The Dyslexia-associated gene *KIAA0319L* is involved in neuronal migration in the developing chick visual system

JASON CHARISH[#,1,2,3], HIDEKIYO HARADA[#,*,1,2], XIAOYAN CHEN[1,2,3],
THOMAS WÄLCHLI[4,5,6,7], CATHY L. BARR[1,3,8], PHILIPPE P. MONNIER[*,1,2,3,9]

[1]*Krembil Research Institute, Toronto, Ontario, Canada,* [2]*Donald K. Johnson Research Institute, Toronto, Ontario, Canada,* [3]*Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada,* [4]*Group Brain Vasculature and Perivascular Niche, Division of Experimental and Translational Neuroscience, Krembil Brain Institute, Krembil Research Institute, Toronto Western Hospital, University Health Network, University of Toronto, Toronto, Ontario, Canada,* [5]*Division of Neurosurgery, Department of Surgery, Toronto Western Hospital, Toronto, Ontario, Canada,* [6]*Group of CNS Angiogenesis and Neurovascular Link, Neuroscience Center Zürich, and Division of Neurosurgery, University and University Hospital Zürich, Switzerland,* [7]*Division of Neurosurgery, University Hospital Zürich, Zürich, Switzerland,* [8]*Program in Neurosciences and Mental Health, Hospital for Sick Children, Toronto, Ontario, Canada,* [9]*Department of Ophthalmology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada*

ABSTRACT **The gene KIAA0319-Like (KIAA0319L) is thought to confer susceptibility for developmental dyslexia. Dyslexia may be caused by alterations in neuronal migration, and in utero knockdown of KIAA0319L in rats indicated migration errors. However, studies carried out with KIAA0319L knockout mice did not reveal an altered neuronal migration phenotype. Gene knockout may activate compensatory mechanisms to buffer against genetic mutations during development. Here we assessed the role of KIAA0319L on migrating neurons in the chick developing tectum. Whole mount in situ hybridization was performed for KIAA0319L on embryonic day (E)3 – E5 chick embryos and in situ hybridization on sections was performed at later stages. The specificity and efficiency of engineered microRNA (miRNA) constructs targeting KIAA0319L for knocking down KIAA0319L were verified. miRNAs were electroporated into E5 chick optic tecta. Our studies demonstrate that KIAA0319L is expressed in the developing chick visual system, as well as in the otic vesicles. Knockdown of KIAA0319L in the optic tectum results in abnormal neuronal migration, strengthening the argument that KIAA0319L is involved in this developmental process.**

KEYWORDS: *cell migration, brain development, KIAA0319L, optic tectum*

Introduction

KIAA0319-like (KIAA0319L) has been identified as a susceptibility gene for developmental dyslexia (Couto *et al.,* 2008). Dyslexia is one of the most common of the complex neurobehavioral disorders, affecting 5-10% of school aged children (Katusic *et al.,* 2001). *KIAA0319L* has also been associated with language impairment and IQ in autism spectrum disorders (Wang *et al.,* 2013; Eicher and Gruen 2015). *KIAA0319L* shares homology with *KIAA0319*, which is one of the four initial identified candidate dyslexia susceptibility genes: *KIAA0319 (*Cope *et al.,* 2005), *DYX1C1 (*Taipale *et al.,* 2003), *ROBO1* (Hannula-Jouppi *et al.,* 2005) and *DCDC2* (Schumacher *et*

al., 2006). In addition to dyslexia, variations in *KIAA0319* are also associated with cortical thickness reductions in language areas of the brain in patients with frontotemporal dementia (Paternicó *et al.,* 2016).

The protein product of the *KIAA0319L* gene (*KIAA0319L*; AU040320 in mice), which has been identified as the entry receptor for adeno-associated virus (Pillay *et al.,* 2016), is predicted to be a single-pass transmembrane protein with an N-terminal cysteine rich domain and an extracellular region consisting of several PKD domains. PKD domains are involved in strong calcium independent homophilic interactions and intercellular adhesion (Ibraghimov-Beskrovnaya *et al.,* 2000). Little is known regarding

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[#]These authors contributed equally to this work.

*Address correspondence to: Hidekiyo Harada. Krembil Research Institute, KDT 8-417, 60 Leonard St., Toronto M5T 2S8, Ontario, Canada.
E-mail: hidek.harada@gmail.com | https://orcid.org/0000-0001-6758-4141
Philippe P. Monnier. Krembil Research Institute, KDT 8-417, 60 Leonard St., Toronto M5T 2S8, Ontario, Canada.
E-mail: Philippe.Monnier@uhnresearch.ca | https://orcid.org/0000-0002-6221-4130
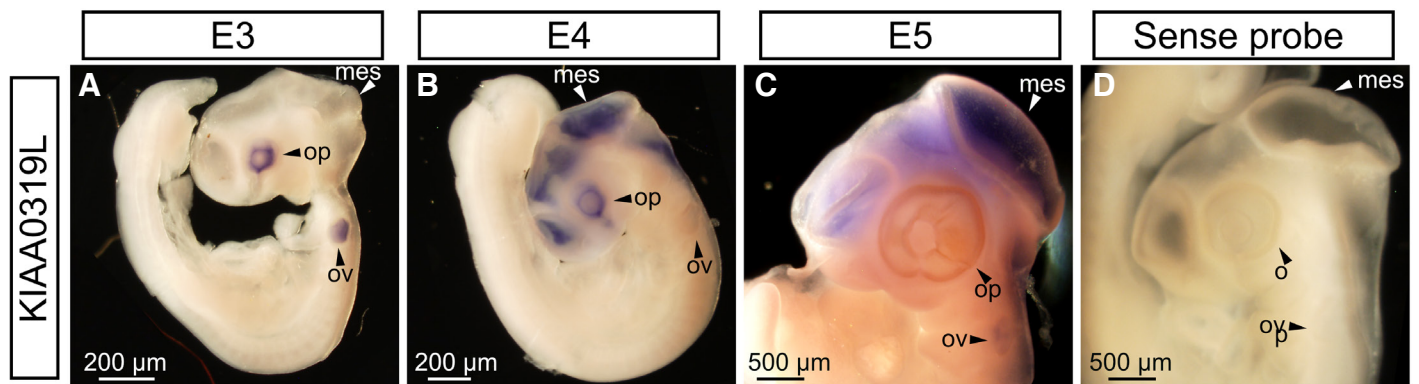
Fig. 1. KIAA0319 expression in early stage chick embryos. (A) *KIAA0319L* is expressed in the optic cups and otic vesicles at E3. **(B)** At E4, *KIAA0319L* displays strong staining in the diencephalon, mesencephalon, telencephalon, metencephalon and optic vesicle. **(C)** At E5, signal persisted in the telencephalon, mesencephalon and metencephalon. Expression in the optic vesicle is diminished. **(D)** Control sense probes gave no visible signal. mes, mesencephalon; ov, otic vesicle; op, optic vesicle.

signaling mechanisms of *KIAA0319L*, though it has been shown to interact with Nogo receptor 1 (Poon *et al.*, 2011). It was found that silencing of *Kiaa0319l* or *Kiaa0319* impaired post-mitotic cortical neuron migration in rats (Paracchini *et al.*, 2006; Platt *et al.*, 2013; Peschansky *et al.*, 2010; Szalkowski *et al.*, 2012), however knockout mouse models showed no indication of abnormal cortical neuron migration (Martinez-Garay *et al.*, 2017; Guidi *et al.*, 2017). However, *Kiaa0319* and *Kiaa0319L* knockout (KO) mice exhibited deficits in auditory processing (Guidi *et al.*, 2017). Altered auditory processing has been reported in individuals with RD (Ramus 2006; Tallal *et al.*, 1996; Strehlow *et al.*, 2006; Kujala *et al.*, 2001). To gain relevant insights on the role of *KIAA0319L*, we decided to study its role in the visual system.

For visual circuitry to function normally, the proper development of both the retina and retinorecipient targets in the brain are indispensable. The optic tectum is the major visual processing center in the non-mammalian vertebrate brain and is the primary synaptic target of retinal ganglion cell axons. In chick, the adult optic tectum consist of sixteen laminae: SO (stratum opticum – fibers of the optic tract), SGFS (stratum griseum et fibrosum superficiale, a, b, c, d, e, f, g, h, i, j), SGC (stratum griseum central – the principle efferent), SAC (stratum album central), SGP (stratum griseum periventriculare), SFP (stratum fibosum periventriculare) and the ependymal layer (LaVail and Cowan 1971; Mey and Thanos 2000).

The manner of postmitotic cell migration in the developing chick optic tectum differs from that seen in the cerebral cortex. In the cortex, an "inside-out" pattern of migration generally occurs where the earliest born neurons are found in the deepest cortical layers, while later born neurons are found in the more superficial layers (McConnell 1988; Jackson, Peduzzi, and Hickey 1989). The laminar structure of the chick optic tectum on the other hand is the result of three migration waves (LaVail and Cowan 1971). The first wave takes place between E4 and E6 and forms the inner laminae (SGC, SAC, SGP, and SFP), while the second wave takes place between E4 and E8 and forms the outer laminae (SGFS a–g); together, neurons from these first two waves are termed early migratory neurons. The third wave (E5-E9) splits the neuronal layers formed by the first and second waves in order to make up the middle lamina. These neurons are termed late migratory neurons and form laminae h–j of SGFS (LaVail and Cowan 1971; Sugiyama and Nakamura 2003).

Here we show that *KIAA0319L* is expressed in the developing chick visual and auditory systems during time points suggestive of roles both within and possibly outside of neuronal migration. Using silencing with specific miRNAs, we provide evidence that KIAA0319L is involved in neuronal migration in the chick visual system development.

Results

KIAA0319-like is expressed in the visual system in developing chick embryos

To explore *KIAA0319L* expression in the developing chick, we first performed *in situ* hybridization on whole mount embryos. At E3, *KIAA0319L* mRNA was observed predominantly in the optic cup as well the otic vesicles (Fig. 1A). This time point corresponds to the onset of neurogenesis in the developing chick eye and ear (Aburto *et al.*, 2012; Doh *et al.*, 2010). Sense probe controls did not show any signal, indicating that the anti-sense probe specifically detects *KIAA0319L* mRNA (Fig. 1D). By E4, *KIAA0319L* had commenced expression in the brain, with signal present in the myelencephalon, metencephalon, mesencephalon (optic tectum), diencephalon, telencephalon and the neural tube. This time point corresponds to the beginning of radial migration in the optic tectum (LaVail and Cowan 1971; Sugiyama and Nakamura 2003; Guidi *et al.*, 2017). Expression in the developing eye remained strong at E4, whereas expression in the otic vesicles became reduced relative to the expression in the eye and brain (Fig. 1B). By E5, expression in the developing eye became reduced relative to expression levels in the brain, with the most prominent signal being in the developing optic tectum (Fig. 1C). The majority of retinal lamination and neuronal migration takes place following E4 (Doh *et al.*, 2010), suggesting that *KIAA0319L* may be involved in non-migration functions in this developing tissue.

We next sought to analyze expression of *KIAA0319L* mRNA at later ages in the optic tectum in order to gain insight into what function this gene may play in visual system development. We carried out *in situ* hybridization on cryosections of the E9 optic tectum. *KIAA0319L* was expressed in deep and middle layers of the optic tectum uniformly along the rostro-caudal axis (Fig. 2 A-F). Within the middle layer, *KIAA0319L* was localized in the layer iii and layer

iv (Fig. 2 C-F). To determine *KIAA0319L* mRNA localization in the deeper layers, we inverted the *KIAA0310L in situ* hybridization picture taken in bright field and merged it with a nuclear stain (DAPI) (Fig. 2 G-I). This analysis confirmed that *KIAA0319L* was expressed in the ventricular zone and the subventricular layer.

Specific and effective knock down of *KIAA0319L*

To investigate *KIAA0319L in-vivo* function during development, four synthetic miRNA constructs targeting *KIAA0319L* were generated based on modified primers according to Das *et al.*,(Das *et al.,* 2006a). Given that the homologous gene *KIAA0319* is also expressed in the chick visual system (Fig. 3B'), we sought to exclude the possibility that our miRNA constructs have non-specific effects by analyzing whether our miRNA constructs targeting *KIAA0319L* have any effect on *KIAA0319* expression.

To assess the specificity and effectiveness of our synthetic miRNAs, miRNAs targeting *KIAA0319L* were electroporated to the right optic vesicle *in ovo* at E1.5. Optic vesicle electroporation enables quicker miRNA evaluation compared to optic tectum electroporation given *KIAA0319L* expression commences at an earlier time point in the optic vesicle (Fig. 1A). Since the miRNA expression vector contains an RFP expression cassette on the same vector, RFP expression levels indicate the relative strength of the miRNA expression level. Two days after electroporation, we selected embryos with high RFP expression levels in optic vesicle. Next, we carried out *in situ* hybridization against *KIAA0319L* or *KIAA0319* on whole mount embryos.

Among the miRNAs that we screened, two miRNA constructs, namely miKIAA0319L1 and miKIAA0319L2, exhibited effective knockdown of *KIAA0319L* expression (Fig. 3 A,A'). To confirm
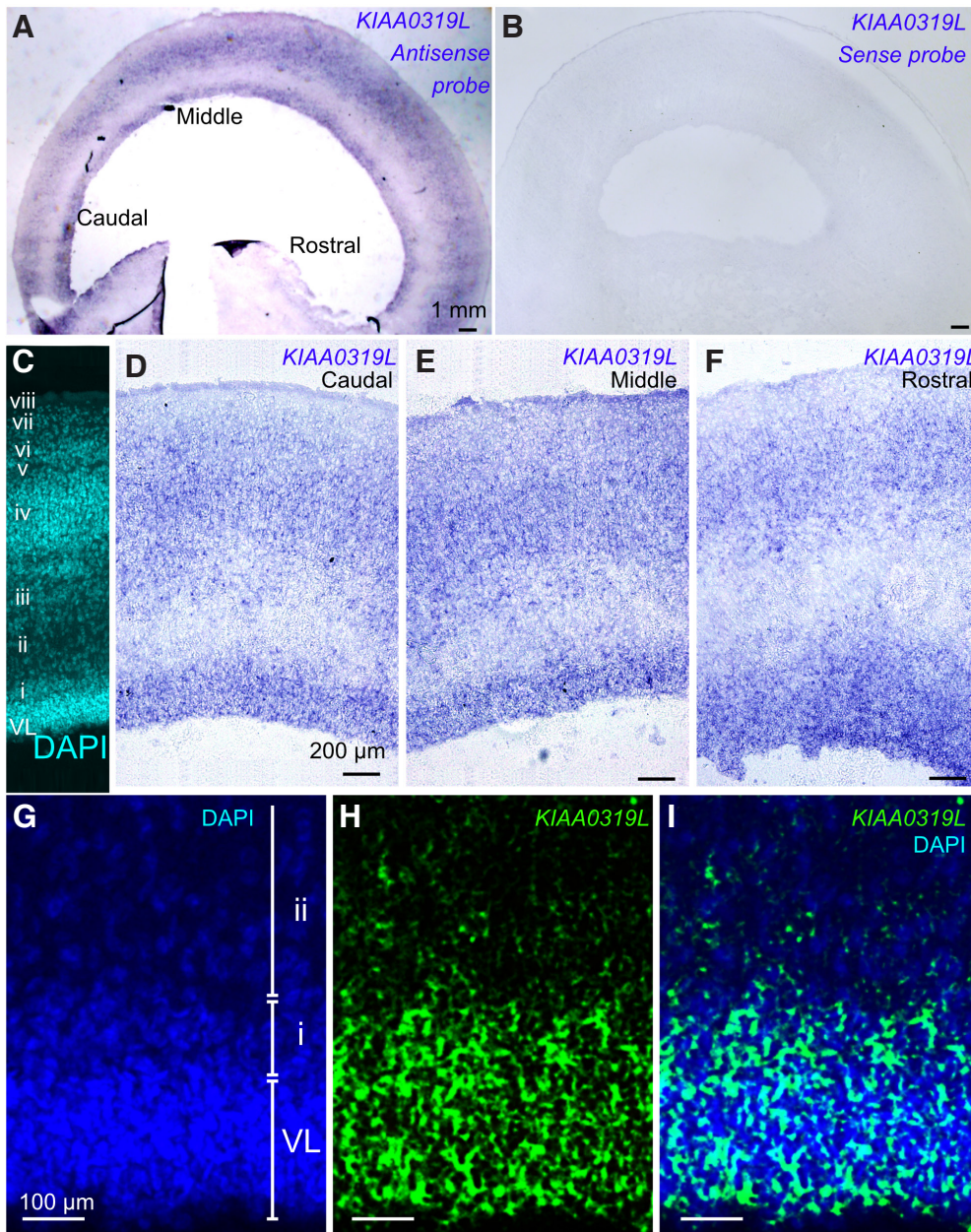

Fig. 2. *KIAA0319L* expression in the E9 chick optic tectum. (A) *KIAA0319L* expression in the E9 chick optic tectum along the rostral-caudal axis. Relatively strong signal is seen in the ventricular layer and in the middle layers as indicated by *in situ* hybridization. **(B)** Sense control does not display any signal. **(C)** DAPI staining (cyan) displays layer structure of the E9 optic tectum. The ventricular layer (VL) and layers i to viii can be observed. **(D-F)** *KIAA0319L* expression as seen under higher magnification in the caudal, middle and rostral position in the optic tectum. *KIAA0319L* expression is observed in the deeper layer, layer iii and layer iv. **(G-I)** High power magnification of the deeper layer. The bright field picture is inverted and is given a pseudo-color (green: *KIAA0319L*) and merged to DAPI (blue). *KIAA0319L* expression overlaps with the ventricular layer as well as with layer i (sub-ventricular layer).
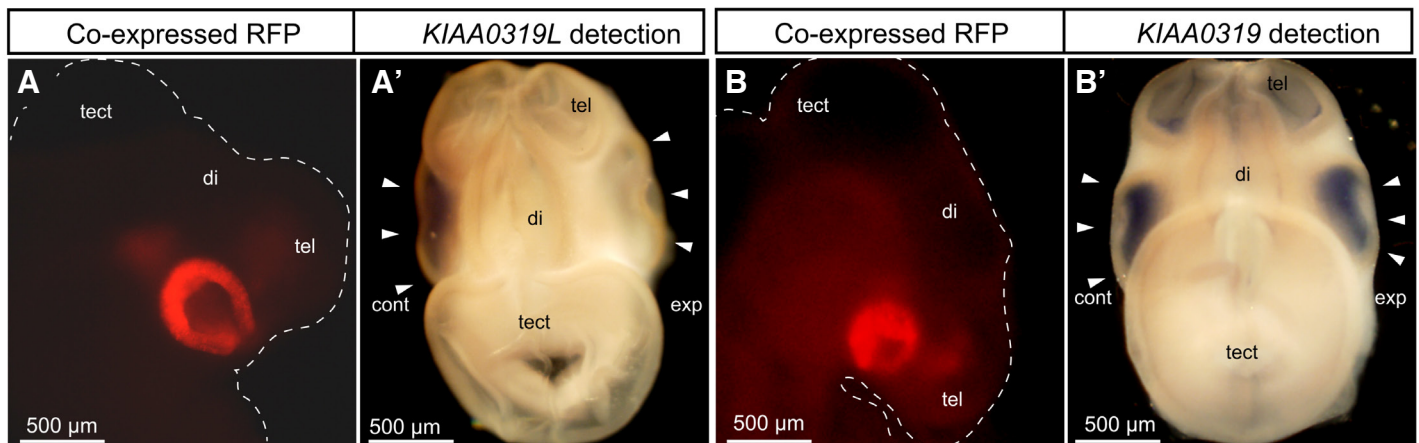
| Co-expressed RFP | *KIAA0319L* detection | Co-expressed RFP | *KIAA0319* detection |

Fig. 3. Specificity and effectiveness of miKIAA0319 constructs. (A) A lateral view of an E3.5 miKIAA0319L electroporated embryo. Red Fluorescent Protein (RFP) indicates successful electroporation. **(A')** A dorsal view of the miKIAA0319L electroporated embryo. KIAA00319L expression is not detected in the right eye (arrowhead), while strong signal is observed in the control side eye, demonstrating effective knockdown of KIAA0319L in the right eye (arrowhead). **(B)** A lateral view of an E3.5 miKIAA0319L electroporated embryo. RFP indicates successful electroporation. **(B')** A dorsal view of the miKIAA0319L electroporated embryo. KIAA0319 (a gene homologous to KIAA0319L), expression is detected in the miKIAA0319L transfected eye (arrowhead) at the same level of the control side eye (arrowhead), demonstrating specific knockdown of *KIAA0319L*. Abbreviations: cont, control side; di, diencephalon; exp, experimental side; tect, optic tectum; tel, telencephalon.

the specificity of *KIAA0319L* knockdown, we electroporated the *KIAA0319L* miRNAs and subsequently analyzed the effect of these miRNAs on the expression of *KIAA00319*, a homologous gene of *KIAA0319L*. Neither miKIAA0319L1 nor miKIAA0319L2 altered *KIAA0319* expression when the transfected retina was compared to the control side, even when high levels of RFP expression were observed (Fig. 3 B,B'). These results indicate that these miRNA constructs specifically silence *KIAA0319L* gene expression. These experiments also confirm the specificity of the *KIAA0319L* probe used in our *in situ* hybridization analysis.

Silencing *KIAA0319L* affects cell migration during tectal layer formation

In order to study the role of *KIAA0319L* protein during chick visual system development, the two independent miRNAs targeting *KIAA0319*L were electroporated into the ventricular zone of the E5 optic tectum. Since the ventricular zone at the E5 optic tectum is a mixture of early and late migratory cells, miKIAA0319L can be applied to the both cell population by *in ovo* electroporation (Sugiyama and Nakamura 2003). Following electroporation, the presence of RFP is indicative of cells that have been successfully electroporated with the miRNA construct. Five days after electroporation (E12), the embryos were sacrificed and analyzed for the presence of RFP in each layer in of the optic tecta. In controls at E12, the majority of cells labeled at E5 with RFP migrated away from the VZ and were predominantly located in upper layers of the optic tectum (Fig. 4 A-C). In contrast, miRNA treated cells showed a more even distribution pattern at E12 with a significant amount of cells remaining near the VZ (Fig. 4 D-F).

We then carried out statistical analysis in each layer of the optic tectum recognized at this stage of development. The amount of miKIAA0319L1 or mi KIAA0319L2 transfected cells was significantly increased in the SAC, SVL (indicated by **) and VZ (Fig. 4 G, P<0.05) compared to control miRNA electroporated embryos. In controls, 2.6±1.7% of electroporated RFP was located in the SAC, whereas miKIAA0319L-1 and miKIAA0319L-2 had 8.6±1.7%

and 11.8±2.9% respectively. In the control miRNA experiments, 0.2±0.2% of electroporated RFP was located in the SVL, whereas miKIAA0319L-1 and miKIAA0319L-2 had 11.2±4.2% and 4.3±1.7% respectively. With respect to the VL, control miRNA had 0.2±0.2% of electroporated RFP in this layer, whereas miKIAA0319L1 and miKIAA0319L2 had 9.6±3.8% and 13.7±5.4% respectively. In the upper layers, only miKIAA0319L1 showed significant decrease in the layer a-d/SGFS. In the control miRNA experiment, 13.4±0.2% of electroporated RFP was located in the a-d/SGFS, whereas miKIAA0319L-1 and miKIAA0319L-2 had 5.5±2.8% (P<0.05) and 9.1±3.9% (P>0.05) respectively. This quantitative analysis demonstrates that knockdown of *KIAA0319L* resulted in a significant increase in RFP localized in deeper layers of the optic tectum (SAC, SVZ and VZ). Together, these data suggest that *KIAA0319L* might be involved in cell migration to the upper layers and/or neuronal differentiation in the chick optic tectum.

Discussion

Developmental dyslexia is a disorder characterized by difficulties with reading, despite adequate intelligence and education. Here we investigated the expression profile and function of the dyslexia-associated gene *KIAA0319L* in the developing chick embryo. To do this, *in situ* hybridization analysis was performed on whole mount chick embryos aged E3 to E5, and on sections of the E9 optic tectum. Data from these experiments indicate that *KIAA0319L* is expressed at various time points during visual system and otic vesicle development. In the otic vesicle *KIAA0319L* is expressed at E3 and stops at E5. Interestingly, this period corresponds to the initiation of neurogenesis in this organ (Adam *et al.,* 1998). Although, this study focuses on the role of *KIAA0319L* in the developing visual system, this expression pattern may provide some insights into why mice that have loss of *KIAA0319L* present a deficit in auditory processing (Guidi *et al.,* 2017). In the visual system, we observed that *KIAA0319L* mRNA begins to be expressed in the optic tectum during a time point coinciding with
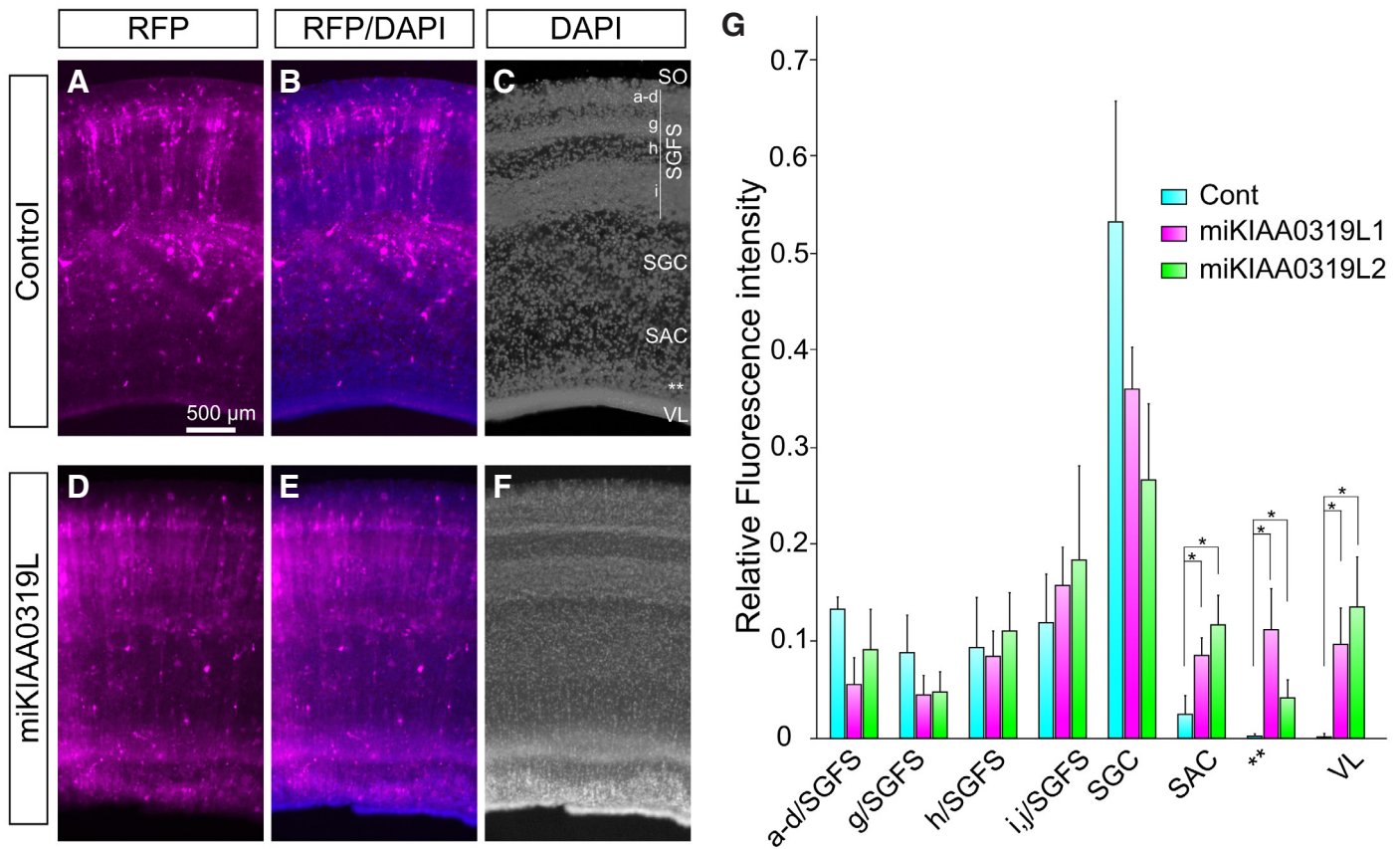
Fig. 4. Location of E5 labeled cells at E12 in control and miRNA transfected optic tectum (OT). *in ovo* electroporation was used to target miRNA constructs to the ventricular layer of the OT at E5. OT were harvested at E12 and the migratory pattern of electroporated cells was assessed. **(A-C)** Control miRNA-transfected OT at E5. Control miRNA was transfected at E5 and the distribution of the control miRNA-transfected cells indicated by red fluorescent protein (RFP) was observed at E12. **(D-F)** miKIAA0319L-transfected optic tectum at E12. miKIAA0319L was transfected at E5 and the miKIAA0319L-transfected cells indicated by RFP were observed at E12. **(G)** Quantification of RFP intensity in the each layer of the OT. RFP intensity was significantly increased in the SAC, subventricular layer (**), and ventricular layer. Abbreviations: RFP, red fluorescent protein; SAC, stratum album centrale; SGC, stratum griseum centrale; SGFS, stratum griseum et fibrosum superficiale; SO, stratum opticum; VL, ventricular layer; **, subventricular layer. * P<0.05.

the onset of radial post-mitotic neuronal migration. On the other hand, retinal expression of *KIAA0319L* mRNA coincides with the beginning of neurogenesis and subsequently diminishes during time points coinciding with laminar development, which suggests *KIAA0319L* participates in additional functions beyond neuronal migration. Within the developing E9 optic tectum, *KIAA0319L* is expressed by the cells in layer iii, layer iv, the SVZ and the VZ (3). Also, as indicated by the WISH experiments, the broad expression profile of *KIAA0319L* is similar to that of *KIAA0319*, suggesting that they may play similar or complementary functions during visual system development.

Between E5 and E12, cells primarily migrate in a radial direction away from the VZ. Therefore, to characterize the involvement of *KIAA0319L* in this process, undifferentiated cells lining the VZ of the E5 optic tectum were electroporated with miRNAs targeting *KIAA0319L*, and the distribution pattern of these electroporated cells was observed at E12. RFP positive cells at E12 were considered to be indicative of cells in which *KIAA0319L* had been knocked down. Significant difference were prominent in the deepest layers, with *KIAA0319L* silencing resulting in significant increase in RFP presence in VZ, SVZ and SAC, with a trend towards decreased RFP in

the SGC as well as a trend towards decreased RFP in superficial layers of SGFS (a-g). This suggests that *KIAA0319L* is involved in waves I and II of tectal migration by early migratory neurons. We did not observe any significant changes in the middle layer cell numbers, indicating that *KIAA0319L* in not required for migration of late migratory neurons in wave III.

It has been shown that Grg4, a co-repressor of Engrailed, confers the late migratory neuron cell in the ventricular layer of the optic tectum. Overexpressing Grg4 changes alters the normal migratory destination of the neurons (middle layers; h, i, and j of the SGFS) (Sugiyama and Nakamura 2003). Moreover, overexpression of Engrailed-2 (En2) alters the normal destination of early migratory neurons from the superficial layers of a-g/SGFS to middle layers (h, I and j of SGFS)(Omi and Nakamura 2015). Grg4 and En2 are critical for post-mitotic cellular migration towards the middle layers (wave III)(Omi and Nakamura 2015), however the downstream genes that serve to regulate cellular migration at the cell surface have yet to be identified. Since the Grg4 and En2 are transcription repressors, one possibility is that KIAA0319L is downregulated by the Grg4/En2 complex in order to switch the default destination from the superficial layers to the middle layers.

With respect to developmental dyslexia, the suggestion that impaired neuronal migration may act as a cellular cause of dyslexia initially came from post-mortem identification of abnormalities in cortical neuron organization in language areas of the brain in individual with dyslexia (Galaburda 1992, 1994). This was followed by the identification of the aforementioned dyslexia susceptibility genes and subsequent RNAi experiments in the rat neocortex, which identified KIAA0319, KIAA0319L, DYX1C1, and DCDC2 as all playing roles in neuronal migration. However, genetic mouse models in which these genes were knocked out, alone (Martinez-Garay et al., 2017; Wang et al., 2011; Guidi et al., 2017; Rendall et al., 2017) or, in the case of KIAA0319 and KIAA0319L, in combination (Guidi et al., 2017), failed to reproduce the expected neuronal migration phenotypes.

It is not uncommon to have discrepancies between knockdown and knockout models, with potential contributing factors including i) interspecies differences, ii) differences in dynamics between RNAi techniques and Cre-recombinase, iii) off target effects of shRNA constructs or iv) compensation in genetic models. For example, it is possible that KIAA0319L knockout mice display genetic compensation through transcriptional adaptation, which has previously been observed in genetic models, but not following RNAi mediated knockdown. Indeed, in a mouse knockout model of Dcdc2, the Dcx gene, a member of the same gene family, functionally compensates for the loss of the Dcdc2 gene (Wang et al., 2011). While it still remains unclear why KIAA00319 and KIAA0319L knockout mice fail to present with altered neuronal migration phenotypes, our present findings add to this picture by indicating that KIAA0319L does play a role in neuronal migration/differentiation in the developing chick visual system.

Materials and Methods

Preparation of chick eggs

Fertilized eggs (White Leghorn) were obtained from a local supplier (Frey's Hatchery, St. Jacobs, Ontario), stored at 4°C for a maximum of 2 weeks. Eggs were incubated at 38 ˚C and 60% humidity.

KIAA0319L constructs

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to generate 500bp *KIAA0319* fragment and 700bp *KIAA0319L* fragment and cloned into pGEM T-easy vector (Promega, Madison, Wisconsin). Antisense probes were linearized with *Kpn*I and transcribed with Sp6 RNA Polymerase (Roche, Basel, Switzerland) while sense probes were linearized with *Hind*III and transcribed with T7 RNA polymerase (Roche).

miRNA hairpin sequences against *KIAA0319L* (target sequence of miKIAA0319L1;ATGTCAGCTGAACTGCTCTGA, miKIAA0391L2; TGTCAACGTTATTGTCAAAGA) was prepared and cloned into a miRNA operon expression cassette (MOEC) vector that contains the reporter gene RFP (Red Fluorescent Protein).

In situ Hybridization

In situ hybridization was performed as described previously (Banerjee *et al.,* 2016). Briefly, for whole mount *in situ* hybridization, embryonic day 3 (E3), E4 and E5 chick embryos were fixed overnight in 4% paraformaldehyde. Embryos were processed as described previously. Developing reaction took place with 125 µg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 250 µg/ml of 4-nitoro blue tetrazolium chloride (NBT) in NTMT (0.1M NaCl,

0.1M Tris pH9.5, 0.04 M MgCl2, 2 mM levamisole, 0.1% Tween20).

For *in situ* hybridization on section, E9 and E12 embryos were dissected and frozen in Tissue Tek OCT compound (Sakura, Torrance, California), then sectioned at 14 µm, then were processed described previously (Banerjee *et al.,* 2016). Color development took place in NBT and BCIP in 0.1M Tris pH9.5, 0.1M NaCl, 50mM MgCl2, levamisole. Tissue sections were then stained with DAPI (0.1ug/mL).

miRNA constructs

miRNA hairpin sequences were prepared with PCR using modified primers from (Das *et al.,* 2006b). The PCR product was then inserted into a miRNA operon expression cassette (MOEC) inside a vector that also contains red fluorescent protein (pRFPmiRNA). The pRFPmiRNA plasmids targeting KIAA0319L were sent for sequencing. Midipreps were performed on DNA so as to obtain the plasmids with a concentration of 1.5 µg/µl, which is ideal for electroporation.

In ovo electroporation

In ovo electroporation in optic vesicle at E1.5 (HH10-12)

The use of fertilized eggs is not subject to any ethic approval. Electroporation of miRNA plasmid vectors was carried out as we described previously with some minor modifications (Harada *et al.,* 2019). Briefly, eggs were incubated for 36 hours to reach around HH stage 10 (Hamburger and Hamilton) in a well-humidified chamber at 38 ˚C. DNA plasmid solution was injected into the right optic vesicle and three times square pulses were applied with CUY21 (Genetronics, San Diego, CA) at 15V, three 50 ms pulses at 950 ms intervals. The eggs were then re-incubated at 37˚C for two days. Embryos were fixed in 4% PFA at E3.5.

In ovo electroporation in the optic tectum at E5 (HH 25-27)

Eggs were incubated for five days in a well-humidified chamber. DNA plasmid solution was injected into the middle part of the optic tectum. Electroporation was performed at 8V with three 50ms pulses with 50ms intervals (Banerjee *et al.,* 2016). The embryos were resealed and incubated at 37˚C. The embryos were sacrificed at E12, fixed in 4% PFA, then 150 µm sections were prepared using a vibratome and stained with DAPI.

Competing interests
The authors declare that they have no competing interests.

Author contributions
JC performed in situ hybridization and electroporation. HH did the immunostaining and blinded quantitative analyses. XC helped generating miRNAs. TW and CB, contributed to manuscript redaction. PPM designed the study and wrote the manuscript.

Availability of data and materials
All data and materials are available upon request.

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