Abnormal Morphological and Functional Organization of the Hippocampus in a p35 Mutant Model of Cortical Dysplasia Associated with Spontaneous Seizures

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Cortical dysplasia is a major cause of intractable epilepsy in children. However, the precise mechanisms linking cortical malformations to epileptogenesis remain elusive. The neuronal-specific activator of cyclin-dependent kinase 5, p35, has been recognized as a key factor in proper neuronal migration in the neocortex. Deletion of p35 leads to severe neocortical lamination defects associated with sporadic lethality and seizures. Here we demonstrate that p35-deficient mice also exhibit dysplasia/heterotopia of principal neurons in the hippocampal formation, as well as spontaneous behavioral and electrographic seizures. Morphological analyses using immunocytochemistry, electron microscopy, and intracellular labeling reveal a high degree of abnormality in dentate granule cells, including heterotopic localization of granule cells in the molecular layer and hilus, aberrant dendritic orientation, occurrence of basal dendrites, and abnormal axon origination sites. Dentate granule cells of p35-deficient mice also demonstrate aberrant mossy fiber sprouting. Field potential laminar analysis through the dentate molecular layer reflects the dispersion of granule cells and the structural reorganization of this region. Similar patterns of cortical disorganization have been linked to epileptogenesis in animal models of chronic seizures and in human temporal lobe epilepsy. The p35-deficient mouse may therefore offer an experimental system in which we can dissect out the key morphological features that are causally related to epileptogenesis.

Key words: epilepsy; dentate gyrus; granule cell dispersion; heterotopia; neuronal migration disorder; biocytin; EEG

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about the relationship of disturbed neuronal organization to neuronal activity and seizure generation. Here we report that significant structural abnormalities appear in the hippocampus (particularly in dentate gyrus) of p35-deficient mice, similar to pathology observed in resected hippocampi of patients with temporal lobe epilepsy (tLE) (Houser, 1990, 1999; El Bahh et al., 1999). Furthermore, a high proportion of p35 knock-out mice exhibits spontaneous behavioral and/or electrophographic seizures reminiscent of limbic seizures. These observations provide an initial basis for relating epileptogenesis to dysplastic malformations resulting from errors in brain development.

Preliminary data have been reported previously in abstract form (Wenzel et al., 1998; Robbins et al., 1999).

MATERIALS AND METHODS

Animals

A complete description of the gene deletion protocol and initial characterization of these mice has been published (Chae et al., 1997). Morphological analysis of hippocampal neurons was performed in brains of p35 knock-out mice (p35−/−). The p35−/− colony was maintained via brother–sister matings, and wild-type controls (+/+). Mice derived from the same stock were used for all experiments. Mice were monitored for 40–50 hr over the course of 3–4 weeks. The brains were immediately removed and placed in the same solution consisting of 2–4% PFA and 0.1–2% glutaraldehyde was used for the detection of synaptic vesicular zinc [particularly enriched in mossy fiber (MF) boutons]. After initial fixation with 4% paraformaldehyde as described above, the brains were transferred to a solution containing 3–4% glutaraldehyde, 0.1% Na₂S, and 0.136 mM CaCl₂ in 0.12 M Millonig’s PB, pH 7.3, for 48 hr at 4°C, followed by cryoprotection in 30% sucrose. Frozen sections were cut at 30 µm and then mounted on slides, air-dried, and transferred to a fresh developer solution containing 30 ml gum Arabic (50%), 5 ml 2 mM citrate buffer, 15 ml hydroquinone (5.76%), and 250 µl silver nitrate (0.73%) for 1 hr in the dark. These sections were then cryoprotected with cryosolution, dehydrated, cleared in toluene, and coverslipped.

Quantitative analysis of granule cells within the dentate gyrus. The number of granule cells in the granule cell layer, as well as the number of heterotrophic granule cells within the molecular layer, were estimated per entire dentate gyrus using the optical fractionator method (West et al., 1991). In Nissl-stained preparations, a granule cell was defined as a neuronal cell body and an oval or round nucleus. The Nissl-stained cytoplasm is found at the apical and basal portions of the cell body (Seress and Pokorny, 1981; Wenzel et al., 1981; Seress, 1992). The granule cell and molecular layers also contain the cell bodies of glial cells, basket cells, and other interneurons (for review, see Freund and Buszaki, 1996). The nuclei of glial cells were easily identified and excluded from the analysis. The nuclei of basket cells are similar in appearance to those of granule cells, but the cell body of basket cells (and also other interneurons) in these layers is larger and more intensely stained for Nissl substance. On the basis of these criteria, nongranule cells could be identified and excluded from the counting.

Cell counting was performed by an investigator who was blind to the genotype of the animals. Total section thickness was used for dissector height, and only “caps” located within counting frames were counted (Buckmaster and Dudek, 1997): caps were defined as the nuclei of granule cells that came into focus while focusing down through the dissector height. mounts were cut at 10× 10 µm were distributed systematically and randomly over the dentate granule cell layer, according to the method described by West et al. (1991). Using a camera lucida-like microscope/computer interface (Nikon Optiphot-2 Microscope with a Hamamatsu C4000 Camera; Nikon, Tokyo, Japan) and NIH Image 1.62 b4 morphometry software package, the numerical density of granule cells, the volume of the granule cell and molecular layers, and the total number of granule cells for a given hippocampus were estimated. Adequacy of sampling was assessed by determining the intra-animal coefficient of variation (CV) as well as the inter-animal coefficient of variation (CV) for each measure (West and Gundersen, 1990).

Immunocytochemistry. Different sets of sections were processed for immunocytochemistry (ICC), using a modification of the avidin–biotin complex (ABC)-peroxidase technique (Hsu et al., 1981). Immunocytochemical procedures were performed as described previously (Wenzel et al., 1997). Briefly, sections were rinsed in PB, followed by 0.1 M Tris-HCL buffer (TB), pH 7.4; endogenous peroxidases were then inactivated with treatment of 0.5–1% H₂O₂ in TB for 2 hr. Sections were then incubated with 3% bovine serum albumin (BSA) (Boehringer Mannheim, Indianapolis, IN), 3% goat or horse serum (Sigma, St. Louis, MO), and 0.25% Triton X-100 (TX) in 0.05 M TB, 0.15 M NaCl, pH 7.4 (TBS) for 1 hr to reduce nonspecific staining. Sets of alternating sections were rinsed in TBS for 30 min and incubated for 24 hr at 4°C in the various antisera and dilutions in TBS containing 1% goat or horse serum, 2% BSA, and 0.1% TX-anti-neuron-specific nuclear protein (Neun) (Chemicon, Temecula, CA), 1:20; anti-glial fibrillary acidic protein (GFAP) (Dako Corporation, Carpinteria, CA) 1:4000; anti-glutamate decarboxylase 67 (GAD67) (Chemicon, Temecula, CA) 1:100; anti-calretinin (Chemicon), 1:4000; anti-parvalbumin (Chemicon), 1:4000; anti-somatostatin (SOM) (Penin-
uda Laboratories, Belmont, CA), 1:5000, and anti-zinc transporter ZnT3 (provided by Dr. R. D. Palmiter, University of Washington, Seattle, WA), 1:250. After rinses for 2 hr in TBS, sections were incubated in biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), diluted 1:500 for 24 hr at 4°C, rinsed for 2 hr in TBS, and then incubated in ABC (Elite ABC Kit, Vector Laboratories), diluted 1:500 in 1% goat or horse serum, 2% BSA, 0.25% TX, and TBS for 24 hr at 4°C. Sections were rinsed thoroughly in TB, pH 7.6, and then incubated for 15 min in 0.025% 3,3’-diaminobenzidine (DAB; Sigma) in TB. After reaction for 5–10 min in fresh DAB with 0.003% H2O2, sections were rinsed in TB, followed by PB. Specificity of the immunostaining was evaluated by omitting primary antibodies from the regular staining sequence. ZnT3 immunoreactivity in synaptic vesicles of mossy fiber boutons was also localized at the ultrastructural level using a protocol described by Wenzel et al. (1997).

Electrophysiology and intracellular labeling with biocytin

Intracellular recording and labeling. Hippocampal slices were prepared conventionally as described previously (Wenzel et al., 2000) for in vitro experiments. Mice were anesthetized with halothane and decapitated, and the brain was removed quickly, cooled briefly in ice-cold oxygenated [95% O2/5% CO2] artificial CSF (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH2PO4, 2 MgSO4, 26 NaHCO3, 2 CaCl2, 10 dextrose, and blocked to contain the hippocampus. Using a vibrisc Wilson, 400-μm-thick slices transverse to the longitudinal axis of the hippocampus were cut into a bath of oxygenated ACSF at 4°C. Sections were then transferred to a holding chamber and allowed to equilibrate for at least 1 hr while submerged in ACSF at room temperature. Slices were then individually transferred to a standard interface recording chamber. In the chamber, slices rested on a nylon mesh over a well that was perfused (1 ml/min) with warmed (33–35°C), oxygenated ACSF. In addition, warmed, humidified air was circulated above the slice. Slices remained undisturbed for at least 15 min in the chamber before recording began.

Intracellular electrodes made from borosilicate glass were pulled using a horizontal puller (Sutter Instruments, San Rafael, CA) and filled with warmed (33–35°C), oxygenated ACSF. In addition, warmed, humidified air was circulated above the slice. Slices remained undisturbed for at least 15 min in the chamber before recording began.

Retegrode labeling with biocytin. Visualization of multiple dentate granule cells was achieved through retrograde labeling via iontophoresis of 4% biocytin (in 0.05 M Tris HCl, pH 7.3) into extracellular space of the hippocampal stratum (s.) lucidum in CA3b subfield (Okazaki et al., 2000). The electrode was introduced through the molecular layer and perpendicular to the granule cell layer (superior blade of the dentate gyrus).

Tissue processing and morphological analysis of biocytin-labeled granule cells (light and electron microscopy). After the intracellular recording procedure and iontophoretic injection of biocytin, slices were removed from the recording chamber and immersion-fixed in a solution of 4% paraformaldehyde and 0.1–1% glutaraldehyde in 0.1 m sodium PB, pH 7.4, for 2–4 hr at 4°C. The slices were rinsed in 0.1 m PB, then infiltrated for cryoprotection with 10% sucrose in 0.1 m PB for 1 hr, followed by 30% sucrose for 8–12 hr. Frozen sections were cut (60 μm) and further processed with a histochemical procedure. Sections of a total of 99 slices with biocytin-filled granule cells were subsequently processed for light and electron microscopy, as follows.

Sections were rinsed in 0.1 m PB, pH 7.4, and then in 0.1 m TB, pH 7.4. Endogenous peroxidases were suppressed with 0.5–1% H2O2 in 0.1 m TB for 2 hr. Sections were pretreated with 2% BSA, 0.25–0.4% dimethylsulfoxide (DMSO) (Sigma), and 0.05 m TBS, pH 7.4, for 1 hr to reduce nonspecific background staining and to permeabilize membranes. Sections were rinsed in 0.1 m TBS for 30 min and then incubated in ABC (Elite ABC Kit, Vector), diluted 1:500 in 2% BSA, 0.25–0.4% DMSO, and 0.05 m TBS for 36–48 hr at 4°C. Sections were then rinsed throughly in 0.1 m TBS followed by 0.1 m TB, pH 7.6, and preincubated in 0.025% DAB with 0.005% NiNH4SO4 added to increase the density of stain for 15 min. Subsequently, the sections were reacted with fresh DAB/ NiNH4SO4 solution containing 0.01% H2O2 for 15–60 min. The reaction was stopped by rinses in 0.1 m TB. Sections were further processed for electron microscopy (EM) using a method that included post-fixation in 1% osmium tetroxide in 0.15 m PB, pH 7.4, for 1 hr at room temperature, alcohol dehydration, and flat-embedding in Eponate 12 Resin (Ted Pella, Redding, CA) between two alar sheets. The biocytin-filled granule cells (and their dendrites and axon arborizations) were visualized at the light microscopical level and photographed before remounting and further sectioning for EM. Camera lucida drawings were made of each biocytin-filled granule cell, and its dendrites and axon arborizations were reconstructed by superimposing all sections of the hippocampal slice. Serial thin sections from various slices containing different portions of the biocytin-filled granule cells (e.g., mossy fiber axon collaterals in the dentate inner molecular layer) were stained with uranyl acetate and Reynold’s lead citrate and examined on a Philips 410 electron microscope.

RESULTS

The gene-targeting strategy and generation of p35−/− mice have been reported previously (Chae et al., 1997). The initial histological study demonstrated a general disorganization of the forebrain in these mice, with a major defect in the development of the normal lamination pattern of the neocortex (Kwon and Tsai, 1998). In the present study, histological examination of the neocortex of p35−/− mice reconfirmed this observation of severe defects in the lamination of neocortex, associated with formation of aberrant fiber fascicles. Our observations also revealed structural abnormalities within the hippocampus, particularly associated with the appearance of dispersed granule cells and abnormal mossy fiber localization. We have focused on these hippocampal abnormalities and their functional correlations, with respect to the occurrence of spontaneous seizures.

P35−/− mutation is associated with low seizure threshold and with occurrence of spontaneous electrophoretic and behavioral seizures

Seizure susceptibility was compared in p35−/− and wild-type mice through flurothyl seizure testing. In all animals, exposure to flurothyl elicited seizure activity. Statistical analysis of the latencies to the flurothyl-induced seizures revealed that the p35−/− mice had significantly shorter latencies than wild-type to both the first (352.2 ± 1.6 vs 423.7 ± 12.0 sec, respectively) and second seizures (590.6 ± 14.3 vs 688.4 ± 13.8 sec) (Fig. 1A). All statistical data are presented as means ± SEM. These results represent a 16% reduction in mean latency to the first seizure and a 14% reduction to the second seizure in p35−/− mice.

Video/EEG recording confirmed the occurrence of spontaneous seizures in many p35−/− mice. A typical example of EEG recorded from a p35−/− mouse during a tonic–clonic seizure is shown in Figure 1B. Spontaneous epileptiform EEG activity was verified in 75% of the p35−/− mice (8 of 12 animals). Twenty-five percent of p35−/− animals exhibited spontaneous tonic–clonic seizures with behavioral manifestations, whereas an additional 50% displayed intermittent interictal electrographic activity. The spontaneous seizures had durations ranging from 59 to 84 sec and were followed by a prolonged isoelectric postictal period lasting 3–5 min. Ten percent of the p35−/− mice died.
during seizure. No epileptiform activity was observed either behaviorally or electrographically in wild-type mice.

Most of the recorded spontaneous seizures were strong generalized tonic–clonic episodes that appeared to occur simultaneously in cortex and hippocampus. Seizures frequently began from a state of sleep or quiet rest, with a sudden decrease in amplitude of the EEG, sometimes preceded by a spike corresponding to the animal awakening; the mouse then lifted and turned its head back and forth. As the seizure evolved, both electrographic and behavioral activity reflected the tonic phase of a seizure; spiking gradually increased in amplitude and frequency, and the animal's head turned rigidly upward and back concurrent with bilateral forelimb extension. This tonic phase was frequently prolonged and usually progressed into a clonic EEG pattern. The animal's behavior often progressed into vigorous bouncing, produced by rapid bilateral hindlimb thrusting. The seizure ended with the onset of a protracted isoelectric post-ictal period; behavioral inactivity was seen when the cortical EEG flattened. Occasionally the hippocampus would continue to spike for several seconds longer than neocortex.

**Histological abnormalities are characteristic features of the hippocampus in p35−/− mice**

Histological examination of the neocortex revealed striking differences between wild-type and p35−/− mice. Figure 2, A1 and A2, shows the typical lamination of the wild-type neocortex with six layers distinguished by neuronal morphology, cell density, and general cytoarchitecture. This normally laminated cytoarchitecture is not apparent in p35−/− mice (Fig. 2B1,B2). With the exception of lamina I containing a few Cajal-Retzius cells, laminae II–VI represent an inversion of the normal neuronal patterning; i.e., large pyramidal neurons normally localized in lamina V are now present underneath lamina I, and small pyramidal cells are distributed in the deep cortical zone [further details are described by Chae et al. (1997)]. In addition, aberrant fiber fascicles course through the neocortex, leading to further interruptions in the cytoarchitecture.

The most striking morphological finding in the hippocampal formation of adult p35−/− mice was a severe disarrangement of its neuronal architecture (Figs. 2, 3). The principal cell
layers (CA1–3 pyramidal and dentate granule cell layers) are disorganized and show frequent interruptions and aberrantly localized cell somata (Figs. 2B2, 3B1,B2,C1,C2). In particular, pyramidal cells of the medial CA1 subfield and subiculum tend to be dispersed into s. oriens, where they form a distinct cell layer (Fig. 2B2). In the CA3 subfield, pyramidal neurons are found outside the pyramidal cell layer, in both s. oriens and s. lucidum/radiatum (Figs. 2B2, 3C1,D,E). To determine the neuronal nature of these heterotopic cells, the neuron-specific NeuN antibody was used. In hippocampal sections immunoreacted against NeuN, a large proportion of the heterotopic cell population was neuronal.

Most p35−/− mice also show a severe disorganization of the granule cell layer, particularly affecting the superior blade. Granule cells are dispersed and heterotopically localized into the molecular layer and hilus, blurring the borders of the granule cell layer with adjacent layers or forming separate cell clusters deep in the hilus (Fig. 3B2,C2, 4A). Although no statistical comparison was performed, there appeared to be a strikingly higher degree of granule cell dispersion into the molecular layer (and particularly into the hilus) in the ventral than in the dorsal hippocampus (Fig. 3, compare B1–2, C1–2). NeuN ICC confirmed the neuronal nature of these cells, and their shape suggested that most of these neurons are displaced dentate granule cells (Fig. 4B).

To further characterize the dentate region of p35−/− mice, Timm staining was used to label zinc in synaptic vesicles of MF boutons. In the wild-type (Fig. 4C), Timm-stained MF axons normally project from the granule cell layer throughout the hilus toward the CA3 pyramidal cell layer, forming a compact projection in s. lucidum; in addition, MF axon bundles are also localized infrapyramidally and in the pyramidal cell layer. Timm staining in p35−/− mice shows a similar pattern of MF projection in hilus and CA3 subfield (Fig. 4D). However, in contrast to the wild-type, numerous Timm-stained MF boutons/axons are also present in the granule cell layer and particularly within the inner molecular layer (Fig. 4D, arrows). Although there was significant animal-to-animal variability in the degree of granule cell dispersion and MF distribution in granule cell and molecular layers, these abnormal patterns were typical of p35−/− mice and not seen in wild-type animals.

**Analysis of granule cell dispersion**

**Quantitative cell counts**

To assess the extent of granule cell dispersion in the dentate gyrus of p35−/− mice, we analyzed the following histological parameters from 2-month-old p35−/− mice and age-matched wild-type mice: (1) numbers of granule cells and (2) volumes of granule cell and molecular layers; from these measures, we calculated the numerical density of granule cells. Each measure was estimated separately for the granule cell and molecular layers. Because in p35−/− mice the border between granule cell layer (GCL) and inner molecular layer is irregular, the distinction between granule cell and molecular layers was artificially (but consistently) defined in each section. The data (Table 1, Fig. 5A,C) revealed small but nonsignificant differences between p35−/− and wild-type mice for the total number of granule cells per dentate (Fig. 5C) and the number of granule cells in the granule cell layer (Fig. 5A). However, p35−/− mice do show...
significantly more granule cells in the molecular layer than wild-type mice (Fig. 5B). Approximately 29.8% of all granule cells in p35−/− mice are localized within the molecular layer (compared with 14.4% in the wild-type). Statistical comparison of the mean volume of the dentate layers revealed a difference between p35−/− mice and wild-type: molecular layer and the total value of the granule cell plus molecular layers of p35−/− animals are significantly smaller (\( p, 0.016 \) and \( p, 0.03 \), respectively), but the difference in the granule cell layer is not significant (\( p, 0.44 \)).

However, the numerical density of granule cells in the molecular layer, the dispersed granule cells, is significantly higher in p35−/− mice compared with the wild-type. The intra-animal CE for the number of cells counted and points counted (see Materials and Methods), as well as the between-subjects CV and CE/\( CV^2 \) ratio, were in the range that indicated adequate sampling (West et al., 1991).

**Field potential analysis**

A laminar profile of synaptic responses, recorded throughout the granule cell and molecular layers of the superior blade of the dentate gyrus, revealed clear differences between p35−/− and wild-type mice (Fig. 6). The amplitude of the evoked fEPSPs was measured at a fixed latency (Fig. 6A) and plotted as a function of location along a model granule soma–dendrite axis (Fig. 6C). Consistent with the morphological findings of a dispersed granule cell layer in p35−/− animals, the fEPSP positivity of p35−/− dentate evoked by perforant path stimulation was much broader in slices from p35−/− mice (Fig. 6B). This positivity is thought to reflect a “somal” current source [see Sutula et al. (1998) for similar analysis of “sprouted” dentate], suggesting that the heterotopic granule cells are activated by incoming perforant path fibers.

**Immunocytochemistry of astrocytes and interneurons reveals cell-specific differences between p35−/− and wild-type mice**

ICC techniques were used to examine the expression of several markers specific for astrocytes and inhibitory interneurons (Fig. 4E–H). Using an antibody against GFAP (Fig. 4E), we found no obvious differences in distribution and density of GFAP-positive
cells within the hippocampus proper containing heterotopic neurons. However, in the dentate gyrus, a characteristic population of astrocytes that is normally localized along the hilar–granule cell border was absent in p35\(^{-/-}\) mice; furthermore, occasional aberrant astrocyte somata were seen between the granule cells.

The distribution of hippocampal inhibitory interneurons in p35\(^{-/-}\) hippocampus was examined using an antibody against GAD67 (synthesizing enzyme of the inhibitory neurotransmitter GABA) to stain all GABAergic neurons. In addition, we used a set of antibodies—calretinin, parvalbumin, and somatostatin—that were previously associated with various GABAergic subpopulations of interneurons. The pattern of GAD67-positive neuron distribution in p35\(^{-/-}\) and wild-type mice was similar to that seen in other ICC and in situ hybridization studies on rodents, including mice (data not shown) (for review, see Houser and Esclapez, 1994; Freund and Buszaki, 1996; Fukuda et al., 1997). In both p35\(^{-/-}\) and wild-type mice, GAD immunoreactive neurons were found in the hippocampal pyramidal cell layer, s. oriens and s. radiatum/moleculare. In the dentate, GAD-positive cells were seen in the dentate hilus, in the granule cell layer, and scattered in the molecular layer. GAD67 reveals consistent “punctate” (presumed axon terminals) immunostaining surrounding somata of principal cells, including the dispersed granule cells in the dentate molecular layer (data not shown). This expanded region of GABA-containing terminals surrounding granule cells constitutes the major difference in the pattern of GAD67 immunoreactivity distribution between p35\(^{-/-}\) and wild-type animals.
Immunostaining for SOM revealed a large number of neurons in all subfields of the hippocampus and dentate gyrus, with laminar distribution consistent with previous studies (data not shown) (for review, see Buckmaster et al., 1994; Freund and Buszaki, 1996). In agreement with these previous studies on mouse, we found only light labeling of SOM-positive axons in the outer two-thirds of the dentate molecular layer (compared with a mouse, we found only light labeling of SOM-positive axons in the dentate molecular layer of p35 mice (Fig. 4G,H)). In addition, in the hilus of the ventral dentate gyrus of wild-type and p35−/− animals, calretinin immunoreactivity labeled the somata and processes of mossy cells (data not shown) as reported previously by Liu et al. (1996). Axon terminals of these mossy cells form a dense supragranular-IR band in the inner molecular layer in both dorsal (Fig. 4F) and ventral dentate gyrus. In p35−/− mice, this supragranular calretinin-positive band appeared more prominent than in wild-type mice and overlapped with the dispersed granule cells in this layer.

In the hippocampus and dentate gyrus, parvalbumin ICC shows parvalbumin positivity in interneuron somata, dendrites, and axon terminals. Parvalbumin-containing cell bodies are mainly localized in the pyramidal cell layer and s. oriens of the hippocampus, in the granule cell layer and hilus of the dentate gyrus of wild-type mice (Fig. 4G), and only occasionally in other layers (e.g., s. lucidum of the CA3 region). The dendrites of parvalbumin-positive neurons arise parallel to the granule cell dendrites and exhibit prominent varicosities spanning all layers of the molecular layer. In both p35−/− and wild-type mice, the axons and terminals immunoreactive for parvalbumin form a dense axon plexus within the cell layers, surrounding pyramidal and granule cells, including the dispersed granule cells within the dentate molecular layer of p35−/− mice. Although no systematic cell counting was performed, the number of parvalbumin-containing neurons appears to be reduced in p35−/− mice as compared with the wild-type; furthermore, the intensity of the parvalbumin staining of the axon plexus and terminals in the dentate granule cell layer was weaker in p35−/− mice (Fig. 4G,H).

**Electron microscopy of mossy fiber connections**

Electron microscopic examination of the granule cells in p35−/− mice localized in granule cell and molecular layers showed ultrastructural characteristics similar to the wild-type and those described previously for dentate granule cells (Laatsch and Cowan, 1966; Ribak and Anderson, 1980; Wenzel et al., 1981; Seress, 1992). Despite the variable granule cell localization (Fig. 7A) and dendritic orientation in p35−/− mice (see next section), dendrites from both genotypes are covered with spines with a broad range of shape, size, and length. Dendrites and dendritic spines form simple and multiple asymmetric synaptic contacts with axon terminals. In p35−/− dentate, MFs form a widely distributed axon plexus around granule cell somata and proximal dendrites, in both the granule cell and molecular layers; these axons are associated with numerous terminals (i.e., MF boutons) that vary considerably in size and shape (Fig. 5B–E). The identity of terminals as MF boutons is confirmed by their ultrastructural features (packed with clear round synaptic vesicles, occasional dense-core vesicles, a few coated vesicles, and numerous mitochondria) and EM localization of ZnT3 IR of synaptic vesicle membranes in these terminals (identical to those seen in hilus and CA3 s. lucidum). The MF boutons, identified by ZnT3 immunoreactivity in both granule cell (Figs. 7D, E) and molecular layers (Figs. 7B, C), form asymmetric synapses predominantly with multiple simple dendritic spines (Fig. 7B); some MF boutons also form synapses with complex spines and with dendrites of presumed granule cells (Figs. 7B, E). Occasionally, MF boutons were found making synapses with the soma of heterotopic granule cells and aspiny dendrites of interneurons (data not shown). The EM observation on ZnT3-positive MF boutons abnormally localized in the molecular layer (Fig. 7C) is consistent with the Timm staining pattern of p35−/− dentate and was not observed in wild-type mice. However, there is some Timm labeling (seen at the light microscopic level) within the granule cell layer of wild-type mice, suggesting that even in normal dentate there may be some MF synapses in this region.

**Table I. Total number and numerical density of granule cells (GCs), and volume of granule cell layer (GCL) and molecular layer (ML), in the dentate gyrus of 2-month-old wild-type and p35−/− mice**

<table>
<thead>
<tr>
<th>Morphological parameters</th>
<th>Wild-type mouse (n = 4)</th>
<th>p35−/− mouse (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule cell number (N × 10^6) in:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule cell layer</td>
<td>1.98 ± 0.16</td>
<td>1.77 ± 0.12</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>0.33 ± 0.06</td>
<td>0.75 ± 0.06* (p &lt;0.001)</td>
</tr>
<tr>
<td>Total number of GCs</td>
<td>2.32 ± 0.12</td>
<td>2.52 ± 0.14</td>
</tr>
<tr>
<td>Volume (Vol) of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule cell layer (μm^3 × 10^6)</td>
<td>5.71 ± 0.39</td>
<td>5.34 ± 0.38</td>
</tr>
<tr>
<td>Molecular layer (μm^3 × 10^6)</td>
<td>1.57 ± 0.03</td>
<td>1.39 ± 0.06* (p &lt;0.016)</td>
</tr>
<tr>
<td>Total volume (μm^3 × 10^6)</td>
<td>2.14 ± 0.02</td>
<td>1.93 ± 0.09</td>
</tr>
<tr>
<td>Numerical density (Nv) of GCs in:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule cell layer (×10^5/μm^3)</td>
<td>3.48 ± 0.23</td>
<td>3.31 ± 0.08</td>
</tr>
<tr>
<td>Molecular layer (×10^4/μm^3)</td>
<td>0.21 ± 0.04</td>
<td>0.54 ± 0.04* (p &lt;0.0001)</td>
</tr>
<tr>
<td>Total GC density (×10^5/μm^3)</td>
<td>3.69 ± 0.22</td>
<td>3.85 ± 0.11</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM values. *Significant difference between wild-type and p35−/− mice.
intracellular labeling with biocytin reveals abnormal patterns of granule cell localization, axonal and dendritic morphology, and mossy fiber reorganization

Light microscopic examination of individual granule cells obtained after in vitro injection of biocytin into the s. lucidum of hippocampal slices, or intracellular labeling with biocytin, revealed a high degree of granule cell abnormality in p35对着 dentate with respect to the localization of granule cell somata and in the orientation of their dendritic fields and axon arbors (Figs. 8, 9). Granule cell somata of wild-type mice have oval or round-shaped cell bodies, typically giving rise to a spiny apical dendritic tree that arborizes within the molecular layer; the mossy fiber axon arises from the hilar side of the soma and runs toward the CA3 subfield. As shown with Golgi and recent intracellular labeling studies (Spigelman et al., 1998; Ribak et al., 2000), our examination of granule cells of wild-type mice revealed only exceptional abnormalities. In contrast, as shown in Figure 8, granule cells of p35对着 mice are often displaced, with cell bodies located either in the inner molecular zone (close to the GCL) or in clusters within the hilus. Normal-appearing granule cells in p35对着 dentate show the expected monopolar-oriented dendritic branching pattern within the molecular layer (Fig. 8A1,B, cell 1). However, many granule cells exhibit a bipolar dendritic arborization (Fig. 8, cells 2, 3, 5; Fig. 9B1–2, C1–2). The spiny dendrites of some p35对着 突触 cells arose from the hilar side of the cell body and then turned 180° to form “recurrent” basal dendrites ascending through the molecular layer (Fig. 8, cell 2; Fig. 9C); some dendrites originating from the hilar pole of the cell body also extend into the subgranular hilar region (Fig. 8B, cell 5; Fig. 9D1–2). Granule cells heterotopically localized within the hilus gave rise to one or two primary dendrites that ran through the hilus toward the granule cell layer to branch within both granule cell and molecular layers (Fig. 8, cell 4; Fig. 9B).

Abnormally localized granule cells, as well as morphologically abnormal granule cells localized within the granule cell layer, usually displayed an axon that arose from the cell body and ran toward the CA3 s. lucidum, forming typical small “en passant” boutons and large MF boutons with filopodial extensions (Fig. 9B2,C2). These observations suggest that both heterotopic and normally positioned granule cells contribute to the normal MF projection in p35对着 mice. However, many of these displaced cells also showed an unusually extensive MF arborization within the hilus (Fig. 9B2). The axons of some granule cells arose from anomalous locations, e.g., the lateral side of the cell body, the apical dendrite, or the initial portion of a recurrent basal dendrite. Figures 8 (A1 and B, cells 2 and 3) and 9 (C2) indicate some examples of abnormal axon origin. Finally, granule cells of p35对着 mice, either localized within the granule cell layer or displaced into the molecular layer, show recurrent MF sprouting into the granule cell layer and/or inner molecular layer, indicative of MF reorganization. Figure 9C1–2 shows an example of a “bipolar” granule cell forming not only recurrent basal dendrites but also an aberrant MF axon collateral that forms a plexus within the granule cell layer.

**DISCUSSION**

These results expand the previous description by Tsai and her colleagues (Chae et al., 1997; Kwon and Tsai, 1998) showing that normal development of hippocampus and dentate gyrus involves p35-mediated processes. We have, in addition, found that (1) hippocampal pyramidal neurons of the CA1 and CA3 subfields and granule cells of the dentate gyrus are heterotopically localized, (2) a significant proportion of p35对着 mice exhibit spontaneous behavioral and electrographic seizure, and (3) heterotopic granule cells exhibit features (e.g., basal dendrites, abnormal
axon origin, and aberrant mossy fiber collaterals and synapses) often observed in epileptic tissue.

The findings of Kwon and Tsai (1998) in p35 knock-out mice, together with similar cortical migration defects in cdk5-deficient mice (Ohshima et al., 1996; Gilmore et al., 1998) suggest a critical signal transmission function of p35/cdk5 kinase for the migration of cortical neurons (or glia) to their proper destinations. In contrast, Reelin, a glycoprotein secreted by the Cajal-Retzius
cells in the marginal zone, is thought to act as a “stop signal” for migrating cortical plate neurons at the boundary of the marginal zone (Sheppard and Pearlman, 1997; Kwon and Tsai, 1998), thus explaining the abnormal presence of neurons in this zone in the reeler mutant (Frotscher, 1998). Developmental processes in the hippocampus are similar to those in the neocortex, except for the long-lasting neurogenesis of granule cells in the dentate gyrus and their “outside-in” gradient of positioning (Cowan et al., 1980; Soriano et al., 1994). The abnormal positioning of dentate granule cells within the hilus and molecular layer and of hippocampal pyramidal neurons in adjacent layers (and absence of a specific astrocyte population) indicates a defect in the migration of later generated cells. It is yet not clear, however, whether the heterotopic granule cell positioning in the hilus results from a migration defect or whether these granule cells might be generated at a later time point, from progenitor cells within the hilus (Altman and Bayer, 1990; Parent et al., 1997), and do not migrate into the granule cell layer (Schlessinger et al., 1975; Soriano et al., 1994).

The occasional appearance of heterotopic granule cells (displaced granule cells) in the dentate hilus and molecular layer has been observed in wild-type mice and rats (Ramon y Cajal, 1968; Amaral, 1978; Seress and Pokorny, 1981; Martí-Subirana et al.,

Figure 8. Photomicrographs (A) and camera lucida drawings (B) of biocytin-filled granule cells from a p35−/− mouse. Granule cells and mossy fibers were visualized after terminal uptake and retrograde transport of biocytin, after in vitro injection of biocytin into the s. lucidum of a hippocampal slice. A1. Biocytin-labeled granule cell bodies (1-5) are present in the granule cell layer (GCL), inner molecular layer (IML), and hilus (H). A2. Photomicrograph of a biocytin-labeled granule cell (5) with basal dendrites (arrows) in an adjacent section of the same slice. A3. Higher magnification of the same granule cell showing a spiny dendrite (arrows) and the mossy fiber axon (arrowhead). B. Drawings of the biocytin-labeled granule cells (GCs) shown in A, demonstrating abnormal morphology with respect to location and orientation of the cell body, and dendrites and axon arborization: neuron 1, normal-appearing GC; neuron 2, inverted GC with recurrent basal dendrites and an axon (MF, arrowhead) originating from the basal dendrite; neuron 3, GC with dendrites emerging from the apical and hilar pole of the soma, and axon (MF, arrowhead) arising from a dendrite: neurons 4, heterotopic GCs within the hilus (H), with dendrites extending into the molecular layer; neuron 5, GC with basal dendrites branching and extending into the hilus. Dashed line indicates border between “normal” GCL and IML.
Granule cell dispersion in the dentate gyrus, similar to that seen in human epileptic hippocampi (Houser 1990; Lurton et al., 1997; El Bahh et al., 1999), has also been observed in animal models of epilepsy, e.g., after kainic acid injection into the dorsal hippocampus (Cavalheiro et al., 1982; Suzuki et al., 1995; Bouilleret et al., 1999), and in the pilocarpine model (Mello et al., 1992). Two mechanisms have been proposed to explain the genesis of these heterotopic granule cells: (1) a disorder of the migration, occurring early in development (before any seizure event) and perhaps contributing to epileptogenesis (Scharfman et al., 2000), and (2) a consequence of repetitive seizures (as seen after kainic acid or pilocarpine administration), perhaps reflecting enhanced neurogenesis (Mello et al., 1992; Bouilleret et al., 1999). It remains unclear whether the appearance of heterotopic granule cells is related to granule cell loss in the epileptic hippocampus (Babb et al., 1984; Mello et al., 1992; Mathern et al.,
Granule cell dispersion has not been observed when cell loss is minimal (Lurton et al., 1997), but both appear to be directly related to mossy fiber sprouting into the molecular layer (Cavazos and Sutula, 1990; Babb et al., 1991; Houser, 1999). The present study, using Timm staining and intracellular labeling with biocytin to visualize mossy fiber boutons, demonstrates that heterotopic granule cells generate (and receive) aberrant mossy fibers, in both granule cell and molecular layers. In addition, heterotopic granule cells show basal dendrites (entering the hilus) and axons originating from the apical pole of the soma or the apical dendrite. These findings represent abnormal phenomena associated with epilepsy both in human hippocampus (Franck et al., 1995) and in experimental animal models (Spigelman et al., 1998; Ribak et al., 2000). The behavioral observations of the present study substantiate the enhanced susceptibility of p35−/− mice to seizures. Simultaneous video/EEG recordings confirmed spontaneous epileptiform activity, including both intermittent interictal discharge and spontaneous generalized seizures. Although no fatalities were observed during recorded seizures, the severity of observed seizures, together with occasional sudden (unexplained) death of some animals, suggests that the mortality rate of ~10% [see also Chae et al. (1997)] might be attributable to fatal seizures. Previous experiments comparing PTZ-induced seizures in p35−/− and wild-type mice showed that generalized convulsions were only fatal in p35−/− mice (Chae et al., 1997).

Early in postnatal development (before postnatal day 10), before any spontaneous seizure, p35−/− mice exhibit heterotopic granule cells, indicating that this abnormality is not a result of seizure activity and suggesting a causal relationship between structural abnormality and occurrence of spontaneous seizures. Other studies investigating animal models of cortical dysplasia with experimentally induced disruption of cortical development also suggest a causal link between cortical malformations and seizure activity (Chevassus-au-Louis et al., 1999; Walsh; Fleck et al., 2000; Chen et al., 2000). Such animal models include migrational malformations induced by freezing (Dvorak and Feit, 1977; Dvorak et al., 1978; Rosen et al., 1992, 1996; Jacobs et al., 1996, Hablitz and DeFazio, 1998), x-irradiation (Roper et al., 1995, 1997; Hicks et al., 1959), and chemical treatment (e.g., methyloxazymethanol: Singh, 1977; Cattaneo et al., 1995; De Feo et al., 1995; Baraban and Schwartzkroin, 1996; Germaino and Sperber, 1997; Chevassus-au-Louis et al., 1998, 1999; Baraban et al., 2000). Animals with abnormalities paralleling human genetic neuronal migration disorders also show enhanced seizure-sensitivity: e.g., type I lissencephaly (Lis1 mutant) (Reiner et al., 1993; Hirotsume et al., 1998; Fleck et al., 2000), and band heterotopia (tish mutant) (Lee et al., 1997). Although many of these animal models exhibit increased excitability (Baraban and Schwartzkroin, 1995; Jacobs et al., 1996, 1999; Luhmann and Raabe, 1996; Roper et al., 1997; Baraban et al., 2000) (for review, see Chevassus-au-Louis et al., 1999), the presence of a cortical malformation does not necessarily result in a spontaneous seizure phenotype. One might speculate that as in humans, a subtle preexisting focal cortical dysgenesis (e.g., heterotopically displaced neurons) provides a substrate for seizure induction (e.g., from a febrile episode) and may also predispose the animal to a seizure-related pathology (e.g., hippocampal sclerosis in TLE) (Lewis, 1999).

The underlying basis for hyperexcitability (and synchrony) associated with cortical disorganization and reorganization remains a topic of intense investigation. Numerous studies have shown that alterations of GABAergic neurotransmission might be critically involved in underlying epileptic disorders (Schwarzkroin, 1998). Although the present study did not reveal any obvious loss of specific interneuron populations in p35−/− mice, both GAD67- and parvalbumin-containing axon terminals were abnormally distributed within the molecular layer, surrounding somata of heterotopic granule cells, reflecting correct target innervation (Fleck et al., 2000). However, the reduced parvalbumin immunoreactivity within the terminal plexus around the granule cells suggests the possibility of functional abnormality in these inhibitory circuits, either as a direct result of the p35−/− disorganization or as a result of earlier seizures. Loss of parvalbumin immunoreactivity has also been observed after kainic acid-induced seizures (Sloviter 1991, 1994; Best et al., 1993; Buckmaster and Dudek, 1997), although transient parvalbumin loss may not be related to cell death (Scotti et al., 1997). Subtle changes of parvalbumin immunoreactivity in the dentate gyrus of p35−/− mice could be of relevance for seizure sensitivity of these animals (Scotti and Nitsch, 1991). In addition, in both wild-type and p35−/− animals, the calcium binding protein calretinin is expressed in exictatory hilar mossy cells (Liu et al., 1996; Blasco-Ibanez and Freund, 1997; Schurmans et al., 1997), which form a dense axonal plexus within the dentate inner molecular layer (Buckmaster et al., 1996). In p35−/− mice, heterotopic granule cells within the inner molecular layer are surrounded by these calretinin-positive terminals; the abnormal position of excitatory mossy cell synapses onto granule cell bodies (data not shown) could undoubtedly alter the balance of excitation and inhibition in this sensitive circuit.

Physiological studies in epileptic human tissue (Masukawa et al., 1991, 1992; Williamson, 1994; Franck et al., 1995) and experimental animal models (Scharfman et al., 1990; Ribak et al., 1992; Sloviter, 1994; Wuarin and Dudek, 1996; Patrylo and Dudek, 1998; Okazaki et al., 1999) have shown that the dentate gyrus can play an important role in epileptic conditions (Masukawa et al., 1999). Dentate granule cells are ideally situated to control the spread of excitation. Developmental malformations of the dentate, whether genetically based or caused by early events such as trauma, fever or infection, may lead to various complex changes (e.g., cell loss, mossy fiber sprouting, receptor alterations, granule cell dispersion) that result in an imbalance between excitation and inhibition. The p35−/− mutant mouse represents one example of an experimental model in which anatomical disorganization is closely linked to seizures. Further studies are necessary to clarify how the p35 mutation and related structural abnormalities lead to epileptiform activities.

REFERENCES


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