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Changes in the expression of cation-Cl^- cotransporters, NKCC1 and KCC2, during cortical malformation induced by neonatal freeze-lesion

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Abstract

Focal cortical malformations comprise a heterogeneous group of disturbances in brain development, often associated with intractable epilepsy. A focal freeze-lesion of cerebral cortex in newborn rat produces a cortical malformation that resembles human polymicrogyria, clinical conditions that results from abnormal neuronal migration. The change in GABAergic functions that occurs during early brain development is induced by an alteration in Cl^- homeostasis and plays important roles in neocortical development by modulating such events as laminar organization and synaptogenesis. We therefore investigated the relationship between pathogenesis of polymicrogyria and ontogeny of Cl^- homeostasis in developing parietal cortex after creation of a freeze-lesion at P0. We demonstrated, by *in situ* hybridization histochemistry for cation-Cl^- cotrtansporters, that NKCC1 mRNA expression was upregulated and KCC2 mRNA expression downregulated at P4 in ''bridge'' structure (formed in lesion site across the gap in intact exofocal cortex) as compared to exfocal cortex. Immunohistochemical investigation revealed a colocalization of NKCC1 and neuron specific enolase (NSE) within this structure, while BrdU-positive cells express GFAP and NKCC1 appeared beneath it. These results suggest that immature cortical plate neurons might produce ''bridge'' structure during formation of microgyrus, and that altered neuronal Cl^- homeostasis might be involved in neuronal migration disorder that ultimately results in cortical malformations.

Keywords: GABA; Chloride; Microgyrus; Migration; Cation-chloride cotransporter; Na^+; K^+-2Cl^- cotransporter 1; K^+-Cl^- cotransporter 2

1. Introduction

Neocortical structural abnormalities due to developmental disturbances are often the cause of therapy-resistant epilepsy (Palmini et al., 1991; Raymond et al., 1995; Guerrini et al., 1999). These cortical malformations are believed to develop after an insult to the developing cerebral cortex, but only if this occurs during the period of neuronal migration. A focal freeze-lesion to the rat cerebral cortex produces a cortical malformation. Electrophysiological studies on freeze-lesioned animals have demonstrated intrinsic hyperexcitability in the lesion itself and in its structurally intact surroundings, indicating a widespread imbalance between excitation and inhibition (Luhmann and Raabe, 1996; Luhmann et al., 1998; Jacobs et al., 1996, 1999; Defazio and Hablitz, 2000).

In the adult central nervous system, GABA elicits a hyperpolarizing action. In contrast, during early postnatal development GABA produces excitatory responses in cortical neurons (Ben-Ari et al., 1989; Luhmann and Prince, 1991; Owens and Kriegstein, 2003). This GABA-mediated depolarization is thought to play a functional role in activity-dependent

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Cl^-]_i$, intracellular Cl^- concentration; CP, cortical plate; FL, freeze-lesion; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; KCC, K^+-Cl^- cotransporter; NKCC, Na^+, K^+-2Cl^- cotransporter; NSE, neuron specific enolase; PBS, phosphate-buffered saline; VZ, ventricular zone; WM, white matter.

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neuronal maturation and synapse formation during early development (Ben-Ari, 2002) and to promote the migration of newly generated neurons (Behar et al., 1996, 1998). The switch from depolarizing to hyperpolarizing GABAergic responses during development is thought to be attributable to a developmental decrease in the intracellular Cl^- concentration ($[Cl^-]_i$). Cation-Cl^- cotransporters play a critical role in the regulation of $[Cl^-]_i$ (Payne et al., 2003), and of these NKCC1 promotes accumulation of Cl^- into the cell (Yamada et al., 2004), whereas KCC2 extrudes it out of the cell (Rivera et al., 1999). As we have previously reported, the ontogeny of Cl^- homeostasis, as regulated by the differential expressions of NKCC1 and KCC2 (Shimizu-Okabe et al., 2002; Ikeda et al., 2003), may be important in neocortical developmental processes, by modulating such events as laminar organization and synaptogenesis (Ben-Ari, 2002). Functional alterations in Cl^- homeostasis are known to be induced by neuronal insults such as trauma or axotomy (van den Pol et al., 1996; Nabekura et al., 2002; Toyoda et al., 2003).

To investigate alterations in Cl^- homeostasis in the freeze-lesion model of focal cortical malformations, NKCC1 and KCC2 expressions were examined by *in situ* hybridization histochemistry and immunohistochemistry. We also investigated whether after induction of a freeze-lesion, neurogenesis occurs to form a microgyrus, might be involved in microgyrus-formation by detecting BrdU-labelling.

2. Experimental and procedures

2.1. Lesion procedures

Wistar rats (SLC, Japan) were used for the experiments. Focal freeze-lesions were made on the day of birth (postnatal day (P) 0, <24 h) using a method described in detail elsewhere (Luhmann et al., 1996, 1998). Briefly, newborn rats were deeply anesthetized by hypothermia and a freeze-lesion was produced by placing a liquid nitrogen-cooled copper cylinder (diameter, 1 mm) for 5 s on the calvarium above the frontoparietal cortex. To produce a longitudinal freeze-lesion, four identical local freeze-lesions were made in a line parallel to the midline, with a distance of 1.5 mm between the lesions. The wound was closed with the aid of histoacryl tissue blue (Braun-Dexon, Germany). Sham-operated rats were treated in the same way, but without cooling the copper cylinder.

2.2. In situ hybridization histochemistry

Male wistar rats at various postnatal ages (P1, 2, 4, 7, 10, 14, 21, 28; $n = 3$–4 at each age) were decapitated under diethylether anesthesia. The fresh brains were quickly removed and immediately frozen on powdered dry ice. The *in situ* hybridization histochemical technique for detecting KCC2 and NKCC1 mRNAs has been described in detail elsewhere (Kanaka et al., 2001). Briefly, hybridization was performed by incubating formaldehyde-fixed, dehydrated sections with a buffer (0.6 M NaCl and 0.06 M sodium citrate, 50% deionized formamide, 0.12 M phosphate buffer, 0.025% tRNA, 10% dextran sulfate in Denhardt's solution) containing [^{35}S]dATP (37–55.5 TBq/mmol, Perkin Elmer)-labeled probes (1–2 × 10^7 dpm/ml, 0.2 ml/slide) for 24 h at 42 °C. Detection of hybridization probes was carried out using emulsion microautoradiography. The sections were counterstained with thionin solution for the purposes of morphological identification.

2.3. BrdU injection

Wistar rats pups were randomly assigned to receive either a freeze-lesion or a sham-operation at P0. They were injected intraperitoneally with BrdU (Sigma; 50 μg/g body weight) in 0.1 M PBS (pH 8.3) within 1 min after surgery, sacrificed at P11, intracardially perfused with fixative, and processed as described below.

2.4. Immunohistochemistry

Male wistar rats at P4 were anesthetized with barbiturate and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed immediately after the perfusion and post-fixed in the same fixative at 4 °C. All samples were then cryoprotected in 0.1 M phosphate buffer containing 30% sucrose for 1–2 days. The brains were cut into coronal sections (32 μm) on a freezing microtome. The free-floating method was used: after washing with PBS, the sections were incubated with a blocking solution (10% normal goat serum, 0.2% Triton X-100 in PBS) for 1 h at room temperature. Sections were then incubated overnight at 4 °C with various combinations of the following: T4 monoclonal antibody against the human colonic T84 epithelial Na^+-K^+-$2Cl^-$ cotransporter (1:100–300; Departmental Studies Hybridoma Bank, Iowa City, IA) (Lytle et al., 1995), anti-neuron specific enolase (NSE) polyclonal antibody (1:300; Chemicon, Temecula, CA), anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:10000; DAKO, Denmark), and anti-GFAP monoclonal antibody (1:400; Chemicon, Temecula, CA). After rinsing in 0.1 M PBS, the sections were incubated with Alexa Fluor 546 goat anti-rabbit IgG (1:2000; Molecular Probes, Eugene, OR) and Alexa Fluor 488 goat anti-mouse IgG (1:1000; Molecular Probes, Eugene, OR) for 1 h at room temperature.

The processing for BrdU staining was modified from Rosen et al. (1996). After washing with PBS, the sections were incubated with 2 M HCl at 40 °C for 30 min. After again washing with PBS, the sections were incubated with a blocking solution (10% normal goat serum, 0.2% Triton X-100 in PBS) for 1 h at room temperature. The sections were then incubated with anti-BrdU antibody (1:100; Chemicon, CA) and either NSE polyclonal antibody (1:300) or GFAP polyclonal antibody (1:10000) at 4 °C overnight. After rinsing in 0.1 M PBS, the sections were incubated with Alexa Fluor 546 goat anti-rabbit IgG (1:2000) and Alexa Fluor 488 goat anti-mouse IgG (1:1000) for 1 h at room temperature.

3. Results

At P1, the site of the lesion was characterized by necrotic tissue, which occasionally extended through the subplate to the white matter (Fig. 1A). By P4, a dense band of neurons forming a cortical ''bridge'' structure (Fig. 1B) could be identified superficial to the lesion site in all freeze-lesioned animals ($n = 4$). At P7, the typical microgyric architecture had already emerged (Fig. 1C). After P14, all animals exhibited a microgyrus consisting of three or four layers, as reported previously (Fig. 1D) (Dvorak et al., 1978; Rosen et al., 1992, 1998; Luhmann et al., 1998; Zilles et al., 1998; Jacobs et al., 1996, 1999). In the sham-operated animals, no abnormalities were detected (data not shown).

In the intact neocortex of both sham-operated and freeze-lesioned rats, KCC2 mRNA was only weakly expressed at P1 (Fig. 2 top), whereas a strong NKCC1 mRNA expression was observed at at this age (Fig. 2 bottom). The KCC2 mRNA expression showed a marked increase after the first week of postnatal life (Fig. 2 top), whereas the expression level of NKCC1 mRNA decreased with a corresponding time course (Fig. 2 bottom).

In the freeze-lesioned area, KCC2 and NKCC1 mRNAs were not expressed at P1 (Fig. 2), most probably because of the clustering of necrotic cells. At P4, the NKCC1 mRNA expression level in the ''bridge'' structure appeared to be higher than in the adjacent exofocal areas (Fig. 2 bottom). In contrast, although KCC2 mRNA reemerged in the ''bridge'' structure, its expression level was weaker than that in the

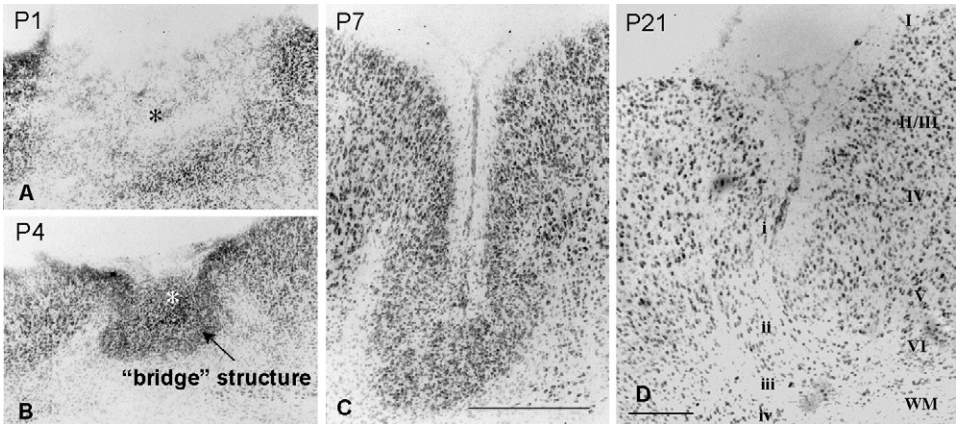

Fig. 1. Thionin staining photomicrographs of cortical malformations. In bright-field photomicrographs, the focal freeze-lesion is indicated by an asterisk. (A) P1, (B) P4, (C) P7, (D) P21. Note that the cortical plate thrusts into the lesion from the surrounding area and forms a ''bridge'' structure at P4 (arrow). At P7, the typical microgyric architecture has emerged. The photomicrograph of a thionin-stained section from the cereberal cortex of a P21 rat illustrates a typical microgyrus. In contrast to the typical six-layered normal cortex (uppercase Roman numerals), the microgyric region contains three or four layers (lowercase Roman numerals). Bar = 500 μm (C), 250 μm (D).

exofocal cortex (Fig. 2 top). No KCC2 mRNA signals could be detected in the tissue between the ''bridge'' structure and the white matter, whereas NKCC1 mRNA was expressed in this area. At P7, this KCC2 mRNA deficient region beneath the ''bridge'' structure had become smaller (Fig. 2 top). After P7, an increase in the KCC2 mRNA signal could be detected. The expression of KCC2 mRNAin the microgyric region was as strong as that in the intact surrounding cortex (and that in the sham-operated cortex). The expression of NKCC1 mRNA had decreased to very low levels at P14-21 in the focal and exofocal cortices, as well as in the sham-operated controls (Fig. 2 bottom). No alterations in NKCC1 and KCC2 mRNA expression levels could be seen in the contralateral cortex.

We next studied the expressions of KCC2 and NKCC1 mRNAs in more detail in the ''bridge'' structure of P4 animals. Dark-field microphotographs showed that KCC2 mRNA expression was weaker in the ''bridge'' structure than in the exofocal cortical plate (CP) (Fig. 3A). Bright-field photomicrography confirmed that the expression level of KCC2 mRNA was weaker in the ''bridge'' structure than in the CP or

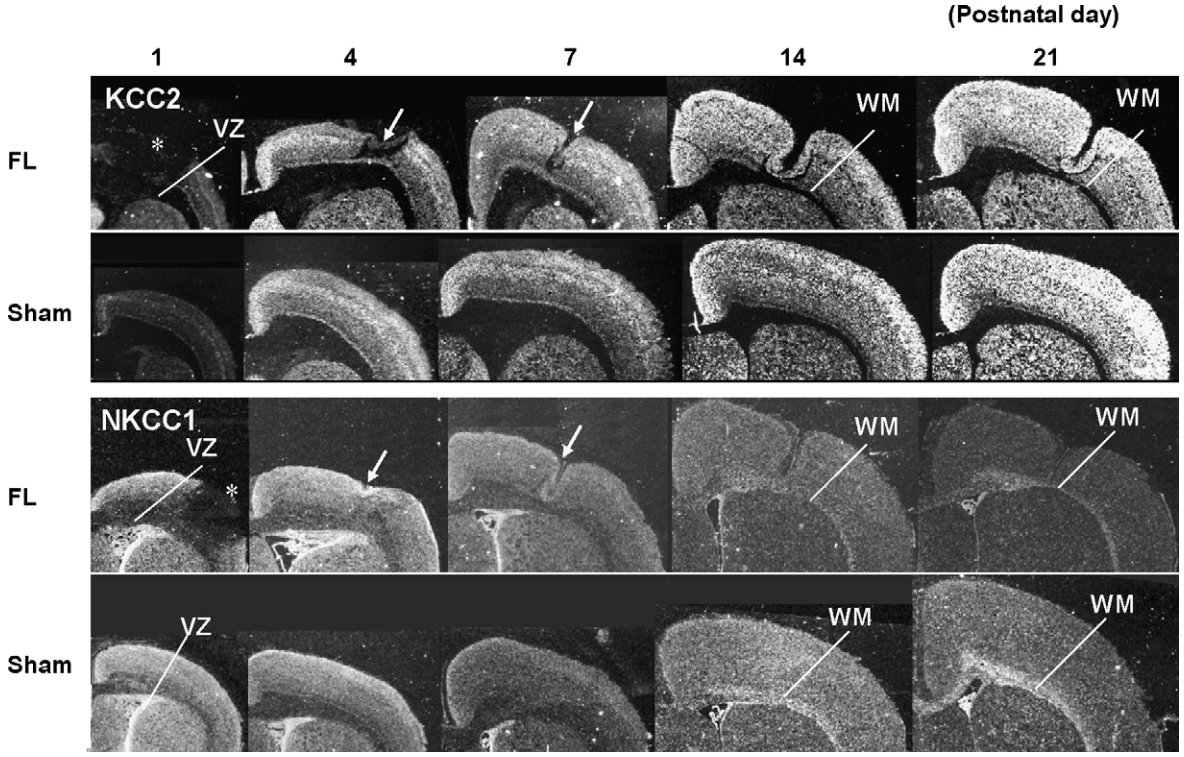

Fig. 2. Dark-field photomicrographs showing expressions of KCC2 and NKCC1 mRNAs in cortical malformations. Sections were hybridized to probes specific for each gene. At P1, KCC2 and NKCC1 mRNAs were not expressed in the freeze-lesioned area (asterisk). Note that in the ''bridge'' structure of the freeze-lesioned cortex, NKCC1 mRNA expression was upregulated and KCC2 mRNA expression downregulated from P4 to P7 (arrows).

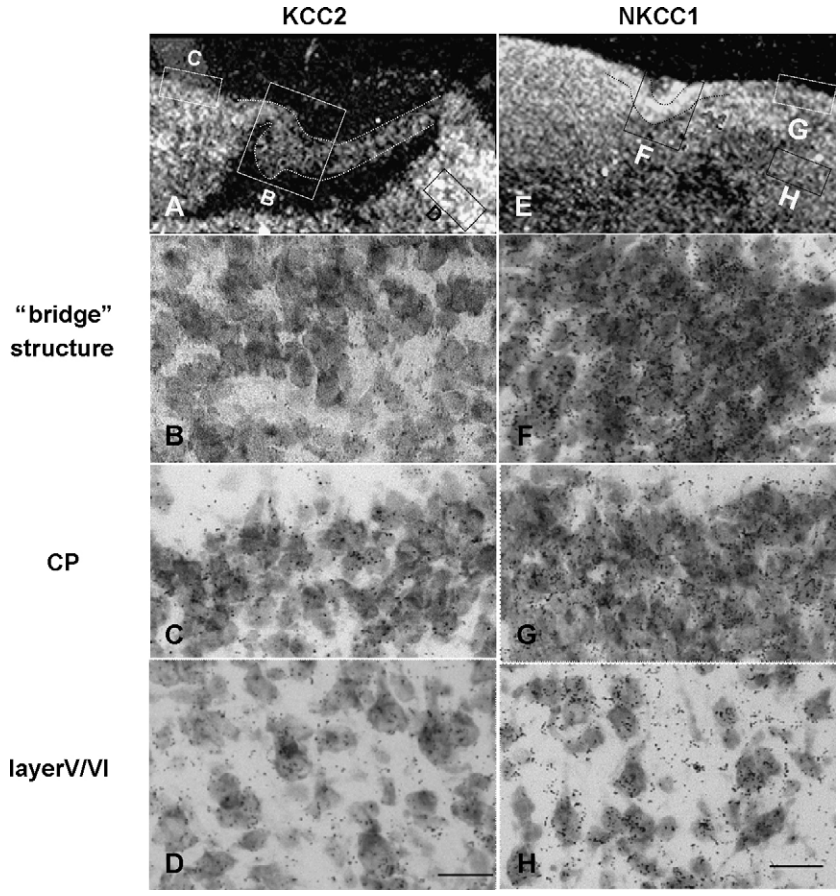

Fig. 3. Bright-field photomicrographs showing expressions of KCC2 and NKCC1 mRNAs in the cortical malformation at P4. Dark-field photomicrographs show expressions of KCC2 mRNA(A) and NKCC1 mRNA (B) in freeze-lesioned cortex. Bright-field photomicrographs show expressions of KCC2 mRNA (B) and NKCC1 mRNA (F) in the ''bridge'' structure of the microgyrus. Note that KCC2 mRNA expression was strongly downregulated (B) and NKCC1 mRNA expression was strongly upregulated within the ''bridge'' structure (as compared to the exofocal cortex) (F). KCC2 mRNA was expressed in both CP (C) and layer V/VI (D) in the surrounding microgyrus. NKCC1 mRNA was expressed in both CP (G) and layer V/VI (H) in the surrounding microgyrus. Scale bar = 20 μm.

layer V/VI (Fig. 3B–D). Both dark-field and bright-field photomicrographs revealed a strong expression of NKCC1 mRNA in the ''bridge'' structure (Fig. 3E and F), but a relatively weak expression in the exofocal CP (Fig. 3G) and layer V/VI (Fig. 3H). In the exofocal cortex, KCC2 mRNA expression was weaker in CP than in layer V/VI (Fig. 3C and D), whereas the reverse was true for NKCC1 mRNA expression (Fig. 3G and H).

We further semi-quantified the expression level of KCC2 and NKCC1 mRNAs by comparing hybridized-signals (e.g., number of silver grains/neuron). All values were shown as means ± S.E. Statistical analyses were performed with ANOVA followed by Sheffe's test (16 neruons). The number of grains for KCC2 in neurons locating in ''bridge'' structure (1.2 ± 1.8, $n = 16$) was significantly decreased as compared to that in exofocal CP neurons (3.0 ± 2.1, $n = 16$) ($p = 0.020$) and layer V/VI neurons (6.6 ± 2.5, $n = 16$) ($p = 6.457 \times 10^{-9}$). The number of grains for KCC2 in CP was significantly lower than that in layer V/VI ($p = 2.261 \times 10^{-5}$). The number of grains for NKCC1 in ''bridge'' structure (18.2 ± 3.0, $n = 16$) was significantly higher than that of CP neurons (10.9 ± 1.9, $n = 16$) and in layer V/VI (6.4 ± 3.7, $n = 16$) ($p = 9.907 \times 10^{-4}$). The number of grains for NKCC1 in CP was significantly higher than that in layer V/VI ($p = 8.696 \times 10^{-5}$). In summary, these results suggest that with respect to Cl^- homeostasis, the ''bridge'' structure is more immature than both CP and layer V/VI (Shimizu-Okabe et al., 2002; Yamada et al., 2004).

NKCC1 was reported to be expressed in both neurons and astrocytes (Yan et al., 2001). We analyzed the localization of NKCC1 expression in both cell types by means of immunocytochemistry. At P4, the glial marker GFAP was strongly expressed between the ''bridge'' structure and the white matter (Fig. 4A), while immunoreactivity for NSE, a neuronal marker, was observed mainly in the ''bridge'' structure itself (Fig. 4B). In both regions, NSE was not colocalized with GFAP (Fig. 4C). While stainings for NKCC1 and NSE were colocalized in the ''bridge'' structure (Fig. 4G–I, J and K), NKCC1 was also observed in the area beneath the ''bridge'' (where no NSE signals were detected) (Fig. 4G–I). In this area, which was strongly stained for GFAP, NKCC1 was colocalized with GFAP (Fig. 4D–F). Colocalization of NKCC1 and NSE was also found in the exofocal cortex (Fig. 4M–O). In summary, these results suggest that the ''bridge'' structure consists mainly of neurons while the area between this structure and the white

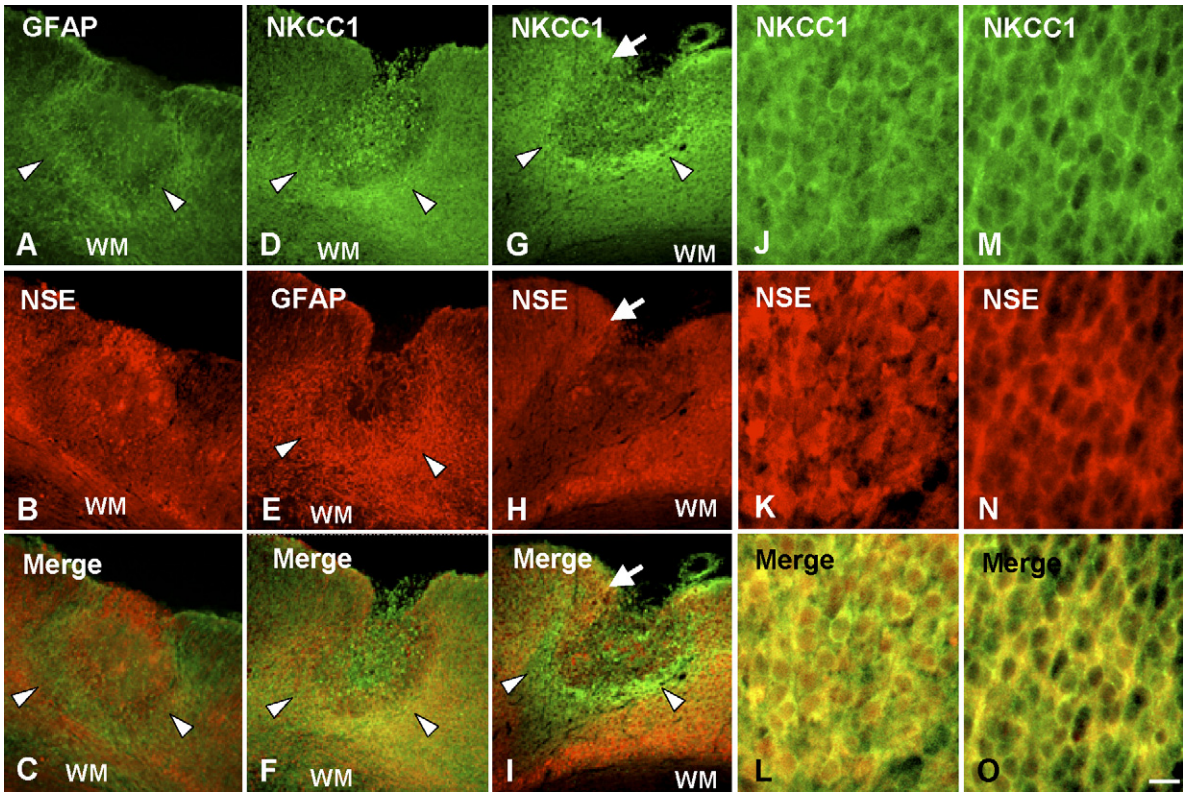

Fig. 4. Colocalization of NKCC1 with NSE and/or GFAP in freeze-lesioned cortex at P4. GFAP was strongly stained between the ''bridge'' structure and the white matter (A, green, arrowheads). The sections were double-stained for GFAP (green, A) and NSE (red, B) in the corresponding section. In this area, NSE was not colocalized with GFAP (C, green, arrowheads). In the area that was strongly stained with GFAP (red, E, arrowheads), NKCC1 (green, D, arrowheads) was colocalized with GFAP (F, yellow, arrowheads). At P4, intensive stainings for NKCC1 and NSE were colocalized in the ''bridge'' structure (G–I, J and K arrows). However, NKCC1 was stained in the area in which NSE was not stained (G–I, arrowheads). Colocalization of NKCC1 and NSE was also noted in the exofocal cortex (M–O). Scale bar = 20 μm.

matter is formed of glial cells, with both cell populations showing NKCC1 immunoreactivity.

To determine whether the microgyric cortex is composed of newly generated neurons or of CP neurons that might have migrated into the lesioned area, we used a BrdU labelling technique by injecting BrdU at P0. Although intensive staining for BrdU was colocalized with GFAP staining in layer iii (Fig. 5A–C), it was not colocalized with NSE (Fig. 5D–F), indicating that neurogenesis does not contribute to the generation of the microgyric cortex. The BrdU-positive cells most likely represent reactive astrocytes.

4. Discussion

The main findings described in the present study in the freeze-lesion model of neocortical abnormal migration were: (i) one day after the creation of the lesion at P0, NKCC1 and KCC2 mRNA expressions were absent from the lesion site, (ii) at P4, a ''bridge'' structure had formed at the lesion site, and this consisted of neurons expressing NKCC1 and (at lower rates than in the exofocal cortex) KCC2, while below this ''bridge'' structure glial cells expressing only NKCC1 appeared, (iii) at P7, the 3–4-layered microgyral appearance typical of the freeze-lesion model emerged, and NKCC1 levels decreased, while KCC2 levels increased, and (iv) following formation of the microgyrus, the NKCC1 and KCC2 expression levels were similar to those in the exofocal cortex after P14.

In this study, the structural appearance of the lesion site (later the microgyrus) was in accordance with previous reports (Dvorak et al., 1978; Rosen et al., 1992, 1998; Jacobs et al., 1996, 1999; Luhmann et al., 1998; Zilles et al., 1998). The ''bridge'' structure we observed at P4 is related to the ''dark zone'' reported by Dvorak and Feit (1978), which they suggested consisted of migrating neurons. We unequivocally demonstrated, by means of NSE immunohistochemistory, that the ''bridge'' structure was composed of neurons. Our BrdU birthdating experiments revealed no evidence that neurogenesis occurred following the creation of the freeze-lesion. This supports a previous report by Rosen et al. (1996) that the cell-dense portions of the microgyrus, corresponding to the ''bridge'' structure and later to layer ii, were formed by neurons generated in embryonic, not postnatal, life. As neuronal migration to the rat neocortex continues for 2–3 days after birth, immature neurons could, however, translocate into the lesion from the surrounding CP to form the ''bridge'' structure, which later develops into layer ii. Accordingly, dysplastic cortices are never formed if lesions are made later than the third day after birth (Humphreys et al., 1991). Previous studies (Zilles et al., 1998; Redecker et al., 2000) have suggested that layer ii of the microgyrus results from the

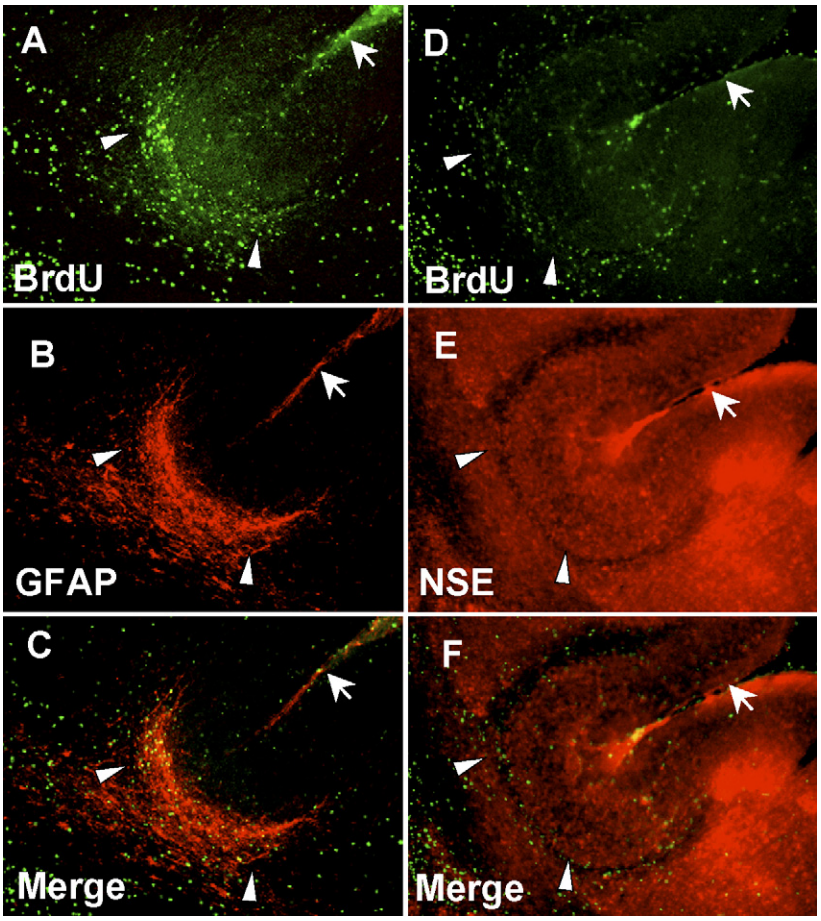

Fig. 5. Detection of BrdU in freeze-lesioned cortex. A, D: BrdU (green); B: same section as in A, but double-stained for GFAP (red). E: same section as in D, but double-stained for NSE (red). Superimposed images of A and B (C), and D and E (F). Arrowheads indicate clusters of BrdU-positive cells with GFAP (C, yellow), but without NSE (F, green). Arrows indicate microgyrus. Scale bar = 20 μm.

tangential growth of the adjacent layer ii, an in agreement with the present result.

In contrast to their lack in layer ii, numerous BrdU-positive cells were detected in layer iii, and these were therefore generated after the creation of the lesion. In this layer, a large number of GFAP-positive cells were found, although it was devoid of neurons. In addition, no obvious correlation between BrdU-staining and the expression of the neuronal marker NSE was found in layer iii, and an expression of KCC2 mRNA, which has been shown to be restricted to neurons and has never been observed in glial cells (Williams et al., 1999), was not found in this region. Taken together, these results demonstrate that the cells in layer iii are glial cells, at least some of which are generated after the creation of the lesion. Indeed, Bordey et al. (2001) found proliferating astrocytes at the base (most probably corresponding to layer iii of freeze-lesion-induced mygrogyri in juvenile rats.

A major finding of the present study was that CP cells might migrate from the surrounding area into the ‘‘bridge’’ structure, in which NKCC1 mRNA expression was upregulated and KCC2 mRNA expression downregulated. It has been demonstrated recently, that NKCC1 mRNA expression is higher in CP than in layer V/VI in the newborn rat, while the reverse is true for KCC2 mRNA, the result being a higher $[Cl^-]_i$ in CP neurons (Shimizu-Okabe et al., 2002; Yamada et al., 2004). These findings led us to propose the hypothesis that migrating cells have higher a $[Cl^-]_i$ than settled cells (Shimizu-Okabe et al., 2002). Accordingly, we demonstrated in the present study that in the ‘‘bridge’’ structure, the expression level of NKCC1 mRNA was stronger and that of KCC2 mRNA weaker than in layer V/VI. This differential NKCC1 and KCC2 mRNA expression in the ‘‘bridge’’ structure indicates that the neurons in this structure have a high $[Cl^-]_i$. Indeed, gramicidin-perforated patch-clamp recordings have revealed that the $[Cl^-]_i$ in these neurons is higher than in the exofocal cortex, and that GABA-induced $[Ca^{2+}]_i$ elevations, which could not be observed in the exofocal cortex, were induced in the ‘‘bridge’’ structure (Sugimoto et al., 2003). In summary, the above findings strongly suggest that neurons in the ‘‘bridge’’ structure retain the properties of migrating neurons.

In cortical neurons, a shift in Cl^- homeostasis toward a higher $[Cl^-]_i$ is implicated in the determination of developmental stage, since depolarizing GABAergic and glycinergic responses mediate various developmental processes, such as neuronal migration, differentiation, and synapse formation (Behar et al., 1996, 1998; Owens and Kriegstein, 2003; Ben-Ari, 2002). Hence, the neurons present in the ‘‘bridge’’ structure, with their immature Cl^- homeostasis, retain their

potential to migrate and form an abnormal network within the cortical malformation. On the other hand, since the ontogeny of Cl^- homeostasis in the microgyrus progresses to adult levels at about P14, the hyperexcitability previously observed in and around the microgyrus (Luhmann and Raabe, 1996; Luhmann et al., 1998; Jacobs et al., 1996) cannot be due to an insufficient GABAergic inhibition (resulting from an increased $[Cl^-]_i$).

In summary, the present study has demonstrated that the formation of a microgyrus after the creation of a freeze-lesion begins with (a) the migration of neurons from the exofocal CP into the lesion site to form a ''bridge'' structure and (b) the appearance of proliferating glial cells between this structure and the white matter. The neurons of the ''bridge'' structure retain an immature Cl^- homeostasis, as a result of which the action of GABA is depolarizing, and this may promote an abnormal migration of neurons and the formation of a disorganized neuronal network.

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