**Dcde2 knockout mice display exacerbated developmental disruptions following knockdown of Dcx**

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**Abstract**

The dyslexia-associated gene DCDC2 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 Dcde2 knockout mouse. Comparisons between Dcde2 knockout mice and wild type 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 Dcde2 knockouts and wild type mice. Consistent with this hypothesis, we found that deficits in neuronal migration, and dendritic growth caused by RNAi of Dcx were more severe in Dcde2 knockouts than in wild type mice with the same transfection. These results indicate that Dcde2 is not required for neurogenesis, neuronal migration or differentiation in mice, but may have partial functional redundancy with Dcx.

**Introduction**

Genetic variation in DCDC2 in humans has been associated with developmental learning disabilities including reading disability (Meng H et al., 2005; Schumacher J et al., 2006), attention deficit hyperactivity disorder (ADHD) (Couto JM et al., 2009), and difficulties in mathematics (Marino C et al.). A genetic variant of DCDC2 associated with dyslexia in some studies is present within an enhancer region that regulates DCDC2 expression (Meng H et al., 2010), further suggesting that altered expression of Dcde2 may be related to developmental learning disability. The specific cellular function or functions of Dcde2 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 Dcdc2 may play a role in neuronal migration.

Dcdc2 is ubiquitously expressed in developing rodent and mature human neocortex (Burbridge TJ et al., 2008; Meng H et al., 2005), and could potentially have roles in several aspects of neural development and/or function. RNAi of Dcdc2 in subpopulations of migrating neocortical neurons in developing rat neocortex causes deficits in neuronal migration indicating that at least one function of Dcdc2, similar to other members of the Dcx family, may be in neuronal migration (Burbridge TJ et al., 2008; Meng H et al., 2005). Similarly, Dcdc2 protein interacts with many of the same cytoskeleton related proteins that other members of the Dcx family interact with, including tubulin, suggesting that Dcdc2 could have a role in mechanisms of cell migration or differentiation that require cytoskeletal dynamics (Reiner O 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 JC et al., 2002; Kerjan G et al., 2009; Pramparo T 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 TA et al., 2006; Koizumi H et al., 2006).

In this study we produced and analyzed the first knockout mouse of Dcdc2 in the mouse (Dcdc2a). Dcdc2 knockout mice are healthy and breed normally. Neurogenesis, neuronal migration, and lamination of neocortex are not significantly different between Dcdc2 knockouts and wild type animals. We also used in utero RNAi targeted against Dcx in developing neocortex in homozygous wildtype and homozygous Dcdc2 mutant animals to investigate a potential shared function between Dcx and Dcdc2. Dcx RNAi created more developmental disruption in Dcdc2 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 Dcdc2 does not alone create abnormalities in neuronal migration or differentiation in neocortex, but that Dcdc2 may have partial functional redundancy with Dcx in regulating neuronal migration and dendritic growth, which is revealed only after both are rendered dysfunctional. The Dcdc2 mutant mouse presents the opportunity for future studies into the role or roles of Dcdc2 in behavior and physiology that are independent of disruptions in neuronal migration.

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, Dcdc2floxed, and a constitutively deleted allele in which exon2 was deleted, Dcdc2del2 (figure 1 A&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 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 figure 1C, consistent with loss of the 52 base pair exon 2, the amplified product from homozygous mutant animals (Dcdc2del2/del2) was approximately 50 bases smaller than that amplified from Dcdc2wt/wt animals (figure 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 (figure 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<sup>wt/wt</sup> 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<sup>del2/del2</sup> mutant mice (figure 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 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<sup>del2</sup> 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 and 1:1 respectively, indicating no evidence of embryonic lethality associated with Dcdc2<sup>del2</sup>. Animals homozygous for the del2 deletion are healthy and are fertile. As an initial screening for behavioral changes in Dcdc2 knockout mice we tested Dcdc2<sup>del2/del2</sup>, Dcdc2<sup>wt/del2</sup>, and Dcdc2<sup>wt/wt</sup> mice on open field behavior and on one configuration of the Hebb-Williams maze (maze 1). As shown in figure 1 F&G, there were no significant behavioral differences in either behavioral test. Measures of spontaneous locomotion and exploratory behavior in the open field test showed no significant differences (Figure 1; p >0.05). Similarly, there was no significant difference in performance on Maze 1 of the Hebb-Williams maze, with no significant interaction between trial and genotype for the number of errors committed during testing (p >005.), and no significant main effect of genotype (p> 0.05) in learning the maze. There was, however, a significant main effect of trial across genotypes, confirming that animals learned the task (p< 0.05).

**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<sup>wt/wt</sup> and Dcdc2<sup>del2/del2</sup> mice (figure 2 A,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 (figure 2 C&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<sup>wt/wt</sup> and Dcdc2<sup>del2/del2</sup> mice. Neuronal cilia can be identified immunocytochemically due to their enriched expression of type III adenylyl 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<sup>del2/del2</sup> and Dcdc2<sup>wt/wt</sup> mice. However, the structure, numbers, and lengths of neuronal cilia in neocortex and hippocampus did not differ between Dcdc2<sup>del2/del2</sup> and Dcdc2<sup>wt/wt</sup> mice (figure 3). Further studies are needed to determine whether signaling to or from these cilia is altered in Dcdc2 mutants.
Dcdc2 mutants display normal neocortical neurogenesis and neuronal migration

Recent reanalysis of Dcx knockout mice, which were initially shown to have undisturbed neocortical lamination (Corbo JC et al., 2002), revealed significant changes in both neurogenesis and neuronal migration in fetal development in Dcx mutants that were largely resolved and no longer apparent by later postnatal periods (Pramparo T et al., 2010). We therefore investigated both neurogenesis and migration during the fetal period in Dcdc2 knockouts. To assess neurogenesis, we compared the percentage of neocortical progenitors within the VZ that were in M-phase of the cell cycle (phoH3+ cells) at embryonic day 15 (E15). We found no significant differences between wild type and Dcdc2 knockout mice (figure 4A). We also tested whether Dcdc2 loss altered the fraction of neocortical progenitor cells that exit the cell cycle. For this analysis, BrdU injections were made at E15, and brains were harvested and processed for BrdU and Ki67 immunohistochemistry 24 hours later. The percentage of cells that had exited the cell cycle after 1 day, BrdU+ and Ki67− cells, did not significantly differ between Dcdc2wt/wt and Dcdc2del2/del2 mice (figure 4A). Together these results indicate that the number of mitotic neural progenitors or the rate at which neocortical neuronal progenitors become postmitotic is not altered by the Dcdc2 deletion mutation.

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 Figure 3C, in both wild-type and knockout 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 (figure 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 Dcdc2flox2 allele and transfected these Dcdc2flox2/flox2 animals at E15 with plasmids expressing cre, pCAG-Cre, and a conditionally gated GFP, pCALNL-GFP. Cre-transfected cells in wild type and in Dcdc2flox/flox animals were marked by the expression of GFP and these cells migrated similarly from the VZ to superficial layers of neocortex (figure 4E) in both Dcdc2flox2/flox2 and Dcdc2wt/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 wildtype 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 TAS et al., 2006; Koizumi H 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 Dcdc2del2/del2 and Dcdc2wt/wt mutant mice. As we previously showed for Dcx RNAi in mice (Ramos RL 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 RL et al., 2006). Similarly, in this study, subcortical band heterotopia failed to form in any wild type mice (n=8) transfected with Dcx RNAi (figure 5A). In contrast to the effects of Dcx RNAi in Dcdc2wt/wt animals, 4 of 9 Dcdc2del2/del2 mutants transfected with Dcx RNAi developed prominent subcortical band heterotopia in the white matter underlying neocortical lamina (figure 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 Dcdc2del2/del2 and Dcdc2wt/wt mice. As shown in the histogram in figure 5C, there was no significant difference between the distribution of neurons in P14 brains transfected at E15 with the scrambled control RNAi vectors in Dcdc2del2/del2 and Dcdc2wt/wt 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 knockouts 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 hippocampus (Kerjan G 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 5 brains within each of four conditions: Dcx shRNA in Dcdc2wt/wt, Dcx shRNA scramble control in Dcdc2wt/wt, Dcx RNAi in Dcdc2del2/del2, and Dcx shRNA scrambled control in Dcdc2del2/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 Dcdc2del2/del2 Dcx RNAi treated mice. The results of these experiments shown in Figure 6 A-F show that Dcx RNAi compared to control RNAi had no effect on the mean number of primary or secondary basal dendrites in Dcdc2wt/wt mice (figure 6D). Dcx RNAi in Dcdc2wt/wt mice, in contrast, significantly decreased the length of basal processes both total dendritic length and length of primary, secondary and tertiary processes (figure 6 E&F; p<0.01). The same Dcx RNAi treatment in Dcdc2del2/del2 mice, created an even greater decrease in all measures of basal dendritic process number and length (p<0.01). The increased severity of the Dcx RNAi in Dcdc2del2/del2 mutants consisted of a complete absence of tertiary basal processes following Dcx RNAi (figure 6F). All measures of basal process length and number were most reduced in Dcdc2del2/del2 mice receiving RNAi targeting Dcx (figure 6 D-F; p<0.01). Thus, similar to neuronal migration, 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 indicate 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 H 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 JC et al., 2002; Deuel TA et al., 2006; Kerjan G et al., 2009; Koizumi H et al., 2006), than when mutations are combined. The compound mutants show perinatal lethality, disorganized neocortical layering, and disorganization of hippocampus (Deuel TA et al., 2006; Koizumi H et al., 2006). In addition, Dcx and Dclk2 double knockout mice display frequent spontaneous seizures and disrupted lamination of hippocampus (Kerjan G 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 O 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 TJ et al., 2008; Meng H 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 AM 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 TJ et al., 2008; Meng H 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 KU et al., 2008; Meng H et al., 2005; Schumacher J et al., 2006; Wilcke A et al., 2009), reading ability (Lind PA et al., 2010), mathematical ability (Marino C et al.), ADHD (Couto JM et al., 2009), and speed of information processing (Luciano M et al. 2010), 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.

**Methods**

**Gene targeting and genotyping**

Mice carrying the loxp-exon2-loxP conditional allele of Dcdc2 (Dcdc2\(^{\text{lox2}}\)) 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 two 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

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targeted ES cell clones. A single positive colony was expanded and used for embryo re-aggregation to produce 5 chimeric mice. Three of these mice were shown to germline transmit the targeted allele to offspring in a cross with C57BL6 mice. The PGK-Neo cassette in the targeting construct was then removed by crossing these mice with 129S4/SvJaeSor-Gt(Rosa)26Sor^{tm1(FLP1)Dym} mouse (JAX labs). These offspring were used to generate a colony of Dcdc2^{floxed/floxed} mice. In order to generate Dcdc2^{del2/del2} mice with a deletion of exon 2 we crossed Dcdc2^{floxed/floxed} mice with Hprt-Cre mice, C57Bl6-Hprt^{tm1(cre)Mnn} (UCHC). Genotyping was subsequently performed by PCR using two pairs of primers (Loxp F: 5′-agtgatctgcaagtcag, Loxp R: 5′-cttcggtgttaaacagcag; Exon2 F: 5′-gagtggatctgcaagttcaat; Exon2 R: 5′-gagttggatctgcaagttaatc).

RT-PCR analysis

Total RNA of the cerebral cortex was extracted from Dcdc2^{del2/del2}, Dfdc2^{del2/wt} and Dcdc2^{wt/wt} knock-out mice and wild-type littermates by RNAqueous (Ambion). 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). RT-PCR was performed using a forward oligonucleotide primers located in Dcdc2 exon1 (5′-atgaacggtcccagctccag) and reverse primer located in exon5 (5′-ccacacggacagtcttct) to amplify Dcdc2 fragments spanning exon1–exon5. 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 by agarose gel electrophoresis, ligated into the PCR3.1 vector (invitrogen), and then sequenced. For quantitative real time qRT-PCR of Dcdc2, primers to exon 5 (tat gtg gcc gtc ggc aga g) and exon 7 (ccg atg gtt gac ttg gat tgc) were used with SYBR GREEN (Applied Biosystems) and assayed on an ABI 7500 qPCR machine (Applied Biosystems). Product amplification was validated for linearity in a serial dilution, and the expected single 98 bp amplicon was confirmed by gel electrophoresis. To quantify expression of Dcdc2 mRNA the delta-CT from Gapdh expression (CT GAPDH-CT Dcdc2) was computed in triplicate technical replicates and a mean established for each of 6 brains from animals of each genotype. The primers used for GAPDH were forward (ggcaagttcaacggcacagtc) and reverse (tggtggtgaagacgccagtag).

Behavioral tests

The open field apparatus was composed of a white plastic, square-shaped (45.72 × 48.26 × 60.96 cm) enclosure. The floor was divided into a grid containing sixteen, 11.43 × 12.07 cm squares. The number of squares entered during a five minute testing period was measured by a blind observer. An entry into a field was defined as having all four paws cross the line into a square. The apparatus was cleaned with 70% EtOH after each test. A 60 × 60 cm Hebb-Williams maze (maze 1), with attached start and goal box, was constructed using black acrylic plastic; the removable floor and top were made with clear Plexiglass. One week prior to testing mice were given free access to water and restricted to 2 g of chocolate-flavored food pellets per day (Bio-Serv, Frenchtown, NJ). Mice were weighed daily and maintained at 85% of their normal body weight. During habituation, mice were allowed to explore the maze without interior walls for a maximum of 10 trials of 120 s duration. Once the subject left the start box a black plastic guillotine door was closed to prohibit re-entry. The trial ended once the mouse ate one chocolate-flavored food pelte located in the goal box, or 120s was reached. If the subject did not reach the goal box in 120s he was gently guided to the goal box, the door to the goal box was closed, and the mouse remained until one food pellet was consumed. Habituation ended when all 10 trials were completed, or the subject completed three consecutive trials in less than 30s. The next day mice were tested using the Maze 1 configuration (Meunier, 1986). The mice completed 6 trials in a maximum time of 120s per trial. ANY-maze tracking software (Stoelting) and a web camera (Logitech Quick Cam) were used to record and analyze behavior. The number of errors made during each.
trial was measured. Errors were calculated when the center of mass of the subject crossed into an error zone.

**Serial Section analysis and Stereological Estimate of Neuron Number in the Cerebral Cortex**

After perfusion and fixation (4% paraformaldehyde) brains P40-45 were washed in water for 24 hours before being dehydrated in a series of 80%, 95%, 100% ethanol and ethanol/ether. The brains were placed into 3% celloidin for at least a week followed by 12% celloidin for 2–3 days. The celloidin block was trimmed to achieve a stable base and notched on the left side for orientation. The sections were cut coronally on a sliding microtome at 30 μm, segregated into 10 compartments, and stored in 80% ethanol. Every fifth section was stained for Nissl substance with cresyl violet. This procedure entailed washing the sections in distilled water and then placing them in 0.5% cresyl violet acetate solution (which stains the Nissl substance) for 3–5 minutes. Each section was placed in distilled water for 1 minute and then differentiated and dehydrated in 80 and 95% ethanol. A few drops of colophonium were added to the 95% ethanol baths. If differentiation was adequate, the sections were then cleared with terpineol and passed through xylol. Sections were mounted with care to orientation so that left and right were identifiable consistently. The sections were then coverslipped with Permount. 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 μm) are classified as being either neurons or non-neurons (glia, blood vessel-related endothelial cells, pial, and ependymal cells). All counts were performed 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/PBS. Brain sections were prepared with vibratome (Leica) at 60–80 μm and rinsed for 5 minutes in 1× PBS, blocked for 1 hr in blocking solution (1× PBS, 0.3% Triton X-100, and 5% normal goat serum), incubated either for 2 hr 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 hr at room temperature, rinsed three times for 5 min each with 1× PBS, incubated 10 min with Topro3 (Molecular Probes,1:1,000), rinsed with 1× PBS, and coverslipped with Antifade (Molecular Probe, 1:3,000). 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, 1:2,000); rabbit anti-CUX1 polyclonal anitbody, (Santa Cruz Biotechnology, 1:1,000); rabbit polyclonal anti-Tbr1 (Santa Cruz Biotechnology, 1:1,000); rat polyclonal anti-Brdu (Accurate Chemical & Scientific,1:100); rabbit polyclonal anti-Ki67 (Novoacastra, 1:200); rabbit anti-phosH-3 polyclonal antibody, (Millipore, 1:200), mouse monoclonal anti-alpha tubulin (Sigma, 1:2,000). For cilia detection the primary antibodies included a rabbit anti-ACIII (1:1000; Santa Cruz) and mouse anti-pericentrin (1:200; BD Biosciences). Quantification of fluorescent images was performed with ImageJ (NIH), and statistical comparisons were made by t-test for comparision of 2 groups and ANOVA for comparision of more than 2.

**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 volts were administered at 1 s intervals with a BTX830 square-wave pulse generator (Genetronics, Harvard Apparatus). Brains were harvested at postnatal 21 days and preceded to immunostaining and imaging analysis.

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Highlights

- We produced the first knockout mouse of Dcde2.
- We find no evidence of neuronal migration disruption in the Dcde2 mutants.
- Dcx RNAi causes a more severe migration deficit in the Dcde2 mutants.
- Dcx RNAi cause a more severe effect on the growth and elaboration of dendrites in the Dcde2 mutants.
- Dcde2 on its own is not required for neuronal migration in the neocortex, but does render the neocortex more susceptible to migration disruptions caused by Dcx RNAi.
Figure 1.
The Dcduc2 knockout and conditional knockout alleles. A) Schematic of wt and two mutant Dcduc2 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 homozygous for Dcduc2^wt and Dcduc2^del2 alleles. The first pair of primers (F/R) (upper panel in B) gives 227 bp PCR amplification products only in Dcduc2^wt/wt and Dcduc2^wt/del2 animals; the second pair (F/2R) gives 2772 products in Dcduc2^wt/wt and 351 bps products in dcdc^del2/del2 and Dcduc2^wt/del2 mice. C) PCR of cDNA prepared from RNA isolated from Dcduc2^wt/wt or Dcduc2^del2/del2 mice amplified different MW products. PCR products from Dcduc2^wt/wt cDNA were 547 bps and 492 bps from Dcduc2^del2/del2 cDNA consistent with the deletion of exon 2 in the Dcduc2^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 Dcduc2^del2/del2 mice. E) Quantitative Real Time PCR results showing the expression levels of Dcduc2 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 Dcduc2 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 6 learning trials in maze #1 of the Hebb-Williams maze.
Figure 2. 

Dcde2 mutation does not result in significant developmental disruption in brain architecture or in neocortical lamination. A) Histology of coronal sections from adult Dcde2<sup>wt/wt</sup> and Dcde2<sup>del2/del2</sup> forebrain showed normal overall brain structure in Dcde2 knockout. Lamination of neocortex and hippocampus were preserved in the knockout. B) The cerebellum of Dcde2<sup>del2/del2</sup> mice showed the pattern typical for wt cerebellum. Scale bars in A and B is 1mm. C, D) Immunocytochemistry for Cux1 and Tbr1 in wt and mutant neocortex at P21. Images are from somatosensory cortex at the same level and indicate no differences in the thickness of layers containing Cux1 positive cells (C) or Tbr1 positive cells (D) between Dcde2<sup>del2/del2</sup> and Dcde2<sup>wt/wt</sup> mice. Scale bar is 100 um and is the same for all images in C and D.
Figure 3.
Comparable neuronal primary cilia in P54 wildtype and Dedc2 KO cerebral cortex. (A-C) Confocal z-stack images of brain sections immunostained for adenylyl cyclase III (red/green) which is enriched in neuronal cilia axonemes. ACIII positive cilia were abundant in hippocampal dentate gyrus (DG) (A), CA1 (B) and neocortex (Nctx) (C) of both wt and KO. Insets in (C) show examples of neocortical cilia labeled with pericentrin (red arrowheads) and ACIII (green arrowheads) in both wt and KO. Pericentrin is a basal body marker that asymmetrically localizes to the base of ACIII+ axonemes. All nuclei were labeled with DAPI. Scale bars =10μm.
Figure 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 hours 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 hours (bottom graph). There were 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 days later show similar migration to upper layers of neocortex in *Dcdc2*^{wt/wt} and *Dcdc2*^{del/del} mice. **D**) Position of eGFP labeled neurons 6 days following electroporation at E15 in *Dcdc2*^{wt/wt} and *Dcdc2*^{del/del} 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}. The position of neurons examined 6 days later on the day of birth have the same migration pattern into upper layers.
Figure 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 knockout were not included in this 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$^{del/del}$ (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 with Dcx RNAi treated cells in the wild type cortex.
Figure 6.
Dcx RNAi reduces dendritic growth and elaboration in layer 3 pyramidal neurons in Dcdc2 knockouts more than in wildtypes. Images of GFP transfected neurons in neocortex from (A) Dcdc2<sup>wt/wt</sup> and (B) Dcdc2<sup>del2/del2</sup> animals that were electroporated with Dcx RNAi at E15 and examined at P25. Images are from somatosensory cortex. (C) Example of reconstructed layer 3 cortical neurons transfected with Dcx RNAi in Dcdc2<sup>wt/wt</sup> (upper row) or Dcdc2<sup>del2/del2</sup> (lower row) mice. The reconstructions of proximal dendritic arborizations was use to analyze the length and number of dendritic processes following Dcx RNAi is Dcdc2 knockouts and wild type animals. (D-F) Bar graphs of number (D) total number, (E) total length and (F) mean length of secondary and tertiary basal dendrites in the four indicated conditions. The morphology of neurons in wt or knockout did not differ in any measures with control, scrambled RNAi transfections (red and blue bars), and Dcx RNAi transfection in Dcdc2 knockout mice had the greatest reduction in dendritic arborization (purple bars).