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EMBRYONIC DISRUPTION OF THE CANDIDATE DYSLEXIA SUSCEPTIBILITY GENE HOMOLOG *KIAA0319-LIKE* RESULTS IN NEURONAL MIGRATION DISORDERS

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Abstract—Developmental dyslexia, the most common childhood learning disorder, is highly heritable, and recent studies have identified *KIAA0319-Like* (*KIAA0319L*) as a candidate dyslexia susceptibility gene at the 1p36–34 (DYX8) locus. In this experiment, we investigated the anatomical effects of knocking down this gene during rat corticogenesis. Cortical progenitor cells were transfected using *in utero* electroporation on embryonic day (E) 15.5 with plasmids encoding either: (1) *Kiaa0319l* small hairpin RNA (shRNA), (2) an expression construct for human *KIAA0319L*, (3) *Kiaa0319l* shRNA + KIAA0319L expression construct (rescue), or (4) controls (scrambled *Kiaa0319l* shRNA or empty expression vector). Mothers were injected with 5-bromo-2-deoxyuridine (BrdU) at either E13.5, E15.5, or E17.5. Disruption of Kiaa0319l function (by knockdown, overexpression, or rescue) resulted in the formation of large nodular periventricular heterotopia in approximately 25% of the rats, which can be seen as early as postnatal day 1. Only a small subset of heterotopic neurons had been transfected, indicating non-cell autonomous effects of the transfection. Most heterotopic neurons were generated in mid- to late-gestation, and laminar markers suggest that they were destined for upper cortical laminae. Finally, we found that transfected neurons in the cerebral cortex were located in their expected laminae. These results indicate that *KIAA0319L* is the fourth of four candidate dyslexia susceptibility genes that is involved in neuronal migration, which supports the association of abnormal neuronal migration with developmental dyslexia.

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; CDSG, candidate dyslexia susceptibility gene; Chrs, Chromosomes; CP, cortical plate; DAB, 3,3-diaminobenzidine; E, embryonic day; eGFP, enhanced green fluorescent protein; IZ, intermediate zone; mRFP, monomeric red fluorescent protein; P, postnatal day; PFA, paraformaldehyde; PVNH, periventricular nodular heterotopia; RNAi, interference RNA; shRNA, small hairpin RNA; SVZ, subventricular zone; VZ, ventricular zone.

Key words: developmental dyslexia, neuronal migration, cerebral cortex, *in utero* electroporation, RNAi.

INTRODUCTION

Developmental dyslexia, the most common childhood learning disorder, is highly heritable (Peterson and Pennington, 2012). At present, nine loci have been associated with developmental dyslexia, including those on Chromosomes (Chrs) 1p36–34 (DYX8), 2p16 (DYX3), 3p13–11q (DYX5), 6q11–16 (DYX4), 6p21–22 (DYX2), 11p15 (DYX7), 12p14, 15q21 (DYX1), 18p11–q12 (DYX6) (see Scerri and Schulte-Korne, 2010; Skiba et al., 2011; Peterson and Pennington, 2012 for reviews). The DYX8 region on Chrs 1p34–36 was originally identified by linkage analysis (Grigorenko et al., 2001), and was subsequently confirmed in a set of 100 families (Tzenova et al., 2004). A candidate gene, *KIAA0319-Like* (*KIAA0319L*), was identified in this interval, which has shown strong associations with deficits in spelling, phonemic awareness, rapid naming of colors and objects, single-word reading, and lifetime dyslexia diagnosis (Couto et al., 2008). This gene is of particular interest because of its similarity to *KIAA0319*, a candidate dyslexia susceptibility gene (CDSG) previously identified at the DYX2 locus (Francks et al., 2004; Cope et al., 2005; Harold et al., 2006; Paracchini et al., 2006, 2008; Zou et al., 2012).

Studies of post-mortem dyslexic brains revealed that these brains had cerebrocortical neuronal migration disorders ranging from small heterotopia to focal microgyria (Galaburda and Kemper, 1979; Galaburda et al., 1985; Humphreys et al., 1990). More recently, magnetic resonance imaging (MRI) studies have found an association between more severe neuronal migration anomalies (periventricular nodular heterotopia) and developmental dyslexia (Chang et al., 2005, 2007). Further, all CDSGs thus far examined have been shown to function as neuronal migration genes, including *DYX1C1* (Wang et al., 2006), *DCDC2* (Meng et al., 2005), *ROBO1* (Hannula-Jouppi et al., 2005; Andrews et al., 2006, 2008; Gonda et al., 2013) and *KIAA0319* (Paracchini et al., 2006). Specifically, embryonic knockdown of CDSG homolog function in rats using interference RNA (RNAi) disrupts neuronal migration, as evidenced by the presence of white matter heterotopia (*Dyx1c1* and *Kiaa0319*) and ''overmigration'' of neurons past their expected laminar location in the cerebral

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cortex (*Dyx1c1* and *Dcdc2*, Wang et al., 2006; Rosen et al., 2007; Burbridge et al., 2008; Peschansky et al., 2010; Currier et al., 2011). Taken together, these results suggest that neuronal migration errors may contribute to the developmental dyslexia phenotype.

Although the specific function of *KIAA0319L* is unknown, it has been shown to interact with Nogo receptor 1, an axon guidance protein (Poon et al., 2011a) that is similar to *ROBO1*, a CDSG on the DYX5 locus (Nopola-Hemmi et al., 2001; Hannula-Jouppi et al., 2005), which has been shown to modulate axon guidance and neuronal migration (Wong et al., 2001; Hivert et al., 2002; Gonda et al., 2013). In the mouse brain, *Kiaa0319l* is expressed in both astrocytes and neurons, most strongly in the olfactory bulb, hippocampus, and neocortex (Poon et al., 2011b). Because of its similarity to *KIAA0319*, we investigated this gene's function using *in utero* electroporation to transfect a subset of embryonic neuronal progenitor cells with plasmids containing either small hairpin RNA (shRNA) targeted against *Kiaa0319l* or expression constructs *in vivo*. We found that disrupting *Kiaa0319l* function interferes with neuronal migration leading to the formation of periventricular nodular heterotopia. These heterotopias contain late generated neurons destined for the upper neocortical laminae. Interestingly, they also contain large numbers of untransfected neurons, some of which are GABAergic, suggesting that non-cell autonomous effects, too, are involved in the formation of these heterotopias.

EXPERIMENTAL PROCEDURES

In utero electroporation

All procedures were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Pregnant Wistar rats (Charles River, Wilmington, MA, USA) were assigned to one of three experimental conditions: *Kiaa0319l* shRNA, KIAA0319L expression, or rescue (*Kiaa0319l* shRNA + KIAA0319L expression). Within each litter, pups randomly received an experimental treatment or a control electroporation (a scrambled version of the *Kiaa0319l* shRNA, empty expression construct, or *Kiaa0319l* shRNA respectively). *In utero* electroporations were performed at embryonic day (E) 15.5 as previously described (Bai et al., 2003; Burbridge et al., 2008; Peschansky et al., 2010). Experimental constructs were co-transfected with monomeric red fluorescent protein (mRFP), while the control constructs were co-transfected with enhanced green fluorescent protein (eGFP). The concentrations of eGFP and mRFP plasmids were 0.75 μg/μL, and the shRNA and expression construct concentrations used were 1.5 μg/μL.

Plasmids

For the *Kiaa0319l* shRNA condition, plasmids encoding shRNA (prKLshr4) and plasmids encoding mRFP (pCAGGS-RFP) were co-transfected. Littermates were co-transfected with a plasmid encoding a scrambled version of the shRNA (pKLsh1 Scram) along with a plasmid encoding eGFP (pCAGGS-eGFP). In the expression condition, pups were co-transfected with plasmid encoding human KIAA0319L (PWP1KL) and mRFP, while their littermates were co-transfected with an empty version of the expression construct (PWP1) and eGFP. In the rescue condition, subjects were co-transfected with *Kiaa0319l* shRNA, the KIAA0319L expression construct, and mRFP plasmids, while their littermates received the *Kiaa0319l* shRNA and eGFP plasmids. Previous research indicates that co-transfection is highly efficient (Rosen et al., 2007).

5-Bromo-2-deoxyuridine (BrdU) injection

Pregnant rats were anesthetized with isoflurane (5%) and i.p. injected with 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU; Sigma–Aldrich, St. Louis, MO, USA, 10 mg/ml solution) at either E13.5, E15.5, or E17.5.

Histology

Animals were sacrificed at E19.5 or postnatal day (P) 1, P5, P10, or P21. P10 and P21 rats were deeply anesthetized (Ketamine/Xylazine 10:1, 100 mg/ml), sacrificed, and fixed by transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were extracted, post-fixed in PFA for 24 h, and cryoprotected, first in 10% and then 30% sucrose phosphate buffer. Tissue was sectioned frozen at 40 μm on a sliding microtome. Sections were stored in a series of every tenth section in phosphate buffer containing 0.02% sodium azide as a preservative. One series was then mounted on a slide and visualized under fluorescence for the presence of eGFP or mRFP. After fluorescence screening, this slide was used for Nissl staining with Thionin. Pups aged P1 and P5 were anesthetized on ice, then sacrificed by transcardial perfusion as described above. Brains were extracted and post-fixed for 48 h, then cryoprotected as above, and sectioned at 18 μm on a cryostat (Leica CM1900, Leica Microsystems, Buffalo Grove, IL, USA). Six series of every tenth section were saved on positively charged slides (Fisher Scientific, Waltham, MA, USA). One series was visualized under fluorescence for the presence of eGFP or mRFP. One series was dried in a vacuum desiccator overnight, then immediately stained for Nissl substance with Thionin. A cohort of animals was sacrificed at E19.5 by decapitation after Cesarean section. Heads were immersion-fixed for 24 h in PFA, at which point brains were extracted and placed in fresh PFA for 24 h, then cryoprotected in sucrose phosphate buffers, and sectioned on a cryostat as described above.

Immunohistochemistry. Immunoperoxidase activity was detected using 3,3-diaminobenzidine (DAB, Vector Labs, Burlingame, CA, USA) according to ABC protocols. One series adjacent to the Nissl-stained series was used for the immunohistochemical detection of eGFP (AB3080, Millipore Corp., Billerica, MA, USA, 1:1800) or mRFP (18-732-292379, Genway Biotech, San Diego, CA, USA, 1:5000). Adjacent series were stained for the laminar markers CUX1 (sc-13024, Santa