Altered Interneuron Development in the Cerebral Cortex of the Flathead Mutant

One approach to defining mechanisms essential to neocortical development is to analyze the phenotype of novel spontaneous mutations that dramatically affect the generation and differentiation of different neocortical neurons. Previously we have shown that there is a large decrease in the total number of cortical neurons in the flathead mutant rat, and in this paper we show that the flathead (fh/fh) mutation causes an even larger decrease in the number of interneurons. The decrease in relative interneuron number is different in different cortical lamina and for different interneuron subtypes. Specifically, the percentage of GABA and calretinin-positive cells in upper layers of somatosensory cortex is not appreciably decreased in homozygous mutants, while other interneuron subtypes in somatosensory cortex and all GABA-positive interneurons have been lost. In addition, the soma and dendritic arbors of interneurons in flathead are greatly hypertrophied, while those of pyramidal neurons are not. Furthermore, we found that at embryonic day 14, flathead mutants display high levels of cell death throughout both the neocortical and ganglionic eminence (GE) proliferative zones with a larger increase in cell death in the GE than in the neocortical VZ. In addition, we provide evidence that there is widespread cytokinesis failure resulting in binucleate pyramidal cells and interneurons, and the number of binucleate interneurons is greater than the number of binucleate pyramidal neurons. Together, these results demonstrate that the fh mutation causes dramatic alterations in interneuron development, and suggest that the flathead mutation causes differential cytokinesis failure and cell death in different types of neocortical progenitors.

Introduction

Normal cortical function is dependent upon the balanced development of two major neuron types: pyramidal cells and non-pyramidal cells. Non-pyramidal neurons primarily contain the neurotransmitter GABA, and calretinin-positive cells in upper layers of somatosensory cortex is not appreciably decreased in homozygous mutants, while other interneuron subtypes in somatosensory cortex and all GABA-positive interneurons have been lost. In addition, the soma and dendritic arbors of interneurons in flathead are greatly hypertrophied, while those of pyramidal neurons are not. Furthermore, we found that at embryonic day 14, flathead mutants display high levels of cell death throughout both the neocortical and ganglionic eminence (GE) proliferative zones with a larger increase in cell death in the GE than in the neocortical VZ. In addition, we provide evidence that there is widespread cytokinesis failure resulting in binucleate pyramidal cells and interneurons, and the number of binucleate interneurons is greater than the number of binucleate pyramidal neurons. Together, these results demonstrate that the fh mutation causes dramatic alterations in interneuron development, and suggest that the flathead mutation causes differential cytokinesis failure and cell death in different types of neocortical progenitors.

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Matthew R. Sarkisian, Mikhail Frenkel, Weiwei Li, Justin A. Oborski and Joseph J. LoTurco
Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT, USA
severe deficits in interneuron generation and alterations in cytokinesis leading to many binucleated neurons. Moreover, Citron-K is highly expressed throughout proliferating areas of the CNS prior to birth (Di Cunto et al., 2000). In addition, the genetic location of the fh mutation in rat is homologous to the region of human chromosome 12 which contains the Citron gene. Here we report that the fh mutation results in a widespread decrease in the relative numbers of interneurons and in a selective increase in interneuron growth. We also show evidence of cell death throughout the neocortical-VZ and MGE, cytokinesis abnormalities in both precursor cells in MGE, and pyramidal and non-pyramidal cells throughout postnatal neocortex. These observations suggest the fh/fh gene causes a defect in the genesis of CNS precursors due to a failed cytokinesis process that subsequently alters the number and size of neocortical interneurons.

Materials and Methods

Animals

A total of 124 (n = 56 fh/fh’s, n = 68 littermates) rats, ranging in age from E14 to P21, were used in this study. Mutant animals were from either of two colonies: the original Wistar rat colony in which the fh mutation spontaneously occurred (WUC1), and a colony generated from an F1 interstrain cross (Lewis × WUC1), allowing for the genotyping of animals (see below) (Cogswell et al., 1998). All animal protocols were in accordance with the University of Connecticut IACUC guidelines.

Materials

Primary antibodies used in this study were rabbit anti-GABA (Sigma, St Louis, MO), mouse anti-parvalbumin (Chemicon, Temecula, CA), mouse anti-calbindin-D28k (Sigma), mouse anti-NeuN (Chemicon), mouse anti-rat brain pyramidial cells (3Want, Bellinzona, Switzerland) and mouse anti-calretinin (Chemicon). Secondary antibodies included biotinylated goat anti-rabbit and goat anti-mouse (Vector Laboratories, Burlington, CA), Texas red dye-conjugated goat anti-mouse and goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA), and fluorescein-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR). Other materials used were 4 µM 4,6-diamidino-2-phenylindole (DAPI; Sigma), 1,1′-3,3′-tetramethylindocarbocyanine (Dil) (Molecular Probes), normal goat serum (NGS) (Vector), avidin and biotin (Vector), Cytoseal mounting medium (Stephens Scientific, Kalamazoo, MI), Vectashield Fluorescence mounting medium (Vector), Prolong™ anti-fade mounting medium (Molecular Probes), and diaminobenzidine (Vector). Genomic DNA was purified using a kit purchased from Qiagen (Valencia, CA). The MapPair™ primers, D12rat80, were purchased from Research Genetics (Huntsville, AL).

Immunocytochemistry

Standard immunocytochemical procedures were used for the following primary antibodies: mouse anti-calbindin D28k (CAL) (1:1000), mouse anti-calretinin (CR) (1:1000), mouse anti-parvalbumin (PARV) (1:1000), rabbit anti-GABA (1:10,000), mouse anti-NeuN (1:1000), and mouse anti-rat brain pyramidial cells (1:7500). For BrdU ICC, timed-pregnant rats/group). For P0 and P14 rats, all cell counts were obtained from sections of comparable horizontal planes between ut and fh/fh. For each stain, five counting boxes were systematically placed across upper and deeper layers (as defined below) of entorhinal (EC) or somatosensory (SS) neocortices, and all labeled cells within each box counted (areas (in µm2) for boxes at P14: NeuN-EC and SS = 11 880, GABA-EC = 11 880, GABA-SS = 51 984, CAL-EC = 11 880, CAL-SS = 51 984, CR-EC = 51 984, CR-SS = 11 880, PARV-EC = 11 880, PARV-SS = 51 984; areas (in µm3) of boxes at P0: GABA and H&E = 1600, GABA and H&E = 5 400). While the area of the NeuN counting box was smaller than the boxes used for GABA, CAL and CR, consistent neuronal densities were routinely obtained as boxes were systematically placed across the counting region of interest, suggesting that cell density was constant over each counting region. The percentage of GABA+ and PARV+ interneurons was determined on SS and EC regions of cerebral cortex by dividing the density of each interneuron cell type in each region by the density of H&E or NeuN+ cells in each region.

For EC, we used the following histological landmarks to identify upper (II/III) or deeper (primarily VI) layers for NeuN, GABA, CR, CAL and PARV+ cells. For NeuN, we defined upper layers by placing counting boxes centered between the internal border of layer I and lamina dissecans. Boxes were first placed at the anterior part of lamina dissecans with subsequent boxes added posteriorly. Similarly, deeper layers were operationally defined by placing counting boxes centered between lamina dissecans and white matter. For GABA, boxes were similarly placed for upper layers along the internal border of layer I and above white matter for deeper layers. For CR, boxes were placed in upper layers, centered between layer I and lamina dissecans (identified by the thick band of immunoreactivity – see Fig. 2A). For CAL, boxes were placed in upper layers, centered within a white, non-fibrous band such that the bottom edge of the box did not extend below lamina dissecans. For PARV, boxes were placed in upper layers, and were centered within the thick fibrous band characteristic of layer II/III immunoreactivity in EC (Wouterlood et al., 1995).

For SS, a line 300 µm anterior to the tip of CA3 of hippocampus was extended radially toward neocortex in fh/fh and wt rats. This served as an indicator of where to begin placing counting boxes with subsequent boxes added systematically towards the anterior. To place appropriately sized counting boxes within upper (II/III) and deeper (primarily VI) layers of SS, we referred to CAL and cytochrome oxidase staining as landmarks for determining lamination patterns of layers II/III and IV respectively (Roberts et al., 2000). For NeuN, GABA and CR, we defined upper layers by placing boxes such that the upper edges of the counting boxes were adjacent to the internal border of layer I. For the same stains, we defined deeper layers of SS by placing counting boxes such that the bottom edge of the box was slightly (~10–20 µm) above white matter. For FH, we counted deeper layers by placing boxes slightly above white matter and PARV, upper layers were defined by placing counting boxes centered between layer I and the darkly stained puncata of layer IV, a defining feature of PARV immunoreactivity in SS, while in deeper layers, boxes were placed slightly above white matter and beneath the thick band of layer IV labeling (Sánchez et al., 1992).

We found that in upper layers of EC, NeuN density was significantly increased in fh/fh compared to wt [mean wt = 51 ± 10, mean/fh = 40 ± 8; t(1,22) = 19.45, P < 0.001]. In lower layers of SS cortex, we found that neuronal density was decreased in fh/fh compared to wt [mean wt = 57 ± 10 vs. fh/fh = 35 ± 10; t(1,22) = 10.77, P = 0.001] while neuronal density was decreased in deeper layers of fh/fh rats compared to wt [mean wt = 25 ± 6, fh/fh = 30 ± 4; F(1,22) = 5.87, P = 0.05].
10. fh/fh = 33 ± 5; F(1,30) = 73.30, P < 0.001. Neuronal density was also decreased in deeper layers of SS cortex in fh/fh rats [mean wt = 48 ± 7, fh/fh = 42 ± 4; F(1,30) = 9.55, P < 0.01]. At P0 interneuron densities were placed over a grand mean of H&E-stained cells. Neuronal density at P0 was not significantly different in upper or deeper layers of EC [for upper: mean wt = 18 ± 1, fh/fh = 18 ± 2, P > 0.05; for deeper: mean wt = 22 ± 1, fh/fh = 23 ± 2, P > 0.05].

At E14, we quantified the percentage of pyknotic nuclei within semithin sections of MGE and neocortical VZ. Pyknotic nuclei were defined as darkly stained, punctate nuclei (see Fig. 5A) and those nuclei that were fragmented were counted as one nucleus. Images of MGE and VZ sections were imported into Adobe Photoshop 5.0 and the total number of nuclei and pyknotic nuclei were counted within 50 µm zones beginning and extending 400–600 µm away from the ventricular surface. To calculate the percentage of dead cells within each 50 µm zone, we divided the total number of pyknotic nuclei by the total number of nuclei within each 50 µm area counted.

**Analysis of Cell Morphology**

Images of all labeled cells were acquired on a Nikon Eclipse E400 microscope using a Spot digital camera. Soma area, nuclear area, number of primary dendrites and average dendritic width were determined from digitized images. Primary dendrites were considered processes originating from the soma, and dendritic widths were measured at a constant distance of 20 µm from the cell body. All measurements were performed by an observer blind to the experimental groups.

**Retrograde Dil Labeling**

To compare morphologies of projecting pyramidal cells between fh/fh and wt rats, P15 rats (n = 2 fh/fh and 2 wt) were perfused with 1 × PBS followed by 4% paraformaldehyde and then post-fixed overnight at 4°C. Crystals of Dil were then placed into the right cerebral hemisphere using a glass micropipette. The brains were then placed into PBS containing 1 mM sodium azide and incubated at 37°C for 8–12 weeks. Coronal sections (50 µm) were taken and labeled pyramidal cells (primarily those in layers II/III and V) were analyzed in both the ipsi- and contralateral hemisphere.

**PCR-based Genotyping of +/+ fh and fh/fh Mutants**

Genomic DNA was purified from either spleens (P14) or bodies (E14 and E15) using a Qiagen QIAamp® DNA Mini Kit. We performed polymerase chain reaction (PCR) with the MapPair™ marker D12Rat80, which is <1 cM from the fh mutation (Cogswell et al., 1998). This allowed us to genotype wildtype (+/+), heterozygous (+/fh) or mutant (fh/fh) rats. Because +/+ and +/fh animals exhibit similar phenotypes and appear behaviorally normal, we have collectively referred to both of these genotypes as wt phenotype for purposes of immunohistological comparisons.

**Statistical Analyses**

Comparisons of neuronal densities and morphological measurements across ages were evaluated using ANOVA. Comparisons of interneuron densities and soma sizes between different neocortical regions were made using a three-factor ANOVA with repeated measures. A P-value <0.05 was considered significant.

**Results**

**Reduction in the Density of GABA+ Neurons in Cerebral Cortex**

In order to determine the relative number of inhibitory interneurons to total neurons in the cerebral cortex of flathead and wildtype rats, we used immunocytochemistry for GABA and the neuronal nuclear antigen, NeuN. Figure 1A shows examples of GABA immunoreactivity in EC and SS for wt and fh/fh rats at P14. As shown in Figure 1C,E, in both areas, and in all layers, the percentage of GABA+ neurons was reduced. In upper layers of EC, ~30% of neurons in wt compared to 9% of neurons in fh/fh were GABA+, while in deeper layers of EC ~15% of neurons in wt compared to 6% of neurons in fh/fh were GABA+ [for upper: F(1,53) = 308.05, P < 0.001; for deeper: F(1,53) = 104.23, P < 0.001]. In deeper layers of SS ~15% of neurons in wt compared to 4% in fh/fh were GABA+. In contrast, in upper layers of SS there was only a slight decrease in the percentage of GABA+ neurons: ~2% of neurons in wt compared to 9% of neurons in fh/fh [for upper: F(1,53) = 28.99, P < 0.01; for deeper: F(1,53) = 43.46, P < 0.001]. Therefore, the flathead mutation causes a widespread decrease in the relative number of inhibitory interneurons. Moreover, the decrease in interneuron loss suggests death is
By age 1 week before seizures begin (Sarkisian et al., 1999), in upper layers of EC at P0, -16% of neurons in \( \textit{wt} \) were GABA+ , while in deeper layers -13% of neurons in \( \textit{wt} \) were GABA+ [for upper: \( F(1,53) = 178.82, P < 0.001 \); for deeper: \( F(1,53) = 107.54, P < 0.001 \)]. Therefore, the reduction in interneurons was of similar magnitude in EC before seizure activity becomes apparent either behaviorally or electrophysiologically (Sarkisian et al., 1996). As shown in Figure 1B, in the depth of P0 SS cortex, a similar pattern of reduction compared to P14 was also observed. As shown in Figure 1D, in upper layers of SS -15% of neurons in \( \textit{wt} \) were GABA+ , while in deeper layers -25% of neurons in \( \textit{wt} \) were GABA+ [for upper: \( P > 0.05 \); for deeper: \( F(1,8) = 24.21, P < 0.01 \)]. Therefore, the reduction in interneurons appears to be a prenatal effect and is likely to result from the failed genesis, migration or increased death of interneuron precursors.

**Hypertrophy of Interneurons**

While interneurons are greatly decreased in number, the sizes of individual interneurons are markedly enhanced in \( \textit{fh/fh} \). Figure 3A shows examples of CR+, PARV+ and CAL+ interneurons in \( \textit{wt} \) and \( \textit{fh/fh} \) neocortex. Figure 3B shows a quantification of soma

**Significant differences in different cortical regions**

In contrast to upper layers, there is a decrease of CR+ cells in deeper layers of SS cortex. Scale bar = 50 \( \mu \text{m} \).
compared to CAL+ cells in and the width of primary dendrites of the largest PARV+ and and Table 1). We quantified the number of primary dendrites across neocortex at P0 were larger in B general hypertrophy of all three interneuron subtypes (Fig. 3 EC and SS neocortices. For each of these regions we observed size from randomly selected PARV+, CAL+ and CR+ neurons from both wt and fh/fh rats. For both PARV and CAL, there were approximately twice as many primary dendrites and an increase in the thickness of dendritic processes. (C) Quantification of soma sizes of GABA-immunoreactive neurons at P0 shows that the increase in soma size is present by birth. Scale bar = 50 µm.

Table 1

<table>
<thead>
<tr>
<th>Interneuron subtype</th>
<th>Soma area (µm²)</th>
<th>No. of primary dendrites</th>
<th>Primary width (µm)</th>
<th>% of neurons in EC</th>
<th>% of neurons in SS</th>
</tr>
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<tr>
<td></td>
<td>wt fh/fh</td>
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<td>wt fh/fh</td>
<td>wt fh/fh</td>
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<tr>
<td>CR</td>
<td></td>
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<tr>
<td>EC</td>
<td>110 ± 24</td>
<td>207 ± 45****</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>189 ± 46</td>
<td>386 ± 195***</td>
<td>4.3 ± 1.3</td>
<td>1.7 ± 0.1</td>
<td>2.6 ± 0.9**</td>
</tr>
<tr>
<td>PARV</td>
<td>205 ± 45</td>
<td>414 ± 144***</td>
<td>3.3 ± 0.7</td>
<td>7.8 ± 3.1***</td>
<td>2.9 ± 0.6***</td>
</tr>
<tr>
<td>SS</td>
<td>181 ± 38</td>
<td>301 ± 67***</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>184 ± 41</td>
<td>366 ± 82***</td>
<td>ND</td>
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</table>

Somas of CR+ (n = 15 wt, 15 fh/fh) and PARV+ (n = 15 wt, 15 fh/fh) cells are hypertrophied in both EC and SS of P14 fh/fh rats. The largest CAL+ (n = 10 wt, 10 fh/fh) and PARV+ (n = 10 wt, 10 fh/fh) cells in fh/fh have increases in the number of primary dendrites and a corresponding increase in primary dendritic widths. Also shown for comparison are the percentages of CR+, CAL+ and PARV+ neurons in upper and deeper layers of EC and SS at P14. Percentages in some layers of EC or SS were not determined (ND). *P < 0.05, **P < 0.01, ***P < 0.001; P > 0.05 not significant (NS).

Like the reduction in cell number, the increase in cell size occurs before the onset of seizures. As shown in Figure 4A–C, neuronal hypertrophy was not evident in pyramidal cells retrogradely labeled with Dil (Fig. 4A), or labeled immunohistochemically using an antibody that recognizes rat brain pyramidal cells (Fig. 4B). We compared the soma sizes from fh/fh and wt pyramidal neurons that contained only single nuclei. As shown in Figure 4C, we found that soma sizes of retrogradely labeled pyramidal cells in wt (n = 35 cells) are actually slightly larger than pyramidal cells in fh/fh (n = 31 cells) [mean wt = 442 ± 37 µm², fh/fh = 199 ± 41 µm²; F(1,64) = 11.45, P < 0.01]. Additionally, the nuclear areas of the same pyramidal neurons show no difference between fh/fh (n = 31 cells) and wt (n = 35 cells) [mean wt = 115 ± 22 µm², fh/fh mean = 107 ± 27 µm²; F(1,64) = 1.85; P > 0.05], nuclei of single-nucleated GABA+ neurons in fh/fh (n = 32 cells) are almost twice as large as wt (n = 32 cells) [wt mean = 93 ± 17 µm², fh/fh:
mean = 171 ± 34 µm²; \( F(1,62) = 134.30; P < 0.001 \). Therefore, the fh mutation alters cellular growth mechanisms in neocortex specific to interneurons.

**Increased Cell Death and Mitotic Abnormalities in the MGE**

In order to determine if there are cellular defects within the GE at the time when interneurons are generated, we performed an EM analysis. As shown in Figure 5A, an increased number of pyknotic cells are present in the MGE and neocortical VZ in \( fh/fh \) compared to \( wt \). Figure 5B shows an electron micrograph of MGE, revealing numerous apoptotic (Fig. 5B, left) and multinucleate cells (Fig. 5B, right). As shown in Figure 5C, the total percentage of pyknotic cells in the MGE at E14 in \( fh/fh \) compared to \( wt \) is increased by ~6-fold. Similarly, the total percentage of cell death in \( fh/fh \) neocortical VZ compared to \( wt \) is increased by ~3-fold. In addition, \( fh/fh \) shows a significantly greater number of pyknotic cells in the MGE than in the neocortical VZ (6% compared to 3%; \( F(1,10) = 26.28, P < 0.001 \)), and similarly the ratio of pyknotic cells in MGE:VZ is twofold greater in \( fh/fh \) than in \( wt \) (2.08 versus 1.03 respectively).

Moreover, we observed that the location of many multinucleate cells (Fig. 5B, left) in MGE is intermixed and corresponds to the location where cell death is maximal, ~150–300 µm away from the ventricular surface (Fig. 5D) and thus appears more concentrated in the SVZ. Therefore, a failure in cytokinesis near the ventricular surface may precede or be closely linked to the cell death in the MGE.

As in the embryo, in P14 neocortex we observed multinucleate non-pyramidal cells (Fig. 6A). In additional experiments, we injected BrdU at E14 and at P13 we found many non-pyramidal shaped cells containing two nuclei, both equivalently labeled with BrdU (Fig. 6B). In no multinucleate cases was only one nuclei BrdU-labeled. We found that across neocortex in flathead, ~27% (257/944) of GABA+ cells contain two nuclei, and ~1% (9/944) contain three or four nuclei, while ~9% (127/1472 cells) of DiI-labeled pyramidal neurons contain two nuclei (Fig. 6C) and none contain more than two. Therefore, like MGE, abnormalities in cytokinesis are also found in postnatal neocortex and appear to affect interneurons more so than pyramidal neurons.

**Discussion**

The flathead mutation results in a widespread reduction in the relative number of cortical GABAergic interneurons. At E14, high levels of cell death are observed in both the MGE and neocortical proliferative areas compared to \( wt \). This cell death is associated with the appearance of multinucleate cells found in both MGE and throughout postnatal neocortex. The cytokinesis defect appears to affect more non-pyramidal than pyramidal cells in flathead, and there is also a selective and specific increase in interneuron growth. Based on these results we hypothesize that the primary defect in \( fh/fh \) occurs sometime during neurogenesis and that the ensuing cell death in all telencephalic progenitors occurs as a result of failed cytokinetic properties. These observations are consistent with recent data described in Citron-K deficient mice which also have dramatic losses in neocortical interneurons and massive cell death associated with altered cytokinesis (Di Cunto et al., 2000). Since we have recently sequenced and identified a mutation in the Citron-K
gene in flathead (unpublished data), it is likely that similar mechanisms of cell death are occurring in flathead as the Citron-K deficient mouse. Citron-K is expressed throughout CNS progenitor regions, so it is unclear at this time why the size and number of neocortical interneurons in flathead are more severely affected. The overall neocortical phenotype of the flathead mutant, however, would indicate that the flathead gene is critical for the generation of interneurons and later generated neurons such as upper layer pyramidal neurons.

**Regulation of Interneuron Growth and Differentiation**

Unlike pyramidal neurons, interneurons in flathead have larger somas and dendritic arbors than wildtype. One possibility for the interneuron-specific hypertrophy is that interneuron growth may be negatively regulated by interneuron-specific interactions, and that the reduction in interneuron number in flathead results in a corresponding compensatory increase in interneuron growth. If so, then areas of reduced interneuron number should show greater hypertrophy and regions of higher interneuron number should show reduced hypertrophy. However, when we made comparisons of sizes of interneurons across different neocortical regions (EC and SS) we found no significant relationship between the density of GABAergic interneurons in a particular cortical area or lamina with the size of interneurons (three-factor ANOVA; F = 0.446, P = 0.64). Therefore, a reduction in interneuron interactions alone is not the likely cause of the

**Figure 5.** Increased cell death and multinucleate cells in MGE. (A) Examples of semi-thin sections of MGE and neocortical-VZ taken in wt (left panels) and fh/fh (right panels) stained with toluidine blue. For both MGE and neocortical VZ, increased numbers of pyknotic nuclei (arrows) can be observed in fh/fh. (B) Representative EM of fh/fh MGE showing apoptotic cells (asterisks, left panel) and also multinucleate cells (arrows, left and right panels). (C) Quantitative analysis of the percentage of pyknotic cells in MGE and neocortical VZ in wt (open bars) and fh/fh (closed bars). Flathead has significantly more cell death in MGE and neocortical VZ compared to wt, and there is significantly more cell death in MGE compared to neocortical VZ in fh/fh. (D) The location of apoptotic cells in MGE relative to the MGE ventricular surface in fh/fh. Most apoptotic cells are found 150–300 μm away from the ventricular surface. Scale bar (A) 100 μm; (B) 5 μm.
that BDNF mRNA levels are increased in the size of NPY interneurons, and recent unpublished data indicate is not synthesized by interneurons themselves, can increase the hippocampus, brain-derived neurotrophic factor (BDNF), which et al previously shown to be very responsive to increases in neuron cell death in response to other developmental defects, such as pyramidal interneuron-specific hypertrophy; however, there may be a influence on growth.

Another possibility for the interneuron hypertrophy may be a response to other developmental defects, such as pyramidal neuron cell death in fh/fh. For example, interneurons have been previously shown to be very responsive to increases in neurotrophins. Marty et al. (Marty et al., 1996) found that in hippocampus, brain-derived neurotrophic factor (BDNF), which is not synthesized by interneurons themselves, can increase the size of NPY interneurons, and recent unpublished data indicate that BDNF mRNA levels are increased in the flathead neocortex (P. Crino, unpublished observation). Similarly, in BDNF knockout mice, there is a decrease in the number of CAL, PARV and NPY+ cells, and BDNF overexpression increases the number and size of PARV+ cells in neocortex (Jones et al., 1994; Huang et al., 1999). The increased interneuron growth in flathead, which is greater than any previously reported hypertrophy, seems therefore to be the result of an early cell-autonomous increase in cell growth; however, we cannot rule out extrinsic influences at this time. It is not clear why pyramidal neurons with or without double nuclei do not show similar dramatic changes in morphology. However, since multinucleate pyramidal cells do not appear as similarly hypertrophied as multinucleate interneurons may reflect a cell type intrinsic difference in cell growth mechanisms. Further studies in the fh/fh brain need to examine the interrelationship between interneurons and pyramidal neurons and whether or not one cell type may be regulating the growth patterns of the other.

**Relationship to Human Cortical Dysplasias**

Alterations in number, morphology and function of interneurons have been reported in brains of humans with cortical malformations and epilepsy (Ferrer et al., 1994; Spreafico et al., 1998; Garbelli et al., 1999; Hannan et al., 1999; Kerfoot et al., 1999) [for review see (DeFelipe, 1999)]. DeFelipe et al. (DeFelipe et al., 1993) and Marco et al. (Marco et al., 1996) have reported selective decreases in PARV+ and glutamic acid decarboxylase immunoreactivity in human epileptogenic neocortex. Spreafico et al. (Spreafico et al., 1998) reported three patients with cortical dysplasias and intractable seizures in which a decrease in PARV, CAL and CR+ cells was observed within the dysplastic regions. In addition, these authors and others (Ferrer et al., 1992; Garbelli et al., 1999; Thom et al., 2000) have reported cytomegalic CAL+ and PARV+ cells, similar in appearance to the cells in flathead. Finally, since no other animal model shows similar interneuron hypertrophy along with epileptiform activity, the flathead mutant offers a unique opportunity to study the role of cytomegalic interneurons in the generation of seizures.

**Cytokinesis Failure and Cell Death in the Flathead Mutant**

Our electron microscopy results indicate that many cells just outside of proliferative zones have double nuclei, and binucleate neurons are present in mature postnatal brain as well (Figs 5 and 6). These observations suggest that abnormalities in cell cycle progression in CNS progenitors may underlie the flathead phenotype. In particular, the mutation may affect the final phases of cell division which results in failed cytokinesis, and in some cases is followed by cell death. It is difficult to determine whether cytokinesis failure or cell death is most responsible for the decrease in neuron number in the flathead mutant; however, there are several reasons why we believe that cytokinesis defects precede the cell death in the flathead mutant. First, we have seen BrdU-labeled binucleate cells following BrdU injections as early as E14 (Fig. 6B); second, there is no difference in the percentage of S-phase cells in flathead proliferative zones (Roberts et al., 2000); third, the increased cell death in flathead occurs away from the ventricular surface in the same region as the appearance of binucleate precursors (Fig. 5B); and fourth, the flathead mutation is likely to be in the Citron-K gene, which is a known regulator of cytokinesis (Madaule et al., 1998).

The cell death revealed in this study may further explain the resultant cortical phenotype in fh/fh. By genotyping E14 embryos with a microsatellite marker <1 cM from the flathead gene, before animals are micrencephalic, we determined that there is increased apoptosis in homozygous mutants as early as E14. This not only contrasts the cell death previously reported to begin at E17–18, but it is also greater than that observed at E16 in proliferative zones (Roberts et al., 2000). While it is difficult to compare quantitatively cell death determined by TUNEL with that determined by pyknotic nuclei, it is clear that increased cell

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**Figure 6.** Many non-pyramidal cells in fh/fh are multinucleate. (A) EM example of a non-pyramidal shaped cell in SS neocortex with two nuclei at P14. (B) Examples of two Nissl-stained cells in P13 SS cortex double-labeled for BrdU. BrdU was injected at E14. Note that the cells have two nuclei that are equivalently labeled with BrdU. (C) ∼28% (268/944 cells) of all GABA+ cells across neocortex compared to ∼8% (127/1472 cells) of DiI-labeled, upper layer pyramidal cells are multinucleate. Scale bar (A) 5 μm; (B) 10 μm.
death occurs in the proliferative zones of the flathead mutant beginning near the time neurogenesis starts, and then increases further towards the end of the neurogenetic period. This could indicate that the mechanisms for cytokinesis control are different in early neurogenesis and in late neurogenesis. Indeed, it has been shown that the pattern of symmetric and asymmetric divisions changes as neocortical neurogenesis proceeds. The pattern of binucleate cells in mature flathead cortex may also indicate a greater susceptibility to cell death for cells that must migrate long distances. In \( fh/fh \) neocortex, we have observed that higher percentages of cells in deeper layers of both EC and SS are multinucleate compared to upper layers (EC: upper = 13%, deeper = 28%, and SS: upper = 11%, deeper = 47%). Therefore, one possibility is that longer migrating cells (upper layer neurons from neocortical VZ and interneurons derived from GE–VZ) are at greater risk of dying because double nuclei may not be permissive to longer distance migrations. In fact, we observed a concentration of dead cells in MGE which appear to be within SVZ (Fig. 6A) and this death may be associated with a failed attempt to migrate towards neocortex. However, since many of the surviving interneurons in upper and deeper layers of cortex are binucleate, double nuclei must not completely impede all migration.

The Flathead Mutation: Similarities to Citron-K Knockout Mice

Ongoing genetic analysis of the \( fh \) mutation in our laboratory has lead to two candidate genes located on the distal arm of chromosome 12: Musash1 and Citron. Musash1 is an RNA binding protein expressed in CNS progenitors (Sakakibara et al., 1996). Citron-K, the embryonic variant of Citron, is expressed specifically in phenotypic similarity to the flathead gene (Di Cunto et al., 2000). Mice lacking Citron-K, like the flathead mutant rat, show massive apoptosis outside of the VZ surface, early onset and severe seizures, a significant loss of neocortical GABAergic neurons (including dramatic reductions in CAL+ and CR+ subtypes), reduced migration of GABAergic neurons from the GE to neocortex, and altered cytokinesis leading to binucleate pyramidal and nonpyramidal neurons (Di Cunto et al., 2000). We have recently sequenced and identified a mutation in Citron-K in the \( fh/fh \) mutant (unpublished data). In future reports we will describe the exact molecular mutation and describe how it affects cytokinesis of neocortical pyramidal and nonpyramidal neuronal progenitors.

Notes

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Address correspondence to Joseph J. LoTurco, Ph.D., Department of Physiology and Neurobiology, University of Connecticut, U-156, 3107 Horsebarn Hill Road, Storrs, CT 06269, USA. Email: loturco@oracle.pnb.uconn.edu.

References


