Analysis of the role of Reelin in mouse brain development: Reelin positive non-gabaergic populations and impact of haploinsufficiency on neuronal morphology

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Boston University
ANALYSIS OF THE ROLE OF REELIN IN MOUSE BRAIN DEVELOPMENT:
REELIN POSITIVE NON-GABAERGIC POPULATIONS AND IMPACT OF
HAPLOINSUFFICIENCE ON NEURONAL MORPHOLOGY

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ANALYSIS OF THE ROLE OF REELIN IN MOUSE BRAIN DEVELOPMENT:
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MIRANDA ANDERSON

ABSTRACT

Reelin, a large extracellular matrix protein responsible for migration and laminar positioning of neurons during brain development, has been implicated in the pathogenesis of schizophrenia and autism. There are extensive populations that have been identified in the adult mouse brain which contain cells secreting Reelin; previously these neurons were believed to be almost exclusively GABAergic. We used immunohistochemistry to reveal multiple groups of Reelin positive neurons that are not GABAergic. Specifically, we used Reelin and GABA antibodies or Vgat cre::Ai9 tdTomato to analyze whether Reelin positive cells are indeed GABAergic. Populations of Reelin positive, non-GABAergic were found in the olfactory bulb and piriform cortex; the perforant pathway; the entorhinal cortex, stratum lacunosum-moleculare of the hippocampus proper, and the dentate gyrus; lastly a small population was found in layer V of the visual cortex. These results suggest Reelin signaling may directly modulate excitatory synaptic circuits in the postnatal brain. In heterozygous reeler multiple morphological abnormalities were identified compared to wild type littermates. Branched analysis revealed a marked decrease in basal dendrite nodes in layer V cells the heterozygous reeler motor cortex and hippocampus as well as a decrease in basal length in the hippocampus. A detailed Sholl analysis indicated abnormalities in both the cortex and hippocampus of the heterozygous
reeler. In the cortex we found decreased basal nodes and number of intersections as well as length at specific compartments of neuronal dendritic structure. More significant differences were found in the hippocampus, which showed a decreased total number of intersections as well as decreased intersections length of CA1 neurons. Changes in both the cortex and the hippocampus of the heterozygous brains were comparable to the homozygous reeler mutant. These findings point to underlying neuronal morphological correlates for the electrophysiological changes found in homozygous reeler mice and the physiological abnormalities exhibited in heterozygous reeler mice.
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<td>Akt</td>
<td>Protein kinase B</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Apoer2</td>
<td>Apolipoprotein E receptor 2</td>
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<tr>
<td>BU</td>
<td>Boston University</td>
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<td>CA</td>
<td>cornu ammonis</td>
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<td>Dab-1</td>
<td>Disabled-1 gene</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
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<td>DISC1</td>
<td>Disrupted in schizophrenia 1</td>
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<td>EGR3</td>
<td>Early growth response 3</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1/2</td>
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<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
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<td>GAD67</td>
<td>Glutamate decarboxylase</td>
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<tr>
<td>Ipsc</td>
<td>Inhibitory postsynaptic current</td>
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<tr>
<td>mEpsc</td>
<td>Mini Excitatory postsynaptic current</td>
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<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<tr>
<td>OCT</td>
<td>Optimal cutting temperature compound</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>pp</td>
<td>Perforant Pathway</td>
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PPI ................................................................. Prepulse inhibition

Reln ........................................................... Reelin

SEM ............................................................ standard error of the mean

slm ............................................................ Stratum lacunosum-moleculare

Vgat ........................................................... Vesicular GABA transporter

VGLUT2 ........................................................ Vesicular glutamate transporter

Vldlr .......................................................... Very-low-density-lipoprotein receptor
INTRODUCTION

Reelin is a large extracellular matrix protein produced by the Cajal-Retzius cells during early embryonic brain development. Reelin serves as a guidance cue for migrating neurons generated at the ventricular zone, and is required for proper formation of layered structure of the cortex, hippocampus and cerebellum (D’arcangelo et al. 1995). This process requires reelin binding to its lipoprotein receptors apolipoprotein E receptor 2 (Apoer2) or very low-density lipoprotein receptor (Vldlr) ( Förster et al. 2014, Trommsdorff et al. 1998). The signaling pathway involves receptor mediated tyrosine phosphorylation of Dab-1, an intracellular adapter protein expressed in Reelin target cells, through an NPxY motif (D’Arcangelo et al. 1999). Specifically, Src phosphorylation of Dab-1 by Reelin leads to its degradation and down regulation thus stopping the migratory effect of Dab1 and influencing cortical layer formation (Feng et al. 2007).

Spontaneous mutation of the mouse reln gene leads to the 'reeler' phenotype, which display malformations in the cerebral cortex as well as impaired motor coordination and tremors (D’Arcangelo et al. 1995). Inactivation of a single reln allele, as happens with heterozygote reeler mice (HRM), does not produce overt gross anatomical or behavioral abnormalities. Interestingly, Reelin haploinsufficiency has been shown to share some biochemical and behavioral similarities to patients suffering from schizophrenia; a neurodevelopmental and psychiatric condition with prominent genetic contribution to its etiology. Studies examining brains of schizophrenic patients shown that Reelin is decreased by 50% in all brain areas and GAD67, the enzyme that catalyzes
the decarboxylation of glutamate to GABA, was reduced up to 70% (Impagnatiello et al. 1998). These biochemical features are shared by HRM. In addition, HRM exhibits pronounced behavioral abnormality seen with schizophrenic patients, such as impaired pre-pulse inhibition (Tueting et al. 1999, Qiu et al. 2006). On the other hand, overexpression of Reelin can prevent occurrence of the schizophrenia-like behavior in mice models (Rogers et al. 2012). Therefore, HRM has been proposed to be a suitable mouse model to study schizophrenia (Teixeira et al., 2011). However, it is currently not clear how reduced Reelin gene dosage affects neuronal development, the establishment of cortical circuits and their function.

The reelin pathway has also been shown to influence dendrite maturation. It has been shown that both cortical principal neurons interneurons of homozygous reeler mice have abnormal positioning and morphology (Yabut et al. 2005, Niu et al. 2004).

However, there is no strong evidence showing altered morphology in heterozygous reeler mice even given the phenotypic changes reported such as age-dependent decrease in prepulse inhibition of startle (Tueting et al. 1999). Deficiencies in Dab1, an adaptor protein in the Reelin-signaling pathway, has been shown to cause defects in synaptic plasticity specifically Akt and ERK1/2 signaling (Trotter et al. 2013). By conducting electrophysiological studies of homozygous and heterozygous reeler in addition to morphological neuron reconstructions we aim to determine if there any anatomical or functional changes present in heterozygous reeler mice.

In addition to its developmental role, reelin is expressed by the postnatal brain in specific neuronal populations. The role of reelin in postnatal brain function is not clear,
but may be related to glutamatergic synapse development and plasticity (Qiu et al. 2006, Iafrati et al. 2013). Previous studies have suggested that Reelin is almost exclusively expressed in GABAergic neurons in the postnatal brain (Pesold et al. 1998, Alacantara et al. 1998). However, there is also conflicting evidence about whether some Reelin positive cells are glutamatergic instead. Recent studies show that there are subsets of neurons expressing reelin that are glutamatergic (Varga et al. 2010). Previous studies have shown non-GABA labeled Reelin positive cells in layer V of the cortex (Moreno et al. 2006), additionally Reelin positive cells double labeled with VGLUT2 were identified in mice up to age E15 in the preplate and marginal zone of developing embryonic mice (Ina et al. 2007). There have been no conclusive studies defining the GABAergic/glutamatergic nature of all populations Reelin positive neurons. We hope to address this additional issue by performing a detailed whole brain analysis of Reelin distribution and its colocalization with GABAergic and glutamatergic markers.
METHODS

1. Animals

Heterozygous reeler mice (HRM) were purchased from Jackson Laboratory (B6C3Fe strain, stock number 000235), and maintained by breeding with wild type (C57Bl6) mice. Homozygous reeler mice were produced by crossing HRM mice. Animals were kept in a temperature-controlled room with unlimited access to chow and water. Mouse genotypes were determined using PCR analysis on tail snips, using a previously described approach (Qiu et al. 2007).

2. Immunohistochemistry

Reelin/GABA Double Immunohistochemistry

Mice were perfused on post-natal day 21 using PBS followed by 4% PFA. Brains were immediately post-fixed in PFA overnight then transferred to 30% sucrose for 48 hours. The brains were frozen in OCT and sectioned into 40 um sections using a sliding microtome. All viable slices were collected for staining. Sections were washed three times in PBS followed by a 0.2% Triton X-100 + 5% NGS block for one hour. Sections were once again washed three times in PBS and were incubated for 36 hours in primary antibody. A 1:500 dilution of Reelin antibody (G10) and a 1:500 dilution of GABA primary antibodies were used for all preparations. The slices were washed three times in PBS and incubated overnight in a 1:500 dilution of corresponding goat anti-mouse Alexa-488 or goat anti-rabbit Alexa-555 secondary antibodies. Sections were mounted using Vectashield Mounting Medium for Fluorescence with DAPI and coverslipped.
Reelin VGAT cre: Ai9 Immunohistochemistry

The two VGAT cre: Ai9 mice were obtained from Jason Newbern’s laboratory (Arizona State University). In these mice all GABAergic interneurons are labeled by tdTomato. Brains were sectioned as above and incubated with a 1:500 dilution of primary Reelin antibody (G10) for 36 hours. The slices were washed three times in PBS and incubated overnight in 1:500 dilution of corresponding goat anti-mouse Alexa-488 secondary antibody. Sections were mounted using Vectashield Mounting Medium for Fluorescence with DAPI and cover slipped.

3. Brain Imaging and Cell Counting

Images of the brain sections were taken using a Zeiss Axioscope at ten times magnification. Images were analyzed using Zen software and ImageJ software was used for cell counting.

4. Golgi Staining and Neuron Morphology Reconstruction

Brains from six mice, two reeler, two heterozygous, and two wild type littermates, were processed according to the protocol for the FD Rapid GolgiStain Kit and were sliced using a vibratome.

Neurons from the pre-frontal cortex, motor cortex, and hippocampus were reconstructed using Neurolucida manual neuron tracing software. Layer V cells were selected for reconstruction. The reeler brain lacks a clear distinction of the laminar boundary, we selected cells located in the layer V with an identifiable, polarize apical dendritic structure. Sholl analysis was performed on the reconstructed neurons from wild type, heterozygous, and reeler mice.
5. **Statistical Analysis**

Values are expressed as a mean; error bars represent ± SEM. For comparisons between two groups, Student t test was used when data pass normality test. One-way ANOVA tests were used to measure significance between three or more groups. Bonferroni test was used for post hoc comparison between groups. Statistical significance was reported as $p < 0.05$, $p < 0.01$, or $p < 0.001$. 
RESULTS

Since recent studies have reported conflicting evidence on the GABAergic/glutamatergic nature of Reelin positive neurons, our first experiments were designed to test which populations of Reelin positive cells not GABAergic using immunohistochemistry. We determined target populations using evidence from a previous study assessing regional patterns of Reelin expression (Alcantara et al. 1998). We aim to systemically discover non-GABAergic Reelin positive populations in multiple brain structures.
Immunohistochemistry

Motor Cortex, Somatosensory Cortex, and Visual Cortex

Figure 1. GABA/Vgat immunoreactivity in Reelin positive neurons in motor and somatosensory cortex. A, D, Percent colocalization of Reelin with Vgat and GABA in motor cortex and sensory cortex respectively. B, E, Average number of cells per section positive for Reelin/GABA. C, F, Average number of cells per section positive for Reelin/Vgat in the motor cortex and sensory cortex. Data represent mean ± SEM.

The motor cortex we selected includes the primary motor cortex, the premotor and the supplementary motor area. Demarcation of these areas is based on images from The Mouse Brain Atlas (Allen Institute for Brain Science, Paxinos et al. 2008). We also quantified cell numbers in the primary somatosensory cortex (barrel cortex in rodents). Reelin positive cells could be found in all layers of the motor and sensory cortex with the majority residing in layers II through V. Almost all Reelin positive cells in every layer were colocalized with either GABA positive or Vgat positive neurons, all layers had over
95% colocalization. There was no significant difference in percentages between the layers or between the GABA and Vgat tdTomato staining. Overall these results support previous findings that the majority of Reelin positive cells in the sensorimotor cortex are GABAergic.

Figure 2. GABA/Vgat immunoreactivity in Reelin positive neurons in the visual cortex. A, Sagittal section of visual cortex showing Reelin/Vgat staining, Vgat staining is red and Reelin is stained in green. B, Percent colocalization of Reelin with Vgat and GABA in layers of visual cortex. C, D, Average number of cells per section positive for Reelin/GABA and Reelin/Vgat respectively. Data represent mean ± SEM.
We next examined Reelin+ neurons in the primary visual cortex. We found that most (over 95%) Reelin+ cells in superficial layers II/III and IV are GABAergic. However, there was a small population of non-GABAergic Reelin positive cells in layer V. Colocalization of Reelin with GABAergic neurons in layer V was around 85%, colocalization was over 95% in all other layers.

**Olfactory Bulb**

**Figure 3. GABA/Vgat immunoreactivity in Reelin positive neurons in olfactory bulb.**

A, Sagittal section of olfactory bulb showing Reelin/GABA staining, GABA is represented by green staining and Reelin is stained in red. B, C, Average number of cells per section positive for Reelin/GABA and Reelin/Vgat respectively. Data represent mean ± SEM.
The olfactory system, responsible for sense of smell, contains the olfactory bulb, cortical olfactory regions, as well as an accessory olfactory bulb. Olfactory cortical regions are the anterior olfactory nucleus, the piriform cortex, the olfactory tubercle, and the dorsal tenia tectum. The olfactory bulb can be divided into a glomerular layer, an external and internal plexiform layer, a granule cell layer, and a mitral cell layer, with cells expressing Reelin found in the latter two layers (Alcantara et al. 1998). We also confirmed Reelin positive cells restricted to the granule and mitral layers, colocalization with GABAergic neurons in these layers was less than 1% (1906/414 cells counted). Cells in the mitral cell layer project to the piriform cortex, the entorhinal cortex, and the amygdala; non-GABAergic Reelin positive neurons were also found in the former two areas. The cells in the external granule cell layer synapse onto the neurons in the mitral layer (Scott et al. 2005).
Piriform Cortex

Figure 4. Non-GABAergic Reelin positive cells in layer II of the piriform cortex. A, Sagittal section of piriform cortex showing Reelin/Vgat staining respectively, Vgat staining is red and Reelin is stained in green. B, Sagittal section of piriform cortex showing Reelin/GABA staining, GABA is represented by green staining and Reelin is stained in red. C, Percent colocalization of Reelin with Vgat and GABA in layers of piriform cortex. D, E, Average number of cells per section positive for Reelin/GABA and Reelin/Vgat respectively. Data represent mean ± SEM.
The piriform cortex, sometimes referred to as the olfactory cortex, deals with the perception of smells. This structure is part of the rhinencephalon and consists of the cortical amygdala, uncus, and anterior parahippocampal gyrus. It has been previously reported that there is a population of pyramidal neurons in layer II expressing reelin, as well as GABAergic interneurons (Alacantara et al. 1998). In agreement with this past evidence we found of the largest average number of cells per section in layer II. However, Reelin’s colocalization with GABAergic neurons in layer II was under 12% whereas colocalization was over 85% in layer I and over 70% in layer III. This non- GABAergic population of Reelin positive cells in layer II of the piriform cortex aligns with the layers that project to the olfactory bulb (Mohedano-Moriano et al. 2012).
Hippocampus

Figure 5. GABA/Vgat immunoreactivity in Reelin positive neurons in hippocampus.
A, Sagittal section of hippocampus showing Reelin/Vgat staining, Vgat staining is red and Reelin is stained in green. B, Percent colocalization of Reelin with Vgat and GABA in regions of hippocampus. C, D, Average number of cells per section positive for Reelin/GABA and Reelin/Vgat respectively. Data represent mean ± SEM.

The hippocampus in rodents, and humans, is responsible for spatial memory and navigation (Morris et al. 1982). The hippocampus is a major formation in the brain that is divided into the dentate gyrus (DG) and cornu ammonis (CA) 1, 2 and 3. The DG consists of three sections, molecular, granular, and polymorph these layers contain pyramidal neurons and granular cells. The CA regions consist of four layers; the stratum
oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare.

The average number of cells immunoreactive for Reelin was lowest in the stratum pyramidale and the stratum radiatum, which contain pyramidal neurons in the Schaffer collateral pathway and Schaffer collaterals respectively (Buzsaki 2011). Additionally, in both of these layers Reelin was colocalized with GABA or Vgat positive cells over 95% (328/458 cells counted). Average cell number was significantly higher in the stratum oriens, in which a large number of basket interneurons resides, with colocalization at 66% with GABA and 75% with Vgat (801/734 cells counted). In the stratum lacunosum-moleculare perforant path axons synapse onto dendrites of pyramidal neurons, the output from these Reelin+ cells has been shown to be glutamatergic (Quattroloco et al. 2014). This layer contained the largest number of Reelin positive cells with colocalization with GABAergic neurons at less than 34% (2489/1494 cells counted). The DG receives input from layer II of the entorhinal cortex, contributes to the formation of memories and has been implicated in depression. In the DG granule layer and the hilar region we found approximately 50% colocalization with GABAergic neurons (978/570 cells counted). Aligning with this evidence the densely packed granule cells of the DG are the principal excitatory cells of the structure and receive input from layer II of the entorhinal cortex.
Entorhinal Cortex

Figure 6. Non-GABAergic Reelin positive neurons in layers II/III of the entorhinal cortex. A, Sagittal section of entorhinal cortex showing Reelin/Vgat, Vgat staining is red and Reelin is stained in green. B, Sagittal section of entorhinal cortex showing Reelin/GABA staining, GABA is represented by green staining and Reelin is stained in red. C, Percent colocalization of Reelin with Vgat and GABA in layers of entorhinal cortex. D, E, Average number of cells per section positive for Reelin/GABA and Reelin/Vgat respectively. Data represent mean ± SEM.

The entorhinal cortex is located in the medial temporal lobe where it aids in memory and navigation (Hafting et al. 2005). Layers II and III project to the DG and
hippocampus as part of the perforant pathway while the deeper layers receive input from the hippocampus (Witter et al. 2000). In layer II/III of the entorhinal cortex Reelin positive cells form a clear band where colocalization with GABAergic neurons at 4.9% for GABA and 6.6% for Vgat (2111/4444 cells counted). Colocalization with GABAergic neurons in layer I and the deeper layers IV, V, and VI were all over 85%.

The existence of non-GABAergic Reelin positive cells in layer II/III of the entorhinal cortex along with the populations in the DG and SLM suggests that Reelin positive cells in the perforant pathway may be glutamatergic.

**Neuron Morphology**

Deficiencies in the Reelin protein have been linked with PPI deficits, reduced IPSC, and altered synaptic transmission and plasticity (Tueting et al. 1999, Qiu et al. 2006, Varga et al. 2010). This evidence suggests that there may be circuit specific deficits or inhibition/excitation balance disturbances. If these abnormalities occur in heterozygous reeler mice we believe there may be neuronal morphological correlates, for this reason we conducted a detailed morphological study of homozygous reeler, heterozygous reeler, and wild type littermates.
Branching Analysis

Figure 7. Branching analysis of neurons in the cortex of reeler, heterozygous and wild type littermates. A, D, Basal length and apical length of neurons in the pre-frontal and motor cortex of each group. B, E, Basal complexity and apical complexity of neurons in the pre-frontal and motor cortex of each group. C, F, Number of basal and apical nodes. Data represent mean ± SEM. n= 27-32 neurons in each group. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Layer V pyramidal cells in the pre-frontal cortex and motor cortex were analyzed using Neurolucida and a detailed branching analysis was performed. Basal length was not significantly different in reeler and heterozygous mice compared to their wild type littermates. Basal complexity, based on the Neurolucida formula, was also not significantly different in either reeler or heterozygous mice. Neurolucida calculates neuron complexity as [Sum of the terminal orders + Number of terminals] * [Total dendritic length / Number of primary dendrites]. Apical length was not significantly different in either heterozygous or reeler mice compared to wild type. Moreover, apical
complexity was increased in the Reeler mice and no difference was found between heterozygous and wild type littermates.

Our results conflict with previous evidence (Niu et al., 2004) by showing increased dendritic length in homozygous reeler, but not heterozygous, mice compared to wild type. Additionally, this prior study showed increased number of branch nodes in the cortex of reeler mice but not their heterozygous littermates (Yabut et al 2005, Niu et al., 2004). In contrast with these findings we discovered decreased nodes in the basal dendrites of homozygous and heterozygous reeler mice compared to wild type, and found no difference between the number of nodes in the apical dendrites of these neurons.
Figure 8. Branching analysis of neurons in the hippocampus of reeler, heterozygous and wild type littermates. A, D, Basal length and apical length of neurons in the pyramidal layer of the hippocampus of each group. B, E, Basal complexity and apical complexity of neurons in the pyramidal layer of the hippocampus of each group. C, F, Basal and apical nodes. Data represent mean ± SEM. n= 8-13 neurons in each group. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

In selected hippocampus CA1 neurons, the basal length was significantly deceased in reeler and heterozygous mice compared to the wild type (Figure 8). Interestingly, basal complexity, as measured by Neurolucida (using the aforementioned formula) was not reduced in either reeler or heterozygous mice in the hippocampus. Differences in apical length and complexity in the CA1 neurons were not significant. There were significant decreases in both the number of basal nodes in reeler and heterozygous mice compared to wild type mice, with no significant difference in the number of apical nodes between groups.
Sholl Analysis

Figure 9. Decreased intersections and length in homozygous and heterozygous *reeler* compared to wild type littermates in the cortex. A, Image of Golgi stained cortex at 5x magnification. B, Average number of intersections per neuron for each group. C, Number of intersections at increasing radii in pre-frontal and motor cortex of wild type, heterozygous, and *reeler* mice. D, Length of dendrites at increasing radii.

Error bars represent ± SEM. n= 27-32 neurons in each group. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
To determine if any morphological abnormalities existed in heterozygous reeler mice we performed an in depth Sholl analysis. We compared the number of intersections and lengths of the dendrites at increasing radii from the nucleus. To ensure the same cell types are compared across different genotypes, we selected layer V projection neurons for morphology analysis. We found no significant difference in the total number of intersections between groups (n = 27-32, p > 0.05, one-way ANOVA). For the number of intersections at increasing radii we found significant differences between homozygous reeler and heterozygous mice compare to their wild type littermates at 10 and 30 micron from the somata. There was also a significant difference on the length of the dendrites between groups at these radii. The morphological differences could be the underlying evidence of the functional difference found in heterozygous reeler mice.
Figure 10. Decreased intersections and length in homozygous and heterozygous reeler compared to wild type littermates in the hippocampus. A, Image of Golgi stained hippocampus at 5x magnification. B, Average number of intersections per neuron for each group. C, Number of intersections at increasing radii in pyramidal layer of hippocampus of wild type, heterozygous, and reeler mice. D, Length of dendrites at increasing radii. Error bars represent ± SEM. n= 8-13 neurons in each group. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

In the hippocampus differences between groups were more profound than in the cortex. There was a significant increase in the total number of intersections in wild type
compared to homozygous and heterozygous reeler mice (n = 8-13, p < 0.01, one-way ANOVA). Additionally, the number of intersections in the wild type group was significantly greater than the other two groups at radius 30, 50, 70, and 90. The length of the dendrites at radius 30, 50, 70, 90, and 110 were also significantly greater in wild type compared to the other two groups. Postmortem studies in schizophrenic brains have suggested abnormal GABAergic activity in the hippocampus (Benes et al. 2015).

**Neuron Polarity**

**Figure 11.** Neurolucida neuron tracing showing abnormal positioning of neurons in prefrontal/motor cortex of heterozygous and reeler mice compared to wild type littermates. A, B, C, Whole section of Golgi stained brains. D, E, F, 5x magnification of prefrontal/motor cortex. G, H, I, Manually traced contours and neurons showing abnormal positioning in heterozygous and reeler brains.
To further examine potential subtle morphological defects in the heterozygous *reeler* mouse we preformed vector analysis on Golgi stained neurons in the prefrontal cortex and motor cortex of wild type, heterozygous, and *reeler* littermates, we calculated the deviation of primary dendrites orientation from a line that is perpendicular to the pia. Normal dendritic polarity should align well with this perpendicular line, while disorganized dendritic polarity will significantly divert. We measured the diverting degrees of layer V neurons from Golgi stained sections in all three genotypes, and plotted the histogram (Figure 12).

**Figure 12. Abnormal orientation of neuron primary apical process in heterozygous and *reeler* mice compared to wild type littermates.** A, B, C, Images showing Golgi stained cortex in wild type, heterozygous, and *reeler* respectively. Arrows indicate neuron processes chosen for analysis. D, E, F, Degree of apical dendrite angle relative to the perpendicular line to the pia surface in wild type, heterozygous, and *reeler* respectively.
In the wild type mouse the angles of the apical dendrites are clustered close to 0 degrees from the normal, with few outliers above 40 degrees. The heterozygous brain still has many of the dendrites clustered in the 0 to ±40 degree range, however, there are significantly more processes positioned at ±80 to ±160 degrees from the baseline. The position of the apical dendrites in the reeler mouse are scattered with only a small cluster within the 0 to ±40 degree range. A clear representation of this abnormal polarity in layer V pyramidal neurons can be seen in figure 11. Previous studies have conducted similar experiments analyzing the polarity of neurons and interneurons in reeler mutants compared to wild type mice, however, these studies either fail to examine heterozygous mice or include them in the normal (wild type) category (Yabut et al. 2007, Forster 2014). Both of these studies identified a significant difference in the neuronal polarity in reeler mice compared to wild type. Our results extend this data to show abnormal polarity in the layer V pyramidal neurons in the haploinsufficient reeler mutant.
Figure 13. Scattered pyramidal neuron somas in the cortex of heterozygous and reeler mice compared to wild type littermates. A, Image showing neurons with retrobead labeling in the cortex. B, Scattered layer V pyramidal neurons in the reeler mouse. C, Scattered layer V pyramidal neurons in the heterozygous reeler mouse. D, Normal clustered location of pyramidal neurons in wild type mouse.

Wild type, heterozygous, and reeler mice were injected with retrograde tracing beads into the striatum at postnatal day 21. After two to three days brains were sectioned and the cortex was examined for the presence of retrobeads; the retrobeads were detected in layer V pyramidal cells. In the wild type mice these neurons are clustered in their normal location in layer 5A and 5B. As previously reported layer V pyramidal neurons in the reeler mutant were diffusely scattered throughout all layers of the cortex. The point of
interest in our findings lies in the location of the pyramidal neurons in the heterozygous reeler brain. We found layer V pyramidal cells present in layers II/III, additionally, the neurons confined to layer V were more scattered than in the wild type mouse.
DISCUSSION

In this study, we systemically investigated the distribution of reelin+ neurons in the young adult age postnatal mouse brain, and identified major populations of non-GABAergic Reelin positive neurons. Our major findings were populations of non-GABAergic Reelin positive cells in layer V of the visual cortex, the mitral and granule layers of the olfactory bulb, layer II of the piriform cortex, the slm and DG in the hippocampus, and layers II/II of the entorhinal cortex. From these areas we defined two major circuits that consist of non-GABAergic Reelin positive neurons. The first pathway contains areas that function in the olfactory system, including olfactory bulb and the piriform cortex. Schizophrenia is frequently accompanied by deficiencies in the olfactory system and there is down-regulation of Reelin in the olfactory bulb of heterozygous reeler mice and abnormally diffuse and densely packed axonal staining of Reelin in the mitral layer (Pappas et al. 2003). Reelin deficient mice also demonstrate olfactory learning defects and disruption of normal organization of the granule layer (Martin-Lopez et al. 2012). Thus, the identification non-GABAergic nature of the Reelin positive neurons in olfactory system could point to non-GABAergic, possibly glutamatergic, participation in schizophrenia related olfactory defects. Neurons from the entorhinal cortex also project to the olfactory bulb and could be a connection between the two pathways identified in our study (De La Rosa-Prieto et al. 2015).

We also identified these populations in the perforant pathway (pp). Non-GABAergic Reelin positive populations of neurons were found in the slm of the hippocampus, the DG, and the entorhinal cortex. In the entorhinal cortex non-GABAergic
Reelin positive neurons were abundant in layers II and III. Layer II primarily projects to the dentate gyrus and layer III to the hippocampus. Alterations in the pp, specifically the CA1 direct input to the entorhinal cortex, cause impairments in memory and organizational strategies in schizophrenic patients (Siekmeier et al. 2007). Thus, determining the properties of Reelin positive cells in this pathway is critical to understanding, and possibly treating, the dysfunction schizophrenic patients due to disruptions in the pp. We found non-GABAergic Reelin positive neurons in the slm, mostly situated in the CA1 region, and the entorhinal cortex, which indicates that this direct input in schizophrenic patients may be glutamatergic. Indeed it has been found that the slm has the highest concentration of dopamine receptors in the hippocampus and has a strong glutamatergic input (Otmakhova 1998).

Layer II neurons in the entorhinal cortex also project to the dentate gyrus where roughly half of the Reelin positive cells were not colocalized with GABA or Vgat staining. The dentate gyrus, responsible for formation of memories, is also believed to be dysfunctional in schizophrenia due to disrupted pattern separation performance in subjects. It has been proposed that this functional defect is caused by the role the dentate gyrus has in the hippocampal model of psychosis due to reduced glutamate signaling to the DG from the entorhinal cortex (Das 2014). Our results indicate that Reelin may be a component of this dysfunctional transmission due to the presence of non-GABAergic populations in both the DG and the entorhinal cortex.

The final population of non-GABAergic Reelin positive cells that were identified was a small number of cells in layer V of the visual cortex. Projections from all layers of
the entorhinal cortex connect to visual associations areas and it has recently been shown that cooperation of the hippocampus and entorhinal cortex was necessary for rapid visuomotor learning in rhesus monkeys and that inactivation of the entorhinal cortex led to impairments in novel arbitrary associations (Moser et al. 2010, Yang et al. 2014). It is possible that the connections responsible for visuomotor learning are modulated by non-GABAergic signaling from Reelin positive cells. This information could contribute to the understanding of the abnormalities found in visual object exploration and planning of visuomotor responses in schizophrenic patients (Chen et al. 2011, Glover et al. 2002). However, further studies with additional brains, glutamatergic staining, and retrograde labeling would be necessary to determine if this population is significant, the nature of these non-GABAergic Reelin positive cells, and if they connect to the entorhinal cortex or other areas. Overall, these results support pervious findings of certain non-GABAergic populations and identify novel cellular targets of Reelin signaling. Further studies looking at the colocalization of Reelin with glutamatergic neurons are necessary to fully understand the role Reelin positive cells in the pp and the olfactory system have in schizophrenia.

In this study, we also investigated morphological abnormalities in heterozygous reeler mice and compared that with their reeler and WT littermates. The notable abnormalities in heterozygous reeler morphology were decreased basal nodes and number of intersections as well as length at locations close to the neuronal soma in the motor cortex. These decreases were more pronounced in the hippocampus with altered basal length and nodes as well as decreased total intersections and decreased intersections
and length at radii more distal to the soma. These results, combined with previous reports (Niu et al. 2004, Yabut 2005) indicate reelin dosage as a key factor for neuronal dendritic development. Dendritic and spine morphology are known to be regulated by multiple growth factors and are key determinants of neurotransmitter signaling efficiency and critical constituent of circuit function. Therefore, the morphological abnormalities we uncovered here may have significant functional consequences.

Dendrites define the physiological properties of neurons and their connectivity to other neurons, thus altered dendritic morphology in the heterozygous reeler brain is a major point of interest. We found evidence that dendritic morphology is more significantly altered in the hippocampus than in the cortex, this aligns with the idea that dendrites continue to branch in the entire brain after neuronal migration is complete and in the hippocampus dendritic process growth and remodeling occurs in the adult brain. It has been established that in homozygous reeler mutants a Reelin signal is necessary for normal dendritic growth, organization and arborization (Niu et al. 2004). Our evidence confirms these abnormalities and additionally demonstrates similar defects in heterozygous reeler.

In addition to our detailed Sholl analysis, we examined the laminar location and neuron polarity in Reelin haploinsufficiency. As expected in the reeler mutant we discovered layer V neurons distributed throughout all layers of the cortex. Unlike its wild type counterparts, where somas were clustered in the middle of layer 5A and 5B, the soma in heterozygous reeler mice were scattered with some somas located in other layers of the cortex. Thus, like the absence of Reelin, Reelin haploinsufficiency also disrupts the
laminar positioning of layer V neurons. Furthermore, neuron polarity in both heterozygous and homozygous reeler mutants were altered compared to wild type. In the mouse embryo neurons polarize in the radial plane at embryonic day 13, this occurs during the splitting of the preplate. This reorientation during cortical development does not occur in the reeler brain. Then during normal development neurons migrating towards deep layer VI form a radial orientation of their apical dendrite. Once again this process fails in reeler mice (Forster 2014). This failure of neurons to attain proper orientation has been previously demonstrated in the homozygous reeler cortex, which our data supports, moreover, we found similar morphological changes, such as abnormal Sholl analysis, neuron polarity, and laminar location in heterozygous reeler mice. This shows that heterozygous reeler have similar, though sometimes reduced, morphological changes as homozygous reeler mice.

We believe that our findings point to underlying neuronal morphological correlates for the electrophysiological changes found in heterozygous reeler mice. As previously mentioned these differences include PPI deficits, reduced IPSC, and altered synaptic transmission and plasticity (Tueting et al. 1999, Varga et al. 2010). Additionally, coupling of reelin signaling with NMDAR and AMPAR receptors has been shown to alter synaptic strength and plasticity in the hippocampus through Reelin enhancement (Qiu et al. 2006). The morphological abnormalities we reported could be one factor in these electrophysiological differences, however, limited evidence exists showing significant alterations in heterozygous reeler mutants. To the end, further electrophysiological experiments studying wild type, heterozygous, and homozygous
reeler mice are currently underway to test this hypothesis.
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CURRICULUM VITAE

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Education

Masters of Science, Medical Sciences, May 2015 Boston University, Boston, Massachusetts

Bachelors of Science, Physiology, May 2013 The University of Arizona, Tucson, Arizona.

Minor in Spanish, May 2013  
The University of Arizona, Tucson, Arizona.

Minor in Chemistry, May 2013  
The University of Arizona, Tucson, Arizona.

Honors and Awards

University of Arizona Wildcat Excellence Award/Scholarship State of Arizona Early Graduation Scholarship Grant

Member of Omicron Delta Kappa National Leadership Honor Society

Research Experience

Undergraduate Research Assistant in the psychophysiology laboratory under Dr. John Allen Fall 2011-Spring 2012 University of Arizona, Tucson, Arizona  
-Assisted in research examining the relationship between anxiety disorders and decision-making tasks. Ran EEG on participants. Collected and cleaned EEG data.

Undergraduate Research Assistant in the neurophysiology laboratory under Dr. Katalin Gothard Summer 2011, Summer 2012-May 2013 University of Arizona, Tucson, Arizona  
-Assisted in research examining the neural basis of emotion. I worked on scoring different types of data including blinks and eye saccades in image and movie files. Assisted with scoring stimuli in tactile movies and determining mouth area in stimuli movies. I learned about the workings of a primate laboratory, received training on working with rhesus monkeys, and learned about different experimental procedures used in neurophysiological research.
Teaching Experience

Undergraduate Preceptor  Introductory cell and animal biology  Spring 2012- Fall 2012
University of Arizona, Tucson, Arizona
   - Worked alongside the teacher’s assistant in teaching lab activities. Taught
   selected lessons and wrote weekly quiz. Attended weekly preparation meetings. Assisted
   in revising rubrics for student projects and reports.

Volunteer Experience

Postpartum volunteer Chandler Regional Hospital  2007-2009 Chandler, Arizona
   - Restocked medical supplies. Assisted in caring for babies. Cleaned and prepared
   patient rooms. Brought refreshments to patients.

Emergency Department Volunteer for Southern Arizona Veterans Hospital  Fall
2011-May 2013 Tucson, Arizona
   - Restocked patient rooms with supplies. Transfer patients from emergency
   department to x-ray or wards. Cleaned and prepared patient rooms. Shadow nurses and
   doctors.

Clinical Volunteer for MedLife (Medicine, Education and Development for Low-
Income Families Everywhere)  Summer 2011 and Summer 2012 Lima, Peru
   - Set up mobile clinics in at need neighborhoods in Lima, Peru. Assisted in setting
   up medical supplies. Triaged patients and shadowed doctors, dentists, obstetricians,
   nurses, and pharmacists. Assisted communities in completing projects to improve their
   community.

Animal Assisted Therapy Team Escort at Phoenix Children’s Hospital  August 2014-
Present Phoenix, Arizona
   - My responsibilities include preparing items needed for patient visits, escorting
   the handler and dog in the hospital, keeping track of rooms visited, ensuring a sanitary
   environment and monitoring patients mood and safety during visits. I am there to make
   sure the handler and dog can focus on the therapy for the child in a safe environment.

Employment

Computer Lab Monitor, Salt Center (Strategic Alternative Learning Techniques)
Fall 2011-May 2013
   - Responsible for monitoring computer lab. Kept computers updated and the
   computer lab clean. Educate students on the different programs to assist with their
   projects. Assist tutors and students with technical issues. This job allowed me to assist
   many special needs students while they learned about computer programs that were more
   suited to their learning style.
-Monitored testing room for math placement tests. Checked in and out students for placement tests. Answered phones, filed records, restocked supplies.