Electrophysiological Characteristics of Reactive Astrocytes in Experimental Cortical Dysplasia

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INTRODUCTION

The astrocytic response to injury of the CNS is often referred to as “reactive gliosis.” Although not fully understood, this stereotypic, mainly astrocytic, response to injury is characterized by an initial dedifferentiation of astrocytes and enhanced cell proliferation, ultimately leading to a dense astrocytic scar. Astrocytes hypertrophy and show marked upregulation of the intermediate filament protein glial fibrillary acidic protein (GFAP) (Eng 1985; Eng and Ghirnikar 1994). Reactive gliosis is often found in association with diseases and is a prominent feature in stroke and epilepsy. It has been shown that freeze-induced neocortical malformations are intrinsically hyperexcitatory (Hablitz and Defazio 1998; Jacobs et al. 1996; Luhmann and Raabe 1996). Experimental microgyria is also associated with increased immunocytochemical staining for glial markers (Hablitz and Defazio 1998).

While the biochemical and antigenic changes that occur in conjunction with reactive gliosis are well studied (Ridet et al. 1997), the changes in biophysical properties of reactive astrocytes are just being revealed. A decrease in $K_{IR}$ current amplitudes has been reported after injury of cultured astrocytes (MacFarlane and Sontheimer 1997) and after a posttraumatic injury in vivo (D’Ambrosio et al. 1999; Schroder et al. 1999), leading to an impaired $K^+$ homeostasis (D’Ambrosio et al. 1999). However, these findings are still controversial in light of conflicting previous studies. In 1970, Pollen and Trachtenberg showed that $K^+$ buffering in freeze-lesion-induced dysplastic neocortex is facilitated by intracellular coupling of astrocytes through gap junctions (Giaume et al. 1999; Mobbs et al. 1988; Yamamoto et al. 1990). The expression of voltage-activated $K^+$ channels changes during early postnatal development (Bordey and Sontheimer 1997; Steinhauser et al. 1992) in a way that suggests that $K^+$ buffering is not fully established at birth but develops in the first 3–4 wk of postnatal life. During this developmental time period, neuronal activity causes much larger $K^+$ fluctuations than in the adult (Ransom et al. 1986; Sykova 1983; Sykova et al. 1992), again suggesting that $K^+$ buffering by astrocytes develops as these cells mature. Interestingly, in cultured astrocytes, this maturation, where expression of $K_{IR}$ channels is a hallmark, can be reversed by injury (MacFarlane and Sontheimer 1997). Mechanical injury to astrocytes leads to a dedifferentiation and proliferation of astrocytes at injury sites. This process is accompanied by a loss of $K_{IR}$ channels.
suggested that a decisive factor in the development of post-traumatic focal epilepsy was a degradation of glial function, especially with respect to the buffering of $K^+$. A loss of $K_{IR}$ channels was reported in human reactive astrocytes associated with epileptic seizure foci (Bordey and Sontheimer 1998) and in rat hippocampal slices rendered seizure prone following a fluid percussion injury (D’Ambrosio et al. 1999). However, Glötzer (1973), using alumina hydroxide gel-induced epileptogenic lesions in cats, found that neuroglial $K^+$ buffering was even more efficient in transporting $K^+$ away from the epileptic sites than under normal conditions. This finding is not consistent with a loss in $K_{IR}$ currents that would reduce their buffering capacity but could result from an increase in intercellular coupling among astrocytes as has been reported for human epileptic astrocytes (Lee et al. 1995; Naus et al. 1991). Adding even more complexity to the issue is the fact that a subpopulation of reactive astrocytes proliferates (Garcia-Estrada et al. 1993; Guénard et al. 1996; Krushel et al. 1995; Niquet et al. 1994; Ritchie and Rogart 1977). Proliferative astrocytes differ significantly in their biophysical properties from differentiated astrocytes. Most notably, they show reduced $K_{IR}$ channel activity (MacFarlane and Sontheimer 1997).

These conflicting findings in different models of injury could result from examination of different populations of reactive astrocytes. Indeed, the above-mentioned in vivo or in situ studies thus far have focused on elucidating properties of astrocytes associated with epileptic seizure foci. In an effort to characterize properties of reactive astrocytes associated with glial scars, we used a cortical freeze-lesion model of brain injury that is characterized by the development of abnormal microgyria. This maldevelopment is associated with neuronal injury that is characterized by the development of abnormal microgyria (Defazio 1998; Jacobs et al. 1996; Luhmann and Raabe 1996). Briefly, rats were anesthetized using pentobarbital (50 mg/kg) and decapitated. The brain was quickly removed and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1.5 MgCl$_2$, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 10 glucose, and 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and was continuously oxygenated with 95% O$_2$-5% CO$_2$. The brain was hemisected, and a block of cortex containing the microsulcus was glued (cyaanoacrylate glue) to the stage of a Vibratome. Slices (250- to 300-μm thick) were cut in cold oxygenated ACSF and transferred to a chamber filled with ACSF at room temperature. After a recovery period of 2 h in ACSF, slices were placed in a flow-through chamber continuously perfused with oxygenated ACSF at room temperature. The chamber was mounted on the stage of an upright microscope (Nikon Optiphot2) equipped with a ×40 (2-mm working distance), water-immersion objective and Nomarski optics.

**METHODS**

**Freeze lesion**

Freeze lesions were performed as previously reported (Hablitz and Defazio 1998). Briefly, timed-pregnant Sprague-Dawley dams were maintained in our animal facility until parturition. On P1, rat pups were anesthetized via hypothermia. After a midline scalp incision, the skin was retracted, and a 5-mm brass probe cooled to $-60^\circ$C was applied for 3 s to the skull 1.5 mm lateral to the midline. After suturing, animals were allowed to recover under a heating lamp for 30 min. They were then returned to their cage and allowed to survive for 16–24 days prior to experimentation. The institutional review board approved this procedure.

**Slice preparation**

Methods used for preparation of thin cortical slices were described previously (Bordey and Sontheimer 2000; Hablitz and Defazio 1998). Briefly, rats were anesthetized using pentobarbital (50 mg/kg) and decapitated. The brain was quickly removed and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1.5 MgCl$_2$, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 10 glucose, and 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and was continuously oxygenated with 95% O$_2$-5% CO$_2$. The brain was hemisected, and a block of cortex containing the microsulcus was glued (cyaanoacrylate glue) to the stage of a Vibratome. Slices (250- to 300-μm thick) were cut in cold oxygenated ACSF and transferred to a chamber filled with ACSF at room temperature. After a recovery period of 2 h in ACSF, slices were placed in a flow-through chamber continuously perfused with oxygenated ACSF at room temperature. The chamber was mounted on the stage of an upright microscope (Nikon Optiphot2) equipped with a ×40 (2-mm working distance), water-immersion objective and Nomarski optics.

**Whole cell recordings and data analysis**

Whole cell patch-clamp recordings were obtained as previously described (Bordey and Sontheimer 2000; Hamill et al. 1981). Patch pipettes were pulled from thin-walled borosilicate glass (OD, 1.55 mm; ID, 1.2 mm; WPI, TW150F-40) on a PP-83 puller (Narishige, Japan). Pipettes had resistances of 3–7 ΩM when filled with the following solution (in mM): 140 KCl, 0.2 CaCl$_2$, 1.0 MgCl$_2$, 10 ethylene glycol-bis(aminohexyl ether)-$\mathrm{NN',NN'}$-tetraacetic acid (EGTA), 4 Na$_2$ATP, and 10 HEPES, pH adjusted to 7.2 with tris (hydroxymethyl)aminomethane (Tris). To label cells for later morphological identification, 0.1–0.2% Lucifer yellow (LY, dilitium salt) was added to the pipette solution. Voltage-clamp recordings were performed using an Axopatch-200A amplifier (Axon Instruments). Current signals were low-pass filtered at 5 kHz and digitized on-line at 25–100 kHz using a Digidata 1200 digitizing board (Axon Instruments) interfaced with an IBM-compatible computer system. Data acquisition, storage and analysis were done using pClamp version 7 (Axon Instruments). For all measurements, capacitance compensation and series resistance compensation (40–80%) were used to minimize voltage errors. Settings were determined by compensating the transients of a small (5 mV) 10 ms hyperpolarizing voltage step; the capacitance reading of the amplifier was used as value for the whole cell capacitance. After compensation, series resistances ranged from 7 to 12 MΩ.

Capacitive and leak subtraction was done off-line using Clampfit 7 (Axon Instruments). Since voltage- or time-dependent currents were activated at almost every step potentials in cells with a “complex” electrical profile, we used a modified tail protocol to calculate membrane input resistance ($R_{in}$, Fig. 1). This protocol has been commonly used to detect $K_{IR}$ currents. The cell was stepped to 0 mV and then hyperpolarized from 0 to $-160$ mV. Between $-0$ and $-60$ mV, long-lasting outward $K^+$ currents were inactivating, and at potentials more hyperpolarized than $-80$ mV, $K_{IR}$ currents were activated (Fig. 1A). There was thus a small window of membrane potentials ($90$ and $-60$ mV at the beginning and the end of the pulse, respectively) without any voltage-activated currents. This can be seen on the traces and in the current-voltage relationship (Fig. 1B). Membrane resistance was determined from the inverse slope of a linear fit to the current-voltage relationship at the membrane potentials not showing voltage-
A current amplitudes were measured at a hyperpolarizing pulse from a holding potential of $-10\text{mV}$. We measured the membrane input resistance, called $R_m$, by applying a 10-mV hyperpolarizing pulse from a holding potential of $-80\text{mV}$. $R_m$ values are contaminated by the activation of inward currents present at $-80\text{mV}$ and are thus underestimated. To activate transient and long-lasting outward K$^+$ currents, depolarizing pulses were applied from $-70$ to $+80\text{mV}$ following a prepulse to $-50$ and $-110\text{mV}$, respectively. After off-line leak subtraction using $R_m$, $K_{\text{DR}}$ and $K_{\text{S}}$ current amplitudes were measured at $+50\text{mV}$ at the end of the protocol and at the peak value, respectively. For cells displaying a “passive” electrical profile, inward currents were measured at $-160\text{mV}$ on nonleak subtracted protocols. These cells were stepped from $-160$ to $80\text{mV}$ from a holding potential of $-80\text{mV}$. The corresponding conductance, $G$, of each K$^+$ and Na$^+$ currents was calculated using the following equation: $G = I/(V - V_i)$ where $V$ is the membrane command potential, $V_i$ is the equilibrium (Nernst) potential for the ion under consideration. Statistical values (means ± SE, with $n$ being the number of cells tested) were evaluated with a Student’s $t$-test or for data that did not have normal standard deviation distribution. ANOVA was used for multiple comparisons or for data that did not have normal standard deviation distributions.

**Proliferation assay**

Slices from freeze-lesioned and age-matched control animals were incubated for $\approx 2\text{h}$ in ACSF containing 10 µmol/l BrdU (Boehringer Mannheim). After cell recordings, slices were fixed in 4% paraformaldehyde at 4°C for 3–6 h. Slices were rinsed thoroughly with PBS and prepared for BrdU staining according to the adapted protocol of Zupanc (1998). Briefly, slices were permeabilized for 5 min in a PBS buffer containing 0.5% Triton and 0.1% BSA and rinsed. DNA was denatured by 2 M HCl for 30 min at 37°C and neutralized twice with Na$_2$B$_4$O$_7$ rinses (5 min) followed by three PBS rinses (5 min). After a 20-min blocking step in a PBS buffer containing 0.1% Triton, 10% normal goat serum, and 1% BSA, the slices were incubated with 1:100 dilution of mouse anti-BrdU FITC-conjugated antibody (DAKO Corporation). This 2-h incubation in PBS buffer was followed by three rinses (5 min) in PBS. Slices were then mounted on glass coverslips with fluorescent mounting medium (Vector). In some cases, the LY-filled cell was preserved, and BrdU staining was determined for that cell. BrdU immunostaining controls were performed by incubating slices without BrdU and using only the fluorescently tagged antibody to BrdU. Note that we may have underestimated the true extent of cell proliferation as only those cells that were in S phase during slice incubation would have been BrdU labeled.

**GFAP staining**

Cells chosen visually for recordings were archived using a CCD camera (Watec Instruments) in combination with a video printer (Sony) for later (off-line) comparison to LY fills. After recordings, slices were transferred to a fixation medium containing 4% paraformaldehyde in PBS. Slices were washed three times in PBS for 1 h and incubated for 10 min with 0.2% Triton X-100 and 1% normal goat serum (Vector) in PBS. Slices were then incubated for 2 h at 4°C with the primary polyclonal antibody to GFAP (IncStar; rabbit anti-mouse GFAP, dilution 1:100) in PBS in the presence of 1% normal goat serum and 0.2% Triton. Slices were washed three times with PBS for 5 min and incubated with the secondary antibody (goat-anti-rabbit IgG), conjugated to rhodamine (dilution 1:100, Atlantic Antibodies) for 2 h at room temperature. Slices were mounted in Gel Mounting Media (Fisher) on glass coverslips. LY labeling and GFAP staining were visualized on a Leica DM microscope at ×20, ×40, and ×100 magnifications. Images were captured with an Optronics DEI-750 integrating camera and printed on an Epson color printer. No bleed-

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**Fig. 1.** Leak subtraction. A, left: tail protocol obtained by depolarizing the cell to 0 mV for 100 ms before giving an hyperpolarizing voltage step from $-180$ to 0 mV for 40 ms, $\Rightarrow$, on which trace and where on the trace current amplitudes used to determine the cell input resistance, $R_m$, were measured. Right: traces obtained after leak subtraction. The leak subtraction was performed off-line using the input resistance, $R_m$, obtained in C. B: current-voltage relationship of the current amplitude measured at the end of the pulse before and after leak subtraction (● and ○, respectively). C: linear fit of the I-V (●) at particular membrane potentials ($-90$ to $-60\text{mV}$, see A, left). The inverse slope yields the membrane input resistance, $R_m$. Activated currents (Fig. 1C). In the cell illustrated, the membrane resistance was 280 MΩ (coefficient of correlation, $r = 0.999$). After off-line leak subtraction using $R_m$ (Fig. 1A, right), the maximum current measured at about $-150\text{mV}$ is indicative of the true $K_{\text{IR}}$ current amplitude. For cells displaying a “passive” electrical profile, $R_m$ values are contaminated by the activation of inward currents present at $-80\text{mV}$ and are thus underestimated. To activate transient and long-lasting outward K$^+$ currents, depolarizing pulses were applied from $-70$ to $+80\text{mV}$ following a prepulse to $-50$ and $-110\text{mV}$, respectively. After off-line leak subtraction using $R_m$, $K_{\text{DR}}$ and $K_{\text{S}}$ current amplitudes were measured at $+50\text{mV}$ at the end of the protocol and at the peak value, respectively. For cells displaying a “passive” electrical profile, inward currents were measured at $-160\text{mV}$ on nonleak subtracted protocols. These cells were stepped from $-160$ to $80\text{mV}$ from a holding potential of $-80\text{mV}$. The corresponding conductance, $G$, of each K$^+$ and Na$^+$ currents was calculated using the following equation: $G = I/(V - V_i)$ where $V$ is the membrane command potential, $V_i$ is the equilibrium (Nernst) potential for the ion under consideration. Statistical values (means ± SE, with $n$ being the number of cells tested) were evaluated with a Student’s $t$-test or for data that did not have normal standard deviation distribution. ANOVA was used for multiple comparisons or for data that did not have normal standard deviation distributions.
through was observed between the rhodamine and FITC fluorescence channels in control experiments. Chemicals were purchased from Sigma, unless otherwise noted.

RESULTS

Cortical freeze lesions contain reactive astrocytes and a population of proliferative astrocytes

A representative photomicrograph of a cerebral cortex section from a P16 rat that received a freeze lesion on P1 is shown in Fig. 2. The lesion induced an abnormal microsulcus. Layer I of the cortex followed the entire extent of the microsulcus. At the base of this malformation, the cytoarchitectonic organization of the cortical layers was disrupted as has been previously described in this model (Dvorak et al. 1978; Hablitz and Defazio 1998; Humphreys et al. 1991; Jacobs et al. 1996). This base region of the microsulcus showed extensive neuronal cell loss. By contrast, normal cortical lamination was observed adjacent to the microsulcus. At a distance of ~400 μm from the microsulcus, neurons display epileptiform responses following electrical stimulation, and thus this region has been referred to as an "hyperexcitable" region (Hablitz and Defazio 1998; Jacobs et al. 1996; Luhmann and Raabe 1996).

Immunohistochemical staining for the astrocyte-specific intermediate filament protein, GFAP, showed profound changes in GFAP immunoreactivity in postlesion cortical slices (Fig. 3A). These changes are consistent with widespread reactive gliosis. An increase in GFAP immunostaining was particularly evident along and at the base of the abnormal microsulcus. GFAP immunostaining was more pronounced around the microsulcus and in the region immediately adjacent to the microsulcus (<200 μm). At distances >200 μm from the microsulcus including the hyperexcitable region, staining was similar to that observed in the contralateral, unlesioned cortex. Astrocytes appeared to be hypertrophic with thickened processes when examined at higher magnification (Fig. 3B). Animals that were sham-operated did not show these morphological features typically referred to as reactive astrocytes (data not shown). Reactive astrocytes have been shown to be a heterogeneous cell population containing both hypertrophic differentiated cells and proliferating astrocytes. This has also been demonstrated in the hippocampus of kainic acid-injected rats that show both proliferative and nonproliferative cells (Niquet et al. 1994). Similarly, in freeze-lesioned animals, Hablitz and Defazio (1998) showed that some of the reactive astrocytes were positively stained for vimentin, a marker believed to be specific for immature, dividing glial cells (Eng and Ghirnikar 1994). To assess this heterogeneity further, we incubated sections containing the microsulcus with BrdU, a DNA marker that is selectively incorporated by dividing cells during S phase of the cell cycle. Slices were incubated for a minimum of 2 h and then stained with a monoclonal antibody against BrdU. Figure 4 shows a representative example of the BrdU immunoreactivity in a section from a freeze lesion compared with an equivalent unlesioned section from the contralateral hemisphere (Fig. 4, A and B, respectively). BrdU-positive cells show bright red cell nuclei. Nuclear BrDU staining was prominent on the lesioned side of the cortex. The contralateral, unlesioned side showed few if any BrDU-positive cells, with primarily unspecific, nonnuclear background labeling. Although proliferative cells were found throughout the lesioned cortex, there was a tendency to cluster at the base of the microsulcus. Microglial cells also proliferate in response to CNS injury. However, BrdU-positive cells were localized to regions of high astrogliosis (intense GFAP staining, see preceding text) and most likely resembled astrocytes. We have established the time course for microglial and astrocytic proliferation and have found that microglial proliferation predominates at 24-72 h after injury and is essentially absent 1 wk after lesioning, while astrocyte proliferation continues for many weeks (F. Love and H. Sontheimer, unpublished data). This was also demonstrated in an axotomy model of injury (Boucsein et al. 2000). These data suggest that the freeze-lesion gives rise to reactive gliosis composed of both proliferative (BrdU-positive) astrocytes concentrated near the base of the microsulcus and nonproliferative hypertropic reactive astrocytes concentrated in adjacent areas. These phenotypes are reminiscent of gliotic lesions in human biopsy specimens from patients with mesio-temporal lobe sclerosis (Kallioinen et al. 1987; McNamara 1994).

Electrophysiological characterization of astrocytes in unlesioned neocortex

The electrical properties of cortical astrocytes were studied in lesioned and unlesioned brains using whole cell patch-clamp recordings from acutely isolated cortical slices obtained from 16- to 24-day-old rats. Astrocytes were visually identified in different cortical layers as cells with somata of <10 μm diameter. These cells did not fire action potentials during seal formation nor on current injection in current-clamp recordings. Identification of these glial cells as astrocytes was confirmed by filling the cells with LY for subsequent morphological analysis. Astrocytes all exhibited stellate morphology and of-
Whole cell patch-clamp recordings were obtained from 50 control cells presumed to be astrocytes by the preceding criteria. Astrocytes in cortical layer I had a typical complex pattern of ion channel expression (Bordey and Sontheimer 2000). Briefly, they expressed a composite outward potassium current that included transient ($K_A$) and long-lasting delayed rectifier ($K_{DR}$) $K^+$ currents (Fig. 5E). Inward sodium currents and inwardly rectifying $K^+$ currents ($K_{IR}$) were typically expressed (Fig. 5F) as previously described in our studies on hippocampal and neocortical slices (Bordey and Sontheimer 1997, 2000; D’Ambrosio et al. 1998; Steinhauser et al. 1992). Astrocytes in deeper cortical layers either expressed a complex pattern of ion channels as described for cortical layer I astrocytes (Fig. 5) or displayed a more “passive” current pattern (Fig. 8A). This “passive” electrical profile was, however, simply due to extensive gap-junctional coupling between these cells (Fig. 7) preventing proper voltage-clamp and hence activation of voltage-gated channels (more in the following text). Dye coupling could only be observed in cells that were located 50 $\mu$m from the surface of the slice. Astrocytes showing no cell-to-cell coupling as judged by LY injection displayed a complex electrical profile and were located <50 $\mu$m deep into the slice. In thin 100-$\mu$m-thick slices, LY coupling between astrocytes was consistently absent and all recorded cells showed a complex pattern of ion channel expression. This observation may account for some of the differing results in previous studies.
(D’Ambrosio et al. 1998; Steinhauser et al. 1992). We did not further explore the reason for this difference in cell coupling but took care to monitor for each cell the exact location within the slice.

Passive electrophysiological properties of astrocytes in freeze lesions

The electrophysiological changes of posttraumatic astrocytes were studied in acute cortical slices from P16 to 24 freeze-lesioned animals. Reactive glial cells in sections containing the microsulcus are not likely to be reactive microglial cells since, as previously mentioned, reactive microglial cells regain a complete normal immunological and electrophysiological phenotype after 14 days (Boucsein et al. 2000). Moreover, microglia have not been shown to extend endfeet onto blood vessels. Whole cell patch-clamp recordings were obtained from 81 cells in the different layers of slices containing a microsulcus and compared with 50 cells in control slices. Representative recordings of both are illustrated in Fig. 5. The mean resting membrane potential ($V_r$) was $-81.6 \pm 5.8$ mV (mean ± SD, $n = 50$) for control astrocytes and $-79.8 \pm 8.6$ mV for astrocytes in the microsulcus.
mV (n = 81) for postlesion astrocytes. The difference in $V_r$ was not significant. When calculated with a "tail" protocol allowing leak current measurement in complex cells (see METHODS), $R_m$ was significantly different ($P = 0.0001$) with values of 239.5 ± 133.6 and 636.0 ± 509.4 MΩ in astrocytes from control ($n = 36$) and freeze-lesioned animals ($n = 54$), respectively. Mean membrane capacitances were not significantly different between control and lesion groups with values of 42.1 ± 21.5 pF ($n = 50$) and 38.1 ± 23.1 pF ($n = 81$), respectively. To interpret these results, we further analyzed our data by comparing changes between different regions, specifically layer I adjacent to the lesion and deeper layers. In slices containing a microsulcus, the deeper layers can be subdivided into two regions: the proliferative zone (seen by staining with BrdU) and the hyperexcitable zone including dye-coupled and non-dye-coupled cells (Fig. 2).

Mean passive membrane properties are summarized in Table 1. In layer I, while mean $V_r$ values were not significantly different between lesioned and unlesioned animals (−81 vs. −78.5 mV), $R_m$ (597 vs. 218 MΩ), and $C_m$ (27 vs. 41 pF) values were significantly different in cells from lesioned cortex as compared with controls. In the hyperexcitable zone, astrocytes that displayed a “passive” current profile had a significantly more hyperpolarized $V_r$ in postlesion slices (−87 vs. −82 mV in unlesioned slices). Mean $C_m$ values were not significantly different. The difference in mean $R_m$ values were marginally significant. $R_m$ values could not be obtained in these cells since there appeared essentially passive. In the same region, astrocytes with a “complex” current profile display no significant changes in their mean passive membrane properties.

In the proliferative zone, the mean values of all the passive parameters were significantly different from the ones from complex cells in either control or lesion-containing slices. These cells had a more depolarized $V_r$ (−70 mV), a higher input resistance, $R_m$ (788 MΩ), and a smaller cell capacitance (21 pF).

**Layer I astrocytes near the microsulcus display reduced inward potassium current amplitudes**

Differentiated astrocytes express inwardly rectifying K$^+$ currents that have been suggested to be involved in buffering of extracellular K$^+$ (D’Ambrosio et al. 1999; Newman 1993; Newman et al. 1984; Ransom and Sontheimer 1995). These currents have also been shown to be developmentally regulated (Bordey and Sontheimer 1997; Steinhauser et al. 1992) and to decrease in amplitude in reactive astrocytes in culture (MacFarlane and Sontheimer 1997). Changes in $K_{IR}$ current amplitude in layer I astrocytes adjacent to the lesion were therefore evaluated. There was a 48% decrease in the relative $K_{IR}$ current expression in these astrocytes (Fig. 5, C and F). This was determined from conductance densities for $K_{IR}$ currents in which the conductance of $K_{IR}$ currents was divided by the membrane capacitance to normalize for cell size ($g_{KIR}$) (446 pS/pF in unlesioned and 232 pS/pF in postlesion; Table 2). This difference was statistically significant ($P < 0.001$). There was also a small decrease in the number of cells displaying $K_{IR}$ currents. The density of conductance for $K_{DR}$ ($g_{KDR}$) and Na$^+$ channels ($g_{Na}$) was, however, not significantly different between control and postlesion cell groups (Table 2). On the other hand, $K_A$ mean conductance density ($g_{KAA}$) was 39% larger (Table 2) in cells near the microsulcus than in control cells. This difference was statistically significant ($P < 0.05$).

To describe the relative changes in the contribution of outward and inward K$^+$ conductances, the ratio of total outward K$^+$ conductance ($g_{Kout}$) divided by $g_{KIR}$ was computed. This ratio is a direct indicator of the relative contribution of $K_{IR}$ current to the total current and, assuming that inward K$^+$ currents are indeed responsible for diffusion-mediated K$^+$ buffering by astrocytes, a larger ratio reflects improved K$^+$ cell buffering capacity. As can be seen in Table 2, there is an apparent decrease in the buffering capacity of layer I astrocytes adjacent to the lesion with $g_{Kout}/g_{KIR}$ ratios larger than 7.

**Proliferative astrocytes lose $K_{IR}$ currents after a freeze lesion**

De novo proliferation of astrocytes has been shown to be accompanied by a change in K$^+$ current amplitudes in vitro (MacFarlane and Sontheimer 1997), with a parallel decrease and increase in $K_{IR}$ and $K_{DR}$ current amplitudes, respectively. We thus recorded reactive astrocytes at the base of the micro-
sulcus where proliferative astrocytes were prominent as indicated by BrdU staining (Fig. 4). After an incubation period of \( \geq 2 \) h in BrdU, astrocytes were recorded and filled with LY. BrdU-immunostaining of LY-filled cells was then performed to allow identification of ion channel expression in single proliferative astrocytes. Figure 6A shows two representative BrdU-positive astrocytes filled with LY. The cell on the left shows a confocal image in which the LY fill (green, smaller image middle top) and BrDu labeling (red, smaller image middle bottom) were merged showing the nuclear localization of the BrDU label. The example on the right shows a fluorescent micrograph in which several cell nuclei were BrDU positive (red) including the LY-filled cell. None of the BrdU-positive cells showed dye coupling. Of 26 recorded and LY-filled astrocytes in the proliferative zone near the microsulcus, only 5 cells were recovered that still had an intact cell body allowing unequivocal identification using BrDU antibodies. All five were BrDU positive. The other cells had their cell body removed on withdrawal of the patch pipette. However, these cells showed identical current profiles as the BrdU-positive cells and for this reason were presumed to have also been proliferating cells. These cells were included in the pooled data to evaluate changes in \( K^+ \) current amplitudes (Table 2). Specifically, proliferating cells had large outward \( K^+ \) currents but lacked \( K_{IR} \) currents as shown for a representative example in Fig. 6B. A representative "complex cell" recording from the hyperexcitable zone and the corresponding \( I-V \) curve are shown in Fig. 6, C and D. There was a significant 72% decrease in \( g_{KIR} \) between control complex cells in layer II–VI and proliferative cells (460 and 127 pS/pF, respectively, see Table 2). This change was also accompanied by a notable decrease in the number of cells expressing \( K_{IR} \) channels (100% in control vs. 31% in postlesion groups). This apparent loss of \( K_{IR} \) currents was consistent with the more depolarized \( V_h \) from proliferative astrocytes (−71 mV in lesion vs. −84 mV in control). There was a significant 114% increase in \( g_{KDR} \) in cells near the microsulcus, whereas \( g_{KA} \) did not change significantly. These changes resulted in a marked increase in the \( g_{Kout}/g_{KIR} \) ratio (7.7 from proliferative cells compared with 2.3 from control complex cells), suggesting a loss of the \( K^+ \) buffering capacity in these proliferative astrocytes.

Sodium currents have been reported to be increased in reactive astrocytes in situ (Bordey and Sontheimer 1997) and in proliferative astrocytes in vitro (MacFarlane and Sontheimer 1997). Astrocytes near the microsulcus did not show a statistically significant increase in \( g_{Na^+} \). MacFarlane and Sontheimer (1997) also reported a switch from TTX-sensitive to TTX-resistant Na\(^+\) currents in reactive spinal cord astrocytes in vitro. TTX sensitivity was tested in five proliferative astrocytes (2 were recovered for BrdU staining and were positive). In each cell tested, Na\(^+\) currents were completely and reversibly blocked by TTX 100 nM (data not shown). The injury-mediated loss of TTX sensitivity may thus be a phenomenon typical of spinal cord injury.

**Astrocytes in the hyperexcitable zone display increased dye coupling**

Lee et al. (1995) reported that gap-junction coupling was more pronounced in cells isolated and cultured from hyperexcitable tissue surrounding human epileptic seizure foci than from uninvolved comparison tissues. By filling the cells with

### Table 1. Passive membrane properties of control and post-lesion cortical astrocytes

<table>
<thead>
<tr>
<th>Layer</th>
<th>( n )</th>
<th>( V_h, \text{mV} )</th>
<th>( R_{mo}, \text{M} \Omega )</th>
<th>( C_{mo}, \text{pF} )</th>
</tr>
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<tbody>
<tr>
<td>Layer I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
<td>−81.2 ± 5.6</td>
<td>218.2 ± 122.2</td>
<td>41.4 ± 18.1</td>
</tr>
<tr>
<td>Post-lesion</td>
<td>16</td>
<td>−78.5 ± 5.7†</td>
<td>597.5 ± 332.7‡</td>
<td>27.4 ± 10.5928</td>
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<tr>
<td>Hyperexcitable zone “passive” cells</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>−81.7 ± 5.2</td>
<td>22.5 ± 11.6*</td>
<td>58.6 ± 25.4</td>
</tr>
<tr>
<td>Post-lesion</td>
<td>27</td>
<td>−87.3 ± 4.2‡</td>
<td>17.8 ± 6.1†‡</td>
<td>63.5 ± 18.4†</td>
</tr>
</tbody>
</table>

Values are means ± SD. * See METHODS for calculation. †, not significant. ‡, significantly different.

### Table 2. Density of conductance of outward and inward currents in control and post-lesion astrocytes

<table>
<thead>
<tr>
<th>Layer I</th>
<th>( n )</th>
<th>( g_{KIR} ), pS/pF</th>
<th>( g_{KDR} ), pS/pF</th>
<th>( g_{KA} ), pS/pF</th>
<th>( g_{Kout}/g_{KIR} )</th>
<th>( g_{Na^+} ), pS/pF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23</td>
<td>446.2 ± 209.0</td>
<td>165.0 ± 111.1</td>
<td>622.4 ± 313.9</td>
<td>2.3 ± 1.3</td>
<td>99.6 ± 94.8</td>
</tr>
<tr>
<td>Post-lesion</td>
<td>16</td>
<td>232.4 ± 69.3*</td>
<td>191.0 ± 105.5†</td>
<td>865.8 ± 427.3*</td>
<td>7.1 ± 4.7*</td>
<td>153.1 ± 118.7†</td>
</tr>
<tr>
<td>Hyperexcitable area complex cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>460.3 ± 337.8</td>
<td>165.0 ± 111.1</td>
<td>545.0 ± 315.8</td>
<td>2.3 ± 1.3</td>
<td>99.6 ± 94.8</td>
</tr>
<tr>
<td>Post-lesion</td>
<td>12</td>
<td>316.3 ± 115.6†</td>
<td>167.4 ± 86.0†</td>
<td>398.9 ± 306.4†</td>
<td>1.7 ± 0.6†</td>
<td>90.4 ± 61.3†</td>
</tr>
<tr>
<td>Proliferative zone</td>
<td>26</td>
<td>126.9 ± 76.7*</td>
<td>353.2 ± 218.0*</td>
<td>488.2 ± 205.9†</td>
<td>7.7 ± 4.2*</td>
<td>169.1 ± 133.7†</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages. * Significantly different; † not significantly different.
LY, we attempted to assess the extent of cell-to-cell coupling between astrocytes. We counted the number of LY-filled cells surrounding one single recorded astrocyte in the hyperexcitable zone. To include cells in multiple planes, we performed confocal reconstruction of coupled cells, as shown in Fig. 7, A and B. The number of LY-coupled cells was significantly higher in postlesion section as compared with control sections [13.6 ± 4.99 cells (n = 6) in control and 46.5 ± 15.19 cells (n = 5) in postlesion (P < 0.001), Fig. 7B]. This increase in intercellular coupling correlated with a more negative \( V_r \) of postlesion astrocytes and with a decrease in the input resistance. There was, however, no significant increase in \( C_{m} \) (8% increase in postlesion sections). This discrepancy between an increase in \( C_{m} \) and \( C_{m} \) could be explained by the limited capacity of the patch-clamp to detect distal membrane capacitances. LY-coupled astrocytes expressed a passive current profile. The conductance density for inward currents was identical in cells from control and freeze-lesioned cortex (1,115 ± 471 and 1,307 ± 518 pS/pF, respectively). The inward current was sensitive to barium (1 mM), indicating...
that these inward currents were due to $K^+$ channel activation (Fig. 8, A and B). Figure 8C shows the barium-sensitive inward current and its $I-V$ curve. The ratios of outward versus inward conductances were $0.79 \pm 0.23$ and $0.64 \pm 0.12$ in control and postlesion slices, respectively. Overall, these results suggest an increased $K^+$ conductance in astrocytes of freeze-lesioned neocortex and an increase in intercellular coupling, features suggesting enhanced $K^+$ buffer capacity.

Some reactive astrocytes surrounding the microsulcus are proliferative

Our results show that the abnormal microsulcus is surrounded by reactive astrocytes as indicated by increased immunostaining for GFAP. In addition, some of these reactive astrocytes are BrdU-positive, indicating that proliferative cells are located where neuronal loss has occurred. The emergence of proliferative astrocytes is commonly observed after brain injury and often accompanies neuronal loss. As an example, a convulsant dose of kainic acid injected intracerebroventricularly into rats causes degeneration of hippocampal neuronal cells (Ben-Ari 1985). This degeneration is accompanied by astrocytic proliferation (Murabe et al. 1981; Niquet et al. 1994). Although reactive gliosis has been extensively observed in different brain injury models, the pathways leading to the activation and the proliferation of reactive astrocytes are still unknown. Astrocyte proliferation has been shown to be modulated by various factors including neurotransmitters and growth factors (Abbracchio et al. 1994; Gómez-Pinilla et al. 1995; Hodges-Savola et al. 1996; Huff et al. 1990; Sawada et al. 1993; Scherer and Schnitzer 1994; Selmaj et al. 1990; Stachowiak et al. 1997). In the case of the freeze-lesion model, it is believed that the freezing probe induces focal hypoxia resulting in cell death and subsequent abnormal lamination (Dvorak and Feit 1977; Humphreys et al. 1991). A cascade of phenomena might thus occur leading to increased extracellular $K^+$ and hyperexcitability and increased neurotransmitter release, as well as release of various growth factors and cytokines.
Two populations of reactive astrocytes coexist in the freeze lesion model and may differentially affect neuronal development and function. We focused on the electrical properties of these cells, most notably in their $K^+$ conductances, as they are likely to affect the cell’s ability to buffer $K^+$. We recorded astrocytes surrounding the lesion (layer I) and at the base of the lesion focusing on BrdU-positive cells. Although we did not observe a significant increase in GFAP immunostaining in the hyperexcitable region known to be present 2 wk after the initial in vivo lesion, we recorded astrocytes in this region and compared their properties to normal astrocytes.

**Proliferative astrocytes lack $K_{IR}$ currents and expressed large $K_{DR}$ currents**

A functional correlation between $K^+$ channel expression and cell proliferation has been commonly observed (Chiu and Wilson 1989; MacFarlane and Sontheimer 1997; Nilius and Wohlrab 1992; Pappone and Ortitzmiranda 1993; Puro et al. 1989; Woodfork et al. 1995). A recent study of reactive, proliferative astrocytes in vitro showed changes in $K^+$ channel expression similar to the one described in our study (MacFarlane and Sontheimer 1997). In comparison with control astrocytes in all cortical layers except layer I, 70% of the presumably proliferative cells lack $K_{IR}$ channel expression, whereas they all expressed $K_{DR}$ channels. In the cells expressing $K_{IR}$ channels, there was a significant decrease in $K_{IR}$ current amplitude accompanied by a significant increase in $K_{DR}$ current amplitude. These cells also had a significantly more depolarized resting membrane potential and smaller membrane capacitance reflecting their smaller cell size. These changes in ion channel expression mirror those associated with development (Bordey and Sontheimer 1997; Steinhauser et al. 1992), suggesting that these reactive, proliferative astrocytes regain an immature phenotype. In the freeze lesion model, N-methyl-D-aspartate and GABA channels also express subunits more typical of immature neurons (DeFazio and Hablitz 1999, 2000).

Similarly, reactive astrocytes in the portion of layer I overlying the lesion displayed significant changes in their electrical profile. A decrease in $K_{IR}$ current amplitudes and an increase in $K_A$ current amplitudes mainly characterized these changes. There was also a decrease in cell-to-cell coupling. As previously mentioned, such changes inversely mirror changes accompanying gliogenesis and might reflect a delayed maturation of these astrocytes.

**Astrocytes in the hyperexcitable zone express increased intercellular coupling**

Conflicting hypotheses exist concerning the buffering capacity of glial cells in epileptic foci (Bordey and Sontheimer 1998; Glötzner 1973; Pollen and Trachtenberg 1970). Walz and Wuttke (1999) showed that reactive astrocytes in slices containing gliotic hippocampal CA1 still have the capacity to limit the increases in extracellular $K^+$ that are produced by hyperactive neurons following kainic acid injection. Astrocytes from the epileptic foci also have increased gap junction coupling (Lee et al. 1995). This could facilitate the redistribution of $K^+$ from the epileptic site to sites with lower $K^+$ levels. Increased levels of neurotransmitter such as glutamate exist at epileptic foci (Carlson et al. 1992; During and Spencer 1993; Meldrum 1994; Ronne-Engstrom et al. 1992). Since glia actively take up glutamate, enhanced intercellular coupling would help the sequestration and redistribution of glutamate through the glial syncytium. Increased coupling could be seen as an adaptive response of reactive astrocytes to neuronal hyperexcitability. Consistent with this idea of increased gap junctional coupling, propagation of paroxysmal activities is accompanied by either changes in the membrane surface area and/or the interglial communication via gap junction (Amzica and Neckelmann 1999). Our finding of a 240% increase in intercellular coupling demonstrates that astrocytes surrounding hyperexcitable neurons potentially have an increased capacity to allow ionic diffusion between cells and this would likely include $K^+$. These cells, by effectively expanding their communication compartment, could also provide neuroprotective effects following brain injury. Increased intercellular coupling could however have epileptogenic effects by increasing the synchronization of discharges (Naus et al. 1991). Thus taken the loss of coupling at the lesion site and the enhanced coupling in astrocytes together, one might envision a scenario where at the lesion, extracellular ion such as glutamate and $K^+$ may accumulate. If these changes induce an astrocytic response, such as a spreading Ca$^{2+}$ wave (Finkbeiner 1992), its propagation may be facilitated by the enhanced coupling of cells in the surrounding hyperexcitable tissue.

If one equates expression of $K_{IR}$ channels with an enhanced ability of cells to take up $K^+$, our results suggest that, in the core of the freeze-induced malformation and in adjacent areas, there may be a decreased capacity to buffer $K^+$. This would in turn lead to neuronal dysfunction and eventually neuronal death. Similarly, if one equates the expression of gap junctions with an enhanced ability to spatially redistribute $K^+$ through the glial syncytium, our results suggest that in the hyperexcitable zone there is an increase capacity to spatially buffer $K^+$ accompanying neuronal activity, while spatial buffering is lost in the lesion proper. Clearly, further studies are needed to functionally link these changes in astrocytic properties to changes in the neuronal microenvironment at the lesion sites.

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**REFERENCES**


