**Dcdc2 KNOCKOUT MICE DISPLAY EXACERBATED DEVELOPMENTAL DISRUPTIONS FOLLOWING KNOCKDOWN OF DOUBLECORTIN**

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Abstract—The dyslexia-associated gene Dcyc2 is a member of the Dcx family of genes known to play roles in neurogenesis, neuronal migration, and differentiation. Here we report the first phenotypic analysis of a Dcyc2 knockout mouse. Comparisons between Dcyc2 knockout mice and wild-type (wt) littermates revealed no significant differences in neuronal migration, neocortical lamination, neuronal ciliogenesis or dendritic differentiation. Considering previous studies showing genetic interactions and potential functional redundancy among members of the Dcx family, we tested whether decreasing Dcx expression by RNAi would differentially impair neurodevelopment in Dcyc2 knockouts and wild-type mice. Consistent with this hypothesis, we found that deficits in neuronal migration, and dendritic growth caused by RNAi of Dcyc2 were more severe in Dcyc2 knockouts than in wild-type mice with the same transfection. These results indicate that Dcyc2 is not required for neurogenesis, neuronal migration or differentiation in mice, but may have partial functional redundancy with Dcx. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: dyslexia, neocortex, migration, mouse, dendrites.

Genetic variation in DCDC2 in humans has been associated with developmental learning disabilities including reading disability (Meng et al., 2005; Schumacher et al., 2006), attention deficit hyperactivity disorder (ADHD) (Couto et al., 2009), and difficulties in mathematics (Marino et al., 2011). A genetic variant of DCDC2 associated with dyslexia in some studies is present within an enhancer region that regulates DCDC2 expression (Meng et al., 2011), further suggesting that altered expression of Dcyc2 may be related to developmental learning disability. The specific cellular function or functions of Dcyc2 protein in development and physiology are currently not well characterized, although its structural relatedness to doublecortin (Dcx) family members, and results from in vivo RNAi studies in rats, suggest that Dcyc2 may play a role in neuronal migration.

Dcyc2 is ubiquitously expressed in developing rodent and mature human neocortex (Burbridge et al., 2008; Meng et al., 2005), and could potentially have roles in several aspects of neural development and/or function. RNAi of Dcyc2 in subpopulations of migrating neocortical neurons in developing rat neocortex causes deficits in neuronal migration indicating that at least one function of Dcyc2, similar to other members of the Dcx family, may be in neuronal migration (Burbridge et al., 2008; Meng et al., 2005). Similarly, Dcyc2 protein interacts with many of the same cytoskeleton related proteins that other members of the Dcx family interact with, including tubulin, suggesting that Dcyc2 could have a role in mechanisms of cell migration or differentiation that require cytoskeletal dynamics (Reiner et al., 2006). Loss-of-function mutations in mice of members of the Dcx family—Dcx, Dclk1, and Dclk2—cause alterations in neuronal migration, neurogenesis, and/or dendritic differentiation (Corbo et al., 2002; Kerjan et al., 2009; Pramparo et al., 2010). Results from analysis of compound mutants of members of the Dcx family indicate that members of the family genetically interact and may participate in coordinated function during neurodevelopment (Deuel et al., 2006; Koizumi et al., 2006).

In this study we produced and analyzed the first knockout mouse of Dcyc2 in the mouse (Dcyc2a). Dcyc2 knockout mice are healthy and breed normally. Neurogenesis, neuronal migration, and lamination of neocortex are not significantly different between Dcyc2 knockouts and wild-type (wt) animals. We also used in utero RNAi targeted against Dcx in developing neocortex in homozygous wild-type and homozygous Dcyc2 mutant animals to investigate a potential shared function between Dcx and Dcyc2. Dcx RNAi created more developmental disruption in Dcyc2 knockouts than in wt mice. The enhanced disruptions included the appearance of subcortical band heterotopia and disruptions in dendritic growth. These results show that genetic loss of Dcyc2 does not alone create abnormalities in neuronal migration or differentiation in neocortex, but that Dcyc2 may have partial functional redundancy with Dcx in regulating neuronal migration and dendritic growth, which is revealed only after both are rendered dysfunctional. The Dcyc2 mutant mouse presents the opportunity for future studies into the role or roles of Dcyc2 in behavior and physiology that are independent of disruptions in neuronal migration.
EXPERIMENTAL PROCEDURES
Gene targeting and genotyping
Mice carrying the loxp-exon 2-loxP conditional allele of Dcdc2 (Dcdc2lox2/lox2) were made by the University of Connecticut Health Center Gene Targeting and Transgenic Facility by standard methods. Briefly, embryonic stem cells harboring a floxed allele of exon 2 of Dcdc2 were produced by electroporating mouse ES cells (129S6 (129SvEvTac) with a targeting construct, and subsequently drug selected and screened by PCR for correctly targeted ES cell clones. A single positive colony was expanded and used for embryo re-aggregation to produce five chimeric mice. Three of these mice were shown to germline transmit the targeted allele to offspring in a cross with C57BL6 mice. The PKG-Neo cassette in the targeting construct was then removed by crossing these mice with 129S4/SJLaeSor-G(Rosa26Soim(FLP)Dyv) J mice (Jackson laboratories). These offspring were used to generate a colony of Dcdc2floxflox2/lox2 mice. In order to generate Dcdc2floxflox2/lox2 mice with a deletion of exon 2, we crossed Dcdc2floxflox2/lox2 mice with Hprt-Cre mice, C57Bl6-Hprtfltm1cro(Mmny)J (UCHC). Genotyping was subsequently performed by PCR using two pairs of primers (Loxp F: 5'-aggtgatctgtaggatctg-3' and reverse primer located in exon 5 (5'-gagttgatctgtaggatctg-3')) and primer located in exon 5 (5'-cctgattgagccctaggacttt).

RT-PCR analysis
Total RNA of the cerebral cortex was extracted from Dcdc2floxflox2/lox2, Dcdc2floxflox2/lox2, and Dcdc2floxflox2/lox2 knockout mice and wild-type littermates by RNAqueous (Ambion, Austin, TX, USA). Reverse transcription (RT) reactions were performed with 5 μg of total RNA using the SuperScript II reverse transcriptase (200 U per reaction; Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using a forward oligonucleotide primers located in Dcdc2 exon 1 (5'-atgagctgctccagctccag) and reverse primer located in exon 5 (5'-gcacccgagcgagcatc) to amplify Dcdc2 fragments spanning exon 1–exon 5. PCR was performed for 35 cycles with a denaturing step at 94 °C (1 min), followed by annealing at 58 °C (1 min) and extension at 68 °C (1 min). PCR products were then purified using standard procedures and orientation so that left and right were identifiable consistently. The sequences were then crossverified with Permout. All cell estimations were performed under 100× oil-immersion DIC-illuminated objective using the optical fractionator as implemented by Stereo Investigator. Preliminary research has determined the optimal parameters for the optical fractionator. Cells are estimated using a sampling frequency of every 20th section. Using a sampling grid of 530×530 μm, cells that lie within a counting box (15×15×20 μm3) are classified as belonging to neurons or astrocytes (astroglia, microglia, and oligodendrocytes). All cells were counted using standard stereologic procedures (disector/3D counting).

Histology and immunohistochemistry
For fluorescent immunostaining, brains were dissected and drop fixed for embryonic or neonatal brain, or perfusion fixed for adult with 4% paraformaldehyde. Brains were prepared with vibratome (Leica) at 60–80 μm and rinsed for 5 min in 1× PBS, blocked for 1 h in blocking solution (1× PBS, 0.3% Triton X-100, and 5% normal goat serum), incubated either for 2 h at room temperature or overnight at 4 °C with the primary antibodies diluted in blocking solution, rinsed three times for 5 min each with 1× PBS, incubated with the appropriate secondary antibodies (Molecular probe, 1:200) diluted in blocking solution for 1 h at room temperature, rinsed three times for 5 min each with 1× PBS, incubated 10 min with Topro3 (Molecular Probes, Eugene, OR, USA, 1:1000), rinsed with 1× PBS, and coverslipped with AntiFade (Molecular Probe, 1:3000). Confocal images were captured using a Leica confocal microscope and imported into Adobe Photoshop. The primary antibodies were: Rabbit anti-GFP polyclonal antibody (molecular probe, 1:2000), rabbit anti-CUX1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000), rabbit
polyclonal anti-Tbr1 (Santa Cruz Biotechnology; 1:1000), rat polyclonal anti-Brdu (Accurate Chemical & Scientific, Westbury, NY, USA; 1:100), rabbit polyclonal anti-Ki67 (Novoceastra, Newcastle, UK; 1:200), rabbit anti-phospho-H3 polyclonal antibody (Millipore, Billerica, MA, USA; 1:200), mouse monoclonal anti-alpha tubulin (Sigma, Cream Ridge, NJ, USA; 1:200). For cilia detection the primary antibodies included a rabbit anti-ACIII (1:1000; Santa Cruz) and mouse anti-pericentrin (1:200; BD Biosciences, Franklin Lakes, NJ, USA). Quantification of fluorescent images was performed with ImageJ (NIH, Bethesda, MD, USA), and statistical comparisons were made by t-test for comparison of two groups and ANOVA for comparison of more than two.

In utero electroporation

Briefly, pregnant mice were euthanized at E14, and uterus was exposed. Lateral ventricles were injected with pulled glass microcapillary needles with plasmids in a 0.01% Fast Green solution (Sigma). Electrodes were placed on either side of the embryo’s head, and 3×50 ms square pulses at 25 V were administered at 1 s intervals with a BTX830 square-wave pulse generator (Genetronics, Havard Apparatus, Holliston, MA, USA). Brains were harvested at postnatal 21 days and preceded to immunostaining and imaging analysis.

RESULTS

Targeted genetic deletion of Dcdc2

In order to generate Dcdc2 mutant mice we sequentially generated mouse lines bearing engineered Dcdc2 alleles; a conditional deletion or “floxed” allele in which exon 2 was flanked by loxp sites, Dcdc2^floxed*, and a constitutively deleted allele in which exon 2 was deleted, Dcdc2^del2 (Fig. 1A, B). Deletion of exon 2, an exon present in all annotated splice variants of Dcdc2, is predicted to result in a frame shift and premature stop codon when exon 1 and exon 3

Fig. 1. The Dcdc2 knockout and conditional knockout alleles. (A) Schematic of wt and two mutant Dcdc2 alleles produced for this study. The schematic also shows the position of PCR primers used for genotyping (the genomic distances are not to scale). (B) Example of genotyping results distinguishing between mice heterozygous or homzygous for Dcdc2^wt* and Dcdc2^del2 alleles. The first pair of primers (F/R) (upper panel in B) gives 227 bp PCR amplification products only in Dcdc2^wt* and Dcdc2^del2* mice; the second pair (F/R2) gives 2772 products in Dcdc2^wt* and 351 bps products in Dcdc2^del2* and Dcdc2^del2 mice. (C) PCR of cDNA prepared from RNA isolated from Dcdc2^wt* or Dcdc2^del2* mice amplified different MW products. PCR products from Dcdc2^wt* cDNA were 547 bps and 492 bps from Dcdc2^del2* cDNA consistent with the deletion of exon 2 in the Dcdc2^del2* allele. (D) Sequencing spectra of a region of the amplicons shown in (C) indicate an exon 1–3 splice variant and premature stop codon in Dcdc2^del2* mice. (E) Quantitative Real Time PCR results showing the expression levels of Dcdc2 mRNA relative to expression levels in wt mice. Levels were significantly decreased in heterozygous and in homozygous mutants, consistent with potent nonsense-mediated decay. Data are expressed and percent of the mean of wt expression levels and errors are SEM. (F) Knockout mice did not differ from wt mice in exploratory behavior. The number of fields entered was not statistically different across genotypes. Data are presented as Mean±SEM. (G) Deletion of Dcdc2 does not significantly affect ability to learn a simple visuo-spatial working memory task. Plot of the mean errors (i.e. entering an error zone) across six learning trials in maze #1 of the Hebb-Williams maze. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
are spliced together. To verify this aberrant splice variant and stop codon in the mutants, we used RT-PCR with primers to exon 1 and exon 3, sub-cloned and then sequenced the resulting fragments. As shown in Fig. 1C, consistent with loss of the 52 bp exon 2, the amplified product from homozygous mutant animals (Dcdc2(del2/del2)) was approximately 50 bases smaller than that amplified from Dcdc2(wt/wt) animals (Fig. 1C). Furthermore, the sequence of the amplified fragment from homozygous mutants indicated that the exon 1-to-exon 3 spliced sequences contained the expected premature stop codon (Fig. 1D). Introduction of premature stop codons by mutation often result in transcripts that are degraded by nonsense-mediated mRNA decay. We tested for such decay by quantitative rt PCR (qPCR) to determine whether Dcdc2 mRNA levels were lower in mice with alleles missing exon 2. In cDNA prepared from RNA isolated from the brains of homozygous mutants we found evidence of approximately 10 fold decrease in Dcdc2 mRNA than in Dcdc2(wt/wt) animals. Similarly, in heterozygous animals, with one mutant and one wt allele, we found intermediate levels of Dcdc2 mRNA. These results are consistent with potent nonsense mediated decay and loss of Dcdc2 transcripts in Dcdc2(del2/del2) mutants (Fig. 1E; n=21, P<0.001). We also attempted to confirm decreased expression at the protein level, however five commercially purchased and tested antibodies failed to identify bands of the appropriate molecular weight (MW) for Dcdc2 even in wild-type brains. Nevertheless, the aberrant splice variant, premature stop codon, and potent nonsense-mediated decay in the mutant provide substantial evidence that the Dcdc2(del2) is a loss of function mutant allele for Dcdc2.

In crosses between mice heterozygous for the del2 and wt alleles of Dcdc2, as well as in crosses between animals homozygous and heterozygous for the del2 alleles, we observed the expected mendelian ratios of 1:2:1 for the animals homozygous and heterozygous for the respective alleles. No significant differences (Fig. 1E; n=21, P>0.05). Similarly, there was no significant difference in the percentage of cells that had exited the cell cycle (phoH3+) in neocortical progenitors within the VZ that were in M-phase (phoH3+) at embryonic day 15 (E15). We found no significant differences between wild-type and Dcdc2 knockout mice (Fig. 1F). We also tested whether Dcdc2 loss altered the number of neuronal cilia and alters cilia signaling (Massinen et al., in press), we performed an assessment of neuronal cilia in hippocampus and cerebellum in P54 Dcdc2(del2/del2) and Dcdc2(del2/wt) mice. Neuronal cilia can be identified immunocytochemically due to their enriched expression of type III adenyl cyclase and pericentrin, proteins localized to the axoneme and basal body, respectively (Bishop et al., 2007; Anastas et al., 2011). We therefore performed an immunocytochemical assessment of neuronal cilia in hippocampus and neocortex of 54 day old Dcdc2(del2/del2) and Dcdc2(wt/wt) mice. However, the structure, numbers, and lengths of neuronal cilia in neocortex and hippocampus did not differ between Dcdc2(del2/del2) and Dcdc2(wt/wt) mice (Fig. 3). Further studies are needed to determine whether signaling to or from these cilia is altered in Dcdc2 mutants.

**Dcdc2 mutants have structurally normal brains**

Dcdc2 knockout mice showed no defects in brain morphology as assessed by comparison of serially sectioned brains from Dcdc2(wt/wt) and Dcdc2(del2/del2) mice (Fig. 2A, B). Laminated neural structures, including neocortex, hippocampus, and cerebellum, all showed typical morphologies. Similarly, the size and organization of major white matter tracts showed no evidence of disruption. There was also no evidence of focal developmental disruptions in neocortex, including neither periventricular heterotopia nor layer 1 ectopia. In addition, the numbers of total neuronal and non-neuronal cells in the cerebral neocortex, as assessed by non-biased stereology of Nissl-stained sections, showed no significant differences between wild-type and knockout mice. Furthermore, immunohistochemistry of two neocortical-layer specific markers, Cux1 (layers II–IV) and Tbr1 (layer V and VI) revealed no significant differences in neocortical lamination patterns (Fig. 2C, D).

As Dcdc2 has been shown to bind to microtubules, and Dcdc2 expressed in hippocampal neurons, localizes to neuronal cilia and alters cilia signaling (Massinen et al., in press), we performed an assessment of neuronal cilia in hippocampus and cerebral cortex in P54 Dcdc2(wt/wt) and Dcdc2(del2/del2) mice. Neuronal cilia can be identified immunocytochemically due to their enriched expression of type III adenyl cyclase and pericentrin, proteins localized to the axoneme and basal body, respectively (Bishop et al., 2007; Anastas et al., 2011). We therefore performed an immunocytochemical assessment of neuronal cilia in hippocampus and neocortex of 54 day old Dcdc2(del2/del2) and Dcdc2(wt/wt) mice. However, the structure, numbers, and lengths of neuronal cilia in neocortex and hippocampus did not differ between Dcdc2(del2/del2) and Dcdc2(wt/wt) mice (Fig. 3). Further studies are needed to determine whether signaling to or from these cilia is altered in Dcdc2 mutants.
In order to test whether there were any defects in neuronal migration in Dcdc2 mutants, we performed three different experiments. First, we injected pregnant females at gestational day 15 with BrdU and examined the positions of BrdU positive neurons within neocortex on the day of birth. As shown in Fig. 3C, in both wild-type and knock-out mice, BrdU labeled cells reached the top of the cortical plate revealing no apparent migration delays or arrest. In an additional assay for migration, we used electroporation of VZ progenitors at the ventricular zone at E15 to label migrating neurons with GFP and then assessed the position of neurons on the day of birth. Similar to the BrdU assay, GFP labeled neurons were present in upper layers in both knockouts and wt mice (Fig. 4D). Lastly, to test whether cells lacking a functional copy of Dcdc2 would migrate more slowly if migrating within the context of a population of cells with functional Dcdc2 alleles, we used animals homozygous for the Dcdc2\textsuperscript{lox/lox} allele and trans-fected these Dcdc2\textsuperscript{lox/lox} animals at E15 with plasmids expressing cre, pCAG-Cre, and a conditionally gated GFP, pCALNL-GFP. Cre-transfected cells in wild-type and in Dcdc2\textsuperscript{lox/lox} animals were marked by the expression of GFP and these cells migrated similarly from the VZ to superficial layers of neocortex (Fig. 4E) in both Dcdc2\textsuperscript{lox/lox} and Dcdc2\textsuperscript{wt/wt}. Together, the results of these three neuronal migration assays indicate that genetic deletion of Dcdc2 in mice does not result in impaired neuronal migration of pyramidal neurons in mouse neocortex.

Dcx RNAi impairs neuronal migration more in Dcdc2 knockouts than in wild-type mice

Previous studies of compound Dcx and dclk1 mutations in mice indicated that loss of combinations of these genes results in greater impairments in neuronal migration and differentiation than does loss of any single gene alone.
Deuel et al., 2006; Koizumi et al., 2006). In order to test for evidence of a similar functional relationship between Dcdc2 and Dcx, we compared the effects of Dcx RNAi on Dcdc2<sup>del2/del2</sup> and Dcdc2<sup>wt/wt</sup> mutant mice. As we previously showed for Dcx RNAi in mice (Ramos et al., 2006), we found that Dcx RNAi delivered at E14 to wt type mice causes some cells destined for upper layers to be distributed into deeper layers in mouse neocortex, but does not lead to the formation of subcortical band heterotopia as it does in rat neocortex (Ramos et al., 2006). Similarly, in this study, subcortical band heterotopia failed to form in any wild-type mice (n=8) transfected with Dcx RNAi (Fig. 5A). In contrast to the effects of Dcx RNAi in Dcdc2<sup>wt/wt</sup> animals, four of nine Dcdc2<sup>del2/del2</sup> mutants transfected with Dcx RNAi developed prominent subcortical band heterotopia in the white matter underlying neocortical lamina (Fig. 5B). These results indicate that the loss of Dcdc2 function by mutations creates a sensitized condition permissive to the formation of subcortical band heterotopia in mice upon decreased expression of Dcx.

To further confirm that the disruption in migration caused by Dcx RNAi was exacerbated in Dcdc2 knockout mice, we quantitatively compared the positions of neurons within neocortex following transfection of a scrambled control and an effective Dcx RNAi in Dcdc2<sup>del2/del2</sup> and Dcdc2<sup>wt/wt</sup> mice. As shown in the histogram in Fig. 5C, there was no significant difference between the distribution of neurons in P14 brains transfected at E15 with the scrambled control RNAi vectors in Dcdc2<sup>del2/del2</sup> and Dcdc2<sup>wt/wt</sup> mice; however, there was a significant shift in the proportion of neurons that resided in deeper positions following RNAi against Dcx in the Dcdc2 knockout mice compared to wt controls (n=5, P<0.01). This difference was seen both in a significant decrease in cells residing in superficial layers, and a significant increase in the number of cells in deeper positions. Thus, Dcx RNAi impairs neuronal migration more in Dcdc2 knockout mice than in wt animals.

**Dcx RNAi impairs dendritic growth and differentiation more in Dcdc2 knockouts than in wt mice**

Results from analysis of compound mutant mice for Dcx and Dclk2 indicate a synergistic function for Dclk2 and Dcx in the maturation of dendritic morphologies in hippocam-
pus (Kerjan et al., 2009). We therefore assessed whether there was a similar synergistic interaction between Dcdc2 and Dcx function in development of dendritic morphologies in the neocortex. For this analysis we measured the basal dendrites of layer III pyramidal neurons in somatosensory cortex in five brains within each of four conditions: Dcx shRNA in Dcdc2^wt/wt, Dcx shRNA scramble control in Dcdc2^del2/del2, and Dcx shRNA Fig. 4. No significant differences in neurogenesis or neuronal migration in fetal neocortex in Dcdc2 knockouts. (A) M-phase cells labeled with phos-H3 in E15 neocortex are shown in the upper panel for both wt and KO. Nuclei are labeled by Topro3. The lower panel in (A) shows BrdU and Ki67 immuno-labeling 24 h after a BrdU injection at E14. (B) Bar graphs of the quantification of experiments depicted in (A) for mitotic cells (upper graph, and the fraction of cells that exited the cell cycle in 24 h (bottom graph). There was no significant difference in either the fraction of cells at the VZ surface that are positive for Phos-H3 (M-phase-index) (n=5, P>0.05), nor was there a significant difference in the fraction of BrdU labeled cells that were negative for Ki67 (cells that exited the cell cycle) (n=5, P>0.05). (C) The position of neurons in neocortex labelled with BrdU at E15 and examined 6 d later show similar migration to upper layers of neocortex in Dcdc2^wt/wt and Dcdc2^del2/del2 mice. (D) Position of eGFP labeled neurons 6 d following electroporation at E15 in Dcdc2^wt/wt and Dcdc2^del2/del2 mice. All labeled neurons were in similar upper layer positions in both wt and knockout animals. (E) Conditional genetic deletion in migrating neocortical pyramidal neurons in Dcdc2^flox2/flox2^ did not result in impaired migration. A Cre-recombinase expressing plasmid (pCAG-Cre) and a reporter plasmid that expresses GFP after Cre recombination (pCALNL-GFP) was transfected into wt and animals homozygous for the floxed allele Dcdc2^flox2^2. The position of neurons examined 6 d later on the day of birth have the same migration pattern into upper layers. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
scrambled control in Dcdc2\(^{del2/del2}\). We restricted the analysis to layer III neurons to avoid the possible confound of comparing displaced cells that reside in deeper layers in increased number in the Dcdc2\(^{del2/del2}\) mutant. We found that there was an aggregation of neurons in the white matter that formed a subcortical heterotopia (between dotted line in Fig. 6B). Histograms show the percent of transfected (eGFP+) neurons contained within each of the position deciles the layer VI white matter boundary (0.1) to the pial surface (1) (neurons in subcortical heterotopia present in knockouts were not included in analysis because they fall below the white matter layer 6 boundary). Statistical analysis of the distribution revealed a significant difference in the pattern of neuronal positions between Dcx RNAi (green and purple bars) and control RNAi (red and blue bars) in both genotypes (ANOVA, position as a repeated measure, \(P<0.001\)), and a significant difference in the distribution between the position of Dcx RNAi treated cells in Dcdc2\(^{wt/wt}\) (green bar) and Dcdc2\(^{del2/del2}\) (purple bars) (ANOVA, position as a repeated measure, \(P<0.01\)). In particular, in Dcx RNAi in Dcdc2 knockouts there were significantly greater fractions of neurons in the lower deciles with a smaller fraction in the upper decile, compared to Dcx RNAi treated cells in the wild-type cortex. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 5. Enhanced migration disruptions by Dcx RNAi in Dcdc2 KO mice. (A, B) P14 cortex in the region of somatosensory neocortex following transfection of a Dcx shRNA plasmid and a GFP expression plasmid at E14 in a wt (A) and Dcdc2 knockout littermate (B). In both transfections, cells are not within the expected upper layer positions, and in the Dcdc2\(^{del2/del2}\) mutant there is an aggregation of neurons in the white matter that form a subcortical heterotopia (between dotted line in B). (C) Histograms showing normalized distributions of neurons in neocortical at P14 following transfection of Dcx shRNA and a control shRNA in wt and Dcdc2 knockout animals (\(n=5\) for each condition). Histograms show the percent of transfected (eGFP+) neurons contained within each of the position deciles the layer VI white matter boundary (0.1) to the pial surface (1) (neurons in subcortical heterotopia present in knockouts were not included in analysis because they fall below the white matter layer 6 boundary). Statistical analysis of the distribution revealed a significant difference in the pattern of neuronal positions in Dcx RNAi (green and purple bars) and control RNAi (red and blue bars) in both genotypes (ANOVA, position as a repeated measure, \(P<0.001\)), and a significant difference in the distribution between the position of Dcx RNAi treated cells in Dcdc2\(^{wt/wt}\) (green bar) and Dcdc2\(^{del2/del2}\) (purple bars) (ANOVA, position as a repeated measure, \(P<0.01\)). In particular, in Dcx RNAi in Dcdc2 knockouts there were significantly greater fractions of neurons in the lower deciles with a smaller fraction in the upper decile, compared to Dcx RNAi treated cells in the wild-type cortex. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
tion, the effects of Dcx RNAi on dendritic elaboration is more severe in Dcdc2 mutants than in wild-type mice.

**DISCUSSION**

We report the first phenotypic description of a Dcdc2 knockout mouse. Our assessment indicates that mutation of Dcdc2 does not cause gross neurodevelopmental defects on its own. Dcdc2 knockout mice breed normally, show no embryonic lethality, and display no gross disturbances in neural architecture. Consistent with normal neuroanatomic patterns in the postnatal neocortex, neurogenesis, and neuronal migration in neocortex do not differ between knockouts and wild-type mice. The lack of clear neurodevelopmental deficits indicates that the Dcdc2 gene on its own is not critical to neuronal migration or neurogenesis in mice. Although there was no first order deficit in migration or neural differentiation in mouse neocortex, we did find that the effects of RNAi against Dcx were more severe in Dcdc2 knockouts. This suggests that in the mouse, Dcx function may partially compensate for the loss of Dcdc2.

Members of the doublecortin family of proteins encode microtubule associated proteins that regulate cytoskeletal dynamics in developing neural cells (Koizumi et al., 2006). Genetic loss-of-function mutations in members of the DCX superfamily, Dcx, Dclk, or Dclk2, in mice have been found to cause far less severe developmental defects (Corbo et al., 2002; Deuel et al., 2006; Kerjan et al., 2009; Koizumi et al., 2006), than when mutations are combined. The compound mutants show perinatal lethality, disorganized neocortical layering, and disorganization of hippocampus (Deuel et al., 2006; Koizumi et al., 2006). In addition, Dcx...
and Dclk2 double knockout mice display frequent spontaneous seizures and disrupted laminination of hippocampus (Kerjan et al., 2009). These studies indicate that Dcx superfamily members may sometimes function in synergistic or partially redundant fashion in mice. We find a similar relationship between Dcdc2 and Dcx in this study by combining RNAi of Dcx with Dcdc2 mutation. The mechanism through which DCX family members cooperate is not completely clear, however, in vitro experiments show that all members of the family share interactions with microtubules, JIP, and neurabin, and these may serve as points of functional convergence (Reiner et al., 2006).

Interpretation of our results with Dcdc2 knockout mice in terms of developmental learning disorders associated with Dcdc2 should be approached with caution. The results using RNAi for three dyslexia susceptibility candidates (Dyx1c1, Dcdc2, and Kiaa0319) in developing rat neocortex have all suggested a connection between these candidate dyslexia susceptibility genes and neuronal migration (Burbridge et al., 2008; Meng et al., 2005). These findings combined with previous correlations between disruptions in neuronal migration and reading disability in humans have strengthened a hypothesis of neuronal migration disruption and dyslexia (Galaburda et al., 2006). It remains unknown whether function of Dcdc2 in humans is more similar to that in rat or to that in mouse, where it is not required for migration in neocortex. As the present study is the first direct genetic test for a loss of function of Dcdc2 mutation in any species, our results support the possibility that genetic loss of Dcdc2 function alone need not impair neuronal migration, and that genetic variants of Dcdc2 in humans may or may not be associated with disruptions in neuronal migration.

The Dcdc2 knockout mouse should prove a valuable model for future studies designed to investigate the role of Dcdc2 in neuronal physiology and behavior. As Dcdc2 is expressed in the developing and mature brain (Burbridge et al., 2008; Meng et al., 2005), after neuronal migration to the neocortex has ended, Dcdc2 may have functions in neurons beyond any role in neuronal migration. Genetic variants in Dcdc2 in humans have now been associated significantly with dyslexia risk (Ludwig et al., 2008; Meng et al., 2005; Schumacher et al., 2006; Wilcke et al., 2009), reading ability (Lind et al., 2010), mathematical ability (Marino et al., 2011), ADHD (Couto et al., 2009), and speed of information processing (Luciano et al., 2011), suggesting some as of yet undefined, and potentially pleotropic, role of Dcdc2 in human neocortical function. Conversely, all of these cognitive functions share a functional property or properties, the development of which is affected by Dcdc2 activity. The genetic mouse model described in our present study should facilitate future studies into the role of Dcdc2 in behavioral and neurophysiological contexts that are independent of neuronal migration.

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