Impaired Synaptic Plasticity in the Surround of Perinatally Acquired Dysplasias in Rat Cerebral Cortex

Freeze-lesion induced neocortical dysplasias in rats mimic numerous aspects of human polymicrogyria and are used as a model for the study of developmental migration disorders. Since memory tests have demonstrated learning deficits in rodents with neocortical malformations, we investigated the expression and properties of long-term potentiation (LTP) in neocortical slices from adult freeze-lesioned and control rats. Field potentials, recorded in layer II/III at a distance of 2–3 mm lateral to perinatally induced microgyri, were strongly enhanced following theta-burst stimulation in layer VI (amplitude: 174 ± 4%) compared to controls (110 ± 2%). In contrast, in layer IV of the freeze-lesioned cortex LTP could not reliably be induced. Histochemical analysis, performed to elucidate the cellular basis of the impaired plasticity, revealed diminished amounts of the GABA A-receptor subunit γ2 in the paramicrogyral zone, likely representing a diminished GABA-ergic filter, which is thought to prevent LTP induced in layer VI under normal conditions. Cytochrome-oxidase staining after electrophysiological examination disclosed that LTP in layer IV of the freeze-lesioned cortex could only be elicited, when stimulation was applied within a preserved barrel cortex. Our study provides evidence that focal cryolesions during cortical development cause an impaired synaptic plasticity that may underlie learning disabilities.

Keywords: developmental migration disorder, learning disabilities, long-term potentiation, neocortical malformation, polymicrogyria

Introduction
Malformations of the cerebral cortex comprise a heterogeneous group of genetic and acquired disturbances of cortical development, which are frequently associated with severe epilepsy syndromes (Palmini et al., 1991; Guerini et al., 1999; Sisodiya, 2000) and neuropsychological deficits, such as developmental delay, mental retardation and dyslexia in humans (Humphreys et al., 1990; Brodtkorb, 1994; Nordborg et al., 1999). Neonatal freeze-lesions of the rat neopallium (Dvorak and Feit, 1977; Dvorak et al., 1978) induce focal cortical malformations, which mimic morphological and pathophysiological aspects of four-layered human polymicrogyria. Freeze lesions cause an aberrant development of cortical connectivity within the paramicrogyral zone (Giannetti et al., 1999; Rosen et al., 2000). Ipsilateral and contralateral to the lesion malformed barrel structures in the somatosensory cortex have been observed (Jacobs et al., 1999b; Rosen et al., 2001), but also widespread changes in cortical function und excitability occur. Autoradiographic studies demonstrated an imbalance between excitatory and inhibitory receptor binding in the surround of the lesion with an upregulation of the binding to AMPA- and kainate-receptors and a downregulation of the binding to GABA A-receptors (Zilles et al., 1998). Additionally, immunohistochemical examinations of different GABA A subunits have revealed a substantial downregulation of the γ2-subunit lateral to the dysplastic cortex and an even stronger decrease of other functionally important subunits medial to the lesion (Redecker et al., 2000). In contrast, widespread changes of glutamate-receptor subunit distribution could not be detected (Hagemann et al., 2003). Alterations in the neocortical architecture, transmitter and receptor properties have been shown to be functionally associated with hyperexcitability and epileptogenesis (Luhmann and Raabe, 1996; Luhmann et al., 1998a,b; Jacobs et al., 1999a).

In addition to structural and functional abnormalities, learning disabilities have been reported in humans showing cortical microdysgenesis, as well as in rodent models of neocortical dysplasias. In individuals with developmental dyslexia, functional deficits in rapid auditory processing have been described (Merzenich et al., 1996; Slaghuis et al., 1996), which are assumed to be related to minor cortical or subcortical malformations (Galaburda, 1993; Galaburda et al., 1994). A similar impairment of fast auditory processing, probably attributable to functional alterations in the surround of the cortical malformation, was observed in the freeze-lesion model in rats (Fitch et al., 1994; Galaburda, 1994). Furthermore, freeze-lesioned mice have been shown to perform poorly in a test of discrimination learning when compared to controls (Rosen et al., 1995).

Long-term potentiation (LTP) is a long-lasting enhancement of the efficacy of synaptic transmission following brief, tetanic stimulation of afferent pathways and is thought to represent a synaptic model of learning and memory in the mammalian brain (Kuba and Kumamoto, 1990; Bliss and Collingridge, 1993). To determine whether learning variances are accompanied by changes in neocortical plasticity in the freeze-lesion model, we tested the ability of different neocortical layers and their synaptic connections to generate LTP.

Materials and Methods
Freeze lesions were induced in 25 newborn (P0, <24 h) male Wistar rats (Experimental Animal Laboratory, Heinrich-Heine-University, Düsseldorf, Germany) by a method, which was originally described by Dvorak and Feit (1977). Animals had free access to food and water and institutional guidelines for animal safety and comfort as well as local government requirements were adhered to. In brief, newborn rats were anesthetized by hypothermia and a copper cylinder (OD: 1 mm) cooled with liquid nitrogen was brought into contact for seconds with the exposed calvarium in the area of the primary somatosensory cortex (for details, see Luhmann and Raabe, 1996). This procedure resulted in a cortical malformation of ∼5 mm length in the rostro-caudal direction in mature animals (for macroscopic presentation, see Redecker et al., 2000). Since it is known from former studies that sham-operated controls do not show any morphological or physiological abnormalities (Luhmann and Raabe, 1996; Rosen et al., 2001), we
abstained from the control-preparation procedure for shams and used untreated animals instead. All electrophysiological and histological experiments were performed on 8–12 weeks (P56–84) old rats.

For electrophysiological measurements animals were anesthetized with enflurane and decapitated immediately after disappearance of the corneal reflex. The brain was rapidly removed and immersed in ice-cold artificial cerebrospinal fluid (aCSF). A block of the frontoparietal cortex (bregma -1, 8 to -3, 5) was removed containing the cortical malformation easy identifiable on the brain surface. Coronal neocortical slices (400 µm) were prepared (VT 1000 S; Leica) and stored at room temperature in aCSF containing (in mM): NaCl 124, NaHCO3 26, KCl 5, CaCl2 2, MgSO4 1, NaH2PO4 1.25 and glucose 10, equilibrated with 95% O2 and 5% CO2 to pH 7.4.

**Extracellular Field Potential Recordings**
The slices were transferred to an interface-type recording chamber, where they were maintained in a moisty atmosphere of 95% O2 and 5% CO2 and superfused with 1.5 ml aCSF per minute at 33°C. Slices were left undisturbed for at least 1.5 h before electrophysiological measurements were started. In a first series a concentric bipolar stimulation electrode (OD: 0.2 mm) was placed in layer VI at the border to the white matter lateral to the lesion (primary somatosensory cortex, Par I, Fig. 1A,B). In a second series the stimulation electrode was placed lateral from the microgyrus just above the middle of the cortical thickness, corresponding to layer IV (Fig. 1D). The recording electrode was filled with aCSF (4–8 Ml2) and placed in layer II/III strictly vertical to the stimulation site (Fig. 1A). In control slices the electrodes were placed at corresponding locations in the primary somatosensory neocortex (Fig. 1B). Synaptic responses were evoked with 50 μs pulses. In each slice the most viable position was selected, based on the field potential (>3 mV) and the ability of the response amplitude to increase substantially by small increments of stimulus intensity. Baseline responses were obtained once per minute using a stimulation intensity that yielded up to 2/3 of the estimated maximal response. The LTP-induction protocol was only started in case a stable baseline for at least 15 min was achieved. The theta-burst for LTP induction consisted of six series of pulses delivered at 0.1 Hz. Each series contained 10 sequences delivered at 5 Hz and one sequence included four 200 µs pulses at 100 Hz (Kirkwood and Bear, 1994; Castro-Alamancos et al., 1995; Hagemann et al., 1998). Pharmacological interference of LTP induction was performed by bath application of NMDA-receptor antagonists (APV: 50 µM; ifenprodil: 20 µM) or by leakage of the GABA-receptor antagonist bicuculline from the recording pipette.

**Histology**
For combined immunohistochemical (IH) and cytochrome oxidase (CO) examinations of the barrel cortex rats (n = 3) were deeply anesthetized and perfused through the ascending aorta with paraformaldehyde (4%) and saturated picric acid solution (15%) in phosphate buffer (0.15 M). Brains were removed immediately after the perfusion and post-fixed for 3 h in the same fixative at 4°C. Additional slices (n = 3) on which electrophysiology was performed, were prepared in the same solution. Samples were cryoprotected in phosphate buffered saline (PBS) containing 30% sucrose for 24 h and stored at –75°C for further processing. Coronal sections were cut at 50 µm (IH and CO) or 80 µm (CO) with a freezing microtome and collected in ice-cold 0.1 M PBS. The GABA receptorsubunit γ2 was visualized using subunit-specific antisera raised in guinea pigs against synthetic peptides derived from rat subunit CDNA and a standard peroxidase staining protocol (for details, see Fritschy et al., 1998). In order to allow receptor immunohistochemistry and CO staining of adjacent brain sections, the tissue was not microwave-treated as described previously (Redecker et al., 2000). For CO staining, free-floating sections were first treated for 15 min with a cobalt chloride intensification (27.5 mg CoCl2 and 10 g sucrose per 100 ml 50 mM Tris buffer), washed twice in Tris buffer, once in PBS and then incubated for at least 1 h at 37°C in a solution containing 4 g sucrose, 50 mg diaminobenzidine (DAB) and 40 mg cytochrome C per 100 ml PBS. All sections were mounted onto gelatin-coated slides, air-dried, dehydrated with ascending series of ethanol, cleared and coverslipped with toluene (Entellan; Merck).

**Statistics**
Data are given as mean ± SEM of each time point before and after TBS. For calculation of differences between amplitudes and slopes measured at different stimulation sites Student’s t-test was employed, values P ≤ 0.05 were regarded to be significant. If not indicated otherwise, number of experiments equals number of animals.

**Results**
All freeze-lesioned animals tolerated the perinatal preparation and grew up as well as untreated control animals (freeze lesioned, 277 ± 46 g, n = 25; controls, 275 ± 52 g, n = 10). In 23 of 25 lesioned animals the contact with the liquid-nitrogen-cooled copper cylinder resulted in a typical, macroscopic visible microgyrus (for details, see Redecker et al., 2000; Hagemann et al., 2003). Two animals had just a slight irregularity in the corresponding area, most likely attributable to insufficient contact with the freezing probe, and were excluded from the study. The microgyri were always located within the medial part of the primary somatosensory neocortex (Par I) or in the lateral part of the hindlimb representation cortex (HL). A spontaneous seizure was observed in one freeze-lesion animal during the beginning of anesthesia with enflurane. As described previously, electrical stimulation

**Figure 1**
Semi-schematic drawing of the neocortical slice preparation and the placement of stimulation- and recording-electrodes in the paramicrogyral region. (A) Long-term potentiation (LTP) was measured either 2 or 3 mm lateral (dotted lines) from the midline of the macroscopic visible microgyrus (straight line). Recording electrodes were always placed in layer III strictly perpendicular to the stimulation electrode. On the left typical microgyrus, on the right the trace denotes the region from which slope (S) was calculated, the amplitude (A) is indicated by the doubled arrow. (B) Schematic drawing of the positions where theta-burst stimulation (TBS) was performed. Four different stimulation sites were chosen in control (slices in the rear, C1–C4, Par I) and in freeze-lesion animals (slices in the front, F1–F4, corresponding area to Par I). On the left all positions tested in layer VI and on the right side all positions tested in layer V, are shown.
LTP in the Freeze-lesioned Cortex can be Achieved by Stimulation of Layer VI/WM

In control animals TBS in layer VI/WM of the somatosensory cortex (positions C1 and C2 in Fig. 1B; each position n = 4) did not induce LTP, but was followed by a slight increase of the FP amplitude and slope (Fig. 3A,B, solid circles). LTP induction in layer VI/WM in control slices was only successful when bicuculine was applied locally by the recording electrode. Typical FP traces, which become substantially broadened by bicuculine, before and after LTP induction are shown in Figure 2A. In contrast to the findings in controls, TBS in the lateral paramicrogyral layer VI of freeze-lesioned slices (positions F1 and F2 in Fig. 1B; each position n = 5) induced an immediate and substantial increase of the FPs. Typical FP traces before and after TBS are shown in Figure 2B (Note the different appearance of FPs before and after TBS in freeze-lesioned slices and controls.) Ten minutes after TBS the amplitude was increased to 1.49 ± 3% of baseline and the slope mounted to 165 ± 14% of control (Fig. 3A,B). In freeze-lesioned slices LTP reached a plateau ~40 min after TBS (amplitude: 174 ± 4%; slope: 208 ± 15%). Results from stimulation sites F1 and F2 (as well as positions C1 and C2 in controls), 2 and 3 mm lateral to the microgyrus (cf. Fig. 1A,B), did not differ significantly and were pooled together. Since contralateral effects of the freeze lesion have been recently reported (Rosen et al., 2001), we tried to elicit LTP in Par I contralateral to the cortical malformation. Interestingly in 2 out of 10 experiments we could initiate a rise of amplitude and slope after TBS in VI/WM comparable to the LTP seen in the paramicrogyral zone. In the remaining eight experiments the results were similar to control animals.

Reproducible LTP Induction in Layer IV Relies on Preserved Barrels

In control animals tetanic stimulation in layer IV of the somatosensory cortex (positions C3 and C4 in Fig. 1B; each position n = 5) was followed by an increase in amplitude and slope of FPs. Ten minutes after TBS a plateau with an average amplitude of 123 ± 2% and slope of 166 ± 8% was reached (Fig. 3C,D; see also the results from single experiments in Fig. 4C). In contrast, TBS in layer IV of freeze-lesioned slices (positions F3 and F4 in Fig. 1B; each position n = 5) led to inconsistent results. Only in 3 out of 10 attempts the amplitudes reached values 20% above baseline during the observation period (Fig. 4D). To investigate the irreproducibility of LTP in layer IV of the freeze-lesioned cortex, we visualized the barrel fields within the somatosensory cortex by CO staining. In three out of seven cases in which LTP induction failed, CO staining of the slice was successfully performed after electrophysiological measurements. The morphology of the barrel cortex disclosed that TBS was applied between dysmorphic barrels in a zone without CO staining (Fig. 4A,B).

Induction of LTP in Layer VI of the Freeze-lesioned Cortex is NMDA-receptor Dependent

To elucidate which receptor type is involved in the enhanced susceptibility to generate LTP in layer VI/WM, we tested the effect of APV, a NMDA-receptor antagonist, which has been reported to block most types of LTP (Bear and Kirkwood, 1993). LTP induction in layer VI/WM of the paramicrogyral zone was completely blocked by superfusion of 50 µM APV (n = 4, Fig. 5A). In parallel a short and transient decrease of the amplitude was noted (88 ± 2%, 5 min after TBS). At least 50 min
of APV wash out were required before a second TBS could elicit LTP. Interestingly application of 50 \( \mu M \) APV ∼ 50 min after successful LTP induction in layer VI/WM of the paramicrogyral cortex led to a significant decrease of the amplitude (\( n = 4, 138 \pm 6\% \), Fig. 5B), indicating that also the maintenance of the LTP in the freeze-lesioned cortex is partially NMDA-receptor dependent. Although we could not demonstrate any gross alterations of glutamate-receptor subunit distribution in the surround of cortical freeze lesions in an earlier immunohistochemical study (Hagemann et al., 2003), there are observations of a functionally enhanced NMDA-receptor subunit 2b (DeFazio and Hablitz, 2000). We therefore tested the hypothesis that this NMDA-receptor subunit is responsible for the strongly enhanced LTP induction in layer VI. Superfusion of the selective NR2B-antagonist ifenprodil (20 \( \mu M \)) did neither prevent induction nor affect maintenance of LTP when applied before or after TBS (\( n = 4 \), data not shown).

**Barrels in the Freeze-lesioned Cortex carry a Diminished Amount of the GABA-receptor Subunit \( \gamma_2 \), which likely Facilitates LTP Induction**

To investigate further the abnormal organized barrel fields and to validate the hypothesis that the inhibitory circuitry in layer IV, which is supposed to act as a GABA-ergic filter (Kirkwood and Bear, 1994), is disarranged in the surround of neocortical freeze lesions, we stained the cytochrome oxidase and in parallel the GABA A-receptor subunit \( \gamma_2 \) in adjacent 50 \( \mu M \) slices (for technical reasons a double-staining protocol could not be performed). Immunohistochemistry showed the previously reported downregulation of the GABA-receptor subunit \( \gamma_2 \) medial and lateral to the freeze lesion (Redecker et al., 2000). Moreover, the comparison of CO staining and immunohistochemistry disclosed, that even preserved barrels in the freeze lesion hemisphere are localized closely to regions with reduced amounts of the GABA-receptor subunit \( \gamma_2 \) (\( n = 3 \), data not shown).

**Discussion**

Our study demonstrates a layer-specific impairment of LTP induction in the ipsilateral cortex of experimentally induced focal malformations and provides evidence that cytovarchitectonic as well as receptor alterations are involved in these functional disturbances.

**Impaired Layer-dependent Synaptic Plasticity in Widespread Areas of the Lesioned Brain**

LTP induced by theta burst stimulation (TBS) is widely accepted as a model for learning and memory in the mammalian brain (Bliss and Collingridge, 1993). Neocortical LTP in neuronal circuits between layer VI and II/III is usually age-dependent and limited to a so-called 'critical period' during the first weeks of life (Vilagi et al., 1992). It has been speculated that a GABA-ergic filter, driven by an inhibitory circuitry that develops during the first weeks, prevents LTP in this model (plastic gate hypothesis; Kirkwood and Bear, 1994). Since we used adult rats (P56–84) beyond the critical period, it is not surprising that in controls LTP by stimulation in layer VI could not be achieved unless bicuculine was applied locally during
the stimulation. In contrast, the corresponding neuronal circuits in freeze-lesioned animals show a robust LTP without pharmacological reduction of the GABA-ergic filter, characterized by a pronounced increase in amplitude and slope after TBS in layer VI/WM.

At least two mechanisms may contribute to this phenomenon: First, layer IV, which is supposed to build the plasticity gate in the neocortex (Kirkwood and Bear, 1994) is morphologically modified in the freeze lesion model. Malformed barrel fields have been demonstrated in this and earlier studies as well (Rosen et al., 2001), revealing that the overall pattern of metabolic staining within the barrel field as well as the formation of individual barrels is abnormal. Adjacent Nissl- or acetylcholinesterase-stained sections demonstrated that both cell clusters and thalamocortical afferents contributed to the abnormally organized paramicrogyral zone identified in cytochrome-oxidase stained sections (Jacobs et al., 1999b). Moreover our data strongly suggest that the disarranged, paramicrogyral barrel cortex contains only diminished amounts of the GABA\(_{\alpha}\)-receptor subunit \(\gamma_2\), which may lead to an ineffective GABA-ergic filter. Additional GABA\(_{\alpha}\)-receptor subunits (\(\alpha_1, \alpha_2\) and \(\alpha_5\)), which were examined immunohistochemically in an earlier study, were also downregulated medial and lateral to the microgyrus (Redecker et al., 2000), while autoradiographic receptor studies revealed that NMDA- and GABA\(_{\alpha}\)-receptor binding was not significantly changed in these regions (Zilles et al., 1998).

Secondly, it has to be taken into account that the lateral paramicrogyral zone is characterized by an increased amount of thalamocortical projections, which have been localized at the border of the malformation and were described as unusually dense and atypical in appearance (Rosen et al., 2000). It is conceivable that in a region of densely packed fibers and synapses, TBS recruits more polysynaptic circuits and thus LTP induction is facilitated. Remarkably the increased synaptic plasticity was not limited to the borderzone of the microgyrus, but was also present 2 and 3 mm lateral from the midline of the freeze lesion, indicating widespread functional alterations. Our finding that in single cases LTP after stimulation of layer VI/WM can also be evoked contralateral to lesion, might be explained by moderate morphological alterations, which have been demonstrated in this area (Rosen et al., 2001). These slight morphological alterations may be associated with subtle distortions of cortical columnar organization, like abnormalities in minicolumns (Buxhoeveden and Casanova, 2002), that have been described to cause dyslexia and Rett syndrome in humans (Casanova et al., 2002, 2003).

NMDA antagonists are known to prevent LTP in most areas of the brain with only a few exceptions (Bear and Kirkwood, 1993). Our measurements show that the induction and maintenance of LTP in the paramicrogyral zone is also dependent on

**Figure 4.** Induction of LTP in layer IV depends on intact barrel structure. (A, B) Cytochrome-oxidase (CO) staining of the barrel fields ipsi- and contralateral to the freeze lesion. Slices taken from one animal in which electrophysiology was performed are shown. For better visualization single CO-stained barrels are surrounded by dotted lines. In the freeze-lesioned cortex typically a distructured barrel field containing zones without CO staining was found. (C, D) Results from single experiments shown in Figure 3C,D. While in the intact barrel fields of controls (not shown) LTP could reproducibly induced, only very few attempts were successful in the paramicrogyral zone of the freeze-lesioned cortex.
Rosen was disturbed, as described previously (Jacobs slices obtained from perfused tissue the typical barrel pattern induction failed, the barrel field was examined after electro-sory cortex using the CO technique. In several cases when LTP impaired plasticity, we stained the barrels in the somatosen-cases LTP could be induced. In search of the reason of this propensity to induce LTP from layer IV was inconsistent. Only in a few paramicrogyral zone of the freeze-lesioned cortex the propen-sity to exceed baseline by 20%; Kimura terms of the definition that within 20 min after TBS responses attempts to induce LTP by TBS in layer IV were successful (in control cortices, which usually do not show a predominant role of NMDA-receptors in the maintenance of LTP.

LTP at vertical synapses onto layer II/III pyramids can also be achieved when the GABA-ergic filter is bypassed by stimulation of layer IV. This paradigm has repeatedly been shown to induce LTP in the adult neocortex (Kirkwood and Bear, 1994; Castro-Alamancos et al., 1995; Hagemann et al., 1999b) and was confirmed in the present study. In controls, 7 out of 10 attempts to induce LTP by TBS in layer IV were successful (in terms of the definition that within 20 min after TBS responses need to exceed baseline by 20%; Kimura et al., 1989). In the paramicrogyral zone of the freeze-lesioned cortex the propensity to induce LTP from layer IV was inconsistent. Only in a few cases LTP could be induced. In search of the reason of this impaired plasticity, we stained the barrels in the somatosensory cortex using the CO technique. In several cases when LTP induction failed, the barrel field was examined after electrophysiological examination. In this tissue and in numerous slices obtained from perfused tissue the typical barrel pattern was disturbed, as described previously (Jacobs et al., 1999b; Rosen et al., 2001). We hypothesize that the disorganization of the local cortical circuitry, which goes along with disturbed columnar organization (Mountcastle, 1997), may prevent LTP in layer IV of the freeze-lesioned cortex. Notably, barrels and inter-barrel septae have been reported to play a major role in the generation of LTP by stimulation of layer IV (Glazewski et al., 1998; Fox, 2002).

**Conclusion**

Our study provides evidence that small cortical lesions induced during early corticogenesis alter synaptic connectivity in wide-spread areas of the ipsilateral hemisphere and thereby may cause a columnar disorganization of the neocortex. We show that connectional reorganization following early injury is functionally associated with a layer-specific impairment to generate LTP in vertical pathways of the somatosensory-cortex. The impaired plasticity, described in the freeze-lesion model, may contribute to the disturbances in cortical processing and alterations in learning and memory in patients with focal cortical dysplasias.

**Notes**

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**References**


**Figure 5.** Induction and maintenance of LTP in freeze-lesioned cortex depends on NMDA receptor activation. (A) Bath application of 50 µM APV prevented the induction of LTP during stimulation in layer VI of the paramicrogyral zone (TBS II). Upon washout of APV, LTP could be induced (TBS II). (B) The maintenance of LTP in the freeze-lesioned cortex is partially NMDA-receptor dependent. Superfusion of APV 50 min after TBS led to reduction of the FP amplitude of ∼50%.

The recently reported enhanced amount of the NMDA-receptor subunit 2b (DeFazio and Hablitz, 2000), does not seem to play a role in this phenomenon, because ifenprodil a selective antagonist for this subunit did neither interfere with LTP induction nor preservation. Surprisingly, the superfusion of APV, ∼1 h after LTP induction, led to a significant decrease of the FP-amplitude. This finding suggests that a NMDA-dependent mechanism is involved in LTP maintenance in the freeze-lesioned cortex and stands in contrast to measurements in control cortices, which usually do not show a predominant role of NMDA-receptors in the maintenance of LTP.