

THE EFFECTS OF EMBRYONIC KNOCKDOWN OF THE CANDIDATE DYSLEXIA SUSCEPTIBILITY GENE HOMOLOGUE *Dyx1c1* ON THE DISTRIBUTION OF GABAergic NEURONS IN THE CEREBRAL CORTEX

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Abstract—Developmental dyslexia is a language-based learning disability, and a number of candidate dyslexia susceptibility genes have been identified, including *DYX1C1*, *KIAA0319*, and *DCDC2*. Knockdown of function by embryonic transfection of small hairpin RNA (shRNA) of rat homologues of these genes dramatically disrupts neuronal migration to the cerebral cortex by both cell autonomous and non-cell autonomous effects. Here we sought to investigate the extent of non-cell autonomous effects following *in utero* disruption of the candidate dyslexia susceptibility gene homolog *Dyx1c1* by assessing the effects of this disruption on GABAergic neurons. We transfected the ventricular zone of embryonic day (E) 15.5 rat pups with either *Dyx1c1* shRNA, *DYX1C1* expression construct, both *Dyx1c1* shRNA and *DYX1C1* expression construct, or a scrambled version of *Dyx1c1* shRNA, and sacrificed them at postnatal day 21. The mothers of these rats were injected with BrdU at either E13.5, E15.5, or E17.5. Neurons transfected with *Dyx1c1* shRNA were bi-modally distributed in the cerebral cortex with one population in heterotopic locations at the white matter border and another migrating beyond their expected location in the cerebral cortex. In contrast, there was no disruption of migration following transfection with the *DYX1C1* expression construct. We found untransfected GABAergic neurons (parvalbumin, calretinin, and neuropeptide Y) in the heterotopic collections of neurons in *Dyx1c1* shRNA treated animals, supporting the hypothesis of non-cell autonomous effects. In contrast, we found no evidence that the position of the GABAergic neurons that made it to the cerebral cortex was disrupted by the embryonic transfection with any of the constructs. Taken together, these results support the notion that neurons within heterotopias caused by transfection with *Dyx1c1* shRNA result from both cell autonomous and non-cell autonomous effects, but there is no evidence to support non-cell autonomous disruption of neuronal position in the cerebral cortex itself. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: developmental dyslexia, heterotopia, cerebral cortex, *DYX1C1*, GABA, genetics.

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Abbreviations: CR, calretinin; eGFP, enhanced green fluorescent protein; GE, ganglionic eminence; mRFP, monomeric red fluorescent protein; NPY, neuropeptide Y; PV, parvalbumin; shRNA, small hairpin RNA; SVZ, subventricular zone; VZ, ventricular zone.

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Approximately 4–10% of the population is diagnosed with developmental dyslexia, a language based learning disability with a strong genetic component. Linkage analyses have identified multiple susceptibility loci, and a number of candidate dyslexia susceptibility genes have been identified. Of the genes proposed, those with the most support are *DCDC2* and *KIAA0319* on Chr 6p22.2 (Francks et al., 2004; Cope et al., 2005; Meng et al., 2005; Paracchini et al., 2006, 2008; Schumacher et al., 2006; Velayos-Baeza et al., 2007, 2008; Wilcke et al., 2009; Lind et al., 2010) and *DYX1C1* on Chr 15q21 (Taipale et al., 2003; Chapman et al., 2004; Marino et al., 2005; Anthoni et al., 2007; Tapia-Páez et al., 2008). Although the functions of these candidate dyslexia susceptibility genes have not been fully elucidated, each has been shown to be involved in neocortical neuronal migration. Thus, knocking down the function of *Dyx1c1*, *Kiaa0319*, and *Dcdc2* gene homologs in rats by electroporation of small hairpin RNA (shRNA) into the ventricular zone (VZ) at embryonic day (E) 15.5 rats results in the disruption of neuronal migration when assessed as early as 4 days post transfection (Meng et al., 2005; Paracchini et al., 2006; Wang et al., 2006).

These results are particularly intriguing as there have been previous reports linking neuronal migration disorders to developmental dyslexia. Thus, examination of post-mortem dyslexic brains revealed the presence of neuronal migration anomalies in the form of molecular layer ectopias, dysplasias, and occasional instances of focal microgyria (Galaburda et al., 1985; Humphreys et al., 1990). More recent research using *in vivo* imaging confirmed these post-mortem findings (Chang et al., 2005, 2007; de Oliveira et al., 2005; Sokol et al., 2006). We have demonstrated that the embryonic knockdown of *Kiaa0319*, *Dcdc2*, or *Dyx1c1* results in similar patterns of cortical disruption when examined postnatally. Specifically, we have reported the presence of heterotopias at the cortex/white matter border in postnatal day (P) 21 brains of animals after *in utero* electroporation of plasmids containing shRNA targeted against either *Kiaa0319* or *Dyx1c1* (Rosen et al., 2007; Peschansky et al., 2009). In addition, embryonic knockdown of *Dcdc2* or *Dyx1c1* results in an “overmigration” phenotype, whereby transfected neurons migrate beyond their expected laminar location (Rosen et al., 2007; Burbridge et al., 2008).

In the above-cited experiments, we saw evidence for disordered neuronal migration as a result of cell-autonomous and non-cell autonomous effects. First, there were large numbers of neurons within the heterotopias that had not been transfected. Second, many of these heterotopic

neurons were born 2 days after the date of transfection. Third, some of these heterotopic neurons stain positive for γ -aminobutyric acid-ergic (GABAergic) antibodies, which are not generated in the dorsal ventricular zone and are therefore not likely to have been transfected.

GABA plays important roles in mature brain function as the main actor in inhibitory action on synapses, and during brain development through its effects on cell proliferation, migration, circuit formation and synaptogenesis (Jelitai and Madarasz, 2005; Ruediger and Bolz, 2007; Wang and Kriegstein, 2009). Furthermore, dysfunction of GABA activity has been implicated in disorders such as epilepsy, mood and anxiety disorders, schizophrenia, autism, and Tourette's syndrome (Petty, 1995; Nemeroff, 2003; Wong et al., 2003; Di Cristo, 2007). In previous work from our laboratory, we reported decreased numbers of GABAergic (parvalbumin-positive) neurons in rodent brains that had undergone induction of cortical microgyria by perinatal freezing injury as a model of human developmental dyslexia (Rosen et al., 1998), and excessive excitatory cortical activity in the form of increased miniature excitatory postsynaptic currents has also been reported in this model (Zsombok and Jacobs, 2007). In human dyslexics, seizures or abnormal electrical activity often accompany cortical malformations (Chang et al., 2005; Papavasiliou et al., 2005; Canavese et al., 2007). Although the implicated GABA dysfunction in dyslexia may have a direct genetic basis (Hisama et al., 2001), non-cell autonomous and other epigenetic effects, as in the freezing lesion model, may also play a role. We have, in fact, demonstrated non-cell autonomous effects on neuronal migration in an intrauterine shRNA model of developmental dyslexia in the rat (Peschansky et al., 2009). A fuller understanding of the neurobiological underpinnings of developmental dyslexia will require a description of the GABAergic system's involvement in this condition.

In the present study we looked for evidence of abnormal GABAergic neuron development by determining the position of such neurons in the cerebral cortex following *in utero* electroporation of plasmids containing shRNA targeted against *Dyx1c1*, and compared the results with littermates transfected with either a scrambled version of the *Dyx1c1* shRNA (control), a DYX1C1 expression construct (overexpression), or a rescue condition where brains are transfected simultaneously with *Dyx1c1* shRNA and the DYX1C1 expression construct.

EXPERIMENTAL PROCEDURES

In utero electroporation

All procedures were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Each of 11 pregnant Wistar rats (Charles River, Wilmington, MA, USA) was assigned to one of three experimental conditions: *Dyx1c1* shRNA, DYX1C1 expression, or rescue. Within each litter, pups randomly received an experimental treatment or a control electroporation (scrambled shRNA). This design was used to control for between-litter variation in gestational age, and so that each of the groups were comprised of rats from multiple litters. *In utero* electroporations were performed at E15.5 as previously described (Bai

et al., 2003; Burbridge et al., 2008; Peschansky et al., 2009). The concentrations of enhanced green fluorescent protein (eGFP) and monomeric red fluorescent protein (mRFP) plasmids were 0.75 $\mu\text{g}/\mu\text{l}$, and the shRNA and expression construct concentrations used were 1.5 $\mu\text{g}/\mu\text{l}$.

Plasmids. For the *Dyx1c1* shRNA condition, plasmids encoding shRNA (pU6DyxHPB) and plasmids encoding eGFP (pCAGGS-eGFP) were co-transfected. Littermates were co-transfected with a plasmid encoding a scrambled version of the shRNA (pU6DyxHPB scam) along with a plasmid encoding mRFP (pCAGGS-RFP). In the expression condition, pups were co-transfected with plasmid encoding human DYX1C1 (pCAGGS-DYX1C1) and eGFP, while their littermates were co-transfected with the scrambled shRNA and mRFP. In the rescue condition, subjects were co-transfected with *Dyx1c1* shRNA, the DYX1C1 expression construct, and eGFP plasmids, while their littermates received the scrambled shRNA and mRFP plasmids. Previous research indicates that co-transfection is highly efficient (Rosen et al., 2007).

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). Four dams received an injection of BrdU at E13.5, four at E15.5, and three at E17.5.

Histology

At P21, transfected animals were deeply anesthetized (Ketamine/Xylazine 10:1, 100 mg/ml), sacrificed, and fixed by transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were extracted, post-fixed for 24 h, and cryoprotected, first in 10% and then in 30% sucrose phosphate buffer. The tissue was frozen and sectioned at 40 μm on a sliding microtome. Sections were stored in series of every tenth section and preserved in 0.4% sodium azide/phosphate buffer. One series was then mounted, coverslipped with VECTASHIELD Mounting Medium (Vector Labs, Burlingame, CA, USA), and visualized under fluorescence for the presence of eGFP or mRFP. Another series of every tenth section was stained for Nissl bodies using Thionin.

Immunohistochemistry. Immunoperoxidase activity was detected using 3,3-diaminobenzidine (DAB; Vector Labs) according to ABC protocols. One series adjacent to the Nissl-stained sections 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 antibodies directed against inhibitory interneurons, namely the GABAergic antibodies Calretinin (CR; AB1550, Millipore, 1:5000), Parvalbumin (PV; MAB1572, Millipore, 1:10,000) and Neuropeptide Y (NPY; ab43871, Abcam, Cambridge, MA, USA, 1:2000). Primary antibodies were labeled with biotinylated secondary antibodies (Vector Labs, all 1:200). Immunohistochemically processed tissue was mounted and coverslipped with Permount mounting medium (Fisher Scientific, Waltham, MA, USA).

For at least one animal from each BrdU injection group (E13.5, E15.5 and E17.5), one series of sections was processed for immunofluorescence of GABAergic cell markers and BrdU (ab1893, Abcam, 1:500; 347580, BD Biosciences, San Jose, CA, USA, 1:100). Primary antibodies were detected with one of the following secondary antibodies: Alexa Fluor 594, Alexa Fluor 586 (Invitrogen, Carlsbad, CA, USA, all 1:200), or DyLight 405 (Rockland Immunochemicals, Gilbertsville, PA, USA, 1:400; Jackson ImmunoResearch, West Grove, PA, USA, 1:200). Tissue processed for immunofluorescence was mounted, coverslipped with VECTASHIELD Mounting Medium, and imaged.

Image processing

Fluorescent images were obtained on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA). Three-dimensional projections of high-magnification image series were obtained in cases of ambiguous co-labeling. Photomicrographs were adjusted for exposure and sharpened (unsharp mask filter) with Adobe Photoshop (Adobe Inc., San Jose, CA, USA). Brightfield images were obtained on a Zeiss Axiophot. Individual images for montages were acquired using the Virtual Slice Module of NeuroLucida (MBF Biosciences, Williston, VT, USA), and were composited in Adobe Illustrator (Adobe Inc.).

Analysis

Assessment of pathology. All brains were analyzed with the experimenter blind to subject condition. Nissl-stained sections were surveyed for the presence of neocortical and/or hippocampal malformations.

Quantitative analysis of position. For each brain, four sections were chosen from the immuno-stained series of each eGFP/mRFP, PV, CR, and NPY. Care was taken to ensure that the four sections were architectonically similar across the different series. The locations of immunohistochemically stained neurons were charted using the program NeuroLucida. The charted sections were then analyzed in a custom Matlab (Mathworks, Natick, MA, USA) program, which determined the location of each marked neuron as a percentage of cortical depth, with 0% corresponding to the white matter/subcortical plate boundary and 100% representing the pial surface. For eGFP/mRFP-stained sections, the region analyzed in Matlab was limited to the area of cortex that was transfected, mainly the somatosensory cortex. The area analyzed in all sections stained for GABAergic antigens stretched from primary motor cortex to granular insular cortex. An unweighted frequency distribution was calculated for each brain (10 bins from 0% to 100% and one bin consisting of neurons with positions below the white matter), and the mean frequency per bin across all animals within each condition was calculated. Differences in neuronal position were assessed using a repeated-measures ANOVA, with Group as the between, and bins as the within, variable. Because the value of the 11th bin could be predicted by the values of the other 10, separate F ratios were determined by systematically removing each one of the bins and these F ratios were averaged. The number of neurons included in this analysis is summarized in Table 1.

RESULTS

Transfection of *Dyx1c1* shRNA results in abnormalities of neuronal position

An initial analysis was conducted to assess the position of neurons in each of the conditions. Brains transfected with shRNA targeted against *Dyx1c1* displayed disruptions in the cortical position of neurons when compared to the

DYX1C1 expression, *Dyx1c1* scrambled shRNA and rescue conditions, with most shRNA-transfected brains displaying heterotopias at the cortex/white matter border (Fig. 1A). As expected from previous studies (Rosen et al., 2007), *Dyx1c1* shRNA-transfected neurons were distributed bimodally, with 6.4% of the neurons positioned in the white matter below the cerebral cortex (Fig. 1B). In comparison, only 0.4% of neurons transfected with the scramble construct and 0.3% of neurons transfected with the expression construct were similarly positioned ($t=2.2$, $df=13$, $P<.05$; $t=2.3$, $df=13$, $P<.05$, respectively). An initial repeated measures ANOVA with Group (shRNA, Expression, Scrambled, and Rescue) as the between measure, and the percent of transfected neurons in each bin as the within revealed a significant Group \times Bin interaction ($F_{27,216}=5.4$, $P<.001$), indicating that the laminar distribution of transfected neurons in the cortex differed among the Groups. Further analysis demonstrated that the distribution of *Dyx1c1* shRNA-transfected neurons differed significantly from both scrambled ($F_{9,124}=11.1$, $P<.001$) and Expression ($F_{9,124}=8.8$, $P<.001$), but not Rescue ($F_{9,115}<1$, NS). More shRNA-transfected neurons were located in upper laminae than were neurons transfected with scrambled shRNA or expression constructs (Fig. 1B).

There is evidence of non-cell autonomous effects of shRNA transfection

As mentioned, gross examination of Nissl-stained material revealed heterotopias at the cortex/white matter border in seven of the eight shRNA-transfected brains. These consisted of collections of neurons that in some cases extended for 6 mm along the rostro-caudal extent of the brains (Fig. 2B). There were also heterotopias in the brains of rats transfected with both shRNA and the expression construct (four out of seven). These malformations were smaller than those seen in the shRNA transfection group, having fewer neurons and extending over smaller distances both medio-laterally and rostro-caudally. There were no heterotopias in brains transfected with either expression or scramble constructs (Fig. 2A).

Comparison of the Nissl-stained heterotopias (Fig. 2C) with adjacent sections immunohistochemically stained for eGFP (Fig. 2D) revealed that only a subset of the neurons in the heterotopia showed evidence of transfection with shRNA, which confirms previous reports (Wang et al., 2006; Burbridge et al., 2008; Peschansky et al., 2009). This suggested that there are non-cell autonomous effects of *Dyx1c1* shRNA transfection. Since transfected neurons

Table 1. Mean (\pm SEM) number of neurons marked in condition. (Number of rats in parentheses)

Transfection group	Immuno-positive neurons			
	eGFP	PV+	CR+	NPY+
<i>Dyx1c1</i> shRNA	2135.3 \pm 388.5 (8)	2361.1 \pm 197.5 (8)	1985.3 \pm 267.4 (8)	822.9 \pm 68.0 (8)
<i>Dyx1c1</i> scrambled	1483.0 \pm 160.1 (7)	2580.1 \pm 284.6 (7)	1818.7 \pm 251.2 (6)	929.6 \pm 158.7 (7)
DYX1C1 expression	1310.6 \pm 191.5 (7)	2864.3 \pm 203.0 (7)	1970.4 \pm 212.2 (7)	1113.3 \pm 148.2 (7)
Rescue	1895.4 \pm 397.3 (6)	2533.7 \pm 263.6 (6)	2034.9 \pm 254.7 (6)	690.1 \pm 55.7 (6)

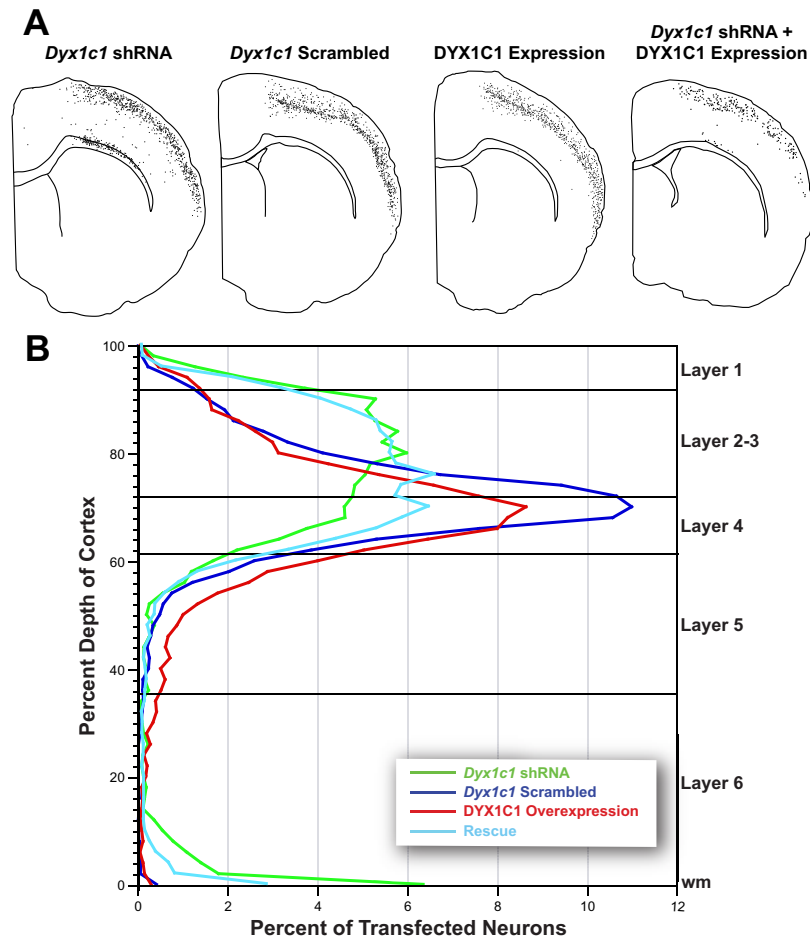


Fig. 1. Qualitative (A) and Quantitative (B) illustration of the position of transfected neurons in each of the experimental groups. (A) Tracings of transfected hemispheres from each of the groups illustrating the positions of transfected neurons. (B) Line graph illustrating the percent of transfected neurons throughout the depth of the cerebral cortex. There is a significant difference in the distribution of transfected neurons between the *Dyx1c1* shRNA group and both the scrambled and overexpression group. This is due to more transfected neurons in the white matter and in upper cortical laminae in the *Dyx1c1* shRNA transfection group ($P < .05$). There is no difference between the rescue and *Dyx1c1* shRNA group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

could have lost their reporter GFP, we examined a population of neurons that could not have been transfected during the initial manipulation, namely those arising from the ganglionic eminences. We found that there were PV+ (Fig. 2E), CR+ (Fig. 2F), and NPY+ (Fig. 2G) neurons in the heterotopias, which confirmed the occurrence of non-cell autonomous effects of *in utero* electroporation of *Dyx1c1* shRNA.

Heterotopic GABAergic cells are born during middle to late stages of neuronal migration

BrdU was injected into pregnant rats either 2 days before transfection of *Dyx1c1* shRNA (E13.5), concomitant with transfection (E15.5), or 2 days after transfection (E17.5) in order to assess the distribution of birth-dated neurons in the brains of their pups. Following E13.5 BrdU injection, BrdU+ neurons were located in cortical layers 5 and 6 (Fig. 3C, G, K). As expected, GABAergic neurons were seen in the heterotopia (Fig. 3B, F, J), and there were scattered PV+ and NPY+ neurons in the cerebral cortex

that were co-labeled with BrdU (not shown), but the majority were not co-labeled.

Transfected neurons both within the heterotopia and in the cerebral cortex were co-labeled following E15.5 BrdU injection (Fig. 4D, H, L). There were a number of non-transfected BrdU+ neurons in the heterotopia, which replicates our previous report, and supports the occurrence of non-cell autonomous effects of shRNA transfection (Fig. 4C, G, K). Finally, we found a number of neurons co-labeled with BrdU and the GABAergic antibodies, both in the cerebral cortex and in the heterotopia (Fig. 4). There were no transfected neurons that were co-labeled with BrdU and any GABAergic antibody.

The majority of E17.5 BrdU neurons were located in layers 2–3 of the neocortex (not shown). In addition, there were large numbers of E17.5 BrdU+ neurons within the heterotopia that were not transfected with *Dyx1c1* shRNA (Fig. 5C, G, K). We detected PV+ (Fig. 5B) and CR+ neurons (Fig. 5F) that were co-labeled with BrdU both within and outside the heterotopia. NPY+ neurons, on the

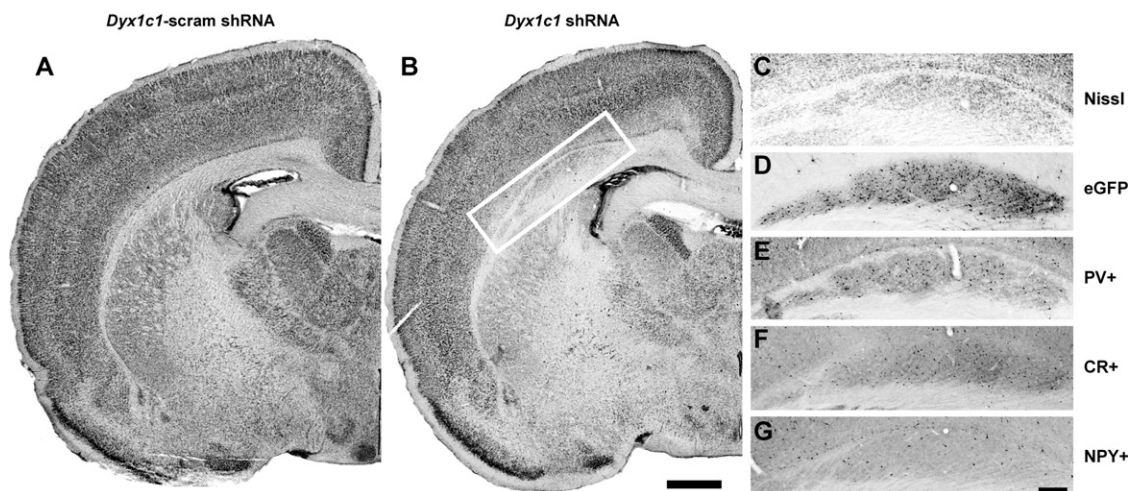


Fig. 2. GABAergic neurons in white matter heterotopias following *in utero* electroporation of *Dyx1c1* shRNA. (A) Low power photomicrograph of Nissl-stained section from a rat embryonically transfected with *Dyx1c1*-scrambled shRNA (control condition). There are no heterotopias visible (compare with B). (B) Low power photomontage of Nissl-stained section from a rat embryonically transfected with *Dyx1c1* shRNA+GFP illustrating large collection of heterotopic neurons in the white matter. White box illustrates enlarged area in panel (C). Bar for (A) and (B)=1 mm. (C) High power photomontage of the Nissl-stained collection of heterotopic neurons shown in panel (B). (D) High-power photomontage of section adjacent to panel (C) immunohistochemically stained for GFP. Comparing with panel (C) indicates that there are large numbers of non-transfected neurons in the heterotopia. (E–G) High-power photomontage of sections adjacent to panel (C) immunohistochemically stained for PV (E), CR (F), and NPY (G). The presence of these GABAergic interneurons in the heterotopia is supportive of non-cell autonomous effects of *Dyx1c1* shRNA transfection. Bar=250 μ m.

other hand, were only co-labeled with BrdU in the cerebral cortex (not shown).

The non-cell autonomous effects of *Dyx1c1* shRNA transfection are limited to the heterotopia

The presence of non-transfected GABAergic neurons in heterotopias in *Dyx1c1* shRNA transfected brains suggests a non-cell autonomous disruption of interneuron neuronal migration. In order to test whether this non-cell autonomous effect extended to the migration of GABAergic cells into the cerebral cortex, we plotted their laminar position in all transfection conditions (Fig. 6).

An initial repeated measures ANOVA with Group as the between measure and the distribution of PV+ neurons as the within measure was significant ($F_{27,216}=2.0$, $P<.01$). Further analysis revealed that there were significant differences in the number of PV+ neurons in white matter among the Groups (Fig. 6A). Specifically, 4.3% of the PV+ neurons are located below the cerebral cortex in the shRNA-transfected group compared to 1.8% and 1.9% in the scrambled and expression groups, respectively ($t=2.3$, $df=13$, $P<.05$ for both comparisons). This is particularly noteworthy, as this takes into account neurons throughout the hemisphere, not just in the transfected region, as did the analysis of transfected neurons (Fig. 1). There was no difference between the percent of neurons in the white matter between the rescue group (2.5%) and the shRNA condition ($t=1.5$, $df=13$, NS).

We did not find evidence to support the hypothesis that migration of PV+ neurons within the cerebral cortex itself was disrupted by *in utero* transfection with *Dyx1c1* shRNA. Using repeated measures ANOVA as above, we found no significant Bin \times Group interactions between shRNA and

scrambled groups ($F_{9,124}=1.3$, NS) nor between shRNA and overexpression groups ($F_{9,124}=1.8$, NS). There was, however, a significant difference between rescue condition and the other three groups (shRNA: $F_{9,124}=3.3$, $P<.01$; Scrambled: $F_{9,124}=3.0$, $P<.01$; Expression: $F_{9,115}=1.9$, $P<.05$). This difference reflects a larger percentage of PV+ neurons in the upper part of layer 5 and layer 4 in the rescue group.

An initial repeated measures ANOVA with Group as the between- and distribution of CR+ neurons as the within measure was not significant ($F_{27,207}=1.4$, NS), suggesting that there is no overall disruption of CR+ neuronal migration (Fig. 6B). The same analysis with NPY+ neurons as the dependent measure found a significant Group \times Bin interaction ($F_{27,216}=3.0$, $P<.001$). This difference is driven by the percent of NPY+ neurons located below the white matter border with the cortex (Fig. 6C). Thus, 9.3% of NPY+ neurons transfected with *Dyx1c1* shRNA and DYX1C1 Expression are located within the white matter, compared to 6.8% of the *Dyx1c1* shRNA, 5.9% of the Scrambled, and 3.9% of the Expression Group.

DISCUSSION

Previous experiments demonstrated that *in utero* electroporation of plasmids containing shRNA targeted against candidate dyslexia susceptibility gene homolog *Dyx1c1* into progenitor cells in the dorsal ventricular zone disrupted neuronal migration to the cerebral cortex (Wang et al., 2006; Rosen et al., 2007). Specifically, we found that neurons transfected with *Dyx1c1* shRNA displayed a bimodal pattern of neocortical distribution in postnatal rats, with collections of neurons near the white matter border and

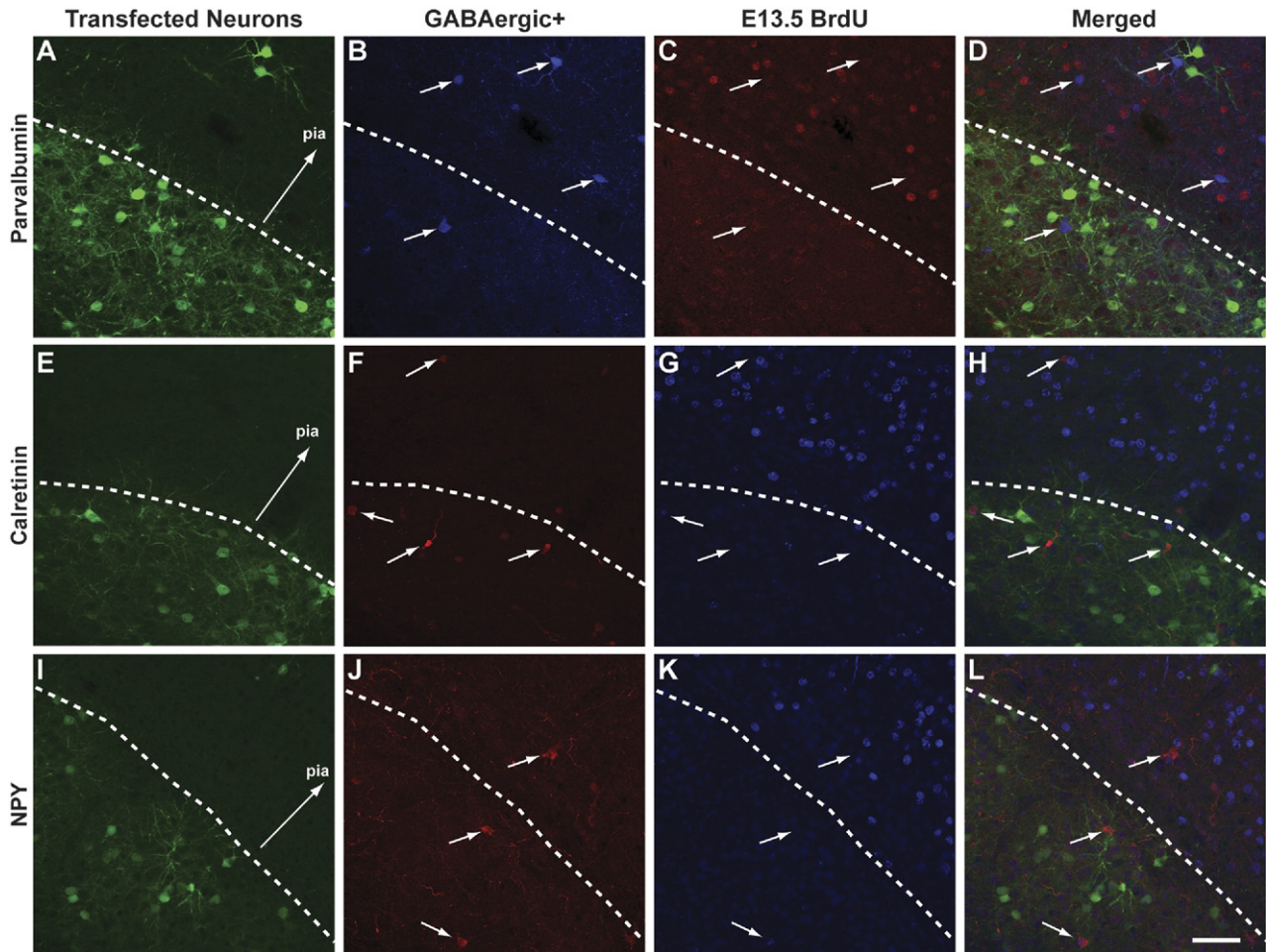


Fig. 3. Confocal microscopy illustrating E13.5 BrdU neurons in white matter heterotopias, and co-labeling with GABAergic neurons. (A–D) Heterotopic collection of neurons containing transfected neurons (A), PV+ neurons (B), E13.5 BrdU+ neurons (C). Arrows indicate PV+ neurons both within the heterotopia and in the overlying cerebral cortex. There are no transfected neurons co-labeled with either BrdU or PV. (E–H) Heterotopic collection of neurons containing transfected neurons (E), CR+ neurons (F), E13.5 BrdU+ neurons (G). Arrows indicate CR+ neurons both within the heterotopia and in the overlying cerebral cortex. There are no transfected neurons co-labeled with either BrdU or CR. (I–L) Heterotopic collection of neurons containing transfected neurons (I), NPY+ neurons (J), E13.5 BrdU+ neurons (K). Arrows indicate NPY+ neurons both within the heterotopia and in the overlying cerebral cortex. There are no neurons that are co-labeled with any of the antibodies. Bar=100 μ m. Long arrows for orientation toward pial surface.

another cohort of neurons positioned beyond their expected location in the upper cortical laminae. The present experiments replicate this finding, as there was a significant difference in the positions of transfected neurons in the cerebral cortex between rats in the *Dyx1c1* shRNA and scrambled shRNA groups. We reported a similar bi-modal distribution of transfected neurons for candidate dyslexia susceptibility gene *Dcdc2* (Burbridge et al., 2008), but not for *Kiaa0319* (Peschansky et al., 2009).

The current experiment is the first to examine the effect on neuronal position of transfection with *DYX1C1* expression vectors. We failed to demonstrate any difference in the position of transfected neurons between the expression and scrambled shRNA groups. These results are in contrast to those following *in utero* electroporation of plasmids expressing *KIAA0319*—where transfected neurons took up abnormal positions subjacent to their expected lamina—but are similar to those reported following embry-

onic transfection with *DCDC2* expression constructs (Burbridge et al., 2008; Peschansky et al., 2009). Therefore, the results of the present study suggest that knockdown, but not overexpression, of *DYX1C1* disrupts the normal pattern of laminar position.

Previous experiments suggested that the abnormal cortical structure resulting from the *in utero* electroporation of shRNAs targeted against candidate dyslexia susceptibility genes included both cell-autonomous and non-cell autonomous effects. Specifically, not all neurons contained within the heterotopia had been transfected with shRNA (Rosen et al., 2007; Burbridge et al., 2008; Peschansky et al., 2009). We found identical results in the present experiment, with large numbers of GFP-negative neurons contained within the heterotopias. There are several hypotheses that could be advanced to explain these results. First, it could be argued that the co-transfection is inefficient, such that not all neurons were transfected with both the

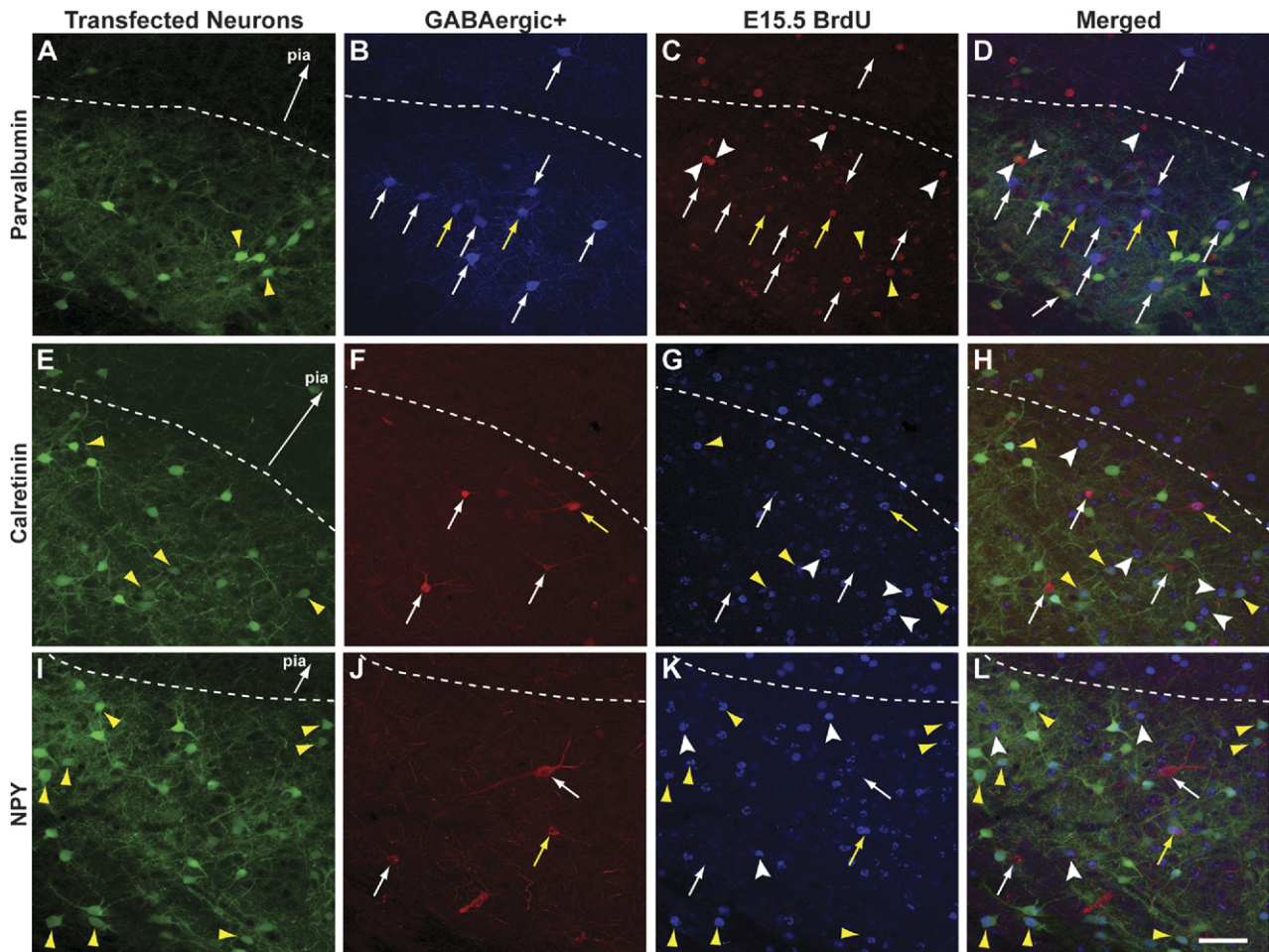


Fig. 4. Confocal microscopy illustrating E15.5 BrdU neurons in white matter heterotopias, and co-labeling with GABAergic neurons. (A–D) Heterotopic collection of neurons containing transfected neurons (A), PV+ neurons (B), E15.5 BrdU+ neurons (C). White arrows indicate PV+ neurons, and yellow arrows indicate neurons co-labeled with PV and BrdU. Yellow arrowheads indicate transfected neurons that are co-labeled with BrdU. White arrowheads are BrdU positive neurons in the heterotopia that are not co-labeled. (E–H) Heterotopic collection of neurons containing transfected neurons (E), CR+ neurons (F), E15.5 BrdU+ neurons (G). White arrows denote CR+ neurons, and yellow arrows indicate neurons co-labeled with CR and BrdU. Yellow arrowheads indicate transfected neurons that are co-labeled with BrdU. White arrowheads are BrdU positive neurons in the heterotopia that are not co-labeled. (I–L) Heterotopic collection of neurons containing transfected neurons (I), NPY+ neurons (J), E15.5 BrdU+ neurons (K). White arrows denote NPY+ neurons, and yellow arrows indicate a neuron co-labeled with NPY and BrdU. Yellow arrowheads indicate transfected neurons that are co-labeled with BrdU. White arrowheads are BrdU positive neurons in the heterotopia that are not co-labeled. Bar=100 μ m. Long arrows for orientation toward pial surface.

Dyx1c1 shRNA and GFP plasmids. However, we have previously demonstrated that nearly 100% of neurons are successfully co-transfected (Rosen et al., 2007). The second possibility is that these apparently non-transfected neurons were, in fact, transfected, but the GFP failed to report in a subset of transfected cells. A number of observations diminish the likelihood of this explanation. For example, we find that there are large numbers of neurons in the heterotopia that were born on E17.5, a full 2 days after transfection, which makes it unlikely that they could have been transfected during the electroporation. In addition, we showed many PV+, CR+, and NPY+ neurons in the heterotopia, despite the fact that these interneurons are not generated in the dorsal ventricular zone and therefore are not likely to have been transfected. Moreover, some of those PV+ and CR+ neurons were generated well after the date of transfection (E17.5). Taken together,

these results support the hypothesis that indeed there are non-cell autonomous effects of embryonic knockdown of *Dyx1c1*.

One additional question raised by the presence of GABAergic neurons in the heterotopia is whether they signal a more general disturbance of GABAergic neuron migration to include abnormalities of positioning within the cerebral cortex itself. To address this question, we marked the positions of PV+, CR+, and NPY+ neurons throughout the transfected hemisphere in all four transfection groups. Although we did find that there were statistical differences among the groups in PV+ neuron position, this was caused by the PV+ neurons in the heterotopias in the shRNA group, which could not be an issue in the scrambled and expression groups for lack of heterotopias in the latter. There was no evidence to support a difference in PV+ neuronal position in the cerebral cortex itself. More-

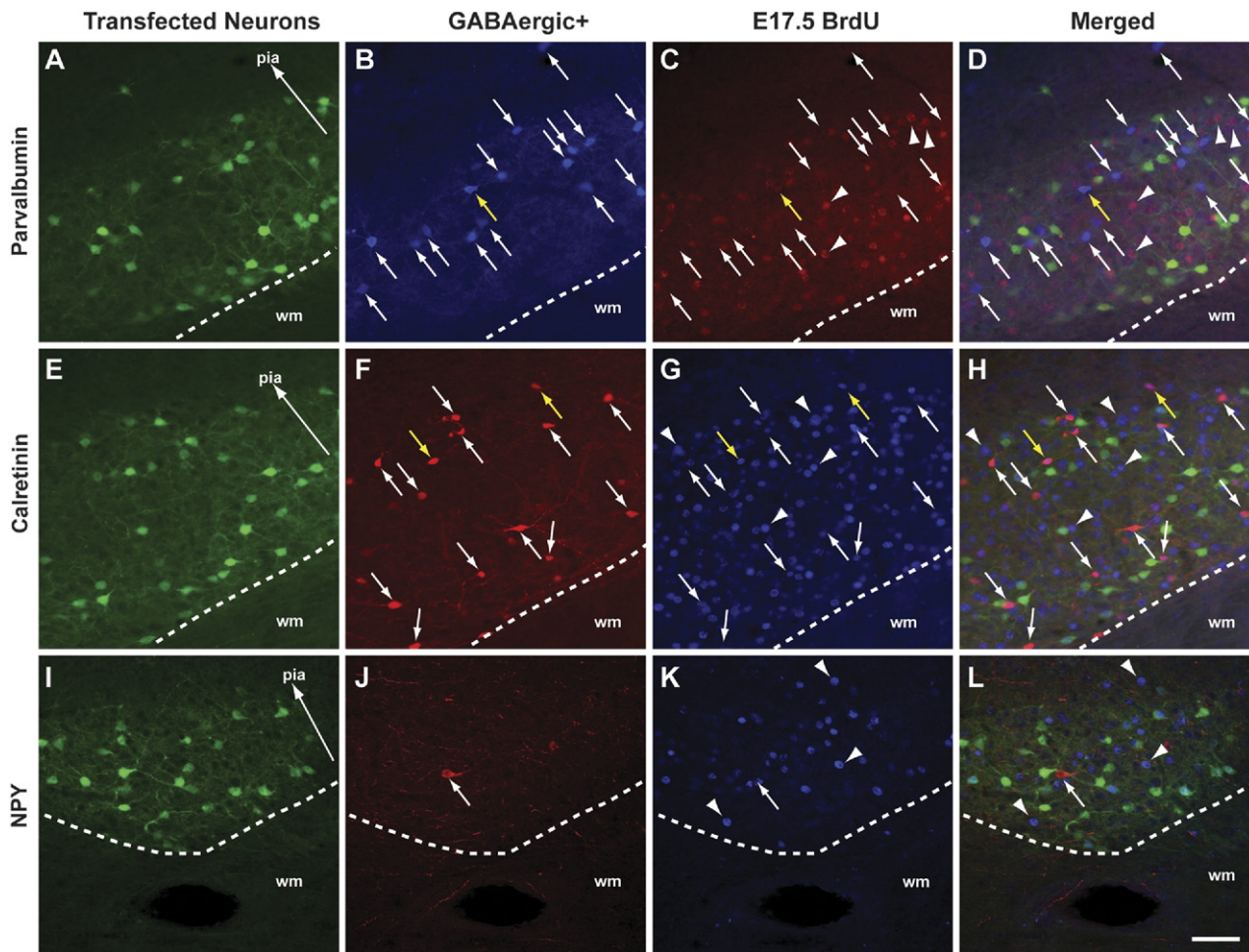


Fig. 5. Confocal microscopy illustrating E17.5 BrdU neurons in white matter heterotopias, and co-labeling with GABAergic neurons. (A–D) Heterotopic collection of neurons containing transfected neurons (A), PV+ neurons (B), E17.5 BrdU+ neurons (C). White arrows indicate PV+ neurons, and the yellow arrow indicates a neuron co-labeled with PV and BrdU. Arrowheads are BrdU positive neurons in the heterotopia that are not co-labeled. There are no transfected neurons co-labeled with either BrdU or PV. (E–H) Heterotopic collection of neurons containing transfected neurons (E), CR+ neurons (F), E17.5 BrdU+ neurons (G). White arrows indicate CR+ neurons, and yellow arrows indicate neurons co-labeled with CR and BrdU. Arrowheads are BrdU positive neurons in the heterotopia that are not co-labeled. There are no transfected neurons co-labeled with either BrdU or CR. (I–L) Heterotopic collection of neurons containing transfected neurons (I), NPY+ neurons (J), E17.5 BrdU+ neurons (K). White arrows indicate NPY+ neurons. Arrowheads are BrdU positive neurons in the heterotopia that are not co-labeled. There are no neurons that are co-labeled with any of the antibodies in the heterotopia. Bar = 100 μ m. Long arrows for orientation toward pial surface. wm, white matter.

over, we did not find any significant differences in the distribution of CR+ and NPY+ neurons in the neocortex among the groups. These results indicate that the non-cell autonomous effects of embryonic *Dyx1c1* shRNA knock-down on GABAergic neuron position are limited to those neurons positioned in heterotopias—those that migrate into the cortex are positioned appropriately.

The literature suggests that the majority, if not all, GABAergic neurons are generated in the ganglionic eminence (GE) of the ventral forebrain and migrate tangentially in a dorsal direction to the cerebral cortex (Anderson et al., 1997). PV neurons are generated in the medial GE, whereas bi-polar CR and NPY neurons are generated in the lateral/caudal GE (Wichterle et al., 2001; Valcanis and Tan, 2003; Fogarty et al., 2007). These interneurons migrate to their final positions via radial mechanisms, either from the ventricular zone (late generated) or from the

marginal zone (early generated) (Nadarajah et al., 2002; Ang et al., 2003; Hevner et al., 2004). Because the GABAergic neurons studied in this experiment are late generated, it is reasonable to assume that they all migrated from the ventricular zone. The question then becomes, why are some of these misplaced within the heterotopia, whereas others apparently migrate to take their appropriate laminar positions?

At present, we do not know the mechanisms underlying these non-cell autonomous effects, but we have speculated that it relates to disruption of the migration apparatus, perhaps because of loss or damage of radial glia (Peschansky et al., 2009). Whatever the mechanism, it could be that those GABAergic neurons that are found in the heterotopia are “trapped” by the disruption caused by the transfection. If this were the case, one would expect that neurons generated around the time of transfection

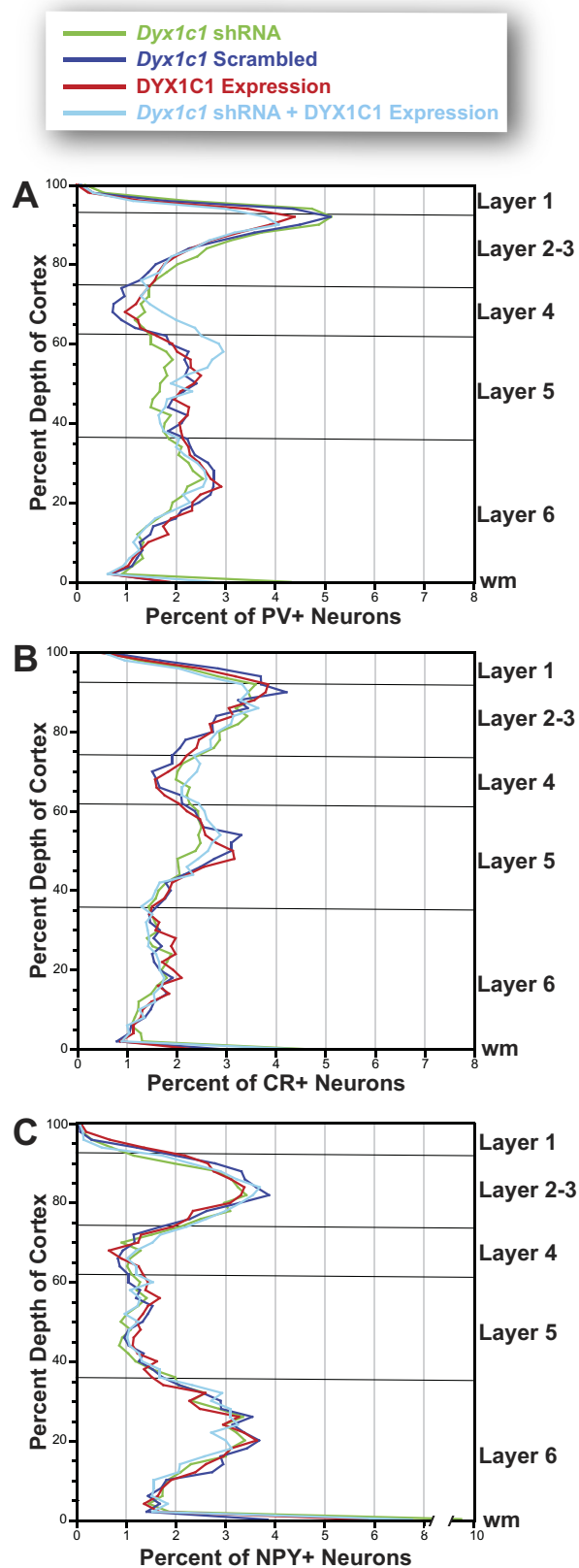


Fig. 6. Line graphs illustrating the percent of PV+, CR+, and NPY+ neurons throughout the cortical depth for all four transfection groups.

would be blocked from their normal pattern of migration, whereas neurons generated post-transfection would be less likely to be affected. This scenario is unlikely as there are a large number of neurons in the heterotopia that are generated 2 days after the transfection and large numbers of neurons in the immediately overlying cortex that were generated at the time of transfection. Alternatively it could be that the non-cell autonomous effects only affect a subset of GABAergic neurons in an “all-or-none” manner. Thus, some small population of non-transfected neurons are vulnerable to the non-cell autonomous effects, and this group ends up positioned within the heterotopia. Those that are unaffected migrate into the neocortex and take their expected laminar position. Experimental verification of this intriguing possibility awaits further elucidation of the likely mechanisms underlying these non-cell autonomous effects.

An important control for off-target effects in RNAi experiments is the co-transfection of the shRNA with a construct expressing the knocked-down protein, termed the rescue experiment. For most of the dependent measures in the current experiment we found little or no difference between the *Dyx1c1* shRNA group and the rescue condition. This was particularly surprising since expression DYX1C1 protein has been shown to effectively rescue the cell-autonomous neuronal migration phenotype when assessed 4–7 days post-transfection (Wang et al., 2006). This suggests that the non-cell autonomous effects caused by *Dyx1c1* shRNA transfection can't be compensated by the simultaneous expression of the DYX1C1 protein. If timing is the issue, then perhaps co-transfecting neuronal progenitors with *Dyx1c1* shRNA and a conditional DYX1C1 expression vector may prove more effective. Along these lines, Manent et al. (2009) demonstrated that aberrantly positioned neurons following *in utero* electroporation of *Dcx* shRNA could be stimulated to migrate again by re-expressing DCX. This treatment reduced neocortical malformations, restored neuronal patterning, and increased convulsant-induced seizure thresholds. We are now conducting experiments with *Dyx1c1* shRNA and conditional DYX1C1 expression constructs.

Finally, the abnormal development of the GABA system in the brain may play a role in subsequent brain development, possibly leading to clinically relevant disorders. GABA neurons are involved in synaptic function and in brain development (Jelitai and Madarasz, 2005; Ruediger and Bolz, 2007; Wang and Kriegstein, 2009). In addition, aberrant GABAergic signaling has been implicated in

(A) There are significantly greater numbers of PV+ neurons in white matter in the *Dyx1c1* shRNA-transfected group compared to the scrambled and expression groups, respectively ($P < .05$). There is no evidence of disruption of neuronal position of PV+ in the cerebral cortex. (B) There is no evidence of disruption of neuronal position of CR+ in the cerebral cortex. (C) There is no evidence of disruption of neuronal position of NPY+ in the cerebral cortex, although there are significantly more NPY+ neurons in the white matter in the *Dyx1c1* shRNA+DYX1C1 expression condition ($P < .05$). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

both acquired and developmental neurological disorders, such as schizophrenia, autism, epilepsy, mood and anxiety disorders, and Tourette's syndrome (Petty, 1995; Nemeroff, 2003; Wong et al., 2003; Di Cristo, 2007). While in the mature brain GABA is involved in synaptic communication, during development GABA plays extrasynaptic roles in neuronal proliferation, neuronal migration, circuit formation and synaptogenesis. While primarily an inhibitory neurotransmitter in the mature brain via GABA_A and GABA_B receptor binding that causes Ca²⁺ influx and membrane hyperpolarization, GABA is excitatory early in development, based on the fact that chloride gradients are changed by increased intracellular chloride, which leads to efflux of chloride and membrane depolarization in young neurons and neuronal precursors.

GABA's role in stem cell proliferation is related to the expression of GABA_A receptors on radial glia and the observation that GABA decreases the rate of DNA synthesis (LoTurco et al., 1995). However, GABA effects seem to promote ventricular zone (VZ) cell division while it inhibits subventricular zone (SVZ) cell division. GABA also has an effect on cell migration (Haydar et al., 2000). Paracrine blocking of GABA_A receptors in hippocampal slice cultures severely reduces migration of neuroblasts (Manent and Represa, 2007). This occurs prior to synaptogenesis in the developing brain. Behar and collaborators (2000) concluded that activation of GABA_{AVC} receptors promoted neuron migration from the VZ/SVZ to the intermediate zone, GABA_B receptors migration from the intermediate zone to the cortical plate and GABA_A receptors ended migration. Furthermore, GABA_A desensitization causes the formation of heterotopias in layer one of the cerebral cortex, presumably through overmigration (Heck et al., 2007). Thus, it appears that some of the anatomical findings of intrauterine electroporation of plasmids containing shRNA targeted against candidate dyslexia susceptibility genes, including abnormal migration of neurons within and outside the cortical plate, may be related indirectly to the changes in GABA neuron development seen with this manipulation, which we report in this study.

As discussed above, GABA activity is initially excitatory, and this excitatory activity precedes any glutamatergic excitatory activity in the developing brain. Therefore, GABA is ideally placed to serve as the main neurotransmitter regulating synapse formation and tuning of neural circuits in early development, without the danger of the excitotoxicity seen with glutamatergic activation (Wang and Kriegstein, 2009). It also appears that GABA regulates the balance between excitation and inhibition in a developing circuit in addition to its role in synaptogenesis (Wang and Kriegstein, 2009). Although all these findings must be tempered by the observation that GABA deficient mice develop brains that appear to be normal (Ji et al., 1999), the situation may be quite different in rats. In addition, compensatory steps available after gene knockout may not be available when changes in GABA function take place later in development. Furthermore, it is likely that subtle alterations of structure and function emerge from the abnormal development of GABA neurons, which are quanti-

tative and require specific hypotheses. Thus, neurons migrating to the wrong layer of the cortex, or mild decreases or increases in the numbers of neurons in the post-migration cortex, may be accompanied by physiological differences that could manifest in physiological and behavioral testing only, such as those reported by our group in the shRNA manipulated animals (Threlkeld et al., 2007).

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