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Neuroanatomical Phenotypes In The *Reeler* Mouse

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Abstract

The *reeler* mouse (*Reln*) has been proposed as a neurodevelopmental model for certain neurological and psychiatric conditions and has been studied by qualitative histochemistry and electron microscopy. Using magnetic resonance microscopy (MRM), we have quantitated for the first time the neuromorphology of *Reln* mice at a resolution of 21.5 μ m. The neuroanatomical phenotypes of heterozygous and homozygous mutant *Reln* mice were compared to those of wild type (WT) littermates using morphometry and texture analysis. The cortical, hippocampal, and cerebellar phenotypes of the heterozygous and homozygous mutant *Reln* mice were confirmed, and new features were revealed. The *Reln*^{rl/rl} mice possessed a smaller brain, and both *Reln*^{rl/+} and *Reln*^{rl/rl} mice had increased ventricles compared to WT controls. Shape differences were found between WT and *Reln*^{rl/rl} brains, specifically in cerebellum, olfactory bulbs, dorsomedial frontal and parietal cortex, certain regions of temporal and occipital lobes, as well as in the lateral ventricles and ventral hippocampus. These findings suggest that certain brain regions may be more severely impacted by the *Reln* mutation than others. Gadolinium-based active-staining demonstrated that layers of the hippocampus were disorganized in *Reln*^{rl/rl} mice and differences in thickness of these layers were identified between WT and *Reln*^{rl/rl} mice. The intensity distributions characteristic to the dorsal, middle, and ventral hippocampus were altered in the *Reln*^{rl/rl}, especially in the ventral hippocampus. These differences were quantified using skewness and modeling the intensity distributions with a Gaussian mixture. Our results suggest that structural features of *Reln*^{rl/rl} brain most closely phenocopy those of patients with Norman-Roberts lissencephaly.

Keywords

Magnetic Resonance Microscopy; reelin; brain; hippocampus; ventricles; cerebellum

Introduction

Diagnosis of neurological and psychiatric conditions is often made according to phenomenological or behavioral criteria (American Psychiatric Association, 2000; Rowland, 2005). Postmortem studies have identified structural features in the central nervous system (CNS) that are characteristic for many neurological syndromes and the approach is somewhat

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successful for certain psychiatric conditions, such as schizophrenia (Roberts et al., 2005; Rowland, 2005). Unfortunately, this analysis is applicable only to deceased individuals and it cannot be used in the initial diagnosis. For this reason, investigators are combining clinical assessment with magnetic resonance imaging (MRI) and/or functional MRI to render a more concrete diagnosis of the disorder (McCarley, 2002). Although this approach has the potential to advance identification and improve the accuracy of diagnoses, in most cases it has not revealed basic mechanisms that underlie the condition so that therapies can be developed to more efficiently ameliorate the severity or expression of the syndrome. Despite this limitation, some researchers are beginning to identify genes and gene products that may underlie structural and/or behavioral features that accompany certain neurological and psychiatric conditions (Katsel et al., 2005).

Alterations in cytoarchitecture of brain may be attributed to sequelae that occur during development and/or maturation of the CNS (Rowland, 2005). Although neurodevelopmental events are often attributed to epilepsy and deficits in learning and memory, they may also underlie autism, schizophrenia, and certain neurological conditions that include hydrocephaly (Rowland, 2005). While investigators are identifying candidate or collections of genes that may predispose a given patient to present a certain syndrome, other scientists are using this information to study pre-existing mutants or to develop genetically-modified animals to model neurodevelopmental morphological features of the condition.

Reln is a mutation that arose spontaneously in mice that displayed ataxia, hypotonia, and fine tremor (Falconer, 1951). Subsequently, reelin was found to be an extracellular matrix glycoprotein that is secreted by certain neurons (D'Arcangelo et al., 1995; Ogawa et al., 1995), such as Cajal-Retzius cells in the cortical marginal zones and granule cells in cerebellum (Tissir and Goffinet, 2003). Reelin appears to act as a stop signal for migrating neurons where it seems to control the positioning and orientation of neurons in their respective target layers. Hence it participates in laminar development and development of architectonic structures within the CNS (Landrieu and Goffinet, 1981; Goffinet et al., 1984; Del Rio et al., 1997; Förster et al., 2006a,b). As a result, *Reln* mice display abnormalities in neural migration that lead to distortions in the dendritic trees and axonal projections, especially in cerebral cortex, hippocampus, and cerebellum. Since these neurodevelopmental alterations bear similarities to certain aspects of those in schizophrenia, autism, and lissencephaly, *Reln*^{rl/+} and *Reln*^{rl/rl} mice have been proposed to represent preclinical structural models for these conditions (Fatemi, 2001).

To date, *Reln* brain has been examined by various neuroanatomical procedures (Caviness and Sidman, 1973; Mikoshiba et al., 1980). These traditional methods are prone to distortion, can cause tissue damage, are typically qualitative, and do not preserve the three-dimensional (3D) spatial relationships between anatomical structures. We have analyzed *Reln* brain using magnetic resonance microscopy (MRM). This procedure obviates many concerns of traditional methods and the sensitivity of the technique is sufficient to detect changes within 0.03% in mouse brain volume (Cyr et al., 2005). In the present study, we have used MRM with a resolution as high as 21.5 μ m to assess morphometric alterations in *Reln* brain.

Materials and Methods

Animal preparation and imaging protocol

The *Reln* mutation originally arose spontaneously in mice (Falconer, 1951). Male and female adult WT, heterozygous, and homozygous mutant *Reln* mice (Jackson Laboratory, Bar Harbor, Maine, USA) were used for this study. The animals were approximately 5 months of age at the time of investigation. Mice were housed in groups of 3–5 animals/cage in a temperature- and humidity-controlled room with a 14:10 light-dark cycle (lights on at 0700 h). Food and water

were provided *ad libitum*. All experiments were conducted in accordance with NIH guidelines for the care and use of animals and with approved animal protocols from the Duke University Institutional Animal Care and Use Committee.

In preparation for morphology studies, mice were anesthetized with 100 mg/kg pentobarbital (i.p.) and transcardially perfused with a solution of 10% formalin and 10% ProHance (Bracco Diagnostics, Princeton, NJ) as described by Johnson and colleagues (Johnson et al., 2002). Heads were stored overnight in formalin and scanned the next day. The brains were placed in fomblin-filled tubes and were scanned in the skull to avoid distortions of brain or damage to tissue due to handling.

All imaging was performed on a 9.4 T Oxford vertical bore magnet with a GE EXCITE Console (EPIC 11.0) that has been specially adapted for MRM. Images were acquired with a radio-frequency refocused 3D spin warp sequence with TE/TR of 5.1/50 ms, a field of view of 22x11x11 mm, and zero filling was used, resulting in a 1024x512x512 matrix and an isotropic resolution of 21.5 μ m. The sequence used nonuniform radial gain and asymmetric data collection resulting in a scan time of 2 h and 7 min per brain (Johnson, in preparation).

Manual segmentation

The brains were manually segmented for ventricles, hippocampus, and cerebellum using Voxstation (MRPath, Durham, NC). Only one plane of orientation was used for segmenting a specific structure; however, a 3D viewer was used to assess the accuracy of segmentation in all three cardinal planes (MacKenzie-Graham et al., 2004). The ventricles were segmented as one system comprising the lateral, third, and fourth ventricles in sagittal slices. Manual segmentation of the ventricles was facilitated by the high contrast that cerebrospinal fluid has in the MRM images, but it was made difficult, especially in WT and heterozygous mice by their thin structure. In several places, an arbitrary cut was required to separate the ventricles from clefts in the brain filled with fluid.

The hippocampus was segmented in coronal slices starting anteriorly at the level of the hippocampal commissure using the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2001) as a guide. The posterior limit was established with the help of the sagittal and horizontal planes, at the level of the ventral nucleus of the lateral lemniscus. In general, the in-plane borders were established using high contrasting white matter tracts like the corpus callosum (external capsule) or alveus for the lateral side, the brachium of the superior colliculus, optic tract, or cerebral peduncles on the medial side. The ventricles provided high contrast for determining the lateral borders of the hippocampus. Aside from hippocampus, the cerebellum was segmented in two mid-sagittal slices in all brains to show the dramatic change in size of the *Reln*^{rl/rl} brain compared to that in the WT and *Reln*^{rl/+} mice.

Layer thickness measurements

Cortical layer measurements were performed in coronal slices at approximately -2.1 mm from bregma and in the region of the primary somatosensory cortex (Hof et al., 2000). Additionally, hippocampal length, from its upper medial to the most distal lateral aspect, and the thickness of hippocampal layers were measured in the same slice.

Image preprocessing

Brain volumes were obtained after stripping the skull using an algorithm based on mathematical morphology and connected component analysis similar to the one described by Badea and colleagues (2003). To increase the time efficiency of the procedure, the image data were down-sampled by a factor of two along each dimension at this step. The algorithm started with smoothing the data, and applying a number of erosions. A seed was selected automatically in

the center of the volume, and region growing was started from that seed. Pixels with values between fixed thresholds and connected to the initial region were added to the brain mask. At the end, dilation was applied for the same number of times as we applied erosion. Empirically, we established that the optimal number of erosions/dilations to be used was 6 for our data, which come as a 512x256x256 matrix.

Image intensity normalization was performed in the following way. The lowest 5% and the highest 2% of the intensity values were discarded and assigned as the minimum (I_{Min}) and maximum (I_{Max}) image values. The original values (I) were mapped to I' after scaling to the same global range of image values ($I_{GlobalMax}$ and $I_{GlobalMin}$):

$$I' = (I - I_{Min}) * \frac{I_{GlobalMax} - I_{GlobalMin}}{I_{Max} - I_{Min}} + I_{Min}$$

The segmented structures were registered with an affine transform using Image Registration Toolkit (Rueckert et al., 2003) to remove global sources of variability, which may arise from changes in the position of a structure within the brain, its size, or its orientation. After thresholding and smoothing the mask volumes, BRAINSUITE (Shattuck and Leahy, 2002) and MATLAB (The MathWorks, Natick, MA) were used to generate surface meshes from the iso-surfaces that separate the binary segmentations from the background and to reduce their dimension to 3000 faces per structure. The meshes were then analyzed using software written in MATLAB.

Morphometry

The total volume of whole brain, ventricles, hippocampus and in particular the dentate gyrus, as well as the mid-sagittal area of the cerebellum were measured. Brain size was not normalized since this parameter was dramatically affected by cerebellar atrophy in the homozygous mutant mice. Absolute volumetric measurements were made for segmented structures using voxel counting and multiplying the number of voxels by the voxel volume (9.94 l). Areas were calculated by computing the sum of areas for elementary patches that make up the surface meshes of the segmented structures. The meshes were used for shape analysis in the following way. Correspondence between meshes was established based on the minimum distance between vertices of two meshes. The mean shapes for each of the structures were generated at each node of the mesh using the linear average of the closest vertices for each of the brains. Distance maps were calculated to give estimates of local shape changes. The root mean square distance between each of the original meshes and their mean was used to pseudocolor the mean mesh and estimate the shape variability within groups. Global parameters like Hausdorff distance (HD), the mean distance between shapes, and the fractal dimension (FD) were calculated for each of the three genotypes of mice. The same morphometric parameters were used for the between group analysis.

The HD measures the degree of mismatch between two shapes, represented by the vertices of the triangulated meshes. The direct HD (h) identifies the point from the first shape ($S1$) that is farthest away from any point in the second shape ($S2$) and measures the distance between the point in $S1$ and its closest neighbor in $S2$. HD was calculated as the maximum of minimum distances (ri to rj) between one shape and another, computed forward and backward.

$$HD_{(S1,S2)} = \max(h_{(S1,S2)}, h_{(S2,S1)}), \text{ where } h_{(S1,S2)} = \max_{ri \in S1} \min_{rj \in S2} \| ri - rj \|$$

This distance metric is, however, sensitive to “outliers.” Hence, the mean distance between shapes was computed as well.

The FD is a measure of the shape complexity of an object. It reflects the rate at which the area increases as the scale of measurement is reduced. The FD was calculated using the box counting method (Barabási and Stanley, 1995). In this method the object is covered with boxes of various sizes. The FD was estimated from the slope of the log-log plot that relates the number of boxes necessary to cover the volume of interest and their size as follows:

$$FD = - \lim_{l_b \rightarrow 0} \frac{\log_{10} N_b(l_b)}{\log_{10} l_b}$$

where $N_b(l_b)$ was the number of boxes with size l_b needed to completely cover the structure.

Grayscale analysis

A grayscale analysis was performed for the hippocampus. The image intensity of each hippocampus was normalized to the same global range of values, according to the gray level distribution of the individual head scans. The minimum and maximum intensities were obtained after discarding the lowest 5% and the highest 2% values of the distribution characteristic of the head scans. The mean, standard deviation, skewness, and kurtosis for the hippocampus were measured. The intensity distribution for the hippocampus was modeled with a mixture of two Gaussian distributions using expectation maximization. In this model, we assumed that one of the distributions would model the intensities characteristic of the cell layers of the hippocampus which appear dark in the images, whereas the second distribution would model the remainder of the hippocampal body. The distance between the means of the two Gaussian distributions was measured. This analysis was performed on whole hippocampus, and separately for the dorsal, middle, and ventral hippocampus.

Statistics

The data are presented as means and standard deviations with some analyses by MATLAB. Most of the data were analyzed using SPSS (SPSS Inc., Chicago, Ill) where univariate ANOVA and repeated measures ANOVA were used; the latter was used to analyze differences in skewness of the intensity distributions for different regions of the hippocampus. *Post-hoc* tests were performed by Bonferroni corrected pair-wise comparisons. A $p < 0.05$ was considered significant.

Results

As a number of morphological features have been identified to be abnormal in neurological and psychiatric patients (Lawrie and Abukmeil, 1998; Rowland, 2005), MRM was used to study the brains of *Reln* mice. A qualitative examination of the images revealed that the appearance of WT and *Reln*^{rl/+} brains differed dramatically from those of *Reln*^{rl/rl} mutants (Fig. 1, see also Supplementary Figs. 1–3). Gross inspection of the images showed the ventricles to be enlarged, the cerebellum atrophied, and the layered organization of the cortex and hippocampus to be greatly disturbed in brains of the homozygous mutants. By comparison, these structures appeared to be similar between the WT and *Reln*^{rl/+} brains.

To perform a quantitative morphometric analysis, brains were semi-automatically segmented and extraneous tissue was removed. The ventricles, hippocampus, and mid-sagittal slices through the cerebellum were manually segmented for all three genotypes. Slices in the three cardinal planes through representative brains are shown (Fig. 1; see also Supplementary Figs. 1–3). The results of segmentations were used to reconstruct the 3D structures (Fig. 2) and measure the volumes, areas, and shape parameters of the brains as detailed below.

Volume measurements

The absolute volumes of the manually segmented structures were measured for brain, as well as for ventricles and hippocampus (Table 1). As expected, the variability of volumes for the total brain and ventricles increased from WT to *Reln^{rl/+}* to *Reln^{rl/rl}* mice. Compared to WT animals, the total volume of brain and hippocampus in the *Reln^{rl/rl}* animals was reduced by approximately 19% and 17%, respectively; whereas ventricular volume was increased by 305% (Table 1). Alterations were also observed in comparisons between WT and *Reln^{rl/+}* mice where total brain, ventricular, and hippocampal volumes in the latter group were enhanced by approximately 6%, 82%, and 7%, respectively.

A univariate ANOVA for brain volumes revealed significant genotype effects [$F(2,9) = 22.58$, $p = 0.0001$] and Bonferroni tests demonstrated that volumes of the WT and *Reln^{rl/+}* brains were similar, but greater than those from *Reln^{rl/rl}* mice ($p < 0.002$). Similarly, univariate ANOVA detected a significant main effect of genotype for ventricular volumes [$F(2,9) = 5.38$, $p < 0.029$] and Bonferroni comparisons found that ventricular volumes for WT and *Reln^{rl/+}* mice were similar, with the WT ventricular volume significantly smaller than that from the homozygous mutants ($p < 0.030$). It should be noted, however, ventricular volumes in the *Reln^{rl/+}* animals were highly variable and were approximately 1.8-fold larger than those in WT littermates. ANOVA for hippocampus revealed statistically significant differences in the volumes for the three groups [$F(2,9) = 18.12$, $p < 0.001$]. A *post-hoc* analysis showed that the hippocampal volumes were not different between WT and the *Reln^{rl/+}* mice, but were greater than those for *Reln^{rl/rl}* animals ($ps < 0.007$). Together, these findings indicate that volumes of total brain, ventricles, and hippocampus from the homozygous mutants were notably different from those of WT mice, whereas only total brain and hippocampal volumes in the *Reln^{rl/rl}* animals were significantly changed from those in heterozygous animals.

Since there did not seem to be a significant difference between WT and *Reln^{rl/+}* volume measurements for hippocampus, we compared volume measurements for the dentate gyrus. The volume for the dentate gyrus for the *Reln^{rl/rl}* mice ($5.71 \pm 1.16 \text{ mm}^3$) represented about 76% of the WT volume ($7.47 \pm 0.35 \text{ mm}^3$); these data were significant [$t(1,6) = 2.908$, $p < 0.027$].

Area measurements

Large variability in areas was noticeable for brain, hippocampus, and ventricular regions, especially for the homozygous mutant (Table 1). For instance, the standard deviation for ventricular area was $\sim 12 \text{ mm}^2$ for *Reln^{rl/rl}* animals, compared to $\sim 4 \text{ mm}^2$ for the WT controls. Despite variability in brain, ventricular, and hippocampal areas in the *Reln^{rl/rl}* mice, Levin's tests for heterogeneity of variance were not significant in any of these incidences. ANOVA for brain areas was not significant. An ANOVA for ventricular areas found a main effect of genotype [$F(2,9) = 10.23$, $p < 0.005$]. Bonferroni tests showed ventricular areas from both the *Reln^{rl/+}* and *Reln^{rl/rl}* mice were significantly different from those of the WT littermates ($ps < 0.02$). ANOVA for hippocampal areas found a main effect of genotype [$F(2,9) = 8.22$, $p < 0.0093$]. Bonferroni corrections indicated that hippocampal areas for WT and *Reln^{rl/+}* mice were smaller than for *Reln^{rl/rl}* animals ($ps < 0.036$), but were not different from each other. Collectively, the analyses of areas revealed high variability within *Reln^{rl/rl}* mice, where ventricular and hippocampal areas were increased relative to the other genotypes.

Since the cerebellum was distorted in shape in the *Reln^{rl/rl}* mice, mid-sagittal slices from this brain region were compared as areas. The mean areas were $10.3 \pm 0.62 \text{ mm}^2$ for WT, $10.7 \pm 0.52 \text{ mm}^2$ for *Reln^{rl/+}*, and $2.6 \pm 0.19 \text{ mm}^2$ for *Reln^{rl/rl}* animals. Hence, cerebellar areas were similar between WT and heterozygous mice, whereas areas were reduced by $\sim 76\%$ in the homozygous mutants. A univariate ANOVA found a main effect of genotype [$F(2,9) = 358.56$, $p < 0.001$]

and Bonferroni corrections revealed the cerebellar area was significantly reduced in *Reln^{rl/rl}* mice compared to that in the WT and *Reln^{rl/+}* animals ($p < 0.001$). Hence, the mid-sagittal cerebellar area was greatly reduced in the homozygous mutants.

Global shape characteristics

Apart from examining differences in volume and areas, ratios of areas to volumes are characteristics of shapes of structures (Table 1). These ratios were found to differ for ventricles for *Reln^{rl/rl}* in contrast to WT and *Reln^{rl/+}* animals, and for hippocampus for WT and *Reln^{rl/+}* compared to *Reln^{rl/rl}* mice. For brain, the differences in the area/volume ratio approached significance [$F(2,9) = 4.07$, $p < 0.055$] and genotype differences could not be discerned with Bonferroni corrections. In contrast, ratios were significant for the ventricles [$F(2,9) = 6.89$, $p < 0.015$] and Bonferroni tests showed that the area/volume ratios for ventricles for *Reln^{rl/rl}* animals were smaller than for WT mice ($p < 0.017$), whereas the ratios for the *Reln^{rl/+}* and *Reln^{rl/rl}* animals approached significance ($p < 0.1$).

Aside from the ventricles, significant differences were also detected for the hippocampal area to volume ratio [$F(2,9) = 29.55$, $p < 0.001$]; *post-hoc* analyses revealed that the ratio was significantly smaller for the homozygous mutants compared to that of the other two genotypes ($p < 0.001$), that did not differ from each other. These ratio results show that the shapes of the ventricles and hippocampus are clearly different between WT and *Reln^{rl/rl}* mice.

Besides examining the area to volume ratio, the fractal dimension (FD) can also be used to quantitatively characterize the global shape and its complexity for segmented structures in brain (Table 2). Analyses of FD for the total brain and ventricles showed these indices to be significantly different [$F(2,9) = 13.23$, $p < 0.002$ for brain; $F(2,9) = 21.39$, $p < 0.001$ for ventricles]. *Post-hoc* analyses indicated differences between WT and *Reln^{rl/rl}* and between *Reln^{rl/+}* and *Reln^{rl/rl}* mice ($p < 0.005$ for brain; $p < 0.02$ for ventricles). Parenthetically, differences in FD for ventricles for WT and *Reln^{rl/+}* mice were also significant ($p < 0.050$). Genotype differences in FD were not detected in hippocampus. Thus, for brain and ventricles the FDs were larger for WT than homozygous mutants, and the ventricular FDs were greater for the WT than the heterozygous animals.

Another way to examine shape characteristics is to analyze boundaries of the segmented structures. The surfaces extracted from the segmentations were used to generate mean meshes for each of the three genotypes (Fig. 3). The HD between the mean meshes for the WT, *Reln^{rl/+}*, and *Reln^{rl/rl}* mice, together with the mean distances are summarized in Table 3. The results indicated larger HD distances between WT and the *Reln^{rl/rl}* mice than between the WT and *Reln^{rl/+}* animals for brain (3.17 mm versus 2.10 mm), ventricles (2.71 mm versus 2.58 mm), and hippocampus (0.75 mm versus 0.36 mm). These same relationships can be seen in comparisons of mean distances, which are less sensitive to noise. To better understand these differences, we evaluated the HD within groups and compared that to the values between groups. The HD between the WT and *Reln^{rl/rl}* mice was greater than the distances within groups for whole brain, ventricles, and hippocampus (Table 3). In summary, when the HD was used as a landmark feature for each genotype, distances were greater between the WT and homozygous mutants than between the heterozygous animals and either of the other two genotypes.

Local shape changes

The analysis of global changes suggested possible local shape changes within total brain, ventricles, and, perhaps, hippocampus for *Reln* mice. Local shape changes were observed after thresholding the distance maps used to pseudocolor the shapes of the segmented structures (Fig. 3). When examining the signed distance maps of total brain, inward deformations of the

cerebellum and olfactory bulbs were clearly evident in the *Reln^{rl/rl}* mice compared to the WT controls (Fig. 4). Shape differences were found between WT and *Reln^{rl/rl}* animals in medial frontal and parietal cortex, and temporal and occipital lobes. Parenthetically, the brain stem areas of WT and *Reln^{rl/rl}* mice appear to be highly variable. These latter results may be due both to removal of the brain from the skull for scanning, as well as true variability in brain structures.

After analyzing total brain and selected regions, we evaluated local shape changes in the ventricles and hippocampus. The ventricles were found to vary mostly in the region of the third ventricle, which was very thin, and along the lateral ventricles—especially on the anterior side and midway along the dorsal to ventral axis at the curvature of the ventricles (Fig. 3). Besides the ventricles, the dorsal and ventral aspects of the hippocampus appeared changed (Figs. 3 and 5). The signed distance map showed a contraction of the *Reln^{rl/rl}* shape along the dorsolateral aspect of hippocampus (Fig. 5). It should be emphasized that brain, ventricle, and hippocampal distances of the *Reln^{rl/rl}* animals showed much greater variability and dispersion relative to those for the WT littermate controls.

Grayscale analysis

It is evident that the organization of the laminated structures in the *Reln^{rl/rl}* mouse brain is greatly disturbed (Caviness and Sidman, 1973). For example, the cell layers of the cerebral cortex and hippocampus appear dramatically different in the homozygous mutant relative to those in the WT animals (Fig. 1; see also Supplementary Figs. 1 and 3). The layers appear disorganized, the contrast between them is reduced, and the cortex and hippocampus appear more homogenous than in the WT controls. In order to quantify differences in texture between genotypes, we performed grayscale analysis and restricted the investigation to the hippocampus because it was the most easy to quantify. Binary masks of hippocampi were intersected with the grayscale images and the range of intensities was normalized to the same global values for all brains included in the analysis. Despite normalization and these precautions, the shapes of the histograms were different. Although mean values and kurtosis of the intensity distributions did not clearly distinguish among the genotypes, analysis of skewness of the distributions was sufficient in this regard. A slice-by-slice analysis along the rostro-caudal axis was performed to examine differences in skewness—which reflected the presence of darkly stained gray matter layers in the hippocampus. A repeated measures ANOVA showed that the skewness of the histogram curves was statistically different for the most dorsal, the middle, and ventral regions of the hippocampus (Fig. 6; see also Supplementary Fig. 4). For rostral hippocampus, the repeated measures ANOVA for multiple levels of grayscale was significant [$F(39,351) = 2.514$, $p < 0.001$], as was the levels by genotype interaction [$F(78,351) = 1.674$, $p < 0.001$]. Bonferroni corrected pair-wise comparisons of the interaction revealed the WT controls had higher levels of skewness than the *Reln^{rl/+}* mice ($ps < 0.052$) and it approached significance for the *Reln^{rl/rl}* animals ($ps < 0.10$). With regards to middle hippocampus, grayscale levels approached significance [$F(69,621) = 1.286$, $p < 0.067$], whereas the levels by genotype interaction was significant [$F(138,621) = 2.048$, $p < 0.001$]. Bonferroni tests of the interaction showed the WT and *Reln^{rl/rl}* mice were different ($ps < 0.045$). Analyses of ventral hippocampus demonstrated levels [$F(69,621) = 6.093$, $p < 0.001$] and the levels by genotype interaction to be significant [$F(138,621) = 2.653$, $p < 0.001$]. *Post-hoc* comparisons of the interaction showed skewness to be significantly higher in the WT than *Reln^{rl/rl}* mice ($ps < 0.042$), with no differences discerned between WT and *Reln^{rl/+}* animals.

Aside from slice-by-slice analyses, the hippocampus was divided into the dorsal, middle, and ventral parts and analyzed by modeling the intensity distributions with two Gaussian distributions, where one Gaussian distribution (Gauss1) represented the stained cell layers and the other distribution (Gauss2) was the unstained areas (cells and fibers). Since the WT and

Reln^{rl/+} mice were not statistically different on the slice-by-slice analyses, only WT and *Reln*^{rl/rl} animals were compared. Gauss1 was significant in the dorsal [$t(1,6) = 2.777, p < 0.032$], middle [$t(1,6) = 2.499, p < 0.047$], and ventral hippocampus [$t(1,6) = 3.003, p < 0.024$]. Similarly, Gauss2 was also significant in these same areas of the hippocampus [dorsal: $t(1,6) = 2.650, p < 0.038$; middle: $t(1,6) = 2.738, p < 0.034$; ventral: $t(1,6) = 2.861, p < 0.029$]. Collectively, these results indicate that the organization of the cell layers in the dorsal, middle, and ventral hippocampus are abnormal in the homozygous mutant *Reln* mouse.

A quantitative analysis of the thickness of layered structures of the hippocampus was performed in a coronal plane approximately -2.10 mm from bregma (Supplementary Fig. 5; see atlas of Hof et al., 2000 as guide). These linear measurements are summarized in Table 4. Overall cortical thickness was diminished [$t(1,6) = 6.66, p < 0.002$] and hippocampal length was reduced [$t(1,6) = 3.89, p < 0.008$] in *Reln*^{rl/rl} mice compared to the WT controls. Layers of the cortex in the homozygous mutants were diffuse and could not be clearly distinguished as layers I to VI in WT animals. In CA1 hippocampus, the pyramidal cell layer was bifurcated and it extended over a larger portion of this region in *Reln*^{rl/rl} than in WT mice [$t(1,6) = 12.6, p < 0.001$]. By comparison, the thickness of the stratum radiatum [$t(1,6) = 4.35, p < 0.005$] was decreased in *Reln*^{rl/rl} hippocampus. Similar to the CA1 pyramidal cell layer, the thickness of this layer in CA3 hippocampus was also greater compared to WT littermates [$t(1,6) = 3.73, p < 0.010$]. In the mutants, the dentate gyrus appeared diffuse and the granular and polymorphic layers were difficult to discriminate. Nevertheless, the upper blade of the molecular cell layer was decreased [$t(1,6) = 4.38, p < 0.005$], while the upper blade of the granule cell layer was thicker in the homozygous mutants than that in the WT controls [$t(1,6) = 6.34, p < 0.001$]. Collectively, these findings show that many features of the cerebral cortex and hippocampus are altered in the *Reln*^{rl/rl} brain.

Discussion

The *Reln* mouse was first described as having an abnormal neuroanatomical phenotype over 50 years ago (Falconer, 1951). Since that time, abnormalities have been observed in the cerebral cortex, hippocampus, and cerebellum (Caviness, 1976; Stanfield and Cohen, 1979; Mikoshiba et al., 1980). In cortex and hippocampus, lamination of cells is perturbed (Ogawa et al., 1995; Gebhardt et al., 2002) and the cerebellum often lacks foliation and appears atrophied due to reductions in Purkinje and granule cells (Mikoshiba et al., 1983; Heckroth et al., 1989; Soriano et al., 1997). Aside from these regions, neurons in the olfactory bulb, cochlear nuclei, inferior olive, and various mesencephalic nuclei are aberrant (Wyss et al., 1980; Martin, 1981; Blatt and Eisenmann, 1985; Yip et al., 2000). Since reelin appears to serve as a stop signal for the migration of cells from the floorplate to the cortical plate (Landrieu and Goffinet, 1981; Goffinet et al., 1984; Del Rio et al., 1997; Hack et al., 2002; Förster et al., 2006a,b), the *Reln* mutant mice have served as a useful neurodevelopmental model to study laminar development and development of architectonic structures within the mammalian CNS. In our own studies we observed changes within many brain regions and concentrated our experiments on whole brain, ventricles, and hippocampus to provide the most robust quantitation of differences among the *Reln* mice. A unique aspect of our studies was to use high resolution (21.5 μ m) MRM to analyze brain structures in *Reln* mice and to provide quantitative analyses in non-distorted brain. Briefly, we compared total brain, ventricles, and hippocampus according to volume, area, and global and local shapes. Grayscale analyses were also applied to hippocampus and thickness measurements were applied to cerebral cortex and hippocampus. Due to dramatic deformation of the cerebellum in *Reln*^{rl/rl} mice, area measurements were applied to mid-sagittal sections in these mutants. Although the cortical, hippocampal, and cerebellar phenotypes of the *Reln*^{rl/+} and *Reln*^{rl/rl} mice were confirmed, some novel features were observed. Brains from both mutants had increased ventricular sizes compared to that of WT controls. *Reln*^{rl/rl} mice also possessed a smaller brain. Shape differences were found

between WT and *Reln^{rl/rl}* brains, specifically in cerebellum, olfactory bulbs, dorsomedial frontal and parietal cortex, certain regions of temporal and occipital lobes, as well as in the lateral ventricles and ventral hippocampus. Additionally, the thicknesses of the cerebral cortex and hippocampus were also altered. These findings suggest the *Reln* mutation exerts heterogeneous effects on brain — perhaps through interactions with other signal transduction systems (Beffert et al., 2004) — where some locations are more affected than others.

Various investigators have emphasized qualitative differences in hippocampus, cerebral cortex, and olfactory bulbs of the *Reln^{rl/rl}* mice (Caviness, 1976; Stanfield and Cowan, 1979; Wyss et al., 1980). Although we saw similar changes, dramatic differences in volumes of the cerebellum and ventricles were observed. Our morphometric analysis also showed *Reln^{rl/rl}* whole brain, hippocampus and its dentate gyrus to be smaller in volume than that in WT controls and *Reln^{rl/+}* animals. Global shape analysis indicated significant differences between WT and *Reln^{rl/rl}* mice, such that the ventricles were enlarged, but hippocampus was reduced in the mutants. Local shape analysis demonstrated significant atrophy of the olfactory bulbs and cerebellum in *Reln^{rl/rl}* animals. In addition, regions of the dorsolateral cortex appeared to be significantly changed. When shape analysis was limited to hippocampus, alterations were localized to its ventral aspect. Although the third and fourth ventricles appeared abnormal, the shape of the lateral ventricles was clearly aberrant in *Reln^{rl/rl}* mice.

Besides regional changes, there is considerable literature supporting disorganization of the laminar structures in the cerebral cortex, cerebellum, and hippocampus of *Reln^{rl/rl}* mice (Mikoshiha et al., 1983; Ogawa et al., 1995; Gebhardt et al., 2002). Although we observed alterations in the cortex and cerebellum, changes within hippocampus were most amenable to quantification. Using active staining, disorganization of cellular layers of the dentate gyrus and hippocampus proper were easy to visualize. By using grayscale analysis, we were able to discern differences between WT and *Reln^{rl/rl}* animals in the dorsal, middle, and ventral hippocampus. The former and latter regions were particularly deformed. In dorsal hippocampus, the pyramidal cell layer in CA1 and CA3 hippocampus were thicker in *Reln^{rl/rl}* than in WT mice. In contrast, in CA1 the thickness of the stratum radiatum was thinner. Although in mutants the dentate gyrus was diffuse and the granular and polymorphic layers were smaller and difficult to discriminate, the upper blade of the molecular cell was thinner, while the upper blade of the granule cell layer was thicker in the *Reln^{rl/rl}* mice than that in the WT controls. In summary, morphometric, grayscale and thickness analyses demonstrated that the hippocampus of the homozygous mutants was very different from the WT mice; many of these same indices were indistinguishable for WT and heterozygous animals.

Structural aberrations in *Reln* mouse brain have been compared to abnormalities in brains of patients with schizophrenia (Ballmaier et al., 2002). These changes typically include increased ventricular volume, especially in the lateral ventricles (Lawrie and Abukmeil, 1998); a smaller cerebellar vermis (Okugawa et al., 2003); and reduced volumes of hippocampus and amygdala (Vita et al., 2006). Changes in shape in the hippocampal tail (Styner et al., 2004) and increased volumes of basal ganglia have been reported (Hokama et al., 1995). At a more detailed level, schizophrenic brains show significant cellular disorganization compared to normal controls (Conrad et al., 1991). These changes are observed in the hippocampus in relation to abnormal pyramidal cell density and orientation (Conrad et al., 1991); smaller neuron size in the subiculum, CA1 hippocampus, and layer II of entorhinal cortex (Arnold et al., 1995); reduced dendritic spine density in CA1 hippocampus and cerebral cortex; and decreased Purkinje cells in cerebellar vermis (Tran et al., 1998). Aside from these changes, analysis of the *RELN* gene in postmortem brain reveals its promoter to be hypermethylated in some schizophrenic patients (Grayson et al., 2005). This result may underlie the reduced expression of *RELN* in layer I neurons in schizophrenic cerebral cortex compared to that of healthy controls (Eastwood and Harrison, 2006). Expression in deeper layers of cortex is unchanged (Eastwood and Harrison,

2003). Due to reduction, but not complete absence of reelin protein, *Reln*^{rl/+} mice have been proposed to represent a structural model of schizophrenia (Costa et al., 2002). In this regard, structural changes have been reported for this mutant in cerebral cortex, hippocampus, and mesolimbic pathways (Liu et al., 2001; Ballmaier et al., 2002). We have observed increased ventricular volume and area in this mouse. Although behavioral studies show prepulse inhibition to be abnormal in *Reln*^{rl/+} mice (Costa et al., 2002), other investigators have failed to replicate this result and find no evidence of a schizophrenia-like phenotype using a large battery of behavioral tests (Podhorna and Didriksen, 2004). Thus, if the *Reln* mouse is considered a structural model of schizophrenia, it must be used with caution and with knowledge of the many limitations of the model.

Besides schizophrenia, the *Reln* mouse has also been proposed as a model for autism (Fatemi, 2001). Structural imaging studies show the ventricles to be enlarged and the hippocampus smaller in autistic patients than in normal controls (Brambilla et al., 2003). Some investigators have also reported the overall brain is larger and the vermis of cerebellum is smaller in autistic subjects; however, these findings are controversial (Palmen and van Engeland, 2004). Hence, relationships of the *Reln* mouse to autism are highly speculative. With regard to genetics, there is little evidence that autistic patients harbor a mutation in *RELN* (Bonora et al., 2003). However, many individuals with Norman-Roberts lissencephaly have mutations at two different locations in *RELN* and some of these patients may present with autistic-spectrum disorders (Hong et al., 2000). Interestingly, the *Reln*^{rl/rl} mouse phenocopies many of the structural attributes of lissencephaly because these patients have enlarged ventricles, small hippocampus, hypoplastic cerebellum, and abnormal cerebral and hippocampal architecture (Kato and Dobyns, 2003). Taken together, these data suggest that the *Reln*^{rl/rl} mouse most closely represents a structural animal model for lissencephaly.

It should be emphasized that the neuroanatomical and behavioral phenotype of the *Reln* mouse is shared by several other mutants. Reelin protein is known to bind the ApoE2 and VLDL receptors and mice that have disruptions in either gene do not display the same phenotype as *Reln* mutants. However, mutants lacking both genes possess a phenotype that is virtually identical to that of *Reln*^{rl/rl} mice (Trommsdorff et al., 1999). Another mutant that possesses similar structural deformations is the *disabled 1* mutant (Howell et al., 1997). This gene encodes an adapter protein for tyrosine kinases that appear in the reelin signaling pathway (Rice et al., 1998). In addition, *fyn* and *src* also seem to participate in this same pathway because disruption of *Fyn* produces an intermediate phenotype (Yuasa et al., 2004), whereas mice lacking both the *Fyn* and *Src* genes phenocopy the *Reln* mutation (Kuo et al., 2005). Furthermore, compound mutant analysis has identified a distal member of the reelin signaling pathway. *Lis1* appears to be important for microtubule stabilization and neuronal migration (Sasaki et al., 2000; Brandon et al., 2004). When heterozygous *Lis1* mice are crossed with animals that lack other genes in the reelin pathway, the *Reln* phenotype is recapitulated (Assadi et al., 2003). Moreover, *LIS1* is a well-known causative gene for human lissencephaly, a condition which may predispose the patient to autistic-spectrum disorders (Kato and Dobyns, 2003). Interestingly, *Lis1* binds to the disrupted in schizophrenia 1 protein and it has been implicated in schizophrenia (Lipska et al., 2006; Tabares-Seisdedos et al., 2006). Hence, interactions of genes involved in reelin signaling may provide insights into etiologies not only for lissencephaly, but also for various neurodevelopmental disorders that include certain aspects of autism and schizophrenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mouse brain structures visible by MRM with resolution to 21 μ m in *Reln* mice. Horizontal (Left), coronal (Middle), and sagittal (Right) sections through the brains of a WT control (Top), *Reln*^{rl/+} (Middle), and *Reln*^{rl/rl} mouse (Bottom). The stain emphasizes cellular layers of the hippocampus where the pyramidal (Py) and granule cells (GrDG) can be visualized. These cell layers have high contrast and a characteristic shape in WT and *Reln*^{rl/+} mice, but appear disorganized in *Reln*^{rl/rl} animals. The ventricles appear enlarged in the *Reln*^{rl/+} and *Reln*^{rl/rl} mice; the cerebellum is atrophied in the homozygous mutant animals.

Figure 2.

Segmentation of the ventricles and hippocampus in *Reln* mice. The lateral ventricles (cyan) and hippocampus (yellow) are depicted in WT (Left), *Reln*^{rl/+} (Middle), and *Reln*^{rl/rl} (Right) animals in a background of the brain. Enlarged ventricles can be seen for the *Reln*^{rl/+} and *Reln*^{rl/rl} mice; the hippocampus is also smaller for the homozygous mutants. The olfactory bulbs appear disorganized in the *Reln*^{rl/rl} animals and they seem deformed in *Reln*^{rl/+} mice relative to the WT controls. Layers of the cerebral cortex can be visualized in the WT animal, but are not as easily seen in *Reln*^{rl/rl} mice.

Figure 3.

Three-dimensional rendering of the whole brain, ventricles, and hippocampus of *Reln* mice. The mean surfaces are pseudo-colored according to the root mean square of the distance within the WT group (Left). The mean surfaces for the *Reln*^{rl/+} (Middle) and *Reln*^{rl/rl} mice (Right) are pseudo-colored according to the distances between the respective mutant group of mice and the WT animals. Histograms of the distance distributions are depicted for whole brain (Top), ventricles (Middle), and hippocampus (Bottom). Most of the values for whole brain in WT and *Reln*^{rl/rl} mice are concentrated in the lower range of distances, whereas those of the *Reln*^{rl/+} animals are more evenly distributed across the whole range of values. With regards to the ventricles, distance values for the WT controls are restricted to 0.01–0.8 mm, while those for *Reln*^{rl/+} and *Reln*^{rl/rl} mice span a much larger range (0.01–2.65 and 0.01–2.77 mm, respectively). A similar pattern is seen for hippocampus for the three genotypes.

Figure 4.

Shape analysis for the surface of the whole brain for *Reln^{rl/rl}* mouse. Signed distance map between the mean meshes for the WT and *Reln^{rl/rl}* mice indicate inward deformations in the regions of the olfactory bulbs and cerebellum. Temporal cortex (blue) is also deformed. The positive distances are shown in the “warm” color scale, whereas the negative distances are depicted in the “cold” color scale. To the right of each brain are the statistical maps of distances based upon Hotelling tests show that shapes are significantly reduced in the *Reln^{rl/rl}* olfactory bulbs, medial frontal and parietal cortex, temporal and occipital lobes, cerebellum, and brainstem.

Figure 5.

Caudal view of whole hippocampus of WT and *Reln^{rl/rl}* mice. (A) The mean shapes for the WT (green) and *Reln^{rl/rl}* (red) hippocampus. Differences can be discerned between genotypes in the dorsal, middle, and ventral hippocampus where this brain area is more compressed in the mutant mouse. (B) Unsigned distance maps quantify differences in shape between WT and *Reln^{rl/rl}* hippocampus. The greatest distance between groups is present in the ventral or tail region of the hippocampus. (C) Signed distance map shows a compression in shape of dorsolateral hippocampus in *Reln^{rl/rl}* mice.

Figure 6.

Grayscale characterization of hippocampus in *Reln* mice. A slice-by-slice analysis of skewness values is used to evaluate genotype differences along the rostral to caudal axis of the hippocampus. Inspection of the histograms reveals that the skewness values for hippocampus are similar for WT and *Reln*^{rl/+} mice and these values are different from those of the *Reln*^{rl/rl} animals. Although differences are observed in the dorsal, middle, and ventral hippocampus, the most robust changes occur in the dorsal (*Inset--Right*) and ventral (*Inset--Left*) hippocampus.

Table 1
Volume and area measurements for the hippocampus, ventricles and total brain.

Structure ^a	Volume (mm ³)		Area (mm ²)		Area/Volume (mm ⁻¹)	
	WT	ReIn ^{d/+}	WT	ReIn ^{d/+}	WT	ReIn ^{d/+}
Brain	552.3 ±14.2	582.8 ±33.5	407.88 ±21.16	443.45 ±38.06	0.74 ±0.05	0.77 ±0.09
Ventricle	3.8 ±0.7	6.9 ±2.2	40.63 ±3.57	62.41 ±8.49 ^b	10.96 ±2.05	9.50 ±1.91
Hippocampus	29.4 ±2.8	31.5 ±0.8	86.56 ±1.09	87.89 ±2.41	2.97 ±0.31	2.80 ±0.11
						3.88 ^{b,c}

^aThe data are presented as means and standard deviations.

^b $p < 0.05$ from the WT control.

^c $p < 0.05$ from the heterozygous mice.

Table 2

Fractal dimension (FD) for the hippocampus, ventricles and total brain.

Structure ^a	WT	Reln ^{rl/+}	Reln ^{rl/rl}
Brain	2.52 ±0.01	2.52 ±0.01	2.49 ±0.01 ^{bc}
Ventricle	2.08 ±0.07	1.93 ±0.05 ^b	1.83 ±0.05 ^{bc}
Hippocampus	2.34±0.04	2.48±0.24	2.35 ±0.01
Dorsal Hippocampus	2.44 ±0.01	2.56 ±0.20	2.47 ±0.01
Ventral Hippocampus	2.38±0.02	2.49±0.23	2.37±0.03

^aThe data are presented as means and standard deviations.^b $p < 0.05$ from the WT control.^c $p < 0.05$ from the heterozygous mice.

Table 3

Global distance measures between the segmented shapes.

Structure	Between Groups				Within Groups					
	WT vs. ReIn ^{a/d}		WT vs. ReIn ^{a/d}		WT		ReIn ^{a/d}		ReIn ^{a/d}	
Distance ^a	MD	HD	MD	HD	MD	HD	MD	HD	MD	HD
Brain	0.24	2.10	0.34	3.17	0.23 ±0.05	2.71 ±0.82	0.27 ±0.16	1.95 ±0.63	0.22 ±0.07	1.66 ±0.80
Ventricles	0.11	2.58	0.29	2.71	0.15 ±0.06	2.13 ±0.94	0.10 ±0.03	0.87 ±0.43	0.16 ±0.07	1.23 ±0.80
Hippocampus	0.12	0.36	0.16	0.75	0.10 ±0.03	0.45 ±0.19	0.11 ±0.04	0.57 ±0.31	0.13 ±0.03	0.68 ±0.10

^a Distances are given in mm.

^b Abbreviations: MD = mean distance, HD = Hausdorff distance.

Table 4

Cortical and hippocampal thickness measurements.

Structure ^a	WT	Reln ^{rl/rl}	p
Somatosensory cortex	1055.70±29.70 ^b	835.92±58.95	0.002
Layer 1 of cortex/	106.69±15.50	102.61±7.99	0.795
Hippocampus (length)	3061.06±28.75	2558.50±256.20	0.008
Stratum oriens (CA1)	146.42±27.98	136.79±24.97	0.626
Stratum pyramidale (CA1)	69.50±6.90	167.27±13.90	0.000
Stratum radiatum (CA1)	214.52±8.16	117.01±44.14	0.005
Stratum lacunosum moleculare (CA1)	61.22±7.23	68.16±6.99	0.106
Pyramidal cell layer (CA3)	84.61±5.12	132.49±25.19	0.010
Molecular cell layer – upper blade (DG ^d)	243.76±14.06	156.04±37.62	0.005
Granule cell layer – upper blade (DG)	72.46±5.71	101.53±7.19	0.001
Polymorphic cell layer ^e (DG)	190.81±13.41	185.98±109.96	0.933
Granule cell layer – lower blade (DG)	61.22±7.23	68.16±6.99	0.217
Molecular cell layer (DG) (lower blade)	130.61±23.47	99.12±33.40	0.174

^aThe structures were named according to the nomenclature of (Hof et al., 2000).

^bThe thicknesses are given in μ m as means and standard errors of the mean.

^cLayer 1 of WT mouse and polymorphic layer of cerebral cortex of Reln^{rl/rl} mouse (Caviness, 1976).

^dDG = dentate gyrus.

^eCorresponds to hilus area