Abstract—Severe brain injuries can trigger epileptogenesis, a latent period that eventually leads to epilepsy. Previous studies have demonstrated that changes in local connectivity between cortical neurons are a part of the epileptogenic processes. In the present study we aimed to investigate whether changes in long-range connectivity are also involved in epileptogenesis. We performed a large unilateral transection (undercut) of the white matter below the suprasylvian gyrus in cats. After about 2 months, we either injected retrograde tracer (cholera toxin, sub-unit B, CTB) or performed Golgi staining. We analyzed distribution of retrogradely labeled neurons, counted dendritic spines in the neocortex (Golgi staining), and analyzed dendritic orientation in control conditions and after the injury. We found a significant increase in the number of detected cells at the frontal parts of the injured hemisphere, which suggests that the process of axonal sprouting occurs in the deafferented area. The increase in the number of retrogradely stained neurons was accompanied with a significant decrease in neocortical spine density in the undercut area, a reduction in vertical and an increase in horizontal orientation of neuronal processes. The present study shows global morphological changes underlying epileptogenesis. An increased connectivity in the injured cortical regions accompanied with a decrease in spine density suggests that excitatory synapses might be formed on dendritic shafts, which probably contributes to the altered neuronal excitability that was described in previous studies on epileptogenesis. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: epileptogenesis, cholera toxin, retrograde staining, sprouting.

INTRODUCTION

Penetrating cortical wounds induce acute seizures that last for hours or days (Dinner, 1993). Then seizures terminate. Vietnam and Croatia postwar epidemiological studies report that 10–15 years after the trauma about 50% of patients with penetrating cranial wounds develop epilepsy (Salazar et al., 1985; Marcikic et al., 1998). The set of latent processes that are triggered by cortical insult and that can lead to the development of epilepsy is called epileptogenesis. The epilepsy itself, on the other hand, is characterized by unprovoked seizures (Rakhade and Jensen, 2009; Timofeev, 2011). The acute phase of epileptogenesis usually occupies the first 7 days after the injury, and this early phase can be characterized with acute seizures (Beghi et al., 2010), immediate early genes response or changes in ion concentration (Rakhade and Jensen, 2009).

The cortical undercut is a well-established model of post-traumatic epileptogenesis; it consists in the partial deafferentation of the neocortex and hence imitates a penetrating brain injury. This model has been used in humans, monkeys, cats, and also in rodents, both in vivo and in vitro (Echlin et al., 1959; Echlin and Battista, 1963; Hoffman et al., 1994; Prince and Tseng, 1994; Nita et al., 2006, 2007; Xiong et al., 2011). In chronic conditions, the anatomical changes in the undercut cortex involve cortical flattening, reduction in cortical depth and delamination (Avramescu et al., 2009). In our previous experiments with the use of undercut in the suprasylvian gyrus of cats, paroxysmal activity could be observed within hours after the injury (Topolnik et al., 2003a,b) and then acute seizures stopped. Electrographic activities starting around the undercut cortex were recorded within tens of hours in cortical isolation experiments (Nita et al., 2007; Timofeev et al., 2013). Within the first 2 months there was a progressive increase in the cortical territories involved in the generation of electrographic paroxysmal activities, which eventually led to a development of full-blown electrographic and behavioral seizures (Nita et al., 2007; Timofeev et al., 2013). Because of the progressive increase in the cortical tissue involved in the paroxysmal activities, we hypothesized...
that the traumatized area might create conditions leading to an increase of connectivity with the rest of the brain. So far, reports pertaining to morphological changes in the cortical undercut model of epileptogenesis mainly showed a local (hundreds of microns) increase in connectivity (Salin et al., 1995; Jin et al., 2006, 2011; Avramescu and Timofeev, 2008), while it remains unknown whether there are any longer range changes in neocortical connectivity. Because in the undercut model of trauma the seizures were not initially generated in the area lying directly above the transection, but in the surrounding areas, and then, with a delay of only a few milliseconds, they invaded also the injured region, (Nita et al., 2007), we hypothesized that excitatory cells in the surrounding areas would form new direct connections with the undercut. Using retrograde tracer cholera toxin (sub-unit B, CTB), we found a significant increase in connectivity of the undercut cortex with more anterior cortical regions. However, within the undercut region, neurons showed a reduced spine density on secondary and higher order dendritic branches, which suggests that new synapses are not formed on dendritic spines, but on dendritic shafts. We also observed a shift in cortical organization from a mainly vertical orientation of neuronal processes in control conditions to a mainly horizontal in the undercut cortex. Together with an overall reduction of neurons, in particular inhibitory interneurons (Avramescu et al., 2009), our results point to major morphological changes of the undercut cortex that contribute to the altered excitability of the epileptogenic tissue.

EXPERIMENTAL PROCEDURES

Undercut surgery

Experiments were performed in accordance with the guideline of the Canadian Council on Animal Care and approved by the Université Laval Committee on Ethics and Animal Research. All efforts were made to minimise the number of animals used and their suffering. The surgery was performed under sterile conditions. Six male cats (8–12 months old) were initially anesthetized with IM administration of a mixture of ketamine (15 mg/kg), buprenorphine (0.01 mg/kg), acepromazine (0.3 mg/kg) and glycopyrrolate (0.011 mg/kg). The anesthesia was maintained with isoflurane inhalation (3–4% for induction and 0.7–2% throughout the surgery), and lactated Ringer’s solution was continuously delivered IV at a rate of 5–10 ml/kg/h. Lidocaine/marcaine (0.3% NaCl and 2.5% dextrose at an approximate rate of 10–15 ml/kg/h) throughout the whole experiment. Frequency of ketamine–xylazine booster IV injections (1/3 of the initial dose) was estimated for each animal separately on the basis of electroencephalogram (EEG) recording and heart rate. An anesthesia booster was injected whenever there was a slight tendency of the EEG pattern to become activated or when the heartbeat increased. The range of time intervals between boosters was approx. 0.5–2 h. Animals were artificially ventilated and paralyzed with 2% gallamine triethiodide; glycopyrrolate (0.011 mg/kg) was administered IV every 6 h.

After opening the scalp and removing the dental acrylic, an craniotomy was performed over the right intact hemisphere to match the size of the opening over the left undercut cortex. The dura matter was removed from both left and right gyri. Cholera toxin in a concentration of 1% (water solution) was injected once in the left and right suprasylvian gyri, approximately 1 cm to the front from the point of knife insertion for the undercut. The tracer was injected with a microinjector (Nanoliter 2000, World Precision Instruments, FL, USA), with a glass micropipette with a diameter of around 40–50 μm.
After fixation, brains were lowered to a depth of about 1 mm below the brain surface. The injection was intermittent, with a single volume of 23 nL and at a slow injection rate (23 nL/s); the single volume was injected five times (into the same place, without moving the micropipette) to a total volume of 115 nL and the interval between consecutive single volume injections was about 10 s. The pipette remained in the tissue for 10 min to prevent leakage of the tracer along the pipette track (Luppi et al., 1990, 1995). We also performed the CTB injection in one control cat, which had not undergone undercut surgery prior to the acute tracer experiment.

It was previously demonstrated that retrograde labeling with CTB is complete after 24 h after injection (Luppi et al., 1990) and remains stable for at least 2 weeks (Ericson and Blomqvist, 1988). In our experiments, 40 h after the tracer injection, ketamine–xylazine anesthesia was overdosed and the animals were perfused with 1 L of saline followed by 1 L of phosphate-buffered paraformaldehyde. Brains were removed and left in the same fixative overnight.

CTB immunohistochemistry. After fixation, brains were cryoprotected in increasing concentrations of sucrose (10, 20 and 30%) dissolved in phosphate-buffered paraformaldehyde, until sinking. Brains were cut in parasagittal plane on a freezing microtome or cryostat, at a thickness of 50 μm. Sections were rinsed with phosphate-buffered saline (PBS), incubated in 0.3% H2O2, and then blocked in 2% normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) and 0.5% bovine serum albumin (Vector Laboratories) with 0.3% Triton X-100 (Sigma, Oakville, ON, Canada) in PBS overnight. The sections were incubated in the primary antibody, goat anti-cholera toxin B subunit (List Biological Laboratories), used in dilution of 1:4000–1:6000, for 3 days at 4 °C, followed by incubation in secondary antibody, biotinylated rabbit anti-goat immunoglobulin G (IgG) (Vector Laboratories) diluted 1:300, overnight at 4 °C. After incubation in ABC (Vector Laboratories, 1:100) with 0.2% Triton, sections were soaked in 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma), enhanced with cobalt and nickel, for 20 min in darkness, and 0.004% H2O2 was then added to the solution for subsequent 20 min for development. The reaction was stopped by rinsing sections in PBS. Brain slices were then mounted in PB onto gelatin-chromalum-coated slides, air-dried, dehydrated in graded alcohols and cover slipped with Permount (Fisher Scientific, Ottawa, ON, Canada).

**Analysis of CTB cell distribution.** The location of stained neurons was reconstructed on every fifth section using Neurolucida (version 9.10.5, MBF Bioscience, Williston, VT, USA). Stained cells were marked under magnification 20x. We have developed functions in Matlab (version 7.10.0, The Mathworks, Natick, MA, USA) to (a) calculate the centroid of the injection site, (b) visualize the density of distribution of the stained cells with the use of color-coded maps with the center of the injection site as the reference point, (c) restrict the area of sections to a region of interest by setting boundaries in antero-posterior and dorso-ventral planes, (d) extract the distribution of cells along the antero-posterior and medio-lateral axes of the brain. The distribution of cells in bins was analyzed in percentage of the total number of cells in the suprasylvian gyrus of every hemisphere. A statistical analysis (analysis of variance (ANOVA) with hemisphere and distance along antero-posterior axis as factors and with Tukey’s post hoc test in SPSS 8.0, SPSS Inc.) was performed to compare the distribution between the hemispheres with undercut, the intact suprasylvian gyri, and the control brain.

**RESULTS**

**Distribution of retrogradely labeled cells in undercut and in intact suprasylvian gyrus**

After injection of the retrograde tracer into the middle part of the suprasylvian gyrus we found CTB-stained cells in the same gyrus, the primary visual cortex, parabasal regions and the thalamus. Fig. 1 depicts an example of a parasagittal section from an undercut hemisphere, with CTB-stained cells, and the reconstruction of this...
section done in Neurolucida; positions of the stained cells are indicated and examples of CTB-containing neurons are shown in magnification. In the slices that were cut perpendicularly to the cortical surface, the stained neurons appeared to be pyramidal, as can be seen on microphotographs with high magnification (Fig. 1, lower panels). From serial reconstructions we identified the average size of the injection site (1.3 mm in the antero-posterior and 1.6 mm in medio-lateral axis). A vast majority of stained neurons was found within the suprasylvian gyrus, therefore further analysis was restricted to this area. Within this gyrus, the maximum distance along which cells were detected was approximately 9000 µm to the front and 7000 µm to the back of the center of the injection site.

We reconstructed the location of stained neurons in every fifth section from every hemisphere and then within a given suprasylvian gyrus we superimposed the reconstructed sections taking the shape of the injection sites, the contours of the sections and of the ventricles and the hippocampus as reference points for alignment. We thus created a 3D image for every hemisphere, preserving the thickness of each section and also the interval between consecutive slices (Fig. 2). Following reconstruction, the difference in cell distribution between the two hemispheres was apparent in the lateral view of the parasagittal sections: in the contralateral hemisphere the cells were distributed symmetrically around the injection site (yellow circle), whereas in the undercut hemisphere there was visibly more cells in the frontal areas of the suprasylvian gyrus. ‘Top view’ was obtained by turning the reconstruction in Neurolucida Explorer in such a way that the cells are seen from the perspective of the top surface (Fig. 2 middle panels in A, B). For quantitative analysis, the cell distribution from the ‘top’ perspective was rendered as a color-coded map (see Experimental procedures section). The boundaries for the reconstruction were set in such a way that only the neocortex in the suprasylvian gyrus was processed. For the analysis, each bin had a size of 250 µm in the antero-posterior axis and 50 µm in the medio-lateral axis (the latter corresponds to the thickness of sections) and the total number of cells per bin was depicted considering the center of the injection site as the reference point. These color-coded maps show that in reference to the injection site (zero points on both axes), the highest numbers of stained cells in the undercut hemisphere were found in the frontal part of the suprasylvian gyrus. Such a color map was done for every analyzed hemisphere. We found the same difference between undercut and contralateral gyri for each brain (data not shown). In every investigated brain
Fig. 2. Difference in CTB-stained cell distribution in undercut versus contralateral suprasylvian gyri. (A) Intact hemisphere. (B) Hemisphere with undercut in the suprasylvian gyrus. Reconstructions from each fifth parasagittal sections are superimposed on one another (1). Each stained neuron is represented by a black dot. The sections were aligned by the position of the centroid of the injection site. (2) Top view of the reconstruction. (3) Color-coded maps of stained cell density are shown for the top view. The maps show absolute numbers of cells in bins, i.e. fragments of reconstruction of the size of 50 \( \mu \text{m} \) (medio-lateral direction) and 250 \( \mu \text{m} \) (antero-posterior direction). Note the more extensive staining in the frontal parts of the undercut gyrus in comparison with the contralateral, intact tissue.
the bin that contained the highest number of stained cells was always localized in front of the injection site in the undercut gyrus and always at the back of the injection site in the contralateral hemisphere; the localization of this peak in stained cell density was usually further from the center of the injection site in the undercut than in the intact suprasylvian area. Fig. 3 shows average color-coded ‘top view’ map obtained for all three brains: in the undercut hemispheres the staining was asymmetrical, i.e. more expanded to the frontal parts and also encompassing more area in the medio-lateral plane in comparison with the contralateral hemispheres.

To analyze the neuronal distribution, we extracted the total number of cells in bins along the antero-posterior direction using the centroid of injection site as the reference point (Fig. 4). For each animal the number of cells in each bin was represented as a percentage in reference to the total number of stained cells in the entire suprasylvian area and the calculation was made for the series of sections composing every hemisphere. In the undercut gyrus the number of stained cells was significantly higher at the front of the injection site (2–3.25 mm from the centroid) and it was significantly lower at the back of the injection (1.5–2 mm from the centroid), as compared to the contralateral hemisphere (ANOVA with Tukey’s post hoc). In comparison with the naive animal the significant changes were more dispersed: 1.75–5 mm at the front and 0.5–2 mm at the back, confirming increase and decrease in the number of CTB-stained neurons respectively. Gaussian distribution was fitted to the data (Fig. 4) and it confirmed the changed pattern of staining, corroborating the shift of the most dense staining to the frontal parts of the injured suprasylvian gyrus.

Dendritic spine density after deafferentation

Because the CTB staining revealed an enhanced connectivity in the frontal parts of the deafferented suprasylvian gyrus, using a Golgi staining approach we analyzed spine density in corresponding cortical areas, i.e. between +7 to +17 coronal planes according to the cat brain atlas (Reinoso-Suarez, 1961). The analysis was restricted to layer 2–3 pyramidal neurons with cell bodies located above 800 μm from the surface (Hassler and Muhs-Clement, 1964). We have found a significant decrease in spine density after the injury (Fig. 5). On the secondary to quaternary dendritic branches stemming from apical dendrites, the reduction in spine number was significant both in the injured and contralateral hemispheres in comparison to control (Fig. 5B, \( p \leq 0.001 \) for the difference between undercut and control and \( p \leq 0.01 \) between contralateral and control). In the case of basal branches, there was a significant reduction in spine density only in undercut hemispheres, as compared with control (Fig. 5B, significance of the difference at a level of \( p \leq 0.01 \)).
Neuronal processes re-orientation after the injury

Apart from the marked spine reduction after undercut, we also observed certain changes in the cortical architecture. In agreement with our previous study (Avramescu et al., 2009), we observed the flattening of the cortical surface in the undercut cortex (Fig. 6A) as well as a reduction in the cortical thickness (not shown). The flattening of the surface was accompanied with a dramatic reorientation of neuronal processes (mainly dendrites) within the undercut cortex (Fig. 6). For systematic analysis of dendritic processes orientation, we collected image stacks from the crown of the gyrus in injured (24 sections from three brains with undercut) and control sections (24 sections from three control brains) stained with Golgi method, and using Neurolucida we automatically reconstructed processes in those fragments (Fig. 6B). Analysis of polar histograms obtained from these reconstructed processes revealed large differences between control and undercut cortices: more processes were detected in the horizontal plane than in the vertical plane in the undercut cortex as compared with control (Fig. 6C). The polar histogram for a mean of all studied sections (Fig. 7A) shows that such differences were systematically present in investigated brains. The independent samples t-test done on percentage of process length in quarters (90° divisions) demonstrate significant differences between the undercut and control in all four quarters.

DISCUSSION

In the present study we investigated morphological aspects of the neocortical connectivity in conditions of penetrating brain wounds.

We have found (a) enhanced long-range connections between the middle part of the undercut cortex and the relatively intact, anterior, part of the cortex; connectivity with the posterior and more severely damaged parts of the deafferented cortex were decreased. (b) The unilateral undercut in the suprasylvian gyrus decreased the number of dendritic spines on basal dendrites in the same hemisphere and on branches of apical dendrites in both ipsi- and contralateral hemispheres. (c) Following undercut, the usually vertical organization of neuronal processes was transformed and horizontal processes dominated.

Previous studies of deafferentation-induced epileptogenesis showed that 2–3 weeks after deafferentation layer 5 pyramidal neurons reveal a highly significant local increase of axonal sprouting, in particular in the perisomatic region, and a tendency toward a reduction of apical dendritic branches (Salin et al., 1995). These data corroborate functional findings of increased neuronal connectivity found in the undercut models of trauma-induced epileptogenesis (Jin et al., 2006, 2011; Avramescu and Timofeev, 2008). However, these studies did not investigate whether long-range connectivity was altered after cortical undercuts.
Following cortical undercut, field potential recordings demonstrated that in acute (Topolnik et al., 2003a,b) and chronic (Nita et al., 2006, 2007) conditions the electrographic seizures start and often remain restricted to the areas surrounding the undercut cortex. However, with time the paroxysmal activity spreads to other cortical areas.
territories and when large cortical areas become involved in paroxysmal oscillations, which takes 1.5–3 months, the behavioral seizures start (Nita et al., 2007). How does the paroxysmal activity involve larger regions remains unclear. Network reorganization is considered to be a part of the chronic phase of epileptogenesis (Rakhade and

Fig. 6. Undercut induces changes in orientation of neuronal processes. (A) An overall change in the neocortical architecture is depicted in representative gyri from partially deafferented (the undercut is marked with arrowheads) and control sections. Areas marked with squares are magnified below. These and similarly located sections in this and other brains served for automatic fiber detection. (B) The automatic fiber detection performed on image stacks (red) is superimposed on the images; scale bar in the inset is 100 μm. (C) Polar histograms obtained for the two slices shown above.
Jensen, 2009). There are at least two non-competitive possibilities of how paroxysmal long-range synchronization can occur. First, the cortical undercut induces a progressive change in the efficacy of the cortical network that enables the involvement of larger regions. Indeed, previous studies have demonstrated that there are

Fig. 7. Differences in orientation of neuronal processes in undercut and control hemisphere. (A) Shows mean radar charts with absolute values of fiber length (in $10^4$ bins) in undercut and control slices, with visible difference in fiber orientation (image stacks from 22 undercut and 24 control slices were used for the analysis). (B) Shows statistical comparison of fiber length percentage: polar histograms were divided into 8 bins of 45° and analysis was then performed on 90° quarters (percentage is presented as mean ± SD). T-test revealed significant increase in the percentage of fibers in the horizontal positions (quarters 2 and 4, 135–225° and 315–45° respectively) and a decrease in the fibers in the vertical plane (quarters 1 and 3, 45–135° and 225–315°) after the injury. $^* p < 0.05$, $^{** *} p < 0.001$. 

Jensen, 2009). There are at least two non-competitive possibilities of how paroxysmal long-range synchronization can occur. First, the cortical undercut induces a progressive change in the efficacy of the cortical network that enables the involvement of larger regions. Indeed, previous studies have demonstrated that there are
Trauma-induced increase in long-range excitatory connectivity (this study) and increase in local connection probability (see above) should be associated with an increase in the number of synapses and most of excitatory synapses are formed on dendritic spines. Similarly to an earlier study (Rutledge et al., 1972) we found that cortical undercut was associated with a decrease in the number of dendritic spines, and in the case of apical dendrites this reduction was found in the suprasylvian gyrus of both partially deafferented and intact, contralateral, hemisphere. Eighty-five percent of intracortical synapses formed by pyramidal neurons are formed on dendritic spines and the remaining contacts are formed on dendritic shafts, a majority of which are formed on dendritic shafts of pyramidal neurons (Kisvarday et al., 1986). These data suggest a possibility that new connections might be formed preferentially on dendritic shafts and not on dendritic spines. This assumption is indirectly supported by the fact that the amplitude of somatic excitatory postsynaptic potentials (EPSPs) is increased in the undercut cortex (Avramescu and Timofeev, 2008) as some, although not major, attenuation is caused by the spine neck that reduces the amplitude of somatic EPSPs (Palmer and Stuart, 2009), implying that an increase in the amplitude of successful EPSPs might be caused by synapses formed on dendritic shafts. A formation of new synapses (before maturation) is characterized by high failure rates (Schiess et al., 2010), which was also observed in our previous study (Avramescu and Timofeev, 2008). Further quantitative electron microscope studies are needed to confirm an increase in the number of synapses on dendritic shafts.

A reduction of dendritic spines on apical dendrites in the hemisphere contralateral to the injured one is intriguing. It might be related to an abolition of callosal axons. Callosal synapses are formed by presynaptic clusters primarily on layer 2–3 or upper layer 4 (Rochefort et al., 2009) on which our study of spine density was performed.

The last important finding of this study was a major change in the dendritic orientation (Figs. 6 and 7). Using an automatic approach we detected neuronal structural processes from image stacks, which included both dendrites and axons. In the intact cortex, the orientation of processes was predominantly vertical due to the major contribution of apical dendrites and vertically oriented axons. However, several weeks after the undercut, the predominantly vertical orientation was lost. Although occasionally apical dendrites could be found in the undercut cortex (Fig. 5), the majority of Golgi stained neurons in layers 2–3 did not reveal a pyramidal structure (Fig. 6B). In agreement with the synaptotrophic hypothesis (Vaughn, 1989; Cline and Haas, 2008), the dendritic structure is very sensitive to the presence of functional inputs. Similarly to our study, a ventral hippocampal lesion in newly born rats removed only a small portion of overall inputs to the medial prefrontal cortex, which resulted in the impairment of the dendritic structure within layer 5 of this cortical area (Ryan et al., 2013). In our study the majority of extracortical inputs to the suprasylvian gyrus was removed, therefore the alteration of the dendritic structure was larger. Such a change in the dendritic structure has multiple consequences on physiological mechanisms. Obviously, it alters the distribution of synapses and therefore properties of synaptic responses. In addition, it might change electrotonic properties of cells. Disappearance of apical dendrite should definitely increase electrotonic compactness of neurons (Rall and Rinzel, 1973), which should increase passive neuronal responsiveness. Even only a change in dendritic geometry, without altering overall dendritic volume, results in changes of neuronal responsiveness (van Ooyen et al., 2002). Thus, our current findings on altered orientation of dendrites corroborate previous observations on overall increased intrinsic neuronal responsiveness in the undercut cortex (Avramescu and Timofeev, 2008).

We conclude that an increased long-range connectivity, a reduced number of spines and an altered neuronal morphology provide the morphological basis for the increased synaptic and intrinsic neuronal responsiveness in the undercut cortex that is a part of trauma-induced epileptogenesis.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.
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