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Knockdown of Kiaa0319 Reduces Dendritic Spine Density

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Developmental Dyslexia is a reading disorder that affects individuals that possess otherwise normal intelligence. Until the four candidate dyslexia susceptibility genes were discovered, the cause of cortical malformations found in post mortem dyslexic brains was unclear. Normal brain development is crucial for the proper wiring of the neural circuitry that allow an individual to perform cognitive tasks like reading. For years, familial and twin studies have suggested that there was a genetic basis to the causation of dyslexia. Kiaa0319 was among the candidate dyslexia susceptibility genes that were ascertained. KIAA0319 is located on Chromosome 6p22.2-22.3 and has been found to exhibit differential spatial-temporal expression patterns in the brain throughout development, which suggests that the polycystic kidney disease (PKD) domain encoded by KIAA0319 facilitates cell-cell adhesion to enable neuronal precursors to crawl up the radial glia during neuronal migration. With the knowledge of KIAA0319 involvement in early neurogenesis, we were interested in determining how different KIAA0319 expression may impact cortical neurons in layer II and III during early adulthood. We show that KIAA0319 knockdown in cortical pyramidal neurons significantly reduces the dendritic spine density. Studies have shown that changes in dendritic spine morphology and density affect properties of neural circuitry. Henceforth, this finding may reveal a link between the Kiaa0319 gene and the deficit of the neural processing task of reading due to reduced spines density. Finding a correlation between Kiaa0319 expression and its influence on dendritic spine development may lead to a greater insight of a direct link between the dyslexia susceptibility gene and the biological mechanism that causes dyslexia.

Introduction

Developmental dyslexia (DD) is a learning disorder that impairs a person's reading ability even though he or she possesses otherwise normal intelligence. Scientists have determined a genetic basis for this learning disorder, which led to the identification of several dyslexia susceptibility genes. KIAA0319, located on Chromosome 6p22.2-22.3 has shown linkage disequilibrium with DD (Parachinni et al., 2006). Previous studies have shown that the protein encoded by this gene participates in neuronal migration (embryonic day 13.5-18.5) (Parachinni et al., 2006). An in situ hybridization study detected a differential spatial-temporal expression pattern for KIAA0319 in the

cerebral neocortex throughout neurogenesis (Parachinni et al., 2006). Also, an in-utero KIAA0319 RNA interference experiment resulted in disruption of normal neuron migration (Paracchinni et al., 2006). Together, these findings suggested that neuronal migration is an important component in the development of the neocortex, because proper placement of the neurons in the cortex is a necessary foundation for higher cognition (Kandel et al., 2000). Furthermore, the inside to outside pattern of neuronal migration during early development determines the morphology of the mature neocortical structure (Kandel et al., 2000). In 1979, Albert M. Galaburda and Thomas L. Kemper published a study that showed that post-mortem brains of a dyslexic individual showed morphological abnormalities localized in the language areas of the brain (Galaburda and Kemper, 1979). The implication of this finding was that developmental dyslexia occurred due to the localized anatomical abnormality, which may have occurred because of disruption in neuronal migration. One plausible explanation for KIAA0319's molecular function in dyslexia may be attributed to cell-cell adhesion properties exhibited by its four Polycystic Kidney Disease Domains(PKD) (Paracchini et el., 2006). The PKD domain of KIAA0319, along with other adhesive proteins, may enable cortical neurons to crawl along the radial glia to its final placement in the cerebral cortex by facilitating interactions between radial glia and neuron (Paracchini et al., 2006).

Based on the knowledge of KIAA0319's involvement in early development, our laboratory conducted additional investigations to see what other phenotypic traits may be influenced by the gene at a later time point in development. The most apparent observation was that KIAA0319 RNAi appeared to influence the morphology and density of dendritic spines on pyramidal neurons in post natal mouse brain. This observation led us to pursue a more thorough examination of KIAA0319's role in spine morphology and density.

Spines are neuronal protrusions that can form in several places throughout the neuronal surfaces such as the soma, dendrites, and axon hillock (Nimchinsky et al., 2002). One of the main functions of spines is to receive input from excitatory synapses (Nimchinsky et al., 2002). In humans, dendrites are relatively bare at birth, however the dendritic protrusions begin to appear along the dendrite process after the first week of life (Nimchinsky et al., 2002). In the second and third week, the density of the dendritic protrusion rapidly increases during synaptogenesis where neurons begin making upwards to 100,000

synaptic connections with neighboring neurons (Nimchinsky et al., 2002; Gilbert, 2002). The dendritic protrusion mature to become the neuronal spines found on pyramidal neurons. Conducting electrochemical signals through synaptic connections is crucial for allowing the brain to perform many highly complex mental and cognitive processes; therefore, studies have suggested that altering the synaptic connections established by spines may result in brain disorders (Widmaier et al., 2008; Nimchinsky et al., 2002). Consequently, alterations in spine density and morphology have been observed in brain disorders like schizophrenia and mental retardation due to Trisomy-21 and Fragile-X syndrome (Nimchinsky et al., 2002). Other studies have shown a decrease in dendritic spine numbers and density as cognitive functions worsen due to aging (Nimchinksy et al., 2002). It would be interesting to see if a structure and function relationship exists betweenKIAA0319's influence on spine density and DD. To modulate Kiaa0319 expression we used *in utero electroporation* in mouse embryonic brain. This gene manipulating technique will enables us to modulate the expression of the Kiaa0319 during embryonic development. Plasmids will be transfected into the neuronal progenitor cells that lie in the VZ surface. Afterwards, the neuronal progenitor cells will migrate away from the VZ along radial glial fibers towards pial surface (Kandel *et al.*, 2000). When the migrating neurons finally stop migrating they will remain in one of the six cortical layers. The earlier the neuron migrated, the deeper the layer of the cortex the neuron will be finally situated at the end of neuronal migration (Kandel *et al.*, 2000).

Materials and Methods

In-utero Electroporation

A pregnant mother wistar rat carrying embryonic day 15 pups was briefly placed under isoflurane inhalation anesthesia then given ketamine/xylazine for anesthesia.

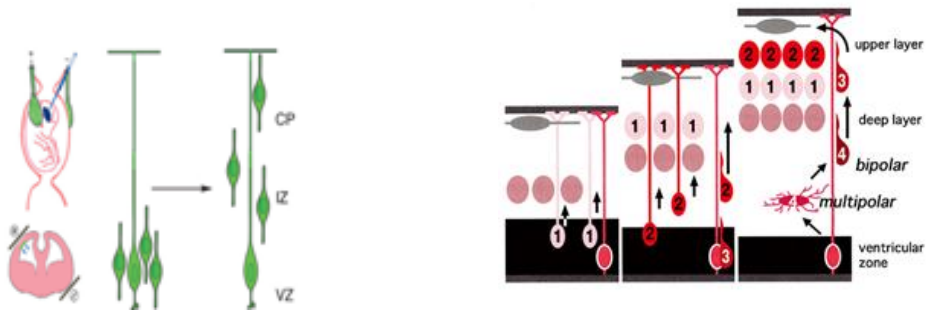


Figure 1: A,B, *Diagram of in-utero electroporation in embryonic mouse brains and the subsequent neuronal migration of the transfected neural progenitor from the VZ layer.*

Glass micropipettes were loaded with 10 µl of plasmids of one condition. Stereotaxic injection dispensed ~1 µl plasmid into the lateral ventricles using an Eppendord Femtojet Pressure Injector. Electrode paddles sent electrical currents across the uterus to permeabilize neuronal progenitor cells linking the VZ surface and allow the cells to uptake the plasmids. The uterus was returned to the IP space and the mother was sutured and placed on a warm isothermal pad to recover. Three vectors at 1.5 µg/ml were used in the experiment: pCA KIAA-RFP (courtesy of Chris Fiondella) which overexpressed the KIAA0319 protein, pU6 KIAA RNAi (Courtesy: Param Murugan) which knocked down KIAA0319 expression, and the control pU6 KIAA Scramble RNAi (Courtesy: James Ackman). All vectors were cotransfected with pCA GAP43 GFP (Courtesy: James Ackman) at 1.5 µg/ml.

Surgical Procedure and Microscopy

The rats were put under isoflurane inhalation anesthesia and euthanized at post natal day 21. The animals were perfused intracardially with 4% paraformaldehyde and then stored in phosphate buffered saline at 4°C. Brains were cut at 80 µm sections using a vibratome (Leica VT1000S). Sections were analyzed with a laser scanning confocal microscope (Leica DMIRE2) using the 433 nm laser. All images were taken at 100x and 1028 x 1028 resolution and zoomed to 2X using the Leica Confocal Software. The images were processed with Adobe Photoshop CS3 and analyzed with NeuronJ v 1.4.1, an ImageJ plugin.

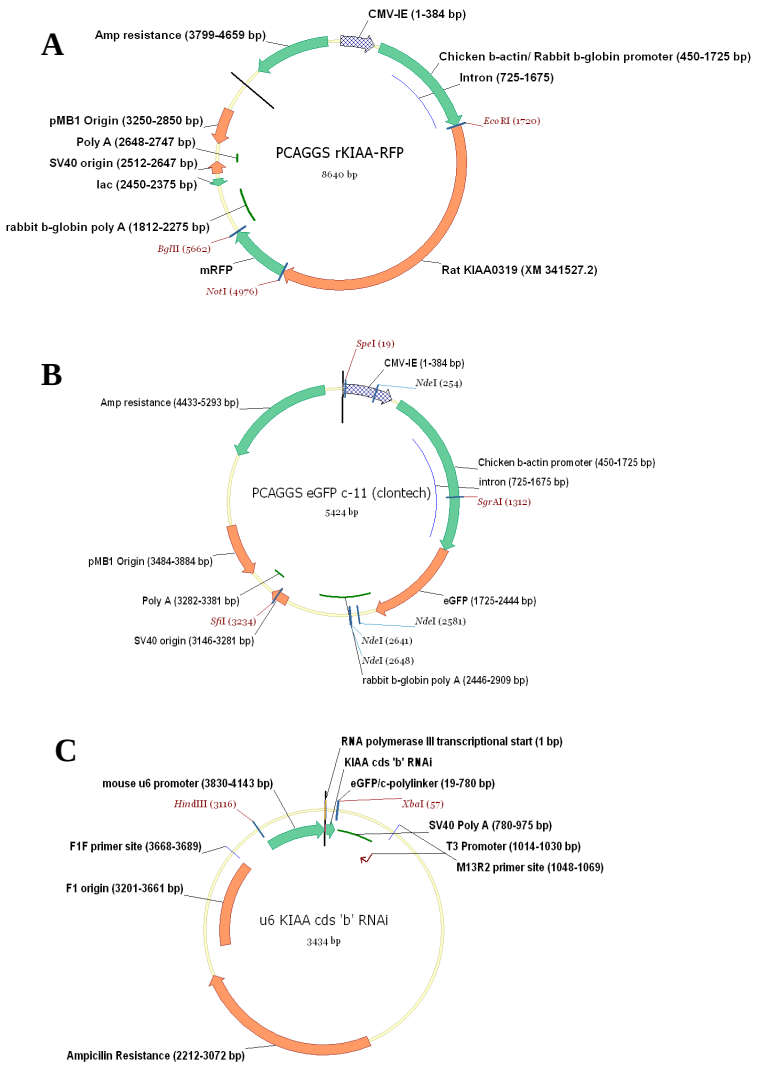


Figure 1. Vector plasmids used for *in utero* electroporation of embryonic day 15 pups and vector plasmids that were trasnfected in the VZ cells. ***A. B,*** Vectors used to modulate the expression of KIAA0319: pCA KIAA-RFP (Kiaa0319 expression plasmid) and pU6 KIAA-RNAi. ***C.*** Membrane bound green fluorescent protein co-electroporated in all conditions: pCA Gap43 GFP

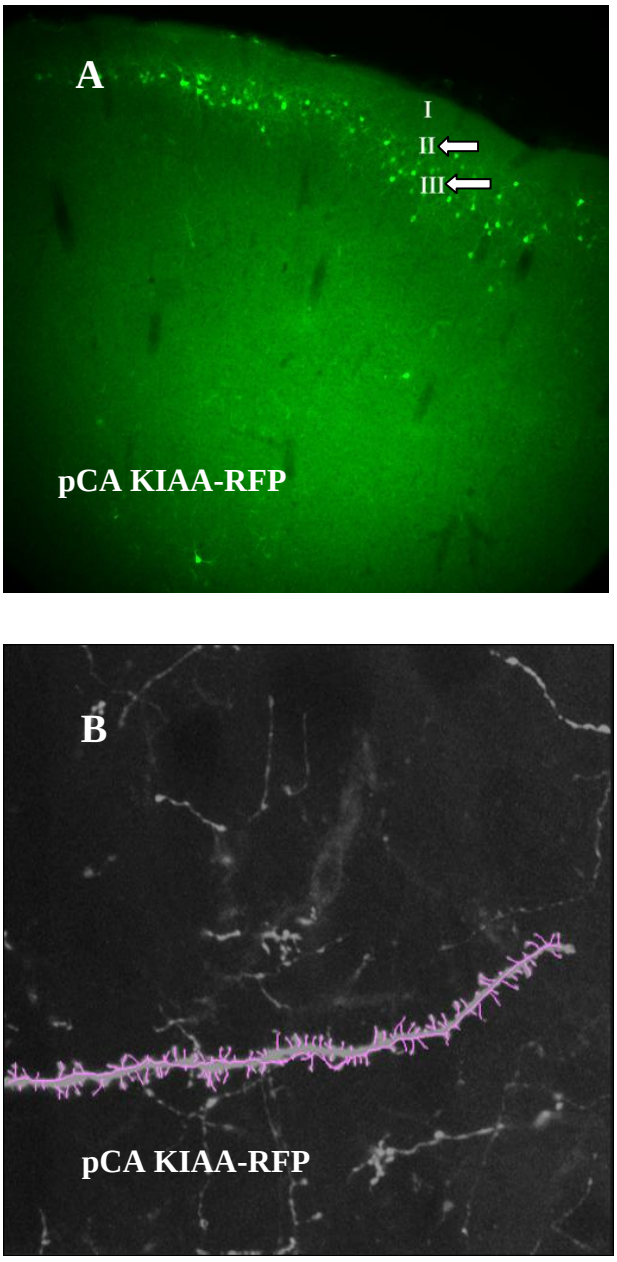


Figure 2. A. Pyramidal neurons in layers II and III were the site of interest for the dendritic spine analysis. B. An example of line tracings that were used to analyze the dendritic spine density and length

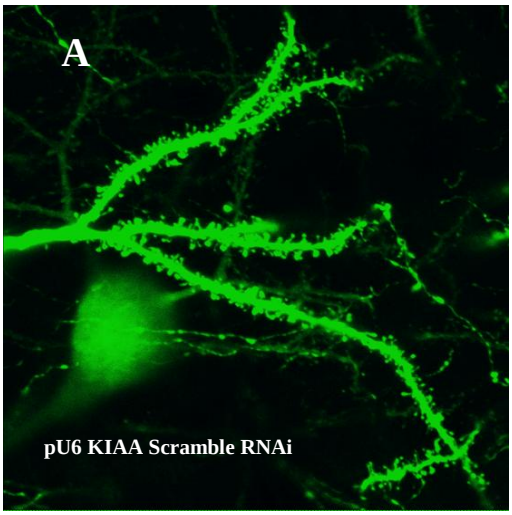
Results

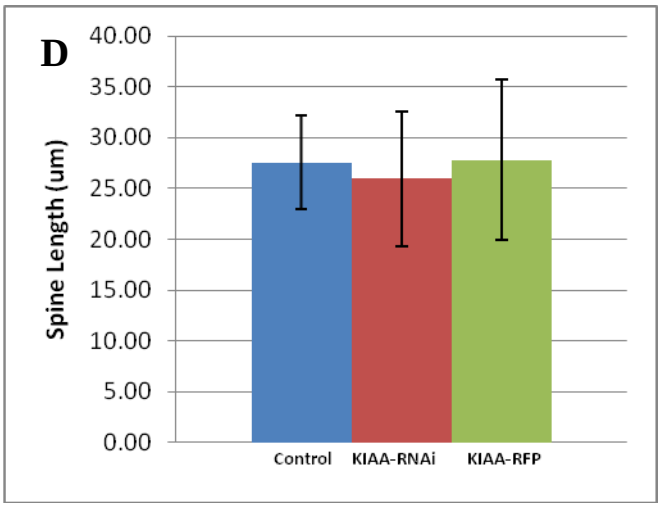
RNAi of Kiaa0319 reduces spine density of Pyramidal Neurons in layer II and III

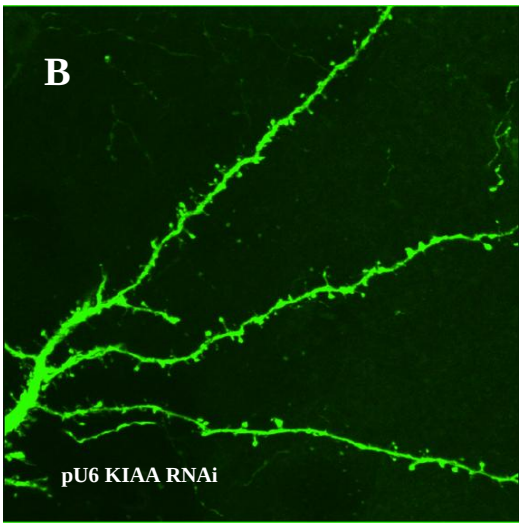
We investigated whether modulating Kiaa0319 expression may influence dendritic spines density and spine length during embryonic development. To investigate the effects of KIAA0319 expression levels on spine density and length, we knocked down and overexperssed Kiaa0319. All three plasmids were co-electroporated with pCA Gap43 GPF expression plasmids. Cells in the VZ were transfected and allowed to migrate along the radial glia towards the pial surface. 42 dendrites in layer II and III neurons across the three conditions were imaged and tracings of the dendrite process and spines were made (see Fig.3B). We found that the mean spine density of RNAi of KIAA0319 (6.33±.77 spines/µm) was significantly reduced ($P = 1.42 \times 10^{-5}$) from that of the control (8.08±0.82 spines/µm). The comparison between mean spine density of KIAA-RFP (7.04 ± 1.88 spines/µm) and the control showed that there was not a significantly difference ($P = 0.24$), indicating that the KIAA0319 expression plasmid had no significant effect on development of dendritic spines.

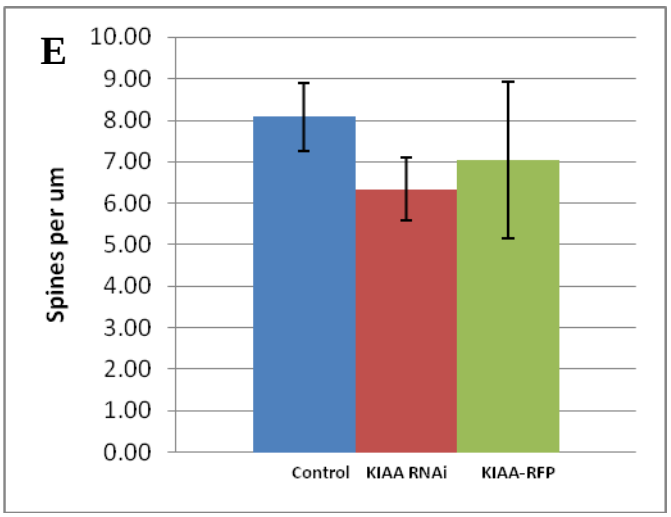
Modulating KIAA0319 expression does not have significant effects on spine length

To examine the effects of KIAA0319 expression on dendritic spines length, the same tracings used to quantify spine density were used to calculate mean spine length. We found no significant difference ($P = 0.287$) between the mean spine length in the









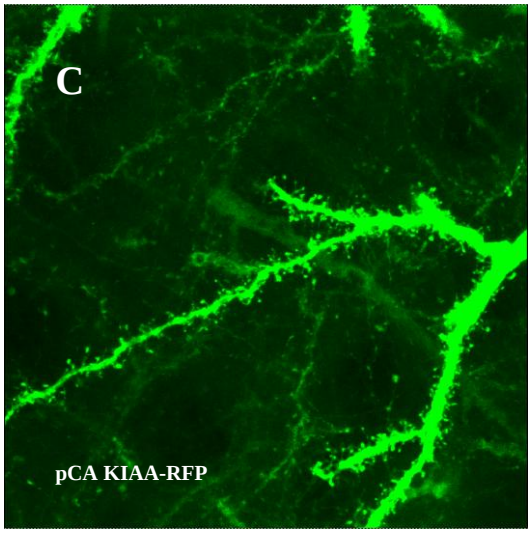


Figure 3. RNAi of Kiaa0319 reduces spine density. ***A,B,C*** 100x images of a single dendrite process, dendritic spines and its corresponding tracings used to analyze spine characteristics. ***D,E,*** Comparison showing the reduced spine density in the RNAi of Kiaa0319.

RNAi of KIAA0319 (23.43±2.29 µm) and control (25.17±1.49 µm). Similarly, the KIAA0319 over-expression plasmid (24.76±3.89 µm) was not significantly different (P = 0.721) from the control.

Discussion

The rationale for modulating Kiaa0319 expression

Although we have gained a greater understanding of dyslexia since candidate dyslexia susceptibility genes like Kiaa0319 were discovered, we still are not entirely certain of what exactly happens in these genes that would lead to the development of dyslexia and the abnormal brain morphology that typify dyslexic brains (Galaburda *et al*., 2006). Current data has suggested that the Kiaa0319 gene codes for proteins that are possibly involved in neuronal migration, which is a critical step for the development of normal brain morphology (Parachinni *et al.,* 2006; Kandel *et al.,* 2000). Normal development of brain morphology is necessary for proper wiring of the neural circuitry that would allow individuals to perform complex cognitive tasks like reading (Kandel *et al.,* 2000). In contrast, failure of normal brain development would interfere with normal brain activity and consequently prevent a person from performing certain cognitive tasks. The cortical malformations found in dyslexic brains are believed to be the result of neuronal migration deficits (Galaburda *et al.,* 2006). Furthermore, neuronal migration deficit and its consequent role in the cortical malformation phenotype are believed to somehow be associated with Kiaa0319 (Paracchini *et al.,* 2006). Studies have not consistently shown that mutation to the Kiaa0319 gene are responsible for this abnormal phenotype, therefore it is possible that neuronal migration deficit may be caused by altered levels of Kiaa0319 expression (Galaburda *et al.,* 2006). Henceforth, we performed both overexpression and knockdown experiments to see if Kiaa0319 played a role in dendritic spine morphogenesis.

Knocking down Kiaa0319 in embryonic mouse brains reduces dendritic spine density of pyramidal neurons in cortical layers II and III

We found that knocking down Kiaa0319 expression in early development resulted in significant reduction in spine density. Although there currently are not any definitive findings that correlate reduced spine density and dyslexia, studies have shown correlation between reduced spines density and certain neurological disorder like schizophrenia and epilepsy (Garey, 1998; Ethell and Pasquale, 2006). The structure and function relationship of dendritic spines are that the spines are small protrusions found along the length of the dendrite processes and are the site of excitatory electrical transmission. Consequently, any alterations in the formation of dendritic spines including

reduced dendritic spine density would likely affect the transmission of excitatory electrical signals between neurons. For example, fMRI studies of patients with the reduced spine density phenotype (i.e. schizophrenia and epileptic patients) have been observed to produce different brain activation pattern from that of a normal brain. Although more experiments will need to be performed before any definitive conclusions can be made, it is possible that the abnormal brain activation patterns observed in fMRI studies of dyslexic brains may be attributed to the reduced spine density phenotype observed in the Kiaa0319 knockdown experiment.

Knocking down Kiaa0319 does not significantly alter the dendritic spine length

We also found that knocking down Kiaa0319 expression did not significantly affect the dendritic spine length. From the current understanding of Kiaa0319, we can only speculate why spine length was not significantly altered by the gene's reduced expression. Current literature has shown that dendritic spine length is regulated by multiple factors. For example, proteins like Rac1, Cdc42, and RhoA have been shown to regulate the spine length through regulating actin, the major cytoskeletal component found in dendritic spines (Ethell and Pasquale, 2005). It would be interesting to see if the Kiaa0319 protein somehow facilitated the dendritic spine length, however the finding from our study suggests that Kiaa0319 expression is probably not involved in regulating the actin in dendritic spine nor any other factor that would otherwise regulate dendritic spine length.

Knocking down Kiaa0319 expression may be more involved in the genetic mechanism causing dyslexia than overexpression

Although we do not know the exact genetic and molecular mechanisms by which Kiaa0319 can cause dyslexia, the collection of current findings seems to suggest that dyslexia is possibly attributed to the knocked down of Kiaa0319 expression rather than an overexpression. For instance, the morphological malformations found in post mortem dyslexic brains have been attributed to neuronal migration deficits, which animal models showed that it was a knockdown of Kiaa0319 that produced this phenotype, not an overexpression (Parachinni *et al.*, 2006). In addition, knocking down Kiaa0319 has also shown to place migrating neurons in an orthogonal position, which has been speculated to be a possible neurological mechanisms in the development of dyslexia (Parachinni *et al*., 2006). In this study, we found that knocking down Kiaa0319 significantly affected the dendritic spine density rather than the overexpression. It is possible that the accumulating data of the various ways RNAi

of Kiaa0319 affect pyramidal neurons during neurodevelopment indicates that a knock down of Kiaa0319 expression is more involved, than the over expression, in the mechanism by which the abnormal phenotypes of dyslexic brain morphology and consequent inability to perform cognitive tasks like reading will be manifested.

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