Focal Damage to the Adult Rat Neocortex Induces Wound Healing Accompanied by Axonal Sprouting and Dendritic Structural Plasticity

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Accumulating evidence indicates that damage to the adult mammalian brain evokes an array of adaptive cellular responses and may retain a capacity for structural plasticity. We have investigated the cellular and architectural alterations following focal experimental brain injury, as well as the specific capacity for structural remodeling of neuronal processes in a subset of cortical interneurons. Focal acute injury was induced by transient insertion of a needle into the neocortex of anesthetized adult male Hooded-Wistar rats and thy1 green fluorescent protein (GFP) mice. Immunohistochemical, electron microscopy, and bromodeoxyuridine cell proliferation studies demonstrated an active and evolving response of the brain to injury, indicating astrocytic but not neuronal proliferation. Immunolabeling for the neuron-specific markers phosphorylated neurofilaments, α-internexin and calretinin at 7 days post injury (DPI) indicated phosphorylated neurofilaments and α-internexin but not calretinin immunopositive axonal sprouts within the injury site. However, quantitative studies indicated a significant realignment of horizontally projecting dendrites of calretinin-labeled interneurons at 14 DPI. This remodeling was specific to calretinin immunopositive interneurons and did not occur in a subpopulation of pyramidal neurons expressing GFP in the injured mouse cortex. These data show that subclasses of cortical interneurons are capable of adaptive structural remodeling.

Keywords: cortical injury, interneuron, neurogenesis, plasticity, regeneration

Introduction

Structural injury to the brain evokes a distinct sequence of events indicative of an attempt to repair and heal, including the activation of microglial, oligodendroglial precursor, meningeal, astrocyte, and stem cell populations (reviewed in Fawcett and Asher 1999). It is now established that neurons can also actively react to injury, either through the formation of reactive axonal sprouts or via altered connectivity of preexisting pathways (reviewed in Chuckowree et al. 2004; Carmichael 2006; Fitzgerald and Fawcett 2007; Macias 2008).

Ultimately, an adaptive brain response to trauma may require the induction of neurogenesis and either appropriate regeneration or compensatory plasticity of neural pathways. Alterations in neural progenitor populations and cell proliferation have been demonstrated in diverse models of experimental brain lesion and stroke conditions and are proposed to contribute to postlesion brain recovery (Clarke et al. 1994; Duggal et al. 1997; Holmin et al. 1997; Kernie et al. 2001; Arvidsson et al. 2002; Chen et al. 2003; Douen et al. 2004; Salmon et al. 2004). However, whether brain injury evokes a neurogenic response that contributes to functional recovery currently remains contentious, with the presence of neurogenic events differing for different injury paradigms (Holmin et al. 1997; Kernie et al. 2001; Kuroda et al. 2002; Rice et al. 2003; Salmon et al. 2004; Yu et al. 2008).

Examples of neuronal plasticity, manifesting as alterations in synaptic strength and wiring, remodeling of axonal and dendritic arbors, and changes in dendritic spine and axonal bouton turnover, have been observed in the naïve brain and in response to alterations in sensory experience and injury/lesion (for a selection of recent examples, see Grutzendler et al. 2002; Knott et al. 2002; Trachtenberg et al. 2002; Majewska and Sur 2003; Portera-Cailliau et al. 2003; Holtmaat et al. 2005; Tailby et al. 2005; Lee et al. 2006, 2008; Majewska et al. 2006; Brown et al. 2007, 2008, 2010; Yamahachi et al. 2009). However, the specificity and degree of remodeling that can occur in response to injury has not been fully elucidated. Cortical neurons demonstrate axonal and synaptic remodeling in response to various models of injury (e.g., King et al. 2001; Knott et al. 2002; Trachtenberg et al. 2002; Majewska et al. 2006). Reorganization such as this may correlate with functional recovery not only in stroke (Carmichael 2003, 2006; Brown et al. 2008; Di Filippo et al. 2008) but comparable plasticity may also account for the recovery observed after forms of spinal cord injury (reviewed in Edgerton et al. 2004; Dunlop 2008).

Recent research indicates that populations of interneurons within the barrel cortex demonstrate adaptive plasticity, specifically with regards to the remodeling of dendritic arbors (Lee et al. 2006, 2008). Modifications of the dendritic tree and synaptic contacts may also occur following injury, with studies showing that the plastic potential of cortical dendrites is enhanced following injury (Kolb and Gibb 1991; Jones and Schallert 1992) lasting over 2 weeks post injury (Jones and Schallert 1992). Interestingly, studies have indicated that not all subpopulations of neurons are equal in their capacity for such adaptive structural plasticity. For example, interneurons in the intact neocortex have been demonstrated to be capable of dendritic arbor remodeling while pyramidal neuronal subpopulations remain stable (Lee et al. 2006).

The current investigation utilized a model of focal brain injury to generate a discrete unilateral lesion in the adult rodent somatosensory cortex. The cellular response to injury was investigated over a time course of up to 14 days post injury (DPI). Acute focal injury to the somatosensory cortex induced an evolving cellular response, initially characterized by tissue destruction and bleeding within the injury site. This was followed by an infiltration of the injury site with glial cells and subsequent tissue remodeling including the formation of a glial scar and neovascularization.
Analysis of the morphological plasticity of resident interneuron and pyramidal neuron populations demonstrated cell-type-specific dendritic arbor remodeling following injury; calretinin positive interneurons reorganized their dendrites with a distal orientation shift away from the lesion site, whereas the dendritic arbors of green fluorescent protein (GFP)-expressing pyramidal neurons were not altered. Additionally, peri-lesion axonal sprouting was observed for pyramidal neurons but not for interneurons adjacent to the injury site. These data suggest that the brain is capable of significant remodeling following injury, specific to neuronal type.

Materials and Methods

**In vivo Brain Injury**

All experimental procedures utilizing adult male Hooded-Wistar rats (250–270 g, corresponding to approximately 8 weeks old) and adult male thy1 GFP-M mice (between 8 and 10 weeks old) were approved by the Animal Ethics Committee of the University of Tasmania and are in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Animals were housed in standard conditions (20 °C, 12/12 h light/dark cycle), with access to food and water ad libitum and monitored daily for signs of illness and stress.

Acute focal neocortical injuries were performed as previously described (King et al. 1997, 2001; Dickson et al. 2005). Briefly, following intraperitoneal administration of anesthetic (pentobarbital sodium, 72 mg/kg, Abbot Laboratories) and analgesic (Carprogen, 4 mg/kg, Pfizer), a burr hole was drilled into the skull, (5-mm anterior and 4.5-mm lateral to lambda for rat, 2.5-mm anterior and 2-mm lateral to lambda for mice), and a focal injury was made in the somatosensory cortex (Par1 region) by lowering a 25-gage Hamilton needle (Reno) to a depth of 1.5 mm into the gray matter for rats and a 29-gage needle to a depth of 1 mm into the gray matter for mice. The needle was left in place for 10 min prior to removal and suturing of the wound. Following recovery, animals were terminally anesthetized (pentobarbital sodium, 140 mg/kg) at a range of postinjury time intervals up to 14 days following injury (n = 5 animals per time point at 1, 7, and 14 DPI), and brains were processed for either immunohistochemistry or transmission electron microscopy as described in the relevant sections below.

To determine the phenotype of cells proliferating within the first 7 days following injury, animals were administered with 12.5 mg/ml solution of the thymidine analog bromodeoxyuridine (BrdU) (Sigma) in 7 mM NaOH/0.9% NaCl by intraperitoneal injection. Animals were injected with BrdU (25 mg/kg/day), between 1 and 6 DPI, and were transcardially perfused (4% paraformaldehyde [PFA]/0.01 M phosphate buffered saline [PBS]) at 7 days following injury. Prior to immunohistochemistry, antigen retrieval was performed by incubating sections in 2 M HCl for 1 h at 37 °C, followed by 3 neutralization washes in 0.1 M borate buffer (pH 8.5).

**Immunohistochemistry**

To determine alterations in specific cell populations following cortical injury, rats were terminally anesthetized and transcardially perfused as above. Noninjured, age-matched controls corresponding to the 1, 7, and 14 DPI time points (n = 5 animals per time point) were also perfused. Brain sections (50-μm vibratome) were collected coronally and also horizontal to the pial surface, through the injury site, from control and experimental animals and were immunohistochemically labeled in single as well as double-labeling combinations, with a range of cell-specific antibodies (Table 1). Primary antibody binding was visualized using species-specific fluorescent secondary antibodies, Alexa488 and Alexa594 (Molecular Probes); however, BrdU labeling was visualized with an anti-mouse rat-adsorbed fluorescein secondary antibody (dilution 1:500, Vector Laboratories). Fluorescence imaging was performed using an inverted DMI1B Leica microscope equipped with a Magnafire (Optronics) digital camera. Adobe Photoshop 7 was utilized to prepare figures.

### Table 1

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Note: R, rabbit polyclonal antibody; M, mouse monoclonal antibody; MC, mouse monoclonal cocktail.

**Ultrastructural Studies**

Animals were injured, as above, and then perfused at 7 or 14 DPI with 4% PFA/2% glutaraldehyde/0.01 M PBS. Brains were postfixed in the same fixative solution overnight at 4 °C and then stored in PBS until sectioned. Small regions of the injury site (or equivalent regions of control noninjured brains) were removed and trimmed to 1-2 mm cubes. Tissue blocks were then osmicated, stained with uranyl acetate, dehydrated, and embedded in Epon resin. Thick (1 μm) plastic survey sections were cut and stained with 1% toluidine blue in 1% borax until a suitable region was found. Thin sections (70-90 nm) were then cut on a Reichert Ultratric ultramicrotome and placed onto copper-palladium mesh grids. Following staining with uranyl acetate and lead citrate, grids were examined using a Phillips CM100 transmission electron microscope.

**Analysis of Dendrite Orientation**

For these investigations, 5 male rats and 5 male thy1 GFP mice at 14 DPI were matched to 5-control animals, for both species, with focal injuries in the somatosensory cortex, as detailed above, performed postmortem. In this line of mice, (thy1 GFP-M), GFP expression is localized to a subpopulation of pyramidal neurons within the cortex (Feng et al. 2000). Vibratome brain sections (80 μm) were serially sectioned through the injury site for each control and experimental animal, and for rat sections, immunolabeled with calretinin, a calcium-binding protein localized to a subset of γ-aminobutyric acidergic (GABAergic) neurons (Pappus and Parnavelas 1990). A series of digital images were captured at optimal z-axes intervals (maximum 2.5 μm), using a Zeiss LSM 510 multiphoton/confocal dual purpose microscope with Zen software. Four 20x fields of view were captured around each injury site for 2 separate sections from each animal and all cells within the field of view identified for analysis. Flattened 3D projection stacks were overlaid with a target that had an 80-μm circumference (e.g., see Fig. 6A,B) and analyzed blinded to experimental conditions, using ImageJ freeware.

To quantify the orientation of the dendrites, the angle of all dendrites intersecting the 80-μm circumference for each neuron was measured, relative to the direction of the injury site. This was calculated by drawing a line from the injury site to the center of each soma and then measuring the acute angle between the intersection point for each dendrite at the 80-μm circumference. Thus, for each dendrite this angle of intersection ranged between 0 and 90°. Therefore, an angle of 0° indicates that the dendrite is orientated directly in line with the injury site (either toward or away from), and angle of 90° indicates that the dendrite is orientated laterally relative to the injury site, with a random distribution having an expected angle of 45° (Fig. 6A). From this data, the mean dendrite angle per neuron (θ) was calculated. Thus, the null hypothesis was that there was no significant change in the mean dendrite angle, relative to the injury site, at 14 DPI in comparison with sham-injured control. Mean dendrite angle in correlation with distance from the injury site was investigated for significance using 2-way analysis of variance (ANOVA), t-tests, and linear regression, respectively with the InStat statistical package (GraphPad). Circular variance of angle distribution was analyzed with the Oriana 3.0 statistical package.
A limitation to analyzing changes in mean dendrite angle is that the statistical analysis can demonstrate significant changes in orientation relative to the control but cannot give an indication of the direction of the alteration. To determine the direction of any significant changes in dendrite orientation, the polarity of all dendrites was determined for calretinin positive neurons. The number of dendrites in 4 quadrants—proximal, lateral 1 and 2, and distal (Fig. 7A), to the injury site, intersecting at 80 μm from the cell body, was calculated to determine dendrite polarity and analyzed using a 2-way ANOVA (InStat, GraphPad).

Confocal and Multiphoton Microscopy
All confocal and multiphoton images collected in this study were obtained on a Zeiss LSM 510 microscope, with Zen software. For analysis of axon sprouting at 7 DPI, images were obtained with an argon 488 laser and a HeLa 543 laser. Both lasers were passed through either a 20×/0.8 NA plan-apochromat (Zeiss) objective to visualize the entire injury site and a 64×/0.8 NA plan-apochromat (Zeiss) oil immersion objective for high-power images. For quantification of potential dendritic changes, calretinin-immunolabeled cells were visualized with the HeLa 594 laser passing through a 20×/0.8 NA plan-apochromat (Zeiss), the laser and pinhole settings remaining constant. GFP cells were visualized with a Mai Tai multiphoton laser, at the wavelength 850 nm, with constant power, passing through a 20×/1.0 DIC VIS IR W plan-apochromat (Zeiss) water immersion objective.

Results

Focal Injury Induces an Active Healing Response Culminating in the Formation of a Glial Scar and Neovascularization
The cellular alterations following localized injury to the neocortex were examined at 1, 7, and 14 DPI. Analysis of cortical postinjury material sectioned in the horizontal plane revealed distinct cellular changes as the response to injury progressed, within this tissue core and surrounding the injury site, that were less evident using traditional coronal sectioning.

Figure 1. Immunofluorescent, light, and transmission electron microscopy demonstrated an active response of the brain following injury. Resin sections cut in the horizontal plane from the control noninjured cortex (A and F) showed that the neuropil of the somatosensory cortex of the right hemisphere was dense unbroken structure comprising of cell bodies and their processes and blood vessels. At 1 DPI (B and G), a discreet, circular injury site was clearly visible in the horizontal sections. The injury site was filled with red blood cells indicating that there had been bleeding within the injury site (G). By 7 DPI, the injury site had dramatically increased in size (C). The red blood cells had cleared, and the injury site was populated with cells (arrowheads) and processes, the area innervated with new blood vessels (arrows) (H). The injury site at 14 DPI remained large in comparison with that at 1 DPI and had become more densely infiltrated with cells and their processes, in comparison with 7 DPI (I). At 14 DPI (J), blood vessels permeated the injury tract (arrow). Transmission electron microscopy analysis at 7 and 14 DPI demonstrated that the injury site was infiltrated with activated microglia (E) with prominent inclusion bodies (arrowhead). Proliferating cells (arrowhead denotes a dividing nuclei) were also present within the lesion site at 7 DPI (J). Immunofluorescent labeling of horizontal sections with an antibody to GFAP demonstrated minimal expression in the uninjured brain (K). There was little GFAP immunoreactivity around the injury site at 1 DPI (L). At 14 DPI, there was a localized increased expression of GFAP around the injury site (M). By 14 DPI, there was substantial GFAP reactivity around the injury site that extended to 500 μm away from the injury (N). Coronal sections labeled with GFAP demonstrated that by 14 DPI a dense glial scar had formed within the injury tract (O). Scale bar: A–D = 250 μm, F–I = 100 μm, E and J = 2 μm, K–N = 150 μm, O = 500 μm, asterisk in L–N denotes injury site.
Focal neocortical injury resulted in substantial tissue destruction at the site of the lesion and subsequent alterations indicative of attempted brain repair. Microscopic analysis demonstrated no detectable histopathological changes in control brains or in brain regions contralateral to the lesion site, evidenced with both resin sections (Fig. 1A,F) and also with immunolabeling for glial fibrillary acidic protein (GFAP) (Fig. 1A). The neuropil in control noninjured resin sections was essentially homogenous, tightly associated and dense (Fig. 1A,F). At 1 DPI, tissue destruction was noted within the injury site, with a clear needle tract being visible (Fig. 1B). This lesion site at 1 DPI was characterized by massive red blood cell infiltration (Fig. 1G). By 7 DPI, the injury site had increased in size and now ranged from 200 to 300 μm in diameter. The blood cells that were present at 1 DPI had cleared, and neovascularization of the lesion site had commenced with the area infiltrated by occasional new blood vessels and sparsely populated with cells and processes (Fig. 1C). The neuropil within the injury site, however, remained significantly less dense than surrounding tissue (Fig. 1H). Immunolabeling for GFAP demonstrated that the injury and peri-lesion site had now become filled with reactive astrocytes with their processes directed toward the central lesion site (Fig. 1M). At 14 DPI, blood vessels permeated the injury tract (Fig. 1D,F, arrows) and a significant proportion of the tract had closed together leaving a central dense GFAP-labeled core (Fig. 1N) (King et al. 2001), with a glial scar extending approximately 500 μm from the edges of the lesion (Fig. 1N,O). GFAP immunolabeling was greatest directly adjacent to the injury site, as indicated by increased immunoreactivity, with this labeling progressively decreasing with increasing distance from the injury (Fig. 1N,O). Ultrastructural studies of the central lesion site at 7 and 14 DPI demonstrate, in support of immunohistochemical analysis, the presence of activated microglia with inclusions (Fig. 1E) and also proliferating cells (Fig. 1J).

**Cortical InjuryInducesNeuralProgenitorProliferationbutnotNeurogenesis**

As noted above, proliferating cells were a prominent feature of the tissue surrounding the injured neocortex (Fig. 1J). These cells were further characterized utilizing the progenitor marker nestin and the mitotic markers, BrdU and proliferating cell nuclear antigen (PCNA). Nestin immunoreactive cells were not evident in the gray matter of the neocortex of control noninjured brains (A), but by 7 DPI were abundant within the lesioned tissue (B). Nestin immunoreactive profiles were present in tissue extending from the SVZ and corpus callosum up to the injury site (C). Double immunofluorescence labeling for BrdU, administered at 1 DPI, (D) and nestin (E) demonstrated localization of these markers to the same cells within peri-lesion tissue. Double labeling for PCNA (F) relative to nestin (G) confirmed the proliferation of nestin-labeled cells within the SVZ. Scale bar: A and B = 100 μm, C = 800 μm, D-G = 50 μm.

Figure 2. Immunofluorescence labeling for nestin was increased within the cortex at 7 DPI. Nestin immunoreactive cells were not evident in the gray matter of the neocortex of control noninjured brains (A), but by 7 DPI were abundant within the lesioned tissue (B). Nestin immunoreactive profiles were present in tissue extending from the SVZ and corpus callosum up to the injury site (C). Double immunofluorescence labeling for BrdU, administered at 1 DPI, (D) and nestin (E) demonstrated localization of these markers to the same cells within peri-lesion tissue. Double labeling for PCNA (F) relative to nestin (G) confirmed the proliferation of nestin-labeled cells within the SVZ. Scale bar: A and B = 100 μm, C = 800 μm, D-G = 50 μm.

**Pyramidal Neurons Respond to Injury with Axonal Sprouting by 7 DPI**

Cortical sections, cut in a parallel/horizontal plane to the pial surface through the injury site at 7 DPI, were immunolabeled administered with BrdU at 1 DPI, or nestin, in addition to markers for astrocytes (GFAP), microglia/macrophages (ferritin), and neurons (NF-M, α-internexin, calretinin, and parvalbumin) (Fig. 3). Neuronal markers were selected to represent pyramidal neuron (NF-M and α-internexin) and cortical interneuron (calretinin and parvalbumin) populations. Examination of injured tissue at 7 DPI showed that both BrdU and nestin immunolabeling colocalized with expression of the astrocytic intermediate filament protein, GFAP (Fig. 3A,B). Additionally, a number of ferritin positive microglia/macrophages were double labeled with BrdU (Fig. 3C) indicating they were mitotically active following injury. Contrary to the widespread colocalization between nestin and GFAP, nestin immunoreactivity was not localized within ferritin-immunopositive activated microglia/macrophages (Fig. 3D). Furthermore, BrdU and nestin immunoreactivity were absent from all neuronal populations examined within peri-lesion tissue, including those immunopositive for NF-M (Fig. 3E,F), α-internexin (Fig. 3G,H), calretinin (Fig. 3I,J), and parvalbumin (Fig. 3K,L).

**Figure 2.** Immunofluorescence labeling for nestin was increased within the cortex at 7 DPI. Nestin immunoreactive cells were not evident in the gray matter of the neocortex of control noninjured brains (A), but by 7 DPI were abundant within the lesioned tissue (B). Nestin immunoreactive profiles were present in tissue extending from the SVZ and corpus callosum up to the injury site (C). Double immunofluorescence labeling for BrdU, administered at 1 DPI, (D) and nestin (E) demonstrated localization of these markers to the same cells within peri-lesion tissue. Double labeling for PCNA (F) relative to nestin (G) confirmed the proliferation of nestin-labeled cells within the SVZ. Scale bar: A and B = 100 μm, C = 800 μm, D-G = 50 μm.
with a range of neuronal markers to examine the neuronal response to injury. The phosphorylated neurofilament marker SMI312 (Fig. 4A, B) and the intermediate neurofilament marker α-internexin (Fig. 4C, D) demonstrated that, although no neurogenesis had occurred, neuronal sprouting of resident neuronal populations characterized the response to injury at 7 DPI (Dickson et al. 2005). SMI312 and α-internexin have been previously reported to be expressed in pyramidal neurons albeit not completely exclusively (Masliah et al. 1993; Dickson et al. 2005). Immunoreactivity for both SMI312 and α-internexin had increased around the injury site (Fig. 4A, C, respectively), and high-power confocal images revealed fine sprout-like structures within the injury lesion (Fig. 4B, D, arrows, respectively). However, immunolabeling with calretinin, a calcium-binding protein that labels a subset of interneurons exclusively, was confined to the intact tissue, with a discreet injury border (Fig. 4F), these calretinin immunopositive neurons did not exhibit a sprouting response (Fig. 4F).

Calretinin Neurons Show Dendritic Remodeling at 14 DPI

At 14 DPI, calretinin labeling in the injured rat brains (Fig. 5A) and the injured thy1 GFP mouse brains (Fig. 5C) confirmed that a tissue core had formed around the injury site (Fig. 5A, C, dotted lines). Immunolabeling for calretinin demonstrated a distinguishable neuronal difference between injured and control sham-injured brains. At 14 DPI, the horizontally projecting dendrites of calretinin-labeled neurons (Fig. 5A, arrows) differed from their sham-injured controls (Fig. 5C). The dendrites of calretinin-labeled cells, at 14 DPI, were radially orientated around the lesion site. Expression of GFP in a subset of pyramidal cells at 14 DPI (Fig. 5B), in comparison with contralateral control sham-injured sections (Fig. 5D), demonstrated no differences in dendrite alignment between the injured and control sham-injured sections, albeit there was an increase in nonspecific immunoreactivity around the injury site.

Calretinin Neurons Had a Significant Change in Dendritic Orientation Around the Injury Site at 14 DPI

To quantitate injury-induced changes in the dendritic arbor orientation of calretinin-labeled interneurons, horizontally projecting dendrites were visualized and their orientation relative to the injury site and control sham-injury site determined. All calretinin-immunolabeled cells within the four 20× fields of view around the injury were included in analysis for both injured and control sham-injured sections. All dendrites intersecting a circumferential point 80 μm from the center of the neural cell body were analyzed. Neurites at this distance corresponded predominately to secondary dendrites that were morphologically elaborate with multiple branch points. Figure 6A,B illustrates a representation of horizontal...
ANOVA demonstrated no significant difference in dendrite length and mean dendrite number were quantified. One-way ANOVA demonstrated that fine sprouts (arrows), immunopositive for SMI312 and α-internexin, were transversing the injury site. However, neurons immunopositive for the interneuronal marker calretinin did not demonstrate the same response. Immunolabeling for calretinin (E and F) was confined to the intact tissue. Furthermore, high-power confocal images confirmed no sprouting response present beyond the lesion edge (V). Scale bar: A, C, E = 200 μm and B, D, F = 50 μm, dotted line denotes edge injury site.

Figure 4. Immunofluorescent microscopy and confocal microscopy of horizontal sections at 7 DPI demonstrated an active neuronal response to injury. Immunolabeling with the pyramidal neuron markers SMI 312 (A) and α-internexin (C) showed a marked increase in immunoreactivity around the injury site. High-power confocal images demonstrated that fine sprouts (arrows), immunopositive for SMI312 (B) and α-internexin (D), were traversing the injury site. However, neurons immunopositive for the interneuronal marker calretinin did not demonstrate the same response. Immunolabeling for calretinin (E and F) was confined to the intact tissue. Furthermore, high-power confocal images confirmed no sprouting response present beyond the lesion edge (V). Scale bar: A, C, E = 200 μm and B, D, F = 50 μm, dotted line denotes edge injury site.

dendrites of neurons most characteristic for the populations at 14 DPI and control sham injuries, respectively.

To investigate dendritic remodeling in this subpopulation of neurons, the orientation of the individual dendrites for each neuron was determined (see Materials and Methods). The distribution of the mean angle of all dendrites for each neuron, from control sham—injured and injured animals, relative to the distance the neuron was from the injury site, is represented in Figure 6CD, respectively. This data demonstrate the distinct shift in dendrite angle relative to injury in neurons from injured brains (Fig. 6D), with a trend for this shift to increase at increasing distances from the injury site; however, linear regression demonstrated that this trend was not significant (p = 0.05). One-way ANOVA of the mean dendrite angle for each injured cell demonstrated a significant (P < 0.001) decrease in mean dendrite angle at 14 DPI (n = 51) in comparison with control sham injured (n = 82) (Fig. 6E). Furthermore, analysis utilizing biaxial θ parameters of circular statistics further identified a difference in plot distributions. Mean dendrite angle for neurons from control animals was 45.50° with a circular standard deviation (SD) of 10.54° and for neurons from injured animals was 19.06° with a circular SD of 17.06°.

To confirm that the alteration in dendrite orientation was not a result of either dendrite damage or growth, the mean dendrite length and mean dendrite number were quantified. One-way ANOVA demonstrated no significant (P > 0.05) difference in the mean number of dendrites between the injured and control sham-injured brains, (mean = 2.97, SD = ±1.90 for control sham injured and mean = 2.87, SD = ±2.16 for 14 DPI). Mean neurite length for all primary, secondary, and tertiary dendrites and other dendrites (with more than 3 branch points) for each interneuron around the injury site in comparison with control, was also analyzed. The number of neurites that branched at more than 3 points was minimal (data not shown). There was no significant (P > 0.05) difference in mean dendrite lengths for primary, secondary, and tertiary dendrites, analyzed with a one-way ANOVA (Fig. 6F).

To determine the directionality of the decrease in mean dendrite angle, the polarity of each neuron investigated was determined. This was analyzed by dividing the quantitation zone of each cell into 4 quadrants (Fig. 7A). For each dendrite, the quadrant in which the dendrite intersects at 80 μm was recorded. The quadrants were divided into proximal, lateral 1 and 2, and distal, relative to the injury site. Two-way ANOVA revealed a significant (P < 0.05) increase in the mean number of dendrites in the distal quadrant (Fig. 7B) at 14 DPI in comparison with the control sham injured. Additionally, there was a significant (P < 0.05) decrease in the mean number of dendrites per neuron in the lateral 1 quadrant.

The Dendrites of Pyramidal Neurons Expressing GFP Did Not Undergo Remodeling Around the Injury Site at 14 DPI

To investigate whether the remodeling response was specific to the calretinin positive interneurons examined or was

Figure 5. Confocal images for calretinin immunolabeling and GFP localization demonstrated a realignment of the horizontally projecting dendrites of calretinin interneurons at 14 DPI. Confocal images for calretinin and GFP horizontal sections at 14 DPI demonstrated that the injury site had filled, forming a dense tissue core (A and B). Horizontally projecting dendrites (arrows) of calretinin-labeled interneurons can be clearly visualized around the injury site in sham-injured control sections (C). At 14 DPI, the horizontally projecting dendrites of calretinin-labeled interneurons had realigned around the injury site (arrows), as demonstrated with confocal microscopy (A). Comparison between GFP localization at 14 DPI (B) and control sham-injured section (D) revealed no detectable differences. Scale bar = 150 μm, dotted line denotes edge injury site.
a stereotypical neuronal response, a subset of pyramidal neurons was also investigated in a transgenic mouse line with GFP expression restricted to this cell type. All GFP positive cells within four 20× fields of view around the injury site were included in analysis for both injured and noninjured control sections. Again, dendrites intersecting a circumferential point 80 μm from the center of the cell bodies were analyzed.

Figure 6. Horizontally projecting neurites of calretinin-immunolabeled cells underwent significant remodeling, with no change in mean length, at 14 DPI. Horizontally projecting dendrites were visualized using flattened z-stacks. A and B illustrate representative flattened z-stacks for calretinin-immunolabeled neurons in control sham-injured (A) and injured (B) horizontal sections. The neuron represented in (A) has been overlaid with the target used to calculate the acute angle of each dendrite relative to the injury site. Plot distribution for the control sham-injured (C) and injured (D) populations in relation to the distance of the cell from the injury site further demonstrated the distinct shift in mean dendrite angle of the injured neurons (D), with a trend for this to be increased with increasing distance from the injury site; however, linear regression demonstrated that this trend was not significant (P = 0.05). The mean of dendrite angle for each cell was significantly (P < 0.01) decreased at 14 DPI in comparison with control (E). The quantification of the mean neurite length of primary, secondary, and tertiary branches at 14 DPI and in control sham injured animals revealed no significant differences between 14 DPI and control (F). Error bars denote standard error of the mean.

Figure 8A,B illustrates a representation of horizontal dendrites of neurons most characteristic for the populations at 14 DPI and control sham injuries, respectively. Measurements of mean dendrite angle, for pyramidal neurons in relation to the distance of the cell from the injury site, demonstrated no significant (P > 0.05) shift in the injured neurons at 14 DPI (n = 86) in comparison with control sham injured (n = 46)
standard error of the mean. Scale bar with dendrites on neurons in control sham-injured tissue (number of dendrites in the distal quadrant away from the injury site, in comparison to control). Error bars denote standard error of the mean. Scale bar = 200 μm, 100 μm for inset.

Figure 7. Horizontally projecting neurites of calretinin-immunolabeled cells underwent a significant increase in distally projecting dendrites and decrease in laterally projecting dendrites, at 14 DPI. A template comprised of 4 quadrants was layered over each neuron under investigation and the quadrant in which each dendrite intersected the 80-μm line (arrows) recorded (B, inset from A). The quantification of dendrite orientation at 14 DPI demonstrated a significant (*P < 0.01) difference in number of dendrites in the distal quadrant away from the injury site, in comparison with dendrites on neurons in control sham-injured tissue (C). Error bars denote standard error of the mean. Scale bar = 200 μm, 100 μm for inset.

Additionally, there was no significant (P > 0.05) difference in mean dendrite lengths for primary, secondary, and tertiary dendrites, analyzed with a one-way ANOVA. (Fig. 8C,D, and E).

### Discussion

The experimental model used in this study produced a discrete lesion, causing structural damage to the neocortical laminae but not surrounding or underlying structures (King et al. 1997, 2001). This form of injury generates a stereotypical sequence of changes that ultimately culminate in wound healing, including phases of acute hemorrhage, necrosis, and edema, followed by glial activation, scar formation, and wound contraction (Maxwell et al. 1990; King et al. 2001). In the present investigation, we highlight distinct mechanisms of injury-induced neuronal plasticity, which are specific to discrete subpopulations of cortical neurons and progenitor cells. Although we found no evidence of neurogenesis within the damaged cortex, we reveal that resident pyramidal and interneurons undergo contrasting alterations in response to structural injury; pyramidal neurons respond by elaborating fine axonal sprouts into the injury site, whereas interneurons undergo dendritic remodeling, whereby the dendritic arbor becomes reoriented away from the injury site. These responses occurred concurrent with a stereotypical gliogenic response.

Several studies have demonstrated evidence of neuronal proliferation in response to experimental brain injury (e.g., see Kernie et al. 2001; Rice et al. 2003), providing support for neurogenesis as a mechanism of brain repair following traumatic brain injury. However, this and other investigations (Holmin et al. 1997; Kuroda et al. 2002; Salman et al. 2004) have indicated that neurogenesis does not lead to nerve cell replacement in the neocortex following an acute focal lesion. Specifically, we observed nestin-labeled cells within the SVZ and corpus callosum by 7 DPI, with nestin immunoreactivity extending up to the injury site. The nestin immunoreactive profiles present within both the SVZ and corpus callosum colocalized with the markers PCNA and BrdU, respectively, indicating that they were mitotically active. Double-labeling studies indicated that nestin positive cells did not express neuronal marker proteins; however, nestin was found to colocalize with the astrocytic marker, GFAP, indicative of a gliogenic, but not neurogenic, response. Whether this upregulation is a result of new cell division or the reexpression of nestin within existing glia (Kronenberg et al. 2005) is unclear.

The lack of postinjury neurogenesis in the present investigation may be due to the location and severity of the injury. Many models of cortical lesion generate widespread neural damage (Posmantur et al. 1996), which often directly affects regions associated with high plasticity, such as the SVZ or hippocampus, or at least damages the cortex more extensively. The model utilized in the current investigation, however, specifically targeted the upper layers of the cortical gray matter and thus may not evoke the same signaling mechanisms initiated in more disruptive forms of trauma. The absence of constitutive cortical neurogenesis has been postulated to result from a lack of appropriate microenvironmental cues rather than a limit of the endogenous precursors themselves (Emsley et al. 2005), and this deficiency may also account for the absence of neurogenesis in the cortex following injury in the present study.

Focal injury to the neocortex evoked a characteristic neurite sprouting response adjacent to the lesion. This sprouting response was likely to be axonal in origin and confined to pyramidal neurons and was not demonstrated to occur from a subpopulation of interneurons immunopositive for calretinin. This induction of axonal growth after injury may be maladaptive, potentially exacerbating a clinically poor outcome. Both in vitro (McKinney et al. 1997; Chuckowree and Vickers 2003; Blizzard et al. 2007) and in vivo (Salin et al. 1995) experimental models indicate that postinjury sprouting may not always be functionally appropriate, with potential aberrant axonal connectivity possibly contributing to the development of epilepsy following brain lesions such as stroke (Carmichael 2003). Furthermore, studies in our laboratory have indicated that regenerative axonal sprouts differ from their developmental counterparts, being tipped by bulbous end structures in vivo and lacking the ability to path find in vitro (King et al. 2001; Dickson et al. 2007; Blizzard et al. 2007, respectively). Recent research indicates that the anatomical and functional plasticity of surrounding intact cortex rather than frank regeneration of
damaged neurons may play a more fundamental role following injury. Hence, understanding the mechanisms underpinning the lack of injury-induced axonal sprouting in interneuron subpopulations may reveal fundamental differences in the intrinsic plastic capabilities of different cortical neuron populations and what implications this may have for brain healing.

Although postinjury axonal sprouting was shown to be specific to pyramidal neurons, interneurons were not without injury-induced remodeling capabilities albeit in a somewhat
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In this investigation, we show the particularly novel property of a subpopulation of interneurons, namely those that were calretinin immunoreactive, to remodel their dendritic arbors in response to acute brain lesion. Cortical interneurons within the barrel cortex have previously been shown to have a capacity for morphological plasticity (Lee et al. 2006); however, this was under physiologically normal conditions. Furthermore, previous investigations have suggested that the potential sites for structural plasticity are the horizontal connections within the superficial layers of the cortex (Kaas et al. 1990; Darian-Smith and Gilbert 1994; Lee et al. 2008). Following acute brain injury in the current study, the dendritic arbor of calretinin positive interneurons was orientated away from the injury site, relative to sham-injured controls. Although it was postulated that this change in dendritic orientation was due to new dendrite growth in response to injury, further analyses of both mean dendrite number and mean neurite length revealed that neither parameter was altered as a result of the injury. Hence, the alteration in dendrite alignment was most likely a result of remodeling of existing arbors, such that those close to the injury retracted, while those distal to the injury grew. These findings are consistent with studies demonstrating that GABA positive interneurons exhibit dynamic arbor rearrangements while pyramidal populations remain stable, under physiologically normal conditions (Frahm et al. 2004).

A subset of pyramidal neurons, specifically those expressing GFP in the transgenic mouse, was also investigated to determine if this remodeling was cell-type specific. While the injury site in these mice had formed a comparable tissue core by 14 DPI, indicating an active brain response, statistical analysis revealed no significant remodeling in the dendrites of these pyramidal neurons. Thus, the dendritic remodeling observed at around the injury site, in our model of brain injury, may be confined to interneurons, specifically the calretinin positive population, demonstrating a cell type-specific response to injury. These findings are in coherence with studies demonstrating that the GABA positive interneurons exhibit dynamic arbor rearrangements while pyramidal populations remain stable, under physiologically normal conditions (Lee et al. 2006).

Previous investigations have demonstrated that the outgrowth of dendrites following injury can be correlated to axonal rearrangement and, interestingly, that alterations in dendritic morphology are associated with synaptic modifications (for recent review, see Macias 2008). Hence, the dendritic reorientation observed in the current study may represent structural plasticity directed toward the intact cortex. The injury-induced generation of aberrant axonal connectivity may contribute to the development of epilepsy following various forms of brain lesion (Carmichael 2003). It is also possible that alterations in the balance of inhibitory synapses, for example, resulting from remodeling of dendritic architecture as observed in the current injury paradigm, may also play a role in the formation of aberrant connectivity following some forms of brain lesion. However, whether this plasticity ultimately culminates in the formation of new functional or dysfunctional connections remains to be elucidated.

Conclusions

The adult brain has a frequently unappreciated capacity for cytoarchitectural remodeling and repair following injury. These studies demonstrate a novel cell type-specific response of neuronal populations to acute injury; a subpopulation of pyramidal neurons elaborated axonal sprouts into the injury site, whereas a subpopulation of interneurons (calretinin neurons) underwent a reorganization of their dendrites to project more distally away for the injury site. These distinct responses, exhibited by different cortical neuron populations, provide novel insight into the plastic potential of the mature brain to injury.

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Notes

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References


