TITLE: Ric-8a is Essential for Regulation of Intercellular Interactions in the Developing Brain

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Interneurons are important cellular components in the brain and they are mainly responsible for inhibitory actions in the central nervous system. Investigation of how interneurons develop is necessary to understand neuronal circuitry and diseases which could be caused by excitatory/inhibitory imbalance. Our laboratory utilizes mouse genetics to study this and related topics on interneurons during embryogenesis. We focused on how Ric8a, an important protein in signaling pathways, contributes to cortical development. Our laboratory is one of the first groups to remove ric8a specifically in interneurons in order to reveal its functions. From my results, it is evident that the ric-8a gene plays a role in interneuron production or survival (but not morphology) at both embryonic and post-natal stages. Our genetic model helps to determine the role that ric-8a plays in cortex development and function, as well as sheds light on possible medical advancements for diseases caused by interneuron defects.

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Abstract

Interneurons are important cellular components in the brain and they are mainly responsible for inhibitory actions in the central nervous system. Investigation of how interneurons develop is necessary to understand neuronal circuitry and diseases which could be caused by excitatory/inhibitory imbalance. Our laboratory utilizes mouse genetics to study this and related topics on interneurons during embryogenesis. We focused on how ric-8a, an important protein in signaling pathways, contributes to cortical development. From my results, it is evident that the Ric-8a gene plays a role in interneuron production or survival (but not morphology) at both embryonic and post-natal stages. Our genetic model helps to determine the role that the Ric-8a plays in cortex development and function, as well as sheds light on possible medical advancements for diseases caused by interneuron defects.

Introduction

To demystify brain function requires detailed knowledge of different types of neurons within this extremely complex structure. Not all neurons in the brain excite their neighbors; in fact, inhibitory actions of some neurons are important to maintain a balanced neural activity in healthy brain. GABAergic interneurons are essential for such purpose due to their capability to form inhibitory synapse with other neurons, propagating information across the cortex to establish proper neuronal communication. They play crucial role in neural processes, as their action potential firing patterns regulate brain rhythms (Gavrilovici *et al* 2012). In addition, interneurons have been linked to neurodegenerative diseases; for examples, patients of Alzheimer's disease have significantly decreased levels of interneuron specific activity (Verret *et al* 2012). However, much remains unknown about the development and function of these neurons.

Mouse molecular genetics is a great tool to study cellular functions of interneurons, and further help us ask fundamental questions about their functions and open new avenues for treating related psychiatric diseases. It is well known that the GPCR (G-protein coupled receptor) pathway plays a critical role in interneuron migration, but our understanding of this pathway in interneuron biology is far from finished, partly because there are hundreds of GPCRs in the vertebrate genome, making any genetic knockout study difficult. Our laboratory established a novel approach to solve such a problem by manipulating Ric-8a, a protein that interacts with multiple GPCR downstream pathways in interneurons followed by phenotypic analysis (Ma et al., 2012). My experimentation will be the first to investigate this gene in the cerebral cortex and gather quantitative conclusions regarding the lack of Ric-8a protein in mammals. We chose to conditionally knockout the Ric-8a gene, which has been found to

regulate different G proteins (Vellano *et al 2011*). It was concluded that the Ric-8a protein is necessary for controlling mitotic spindle orientation downstream of signaling; it is also essential in monitoring and maintaining the spindle within the epithelium (David *et al* 2005). Ric-8a excision in Drosophila has led to more conclusions regarding the proteins function. In mutants without the Ric-8a protein, G alpha proteins as well as their associated beta subunit are localized in the plasma membrane, which ultimately leads to their cytoplasmic degradation (Hampoelz *et al 2005*). In addition to above functions, the Ric-8a gene has been found to have a profound impact in cerebellar foliation. More specifically the excision of this specific gene leads to defective granule cell migration as well as an alteration of Purkinje cell body positioning in the cerebellum (Ma 2012). We further studied the mechanism by which Ric-8a regulates the interactions that exist between different cell types and then they were assessed, especially during interneuron development. This will be the first study on how Ric-8a regulates embryonic interneuron development in mammals.

Methods

DNA analysis and genotyping

In order to analyze both the wild type group along with the ric-8a lacking mutant mice, DNA analysis must be performed first. Using 10 mice to give a proportion of about 7 controls to 3 mutants, I analyzed the DNA according to laboratory procedure using the taq enzyme. Taq is especially critical to this process, and especially expensive, because it is one of very few enzymes that are able to be functional at such high temperatures that are required in the polymerase chain reaction (PCR) process (I specifically used the eppendorf mastercycler for the PCR process). Genotyping occurs when the primer in the PCR machine is able to identify the

target sequence in the DNA and through the cre/flox system operationally excise the existing Ric-8a gene assuming that it has flox on both sides of its genomic location. Once it is determined which mice were mutants and which were wild type, I labeled which mouse belongs to which sub set. Then the DNA from each mouse is placed into a well in the gel electrophoresis set up, after the creation of the gel itself which takes a couple of hours to solidify. In addition to the mouse DNA, ladder DNA must be also placed into the first well to serve as a marker for the length the DNA travels down the gel. All of these steps ultimately lead to the recognition of mutants without the Ric-8a protein through gel electrophoresis.

Neuronal sectioning

The next phase is to section and compare and brains of both mutants and the wild type to contrast their neuronal signaling. In order to sacrifice the mouse, isoflurane was utilized on the animal for euthanasia and removal of the embryonic mice for the experimental specimens, marked P0. The first and foremost step is the removal of the mouse brain, which is to be performed under a microscope to ensure accuracy and completeness. After the removal of the mouse brain, I removed the cerebellum and placed the cortex in a 5% agarose solution to solidify and stabilize the brain. A very specific and important detail in this process is that the olfactory bulbs of the cortex must be pointing upwards in order to ensure proper sectioning later in the process. The following day, be careful in the removal of the gel and brain from the dish as to not damage the combination. Next, cut off all excess gel and glue the cortex to the metal plate of the sectioning mechanism and begin the sectioning procedure depending on the specific machinery. It is very important to have a low speed of the sectioning blade due to the fragility of the cortex, if the blade is too fast it will inhibit the ability of the machine to form accurate and intact cortex

sections. A small note is that the olfactory bulbs do not need to be collected after sectioning because they are of little use to this investigation from their obvious lack of ventricles.

Histology through traditional and fluorescence microscopy

In order to correctly see the phenotype of the microscopic image, sections went through nissl staining in order to clean the image and allowed me to differentiate between the wild type and mutant group. To label interneurons, I used Tuj1, because it binds to beta actin in neurons. Then I used fluorescence-conjugated antibody to bind Tuj1 in order to see neurons under microscope. To label neuron nuclei, I used DAPI, a synthesized fluorescent chemical which strongly binds to DNA in nucleus. In this way, I can see nuclei under the fluorescence microscope. These sections can then be placed onto slides; cover slips were then applied with the appropriate solution, and placed under a fluorescent microscope which will be very useful in the quantitative analysis of different blood vessels and cell types between controls and mutants.

Techniques for statistical analysis

In order to analyze morphological differences between the wild type and mutant groups, a conventional microscope was used and the differences were noted after repetitive visualization repetition of the same physical defects in the mutant. In terms of blood vessel densities, I used NIS-elements software to measure the total length of the blood vessels in the ventral area of both groups, and then divided by the total ventral area to calculate vessel density. Using the same software to analyze branching densities I simply counted the number of branching points, which are where smaller vessels extend out from the original vessel, and then divided it by the ventral area. The quantitative analysis of the interneuron development was a bit more difficult due to the incredible number of neurons in the ventral area. I measured out a small, constant area of the ventral area for both wild type and mutant sections, and then I divided the number of

interneurons by this area to calculate density. To determine both the statistical significance as well as precision, I conducted p-value and standard error measurements for all quantitative analysis in the wild type and mutant group.

Results

Vascular defects in Ric-8a Mutant

One of the primary features that my laboratory and I investigate is the morphological differences that result from the conditional knockout of the Ric-8a gene. Before I specifically began to look at interneuron morphologies in the developing cortex, my lab had already made an interesting finding by deleting Ric-8a in all types of neural cells. As seen in figure 1, when a Nissl stain was performed at P0 of the developing mouse, there appeared to be hemorrhage in the Ric-8a mutant cortex. In addition, when I looked at individual interneuron cells in both the Ric-8a mutant and wild type mice it appears that the individual morphologies were relatively similar with no significant differences, as seen in figure 2. Overall, the excision of the ric-8a gene has an effect in inducing neuronal hemorrhage but appears to play little, if any, role in the morphological development of individual interneuron cell bodies.

Interneuron development defect in Ric-8a Mutant

I began my investigation with the intent to discover the possible role of the Ric-8a gene in interneuron generation throughout the neurodevelopment using the mammalian model. Immunostaining and individual neuron counting were employed to describe final cell densities in the mutant and wild type cortex. In this specific study, I suggested that the Ric-8a plays a novel role in interneuron population in the cortex through development and all the way until adult (1 year old) mice. Preliminary data that I collected showed that the Ric-8a gene has a role in

interneuron development in the mouse cortex. As visualized in graph 1, the Ric-8a mutants show significant reduction in interneuron population in the cortex compared to the wild type in the pre-natal stage of E13.5. I then analyzed neuronal sections using the same methodology and quantified the overall cortical interneuron densities in the adult brain. As observed in graph 3, when the Ric-8a gene is conditionally excised from interneurons, the cortical interneuron density in the mutant is significantly less than in the wild type group in 1 year old mice. Additionally as visualized in the comparison images of figures 3 and 4, it is obvious through the immunostaining that the wild type group had higher interneuron density when compared to the knockout mutant group. These results indicate that the Ric-8a dependent regulation of interneuron development is continuous and persistent throughout all stages of neurodevelopment.

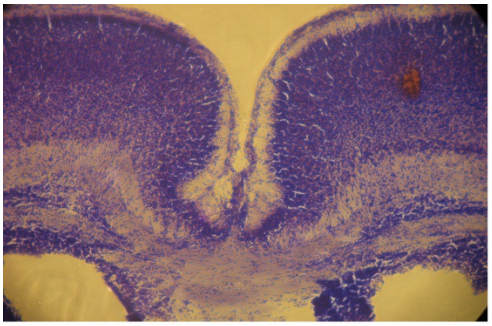
Discussion

My laboratory members and I are one of the first principal investigators of the Ric-8a protein in mammals, and the conclusions that are stated in the results section provide a clear and new basis of the knowledge about this gene encoding the protein. From past research studies it is known that interneurons play a significant role in both neuronal development and overall cognitive functions. In the developing brain the growth of neurons is of the utmost importance, and it is clear that the Ric-8a gene plays a significant role in neuronal development. It is interesting to note that Ric-8a plays multiple roles in neurodevelopment by regulating interneuron development as well as in some aspect of vasculature development. Although examined very carefully, it is interesting to note that the excision of Ric-8a had no effect on the morphology of interneurons. With regard to these conclusions, it is important to note possible caveats when we draw definitive conclusions from my data. Possible errors include unequal sectioning of the mouse cortex leading to partially broken brain sections, which would skew

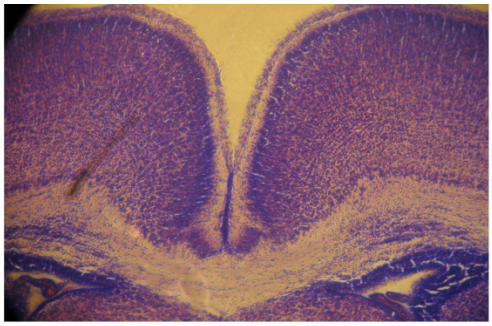
quantification during microscopic imaging. Therefore, more samples and repetition are needed for analyses. Another uncertain factor is essentially human error, and the quantitative and qualitative analysis of phenotypes under a microscope could have been questionable due to simple miscalculations or inappropriate software use. However, application of statistics using multiple samples has reduced such uncertainty to minimal. In conclusion, our laboratory expects that further research will be continued to reveal the role of Ric-8a gene in mammals so that its full functions, even outside of neural tissues, can be uncovered. This discovery could lead to medical advancements to cure humans who are born with neurological disorders pertaining to the Ric-8a gene.

Tables and Figures

Figure 1.



The conventional microscopic image of the p0 embryonic mutant mouse cortex, focused on the anterior cingulate complex.

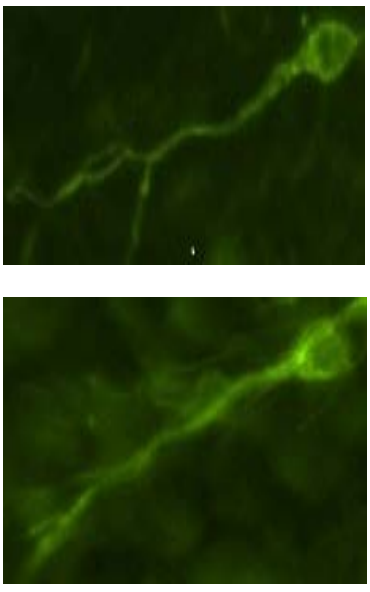


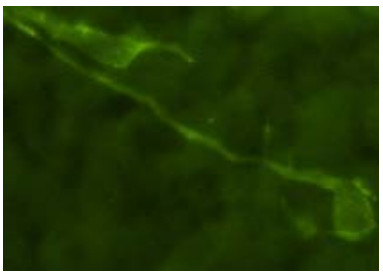
The conventional microscopic image of the p0 embryonic wild type mouse cortex, focused on the anterior cingulate complex.

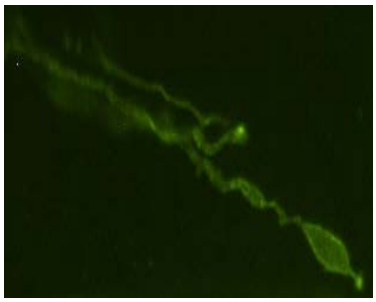
Figure 2.

Mutant

Wild Type

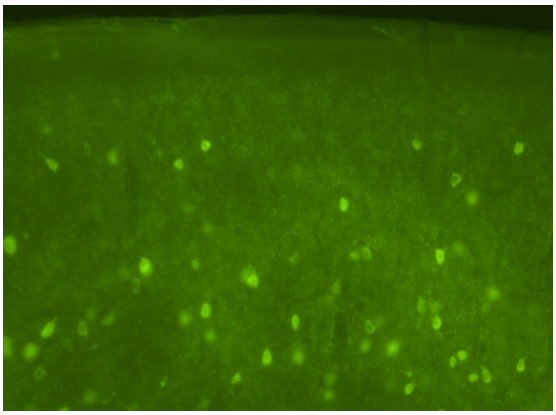




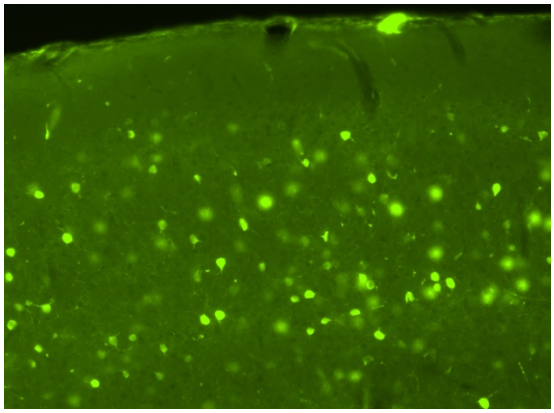


Fluorescent microscopic images of interneurons in the cortex at E13.5 in the developing mouse cortex. Staining was performed using fluorescent antibodies. As visualized, there is no obvious difference in the morphology of the individual interneurons in the mutant or wild type mouse.

Figure 3.

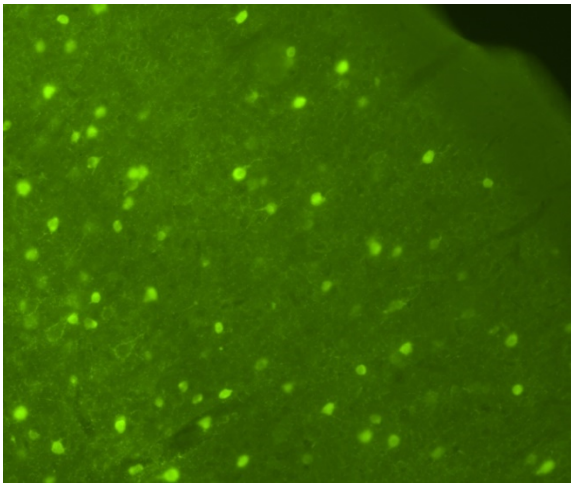


Immunoflurouescent image of a mutant mouse at the 1 year stage. Image utilized fluorescent antibody labeling and was taken under 20X microscopic zoom.



Immunoflurouescent image of a wild type mouse at the 1 year stage. Image utilized fluorescent antibody labeling and was taken under 10X microscopic zoom.

Figure 4.



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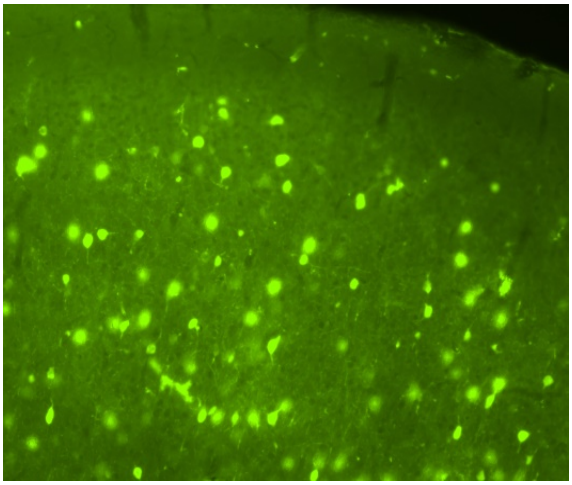
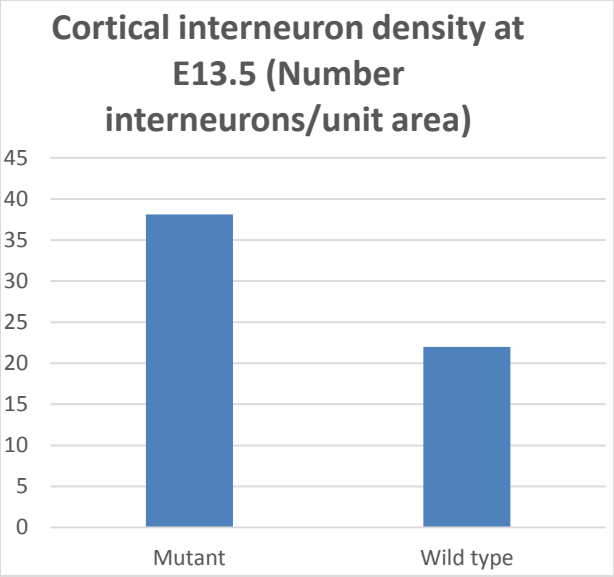
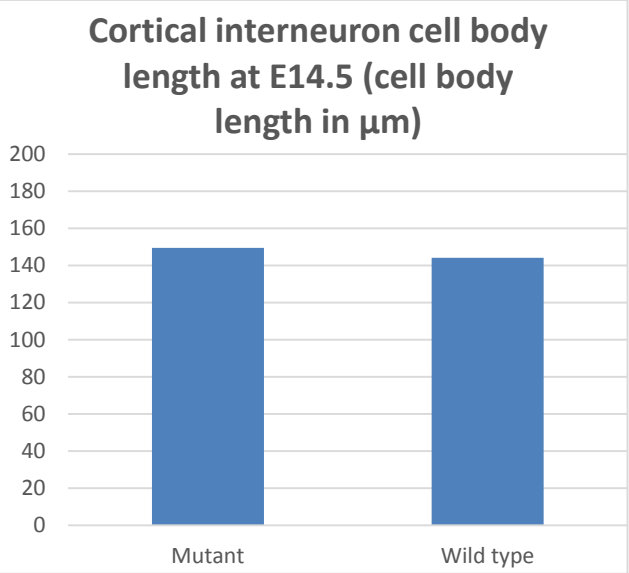


Immunoflurouescent image of a wild type mouse at the 1 year stage. Image utilized fluorescent antibody labeling and was taken under 20X microscopic zoom.

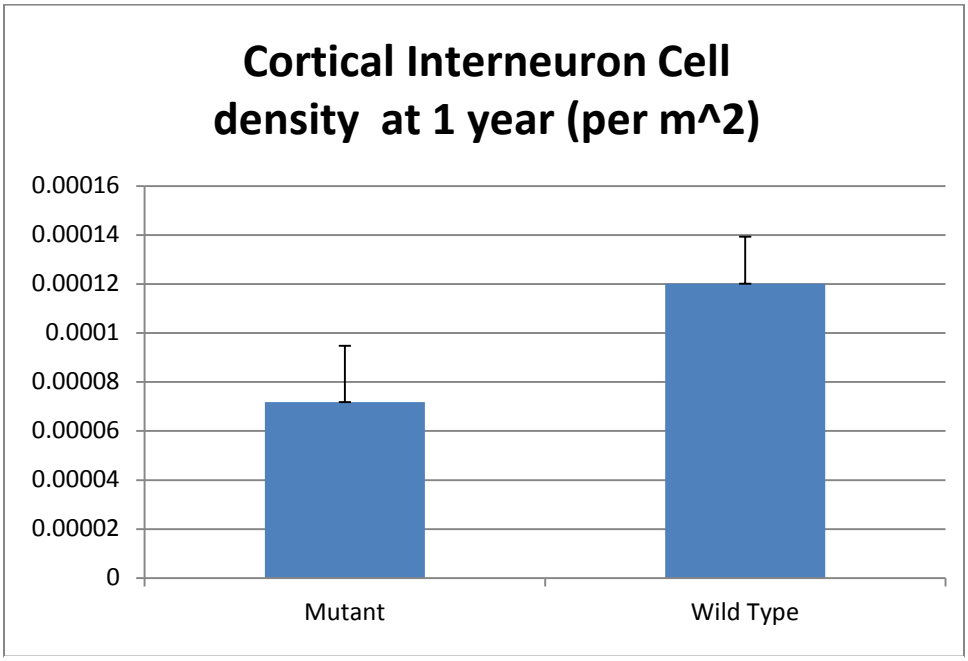
Graph 1.



Graph 2.



Graph 3.



Cortical interneuron densities compared between the mutant and wild type group. Densities were recorded via counting a select number of cells and dividing by the area; area remained almost constant during quantification. P value of .007217.

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