que et al. 1999a) supports this last hypothesis.
4.3 Interneuronal diversity

The above described findings provide new insights into the cellular mechanisms responsible for the impaired GABAergic transmission which characterizes neuronal networks in the surround of focal brain injuries.

The importance of this study arises from the complexity of the GABAergic system in the mammalian brain.

GABAergic neurons are extremely heterogeneous in term of their morphological, physiological, molecular and synaptic characteristics. Based on these features, interneurons are divided into different subclasses, each with a potentially singular functional role. Exploring the effect of a focal cortical lesion on diverse interneuronal subpopulations is therefore fundamental to disclose the consequences of the injury on different cortical neuronal functions.

Furthermore the post-lesion reduction of intracortical GABAergic inhibition seems to share both beneficial (increasing plastic properties of neurons) and detrimental effects (abnormal neuronal activity and epileptic seizures). The discrimination of the cellular mechanisms or the interneurons subclasses mediating one or the other effects, if similarly expressed in human cases of brain injuries, could have, one day, a clinical relevance in the choice of a targeted therapy.

4.4 Beneficial and detrimental effect of reduced intracortical inhibition

It has been proposed that the reduced strength in the GABAergic transmission observed post-lesion could lead to the recovery from functional deficits by unmasking preexisting, subthreshold connections and by promoting neuronal plasticity and consequently the reorganization of cortical circuits.

In this regard, the post-lesion observed reduction in the number of PV expressing cells and the lower sIPSCs frequency resembles a developmentally more juvenile status characterized by more immature but highly plastic cortical networks.

In the visual cortex, during the first postnatal weeks, defined levels of a developing intracortical inhibition have been shown to control the onset and the closure of the critical period for ocular dominance plasticity. The slowly increasing strength of inhibitory transmission is suggested to cross two thresholds during its maturation: crossing the first one starts a period characterized by high ocular dominance plasticity while the second closes the time window for plasticity (Feldman 2000; Hensch 2005).
The reduced strength of GABAergic transmission post-lesion might therefore match an ideal level of inhibition normally found in the critical period thereby enhancing the remodeling capacity of the surrounding cortical networks. However, because the animals used in this study were still going through the critical period, it is difficult to draw a final conclusion on this topic. Nonetheless, manipulations of sensory inputs have been shown to shift the critical period and the maturation of the GABAergic system in young animals (Mower 1991, Morales et al. 2002). Therefore, it is likely that the cortical lesion induced in these juvenile rats could cause similar processes by modulating neuronal activity in response to peripheral inputs. The involvement of the GABAergic system in the formation of functional cortical circuits on the basis of sensory experience is also well established. In the visual cortex, the maturation of intracortical inhibition strongly contributes to the development of visual acuity and it is involved in shaping cortical receptive fields in a visual experience-dependent manner (Fagiolini et al. 1994). Although these observations highlight the significance of the GABAergic system in mediating critical period-dependent plastic processes a question remained: how can the strength of the GABAergic inhibition critically regulate the plasticity in cortical networks?

As early as in the 1987 Artola and Singer (Artola and Singer 1987) proposed that strong inhibitory synapses could prevent NMDARs activation, indispensable for many forms of synaptic plasticity, by reducing excitatory postsynaptic currents. Shortly afterwards, Kirkwood and Bear (Kirkwood and Bear 1994) also suggested that the mature inhibitory circuitry in the cortical layer 4 might act as a kind of filter by limiting the activity pattern able to gain access to layers 2/3 from subcortical structures. This theory explained why the authors were able to induce LTP in layers 2/3 of the visual cortex, by stimulation of the white matter only when GABAergic inhibition was partially pharmacologically suppressed. A reduced/immature GABAergic transmission might therefore act as a permissive substrate allowing sensory experience to remodel structure and function in cortical networks. Recently, PV positive basket cells have been suggested to contribute to the critical period-dependent plasticity. Their fast somatic inhibition could filter the APs able to access the dendritic arbor by back-propagation thereby allowing postsynaptic spikes to meet presynaptic inputs within specific temporal windows appropriate for synaptic
plasticity induction (Bi and Poo 2001). Furthermore PV positive basket cells, being electrically coupled through gap-junctions, are able to detect strong synchronous activity arriving in the cortex, which normally carries relevant information from the periphery (Galarreta and Hestrin 2001). These interneurons are therefore well suited to produce competitive outcome by reinforcing relevant and favoring the elimination of irrelevant connections based on the sensory experience (Hensch 2005).

The modification of the functional properties of PVir cells, which is likely to occur during development as well as a consequence of an altered neuronal activity, might be crucial in the regulation of these plastic processes. Based on these assumptions, the lesion-induced decreased GAD67 and PV expression, together with an impaired synaptic GABAergic transmission, observed in the present study, might be important to guarantee a proper functional reorganization of neuronal circuits in the surrounding cortical area.

It is important to point out that the reduced release of GABA was found to be exclusively functional and not due to a reduced GABAergic innervation. In contrast, during development, cortical activity is believed to shape neuronal circuits by regulating axonal growth and synaptic formation, therefore GABAergic synapses formation might be crucial for the maturation of functional cortical networks. In particular, the increasing number of GABAergic synapses in rat visual cortex during the first postnatal weeks correlate well with the decline of the critical period for visual cortical plasticity (Morales et al. 2002).

Nevertheless, a simple pharmacological reduction of inhibition, which is supposed to functionally reduce intracortical inhibition, has been shown to be sufficient to reconstitute ocular dominance plasticity in the visual cortex of adult animals (Harauzov et al. 2010).

Unfortunately, the potential beneficial effects of a reduced inhibition are often accompanied by the development of abnormal neuronal activity eventually leading to the generation of seizures frequently observed in human cases of focal brain injury. Recent genetic and pharmacological manipulations in the mouse visual cortex proposed the fundamental role of PV positive cells in controlling both this positive and negative effects. However, while basket cells seem to be essential for the induction of ocular dominance plasticity, chandelier cells selectively controlling discharges of principal cells might modulate the excitability of the tissue (Fagiolini et
Future studies are necessary to further explore the effect of the lesion on the activity of these interneuronal subclasses. In the present study seizures were never observed in lesion-treated animals. It might be possible that hyperexcitability did not reach a threshold to trigger observable seizures. The reduced release probability of GABA could also modify neuronal network functions \textit{in vivo} without necessarily inducing abnormal excitability. Upon repetitive stimulations, at relative high frequencies (normally occurring \textit{in vivo}), synapses with a lower initial release probability (observed in lesion-treated animals) show a slower rate of depression, while synapses with a higher initial release probability (observed in sham-operated rats) depress faster with subsequent stimuli (see Fig. 3.19B). This difference in short-term plasticity might critically influence the processing of incoming inputs but it will not necessarily lead to hyperexcitability. In particular the decreased short-term depression of eIPSCs post-lesion, if not coupled with significant changes in short-term plasticity at eEPSCs, will cause an increased amplification of afferent signals (due to the larger transient unbalanced between excitatory and inhibitory signals). This amplification will be however restricted in a more narrow temporal window because subsequent excitatory inputs will be better counterbalanced by less depressed inhibitory synapses (Varela et al.1999). Furthermore, the prolonged kinetics of GABA\textsubscript{A}R-mediated currents together with the higher total charge transfer upon repetitive synaptic stimulations, independently of their origin, could shunt depolarizing inputs more efficiently and for a longer time window, thereby decreasing the probability of generating hyperexcitable neuronal circuits.

\subsection*{4.5 Excitation-inhibition unbalance post-lesion}

In the cerebral cortex the fine-tuned balance between excitation and inhibition is essential to guarantee a proper function of cortical circuits. The profound decline in the spontaneous synaptic GABAergic transmission expressed both in the hemisphere ipsilateral and contralateral to the lesion was not fully counterbalanced by the slightly and spatially restricted reduced spontaneous excitatory transmission. This strongly indicated a perturbation of this balance in favor of excitation.
4.DISCUSSION

Based on these results it was surprising to observe that only neurons from the contralateral intact cortex revealed an enhanced spontaneous firing frequency compared to sham-operated animals, while ipsilaterally even a slight reduction was recorded at 1 mm from the border of the lesion.

In search of additional functional alterations which could explain this discrepancy, prolonged kinetics of eIPSCs as well as a positive shift in the spike threshold were found to affect exclusively the hemisphere ipsilateral to the lesion.

4.5.1 Prolonged kinetics of eIPSCs in the hemisphere ipsilateral to the lesion

The prolonged time course of eIPSCs observed predominantly in the hemisphere ipsilateral to the lesion could have both pre- or postsynaptic origins. At the presynaptic site the GABA uptake blockage has been shown to prolong the decaying phase of IPSCs in hippocampal neurons (Draguhn and Heinemann 1996) while at postsynaptic site the subunit composition of synaptic GABA_ARs (Barberis et al. 2007) and the phosphorylation state of the receptor subunits (Jones and Westbrook 1997) have also been reported to control the kinetics of these inhibitory currents. Independently of the origins, the observed prolonged kinetics of evoked IPSCs could have profound influences on the neuronal network excitability. The increased duration of these inhibitory postsynaptic events will prolong the temporal window during which incoming excitatory inputs will be less effective in depolarizing the postsynaptic cell and therefore in generating APs. sIPSCs did also show an increased charge transfer in the hemisphere ipsilateral to the lesion. Therefore, it is likely that the altered kinetics of IPSCs could be responsible, at least in part, for the lack of any increase in the firing frequency in a tissue characterized by a shifted balance between excitation and inhibition.

Initially neuronal spikes should depend on the spatio-temporal pattern of spontaneous synaptic transmission as well as on some intrinsic properties of the neurons. However, because of the recurrent structure of cortical networks the situation was more complex once the recorded cell started to fire APs. From that moment on recurrent excitatory and inhibitory connections projecting back to the recorded neuron did also contribute to define the firing rate of the recorded cell. This implicates that not only the kinetics
4.DISCUSSION

of small sIPSCs but also the time course of evoked IPSCs, activated in this feedback circuitry, could contribute in defining the firing rate of the cells.

4.5.2 Changes in the spike threshold and amplitude of neurons in the hemisphere ipsilateral to the lesion

The positive shift in the spike threshold, observed in the hemisphere ipsilateral to the lesion, was also considered responsible for the lack of any increase in the spontaneous firing frequency. This shift, accompanied by a decrease in spike amplitude, could be explained by a reduced availability of voltage-activated Na\(^+\) channels in the active state.

Membrane potential fluctuations, mostly due to ongoing synaptic activity, have been shown to influence the threshold for spike induction in the visual cortex of cats in vivo. This was attributed to a different availability of Na\(^+\) channels which was dependent on the rate of membrane depolarization preceding a spike (Azouz and Gray 2000). However, in our in vitro recordings no difference in membrane depolarization was observed before spike generation (data not shown). This was expected, since such differences most probably arise in-vivo depending on the synchronization of excitatory synaptic inputs.

As alternative possibility the strength and the spatio-temporal distribution of inhibitory inputs from chandelier cells (which preferentially innervate the axon initial segment of pyramidal cells, where the APs are initiated) could also locally modulate the membrane depolarization and thereby the opening of Na\(^+\) channels.

The observed shift in spike threshold could be a homeostatic change in response to an altered neuronal activity taking place in vivo after the lesion induction. Similar forms of homeostatic plasticity have been already described, in cortical circuits, in response to altered neuronal activity (Desai et al. 1999; Maffei and Turrigiano 2008). These homeostatic changes seem critically important to guarantee the stability of neuronal networks by dynamically maintaining certain parameters (such as the firing rate or the Ca\(^{2+}\) intracellular concentration) within a physiological range (Turrigiano and Nelson 2000).

Together these findings suggested that the lesion-induced functional alterations of the surrounding neuronal networks might be the result of different mechanisms, some
affecting a large cortical area and expanding into the contralateral hemisphere and others spatially restricted to the ipsilateral cortex.

4.6 Spatial profile of the altered GABAergic transmission post-lesion

One interesting finding of this study was the spatial profile of the lesion-induced alteration of the GABAergic system. In the lesioned hemisphere the reduction of GAD67ir cells was observed in a large part of the primary visual cortex. In parallel, patch clamp recordings of spontaneous and evoked IPSCs revealed a reduced release of GABA in the same cortical area. Surprisingly the lesion effect on the GABAergic system was found to be expressed to a similar extent in the contralateral hemisphere as well.

Previous studies performed in photothrombotic and ischemic cortical lesion models already reported a transhemispheric diaschisis (Buchkremer-Ratzmann et al. 1996; Reinecke et al. 1999; Neumann-Haefelin and Witte 2000). Here the authors, performing in vitro extracellular recordings, reported a reduced strength in the GABAergic transmission which was widespread throughout the all contralateral hemisphere. In these studies the primary lesion had a diameter of about 2 mm.

In the present work, although the lesion was induced by a different mechanism and was smaller in size, similar alterations in the hemisphere contralateral to the lesion were observed.

The deafferentiation or functional alterations of intracortical and transcallosal connections can be the origin of the profound and widespread alterations of inhibition post-lesion, as a consequence of a lesion expanding into the lower cortical layers (the main source of these projections).

In accordance with this hypothesis a previous study performed in photothrombotic model of lesion reported that, when the lesions were confined in the superficial cortical layers, the reduction of GABAergic inhibition was spatially restricted to the proximity of the injury (Buchkremer-Ratzmann and Witte 1997).

Callosal connections are more densely distributed between areas processing adjacent points in the medial part the visual field where they are thought to promote the integration of visual inputs from the two eyes (Olavarria and Van Sluyters 1985).

The lesions performed in this study were however located at medial border of the visual cortex, where inputs from the lateral part of the visual field are normally
processed in rodents. This region should be therefore rather devoid of transhemispheric connections. Nonetheless robust and widespread alterations in the hemisphere contralateral to the lesion were strongly supported by both immunohistochemical and electrophysiological investigations.

Structural changes have been also described in the contralateral intact hemisphere by some authors: an increased dendritic arborization of layer 5 pyramidal neurons (Jones and Schallert 1992; Jones and Schallert 1994) and an increase in the turnover rate of usually stable mushroom-type synaptic spines (Takatsuru et al. 2009) were observed in an electrolytic and in a photothrombotic lesion model, respectively. Furthermore, an abnormal activation of the unaffected hemisphere has been also reported in clinical cases of cortical injury. Accumulating functional Magnetic Resonance Imaging studies, in patients suffering from strokes, reported an increased activation in the contralateral unaffected hemisphere. In particular some normally silent brain regions, ipsilateral to the side of the body involved in a task, were found to be activated (Cramer et al. 1997, Cao et al. 1998).

The hypothesis therefore emerged that the overactivation of the contralateral hemisphere might contribute to the functional recovery post-lesion by compensating, and partially taking over the function of the damaged hemisphere.

4.7 Molecular mechanisms underlying the lesion-induced functional alterations

The molecular mechanisms underlying the functional changes presented in this study are largely unknown. The bihemispheric reduction of GAD67 and PV expression, which might reflect a decrease in the activity of some interneuronal subtypes, could be interpreted as a homeostatic regulation in response to the loss of afferent connections.

Persistent changes in synaptic activity, which could arise from the degeneration of intracortical and callosal fibers post-lesion, can trigger the activation of intracellular signal cascades leading to a differential gene expression or local protein synthesis (from already present mRNAs) which in turn can modulate synaptic functions. Furthermore the secretion of neurotrophic factors (such as BDNF) from neurons and cytokines (such as TNFα) from glia, which might be altered post-lesion, has also been
shown to play an important role in the regulation of synaptic functions as well (for review see Pozo and Goda 2010).

Finally, changes observed only in the cortical hemisphere ipsilateral to the lesion should be instead the result of processes taking place exclusively in the lesioned hemisphere.

A wave of spreading depression characterized by a massive neuronal depolarization, accompanied by a transient suppression of neuronal activity, has been observed few hours after the lesion induction exclusively in the hemisphere ipsilateral to the lesion in other experimental models of cortical injury (Schroeter et al. 1995). If this phenomenon would occur in our model of lesion as well, it might foster the spreading of functional alterations across a large cortical area ipsilateral to the lesion. Further investigations are necessary to verify these hypotheses.
5. References


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REFERENCES


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5. REFERENCES


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6. Appendix

6.1 Curriculum Vitae

**PERSONAL INFORMATION**

Name: Imbroschi Barbara  
Address: Oskar Hoffmann str. 103, Bochum  
Country: Germany  
E-mail: barbara.imbroschi@googlemail.com  
Nationality: Italian  
Date of birth: 27th April 1982

**WORK EXPERIENCE**

Date: January 2007 – Currently

Function and occupation: Working as a PhD student in a laboratory specialized in neurophysiology at the “Department of Neurophysiology” at the Ruhr University Bochum

Principal tasks: Whole-cell patch clamp recordings from acute brain slices, immunostainings


Function and occupation: Internship as a student in a laboratory specialized in Molecular Biology at the “Department of Medical Pharmacology, Chemotherapy and Toxicology” at CNR based in Milan

Principal tasks: Cloning, real time PCR and other molecular biology techniques, culture of various human cell lines, transfection

**AWARDS**

Date and event: 3rd September 2009, poster competition “Neuro-visionen 5”

Prize: 1.000 Euro for the best poster presentation
EDUCATION AND TRAINING

Date: October 2004 – July 2006

Certificate or degree obtained: Master’s degree in “Medical Biotechnology and Molecular Medicine with specialization in neuroscience” with a final grade of 110/110 distinction.

Main courses: Laboratory practise, Neuronal network and artificial intelligence, Neurobiology, Psychology, Neuropsychopharmacology, Neuropsychopathology, Immunology, Genetic Therapy, Medical and Molecular Genetics, Bioinformatics, Physical Technologies

Name of education institute: “Università degli studi di Milano” in Milan (Italy)

Date: October 2001 – July 2004

Certificate or degree obtained: Bachelor’s degree in “Medical Biotechnology “with a final grade of 110/110 distinction

Main courses: Anatomy and Histology, Cellular and Molecular Biology, Biochemistry, Phatology and Immunology, Microbiology, Genetics, Pysiology, Bioethics, Pharmacology

Name of education institute: “Università degli studi di Milano” in Milan (Italy)

LANGUAGE SKILLS

Italian: Mother Tongue

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SOCIAL SKILLS

I am responsible, reliable, well-organised, friendly and able to work on own initiative and as part of a team. I am very interested in learning about foreign cultures and people

SPORT & HOBBIES

Sports: skiing, ice-skating, jogging, swimming, several team sports. Hobbies: reading, traveling, cooking
6.2 List of Publications

Imbroschi B, Eysel UT, Mittmann T. Metaplasticity of horizontal connections in the vicinity of focal laser lesions in rat visual cortex. in revision.

Imbroschi B, Neubacher U, Eysel UT, Mittmann T. Cellular mechanisms underlying the functional reduced GABAergic transmission in rat visual cortex after a focal laser lesion. in preparation.

Palagina G, Imbroschi B, Neubacher U, Eysel UT, Mittmann T. Functional reorganization at the lesion projection zone following retinal lesions in rat visual cortex. in preparation


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