Granule Cell Dispersion and Aberrant Neurogenesis in the Adult Hippocampus of an LIS1 Mutant Mouse

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Key Words

Neurogenesis • Dentate gyrus, cell proliferation/migration/differentiation • Lissencephaly • Hippocampus, adult

Abstract

Human type 1 lissencephaly is a severe brain malformation associated with cognitive dysfunction and intractable epilepsy. Mutant mice with a heterozygous deletion of *LIS1* show varying degrees of hippocampal abnormality and enhanced excitability. Whether a reduction of LIS1 function affects adult hippocampal neurogenesis, and if so, whether aberrant neurogenesis contributes to the generation of a disorganized hippocampus remain unknown. Previous reports indicate the presence of multiple pyramidal cell layers and granule cell dispersion in LIS1 mutant mice. Here we observed disruption of the subgranular zone and glial fibrillary acidic protein-immunoreactive radial astrocytes in the dentate gyrus of adult LIS1 mice. Using pulse-chase bromodeoxyuridine (BrdU) labeling combined with neuronal and glial antibody staining we provide evidence for ectopic adult neurogenesis in LIS1 mice. A gradually decreased survival rate for these newborn granule cells was also demonstrated in LIS1 mice 7 days after BrdU injection. This reduced survival rate was associated with impaired neuronal differentiation 28 days after BrdU administration. Thus, *LIS1* haploinsufficiency can lead to abnormal cell proliferation, migration and differentiation in the adult dentate gyrus.

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Introduction

Type 1 lissencephaly is a severe brain developmental disease characterized by a smooth neocortical surface and the absence of gyri/sulci [Reiner et al., 1993]. Lissencephaly patients exhibit variable degrees of mental retardation and epilepsy [Dobyns et al., 1993]. Impaired migration of neuronal precursors during brain development is presumed to underlie this brain malformation. The defective gene for this disorder, *LIS1*, encodes a brain-specific noncatalytic subunit of platelet-activating factor acetylhydrolase 1b [Cardoso et al., 2002; Reiner et al., 2002]. Accumulating evidence, from different species, suggests that the LIS1 protein binds dynactin/dynein, modulating cell proliferation and neuronal migration [Gambello et al., 1999; Liu et al., 2000; Tanaka et al., 2004]. A knockout mouse further confirmed the developmental importance of the LIS1 protein with homozygous embryonic lethality and heterozygous mice showing migration defects, severe hippocampal dyslamination and hyperexcitability [Hirotsume et al., 1998; Fleck et al., 2000; Cahana et al., 2001]. Of particular interest, in these mice, was the presence of a multilaminated stratum pyramidale and a dispersed granule cell layer (GCL) reminiscent of that seen in patients with temporal lobe epilepsy [Houser, 1990]. How a heterozygous loss of LIS1 contributes to these hippocampal malformations, and specifically how a dispersed GCL originates, remains unknown.

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One possibility is that dispersed granule cells arise from newly born neurons. Indeed, recent work suggests a unique capability for neurogenesis in the subgranular zone (SGZ) of the adult dentate gyrus. In wild-type (WT) mice, these newly born cells (about 6% of the total granule cell population) are suggested to play an important role in learning and memory [Eckenhoff and Rakic, 1988; Schinder and Gage, 2004; Ming and Song, 2005]. More importantly, a variety of factors can modulate the number and phenotype progression of these newly born granule cells [Ming and Song, 2005]. For example, seizures activate a robust granule cell neurogenesis response and the subsequent ectopic migration and integration of these neurons may directly contribute to the generation of a reorganized hyperexcitable network [Parent et al., 1997; Scharfman et al., 2000]. Whether hippocampal neurogenesis is altered in mice with reduced *LIS1* function is unknown. To begin to address these issues, we first analyzed the proliferative SGZ of *LIS1* heterozygote mutants. Using pulse-chase bromodeoxyuridine (BrdU) labeling of newly born granule cells and a series of antibody double-labeling studies, we further examined cell proliferation and phenotype progression of newborn cells in the adult dentate gyrus. Evidence for abnormal SGZ architecture and aberrant neurogenesis in *LIS1* mutant mice is presented.

Materials and Methods

Animals and BrdU Injection

LIS1^{+/-} mice and age-matched WT littermates (2–4 months old, weighing 25 g or more) were used for all experiments. BrdU (Roche) was administered intraperitoneally at a concentration of 50 µg/g body weight (10 mg/ml stock, dissolved in PBS). Animals were perfused at 2 h or 7 days after BrdU treatment received only a single BrdU injection. Animals sacrificed 28 days after BrdU treatment received daily injections on 4 consecutive days. Each experimental time point included 4 animals.

Immunostaining

Animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in PBS. The brains were immediately dissected, postfixed overnight and cytoprotected in 30% sucrose in PBS for 48 h at 4°C. Brains were cut into 40-µm coronal sections. Sectioning began at a random starting point in the hippocampus (approx. bregma -1.8 through bregma -5.8), which yielded tissue for the entire dentate gyrus. For the detection of BrdU-labeled nuclei, tissue sections were incubated in 2 N HCl at 37°C for 30 min, and rinsed in 0.1 M boric acid (pH 8.5) for 15 min. Sections were blocked for 2 h in PBS with 0.1% Triton X-100, 10% donkey serum and 1% bovine serum albumin. For primary reactions, sections were incubated at 4°C for 24 h in various primary antibodies including rat anti-BrdU (1:30, Accurate

Chemical), goat anti-doublecortin (anti-DCX; 1:80, Santa Cruz), mouse anti-NeuN (1:1,000, Chemicon), rabbit anti-glial fibrillary acidic protein (GFAP; 1:500, DakoCytomation) and rabbit anti-Ki67 (1:500, Vector). After several washes, sections were incubated for 2 h with the corresponding secondary antibodies diluted at 1:500 (Alexa-488-conjugated donkey antirat or antirabbit, Alexa-546-conjugated donkey antigoat, Alexa-647-conjugated donkey antimouse). The fluorescent signals were obtained using a Zeiss LSM 510 confocal microscope.

Quantification and Statistics

BrdU-positive cells were counted in a one-in-six series of sections of 40 µm thickness spaced 240 µm apart. For cell counting, results were calculated as the average number of BrdU-positive cells per section and expressed as mean ± SEM. For phenotype analysis, 25 BrdU-positive cells were analyzed for coexpression of NeuN and/or DCX. Ratio of colabeling was determined. Data were analyzed by Student's *t* test, and *p* values were taken as 0.05.

Results

Disruption of the SGZ in LIS1^{+/-} Mice

We first examined SGZ organization in the adult dentate gyrus. Triple-labeling immunofluorescence was performed with antibodies to GFAP (for stem cells and parenchyma astrocytes), DCX (for the intermediate precursors) and NeuN (for mature differentiated neurons) [Seri et al., 2004]. As expected, a tightly packed GCL labeled with NeuN was observed in WT mice (fig. 1B). Radial proliferative units (box in fig. 1A) comprised of GFAP-positive astrocytes with thin perpendicularly oriented radial processes (green in fig. 1A) extending through the entire GCL and DCX-positive cells (red in fig. 1A, C) localized to the SGZ or inner GCL were observed. In contrast, distinct granule cell dispersion was evident in the NeuN-labeled dentate gyrus from age-matched *LIS1*^{+/-} mice (fig. 1E). Radial proliferative units in these mutants were severely disrupted: GFAP-positive astrocytes failed to form regular radial scaffolding, with short processes and aberrant orientation; DCX-positive cells lost their typical SGZ position and penetrated deep into the dispersed GCL (fig. 1D–F), and a clearly delineated SGZ was not observed (compare fig. 1C, F). Schematic panels are presented to highlight normal (fig. 1G) and aberrant (fig. 1H) SGZ architecture.

Aberrant Neurogenesis in LIS1^{+/-} Mice

Expression of DCX-positive cells in outer GCLs raised the possibility that these cells are born in abnormal non-SGZ locations, i.e., ectopic neurogenesis. To evaluate the location of newly born granule cells, a 2-hour BrdU pulse-

Fig. 1. Radial proliferative units are disrupted in LIS1 mice. In WT mice, GFAP-positive radial astrocyte appears to interact closely with clusters of DCX-positive cells, forming radial proliferative units (box in **A**), which are mainly localized in the SGZ and inner GCL (**B**, **C**). In LIS1 mutants, radially oriented GFAP-positive cells are disrupted and DCX-positive cells are dislocated throughout the GCL (**D–F**). The schematic cartoons demonstrate the normal architecture of radial proliferative units in WT (**G**), and aberrant units in LIS1 mice (**H**). Scale bar: 20 μ m.

labeling protocol was used. This approach labels only a portion of cycling cells, i.e., cells that were in the S-phase long enough to incorporate detectable amounts of BrdU. In WT mice, most BrdU-labeled cells were found as single cells located in the SGZ (fig. 2A). Less than 8% of BrdU-positive cells stained with GFAP representative of early progenitor and/or stem cells (fig. 2A–C) and 49% of BrdU-positive cells were DCX-positive late progenitor cells (fig. 2G–I). Interestingly, in LIS1^{+/-} mice, BrdU-labeled single cells or doublets of synchronously dividing cells were consistently found in outer GCLs (fig. 2D–F, J–L) and a few of them were found scattered in the hilus and the inner molecular layer (data not shown). Although present in aberrant locations, similar proportions of BrdU-positive cells coexpressed GFAP (fig. 2D–F; $7.9 \pm 0.1\%$ in WT vs. $8.1 \pm 0.3\%$ in LIS1^{+/-} mice, $p > 0.05$) and DCX (fig. 2J–L; $48.5 \pm 0.2\%$ in WT vs. $52.3 \pm 0.5\%$ in LIS1^{+/-} mice, $p > 0.05$). These results strongly suggest ectopic neurogenesis in the dispersed GCL of LIS1 mutants.

Because of the possibility of BrdU-producing mutated cells in LIS1^{+/-} mice, or the uncertainty of diffusion of BrdU across the blood-brain barrier after intraperitoneal injection, we next used an antibody to Ki67, i.e., an endogenous proliferation marker expressed in dividing cells [Kee et al., 2002]. In WT mice, Ki67-labeled nuclei are primarily located in the SGZ and 56% of these cells double-labeled with DCX (fig. 2M–O). In many cases, these cells were observed closely apposed to blood vessels (asterisk in fig. 2O). In LIS1 mutants, Ki67-positive nuclei were found dislocated within the dispersed GCL (fig. 2P–R), the hilus and inner molecular layer with 58% of these cells colabeled with DCX. To estimate the number of newborn cells, we counted BrdU-positive and Ki67-positive cells in the dentate gyrus 24 h after BrdU injection. As shown in figure 2S and T, there was no significant difference in the overall number of labeled cells (BrdU stained, 10.8 ± 0.9 in WT vs. 11.6 ± 0.9 in LIS1^{+/-} mice, $p > 0.05$; Ki67 stained, 12.8 ± 0.9 in WT vs. 13.5 ± 0.9

Fig. 2. Dividing cells in the dentate gyrus. Confocal micrograph of BrdU-labeled GFAP-positive astrocytes 2 h after BrdU injection were mainly localized at the SGZ in WT mice (**A–C**), but penetrated into the GCL in LIS1 mutants (**D–F**). BrdU-labeled DCX-positive cells 2 h after BrdU injection were dislocated in the GCL in LIS1 mice (**J–L**) compared with WT littermates (**G–I**). Ki67-positive clusters coexpressed DCX and were mainly distributed in the SGZ in WT (**M–O**), whereas displaced in the GCL in LIS1 mice (**P–R**). The number of 2-hour pulse BrdU-positive cells and Ki67-positive cells in the dentate gyrus was not significantly different between WT and LIS1 mice (**S, T**). The asterisk in **O** denotes a blood vessel in the inner GCL. Scale bar: 20 μ m.

in LIS1+/- mice, $p > 0.05$). Because DCX-positive cells accounted for a similar proportion of the total number of BrdU-positive cells in both animals, our results suggest a comparable level of granule cell neurogenesis between WT and LIS1 mutants. It is noted that embryonic neurogenesis in the hippocampus of LIS1+/- mice also showed nearly identical numbers of newborn cells compared to age-matched controls [Fleck et al., 2000].

With regard to the morphology of BrdU-labeled DCX-positive cells, WT mice showed elongated BrdU-positive nuclei with either no process or processes oriented parallel to the GCL (fig. 2H). In LIS1+/- mice, BrdU-labeled

DCX-positive cells demonstrated disorientated nuclei with aberrant apical processes (fig. 2K), previously defined as a later phase of D cells than those with horizontal processes [Seri et al., 2004]. Also, more BrdU-positive doublets were found in LIS1 mutants compared to WT mice (fig. 2), perhaps indicating a shorter cell cycle for LIS1 heterozygote mice.

Immature Migrating Neuroblasts 7 Days after BrdU Injection

To examine the progression of cell phenotype, 7-day pulse labeling after a single BrdU injection was used. In

Fig. 3. Immature migrating neuroblasts in the dentate gyrus. Confocal images of BrdU-labeled DCX-positive cells 7 days after BrdU injection mainly demonstrated SGZ localization in WT mice (**A–C**), whereas aberrant position in the GCL in LIS1 mice (**D–F**). The number of 7-day pulse BrdU-positive cells was significantly decreased in LIS1 mice (**G**). The asterisk indicates a statistical difference between experimental groups ($p < 0.05$, Student's *t* test). Scale bar: 20 μm .

Fig. 4. Neuronal differentiation in the dentate gyrus. In WT mice, most BrdU-positive cells 28 days after BrdU injection were NeuN-positive (**A–C**) and GFAP-negative (**M–O**). In LIS1 mutants, BrdU-positive cells were NeuN-positive, NeuN-positive/DCX-positive (**D–I**, arrow), DCX-positive (**G–I**, arrowhead), neither showing a neuronal nor glial marker (**J–L**, **P–R**). The number of BrdU-positive cells was significantly decreased in LIS1 mutants (**S**). The asterisk indicates a statistical difference between experimental groups ($p < 0.05$, Student's *t* test). Scale bar: 20 μm .

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WT mice, the number of labeled dividing cells increased about 1.5-fold in the SGZ compared to the number observed with 2-hour pulse labeling, reflecting an ongoing proliferation process. BrdU-positive nuclei were oval, aligned within the SGZ and 87% expressed DCX (fig. 3A–C). In LIS1 mutants, elongated BrdU-positive nuclei penetrated deep within the GCL and a similar proportion of cells coexpressed DCX (91%, $p > 0.05$; fig. 3D–F). From these studies, it does not appear that the progression of phenotype for newborn cells is delayed in LIS1 mutants. To examine the short-term survival of newborn cells, we analyzed the number of BrdU-positive cells. As shown in figure 3G, a significant decrease was found in LIS1+/- mice compared with WT mice (11.1 ± 0.7 vs. 15.5 ± 1.1 , $p < 0.05$). This result could reflect the possibility that the BrdU content is diluted in LIS1+/- mice owing to a shorter cell cycle or perhaps an LIS1-mediated disruption of factors necessary for cell survival.

Neuronal Differentiation 28 Days after BrdU Injection

Next, we investigated long-term changes in neurogenesis by analyzing cell fate and phenotype 28 days after the last BrdU injection. For this study, BrdU was injected once a day for 4 consecutive days and animals were allowed to survive for approximately 1 month, a time at which most newly born cells express NeuN [Kempermann et al., 2003]. In WT mice, most BrdU-positive cells migrated out of the SGZ into the GCL with 76% coexpressing NeuN, indicating that BrdU was incorporated into mature neurons (fig. 4A–C). These BrdU-positive cells rarely expressed the astrocyte marker GFAP and the immature neuronal marker DCX at this later time point (fig. 4M–O). In LIS1+/- mice, the differentiation delay was distinct with only 34% of BrdU-positive cells expressing NeuN, 18% expressing DCX (fig. 4G–I, arrowhead), 16% expressing both NeuN and DCX (fig. 4D–I, arrows) and 32% expressing none of the cell type markers used in our experiments (fig. 4J–L, P–R). To further estimate the number of surviving cells, we counted BrdU-positive cells at this later time point. As shown in figure 4S, a 70% decrease in the total number of BrdU-positive cells was found in LIS1+/- mice compared with WT mice (3.9 ± 0.5 vs. 12.4 ± 1.4 , $p < 0.01$). As such, the newborn cells in LIS1+/- mice demonstrated a lower survival rate with an impaired differentiation.

Discussion

Ectopic Neurogenesis and Putative Aberrant Migration Pathway

The present results revealed a significant disruption of the SGZ in adult LIS1+/- mice. Of particular interest was the location of newborn cells within the dispersed GCL. By using 2-hour BrdU and Ki67 pulse labeling, we revealed ectopic neurogenesis in LIS1 mutants with many newly born cells located in outer GCLs. This was in stark contrast to the restriction of newly born cells to the SGZ in WT mice. It has recently been suggested that specific types of brain injury and seizures also lead to ectopic hippocampal neurogenesis [Magavi et al., 2000; Nakatomi et al., 2002; Arlotta et al., 2003; Kralic et al., 2005; Parent et al., 2006]. Reduction of the LIS1 protein might mimic this condition by creating mice prone to injury or seizures (not yet examined), or LIS1 haploinsufficiency might directly disturb the dentate gyrus proliferative matrix (shown here), or both.

The majority of dentate granule cells are born within the first 3 postnatal weeks. These cells are thought to migrate along GFAP-positive radial glial scaffolding in the dentate gyrus [Rickmann et al., 1987; Frotscher et al., 2003]. Our study revealed that GFAP-positive processes were stunted and aberrantly distributed in the dentate gyrus of LIS1+/- mice, strongly suggesting a potential disruption of radial migration. Similar phenotypes were observed in reelin and Wnt signaling mutants [Weiss et al., 2003; Zhou et al., 2004]. Together with the previous findings that the radial migration of cortical projection neurons and tangential migration of GABAergic interneurons are aberrant in LIS1+/- mice [Sun et al., 2002; Gambello et al., 2003; McManus et al., 2004], it would be an intriguing question to ask how the LIS1 protein, known to act on the cellular mechanism of locomotion, disrupts these diverse migratory pathways. In our study, the dislocation of newborn cells per se could affect their ability to join the migration pathway, therefore aggravating the defects.

A Reduction of LIS1 Function Leads to Impaired Neuronal Survival and Differentiation

Our study demonstrated a gradually decreasing survival rate of newborn cells detected from 7 days after BrdU injection. This reduced survival rate was accompanied by impaired differentiation. The underlying reasons could be germinal niche and/or cell-autonomous effects. First, SGZ astrocytes secrete soluble and membrane-bound factors, and the fate choice of newborn cells de-

depends on these local environment cues [Temple and Alvarez-Buylla, 1999; Song et al., 2002]. The abnormal morphology of GFAP-positive astrocytes in our study implicates an aberrant germinal niche in the SGZ and potential disruption of these factors. Second, within the adult hippocampus, hot spots of cell proliferation in the SGZ were often found to be in close proximity to capillaries [Palmer et al., 2000]. In our study, that format was found in WT (fig. 2O) but rarely in LIS1+/- mice. This suggests that a disrupted vascular niche in the dentate gyrus of LIS1+/- mice could be one reason for abnormal neurogenesis. Third, LIS1 is implicated in mitotic progression and spindle orientation [Faulkner et al., 2000; Tsai et al., 2005]. In view of evidence that the signals for neuronal differentiation depend on the plane of cell division, a defect in mitotic progression in LIS1+/- mice could contribute to an abnormal differentiation.

Implications of Aberrant Neurogenesis

Hippocampal malformations, largely resulting from abnormal neuronal migration, are often associated with cognitive dysfunction and epilepsy. For example, one distinct feature of human temporal lobe epilepsy is a distinct dispersion of the GCL [Houser, 1990]. This malformation has also been observed in rodent animal models of temporal lobe epilepsy [Mello et al., 1992; Bouilleret et al.,

1999], mouse mutants lacking reelin, an extracellular glycoprotein and p35, a neuronal activator of cyclin-dependent kinase 5 [Wenzel et al., 2001; Drakew et al., 2002] and LIS1 heterozygote mice. How the GCL becomes malformed is unknown. One implication of our findings is that granule cells in the outer molecular layers are ectopically born in, or near these locations, and 'disperse' outward from the GCL. Because hippocampal neurogenesis has also been associated with learning and memory, the observed decreased survival of newly born cells in an LIS1 mutant mouse may explain some of the cognitive dysfunction observed in lissencephaly patients. Finally, ectopic granule cells in temporal lobe epilepsy patients and rodent animal models of temporal lobe epilepsy may be involved in abnormal network reorganization and seizure genesis [Parent et al., 2006; Scharfman et al., 2000]. Hippocampal slices from LIS1+/- mice exhibit a reduced threshold for the generation of epileptiform-like activity [Fleck et al., 2000; Wang and Baraban, unpubl. obs.]. This hyperexcitability deficit could potentially originate from the aberrant neurogenesis and impaired differentiation observed in these animals. Future efforts should focus on regulatory mechanisms and functional implications of a dispersed GCL with the goal of obtaining a better understanding of hippocampal pathogenesis in lissencephaly.

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