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Persistent spatial working memory deficits in rats following *in utero* RNAi of *Dyx1c1*

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Disruptions in the development of the neocortex are associated with cognitive deficits in humans and other mammals. Several genes contribute to neocortical development, and research into the behavioral phenotype associated with specific gene manipulations is advancing rapidly. Findings include evidence that variants in the human gene *DYX1C1* may be associated with an increased risk of developmental dyslexia. Concurrent research has shown that the rat homolog for this gene modulates critical parameters of early cortical development, including neuronal migration. Moreover, recent studies have shown auditory processing and spatial learning deficits in rats following *in utero* transfection of an RNA interference (RNAi) vector of the rat homolog *Dyx1c1* gene. The current study examined the effects of *in utero* RNAi of *Dyx1c1* on working memory performance in Sprague–Dawley rats. This task was chosen based on the evidence of short-term memory deficits in dyslexic populations, as well as more recent evidence of an association between memory deficits and *DYX1C1* anomalies in humans. Working memory performance was assessed using a novel match-to-place radial water maze task that allows the evaluation of memory for a single brief (~4–10 seconds) swim to a new goal location each day. A 10-min retention interval was used, followed by a test trial. Histology revealed migrational abnormalities and laminar disruption in *Dyx1c1* RNAi-treated rats. *Dyx1c1* RNAi-treated rats exhibited a subtle, but significant and persistent impairment in working memory as compared to Shams. These results provide further support for the role of *Dyx1c1* in neuronal migration and working memory.

Keywords: Brain development, dyslexia, neuronal migration, RNA interference, working memory

Neocortical migrational anomalies have been associated with learning, language and other cognitive deficits in humans (Boscariol *et al*. 2009; Casanova *et al*. 2004; Galaburda *et al*. 1985; 1994; Hage *et al*. 2006). Studies from our laboratory have shown that rodents with cortical malformations exhibit deficits in rapid auditory processing (RAP) abilities, which are similar to those observed in humans with language impairment (Rosen *et al*. 1995; Clark *et al*. 2000; Peiffer *et al*. 2002; 2004a; Threlkeld *et al*. 2006, 2007). In fact, longitudinal assessment of infants, as well as studies of older populations, suggest that the RAP difficulties may represent one facet of the constellation of deficits associated with learning disorders such as developmental dyslexia (Benasich *et al*. 2006; Choudhury *et al*. 2007; Farmer *et al*. 1995; Tallal & Benasich 2002; Tallal *et al*. 1993; Wright *et al*. 1997).

Twin studies first suggested that developmental dyslexia was a genetic disorder and early genetic linkage studies laid the groundwork for recent molecular genetic studies (see Fisher & DeFries 2002 for review). Recent genetic association analyses in human populations have revealed gene variants (*ROBO1*, *DCDC2*, *KIAA0319* and *DYX1C1*) that are associated with increased risk of developmental dyslexia (Brkanac *et al*. 2007; Chapman *et al*. 2004; Cope *et al*. 2005; Dahdouh *et al*. 2009; Francks *et al*. 2004; Hannula-Jouppi *et al*. 2005; Harold *et al*. 2006; Ludwig *et al*. 2008; Marino *et al*. 2007; Massinen *et al*. 2009; Meng *et al*. 2005; Paracchini *et al*. 2006; Schumacher *et al*. 2006; Taipale *et al*. 2003; Wigg *et al*. 2004; Wilcke *et al*. 2009). *DYX1C1*, which is located on chromosome 15, was the first candidate dyslexia risk gene reported, with variants linked to reading-related phenotypes in a dyslexic family in Finland. A linkage disequilibrium analysis linked the same variants of the gene to clinical disability in a larger population of Finnish dyslexics (Taipale *et al*. 2003).

Recent reports have linked variants in *DYX1C1* to impairments in short-term memory in dyslexic individuals (Marino *et al*. 2007). Verbal and non-verbal short-term memory deficits – including visuospatial memory deficits – are often comorbid with the phonological impairments that characterize developmental dyslexia and specific language impairment (Archibald & Gathercole 2006; Baddeley & Hitch 1974; Gathercole *et al*. 2006; Smith-Spark & Fisk 2007).

Animal research employing embryonic (*in utero*) RNA interference (RNAi) has shown that the knockdown of the rodent homolog for *DYX1C1* (*Dyx1c1*) in rats results in aberrant neuronal migration, including the formation of heterotopic and ectopic clusters of neurons throughout the

cortex (Rosen *et al*. 2007; Wang *et al*. 2006). Behavioral assays on rats following *in utero* RNAi of *Dyx1c1* have revealed deficits in RAP. Spatial learning deficits were also present in a subset of animals with neuronal heterotopias in the hippocampus (Threlkeld *et al*. 2007).

This study sought to explore the possibility that early cortical disruption via interference with the rodent homolog of *DYX1C1* may lead to higher order memory and learning impairments paralleling those observed in developmental dyslexia. Specifically, we employed a model using E14 *in utero* ventricular RNAi against *Dyx1c1* to characterize the working memory abilities of embryonically transfected rats.

Methods

A total of 61 male Sprague–Dawley rats (Charles River Laboratory, Wilmington, MA, USA) were used in these experiments (*Dyx1c1* RNAi = 33; Sham = 28). All rats were pair housed in tubs with same-sex littermates and were maintained on a 12 h/12 h light/dark cycle in a temperature-controlled room. Water was available *ad libitum*. All procedures were performed in accordance with guidelines set forth by the National Institutes of Health and were approved by the University of Connecticut's Institutional Care and Use Committee.

Transfection

Transfection of *in utero* RNAi of *Dyx1c1* was performed by Y. W. at the University of Connecticut in accordance with the procedure outlined in Bai *et al*. 2003. Two batches of surgeries were performed. In all *Dyx1c1* treatments, plasmids encoding short hairpin (pU6DyxHPB) RNA (*Dyx1c1* RNAi) were transfected into the fetal (embryonic day 14 or E14) ventricular zone (VZ) by *in utero* electroporation, following externalization of the uterine horn. In Batch 1, the *Dyx1c1* shRNA plasmid was co-transfected with a plasmid encoding enhanced green fluorescent protein (eGFP), as well as fast green dye (a benign dye used to confirm injection into the lateral ventricles during the procedure). In Batch 1, Shams subjects received injections of a solution of fast green dye only. In Batch 2, *Dyx1c1* subjects received injection of the *Dyx1c1* shRNA plasmid and fast green dye. Sham subjects received transfection with plasmids (pCAGGS-RFP) encoding monomeric red fluorescent protein (mRFP) and fast green dye.

Briefly, time-mated Sprague–Dawley dams (Charles River Laboratory) were palpated to confirm gestational age as predicted by timed mating. At E14, dams were anesthetized (ketamine/xylazine; 100/10 mixture, 0.1 mg/g, intraperitoneally) and abdominal incisions were made through the skin and muscle. The uterine horns were exposed and injections were made as follows: Batch 1, *Dyx1c1* RNAi plasmids (1.5 μg/μl) + eGFP plasmids (0.5 μg/μl) + fast green dye, Shams received injections of fast green dye; Batch 2, *Dyx1c1* RNAi plasmids (1.5 μg/μl) + fast green dye, Shams received mRFP plasmids (0.4 μg/μl) + fast green dye. All solutions were microinjected into fetal ventricles by pressure (General Valve Picospritzer, Pine Brook, NJ, USA). This was performed directly through the uterine wall. One randomly chosen lateral ventricle (left or right) of each embryo was transfected, using a pulled glass capillary (Drummond Scientific, Broomall, PA, USA). Electroporation was achieved by discharge of a 500-mV capacitor charged to 50–100 mV. A pair of copper alloy plates (1 × 0.5 cm) pinching the head of each embryo was the conduit for the voltage pulse and the voltage current was discharged through both sides of the brain, transfecting the cortex bilaterally. Since embryos were unable to be sexed at this age and only males were used for testing, equal numbers of Sham and *Dyx1c1* RNAi injections were made, at roughly double the numbers needed. (Transfected females were used in other studies). Note that only males were used in this study based on previous research that has demonstrated that male rodents exhibit more robust functional deficits than females following early disruption of neuronal migration (Fitch *et al*. 1997; Peiffer *et al*. 2002, 2004b). Additionally, there is a higher incidence of dyslexia in males than in females (Flannery *et al*. 2000; Katusic *et al*. 2001; Rutter *et al*. 2004).

Sixty-one male subjects were weaned on postnatal Day 21 (P21) and received right or left ear marking. Treatment could not be identified at weaning; therefore subjects were housed in same-sex littermate pairs. (Treatment was later identified post-mortem via fluorescence of GFP in Batch 1 and via fluorescence of RFP in Batch 2). Note that the numbers of Sham and RNAi-treated subjects were expected to be roughly equal, since equal numbers of Sham and RNAi injections were made and only males were used. Histology later confirmed *Dyx1c1* treatment ($n = 33$) and Sham ($n = 28$) for a total of 61.

Apparatus

The radial arm water maze was housed in a black Plexiglas pool (140 cm in diameter and 40-cm deep) filled with cool water [22 (±2)°C]. The maze consisted of eight removable stainless steel arms painted flat black that could be attached to a central octagonal hub (50 cm across). Each corridor was 14-cm wide and extended 36 cm to the edge of the pool (Fig. 1). A removable black plastic platform (10 cm diameter) served as the escape platform and was submerged 4 cm beneath the surface of the water. The entire apparatus was in a large room with two empty walls, a long table and the cage rack forming the boundaries around the maze. During testing, light was provided by a desk lamp in the northeast corner of the room, for additional spatial cues.

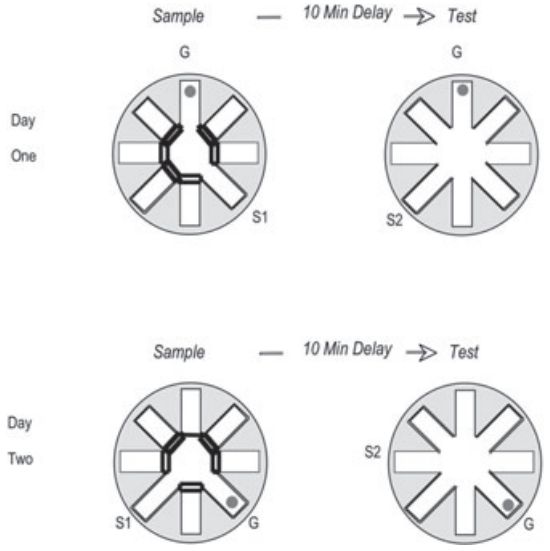

Figure 1: Diagram of the delayed match-to-sample radial water maze task, including sample (left) and test (right) trials. Exemplars for the first and second days of testing are given. S = start arm [location changes between sample (S1) and test trial (S2)] and G = goal arm (location remains fixed). Each day of testing rats were given one forced-choice sample trial in which all arms were blocked except for the start arm and goal arm. A plastic escape platform was submerged at the end of the goal arm. During the test trial (10 min later), all the arms were open and a new start arm (S2) was used to test the animal's memory of the spatial location of the goal arm. Each animal received one sample trial and one test trial each day of testing. The sequences of start arms and goal locations used each day varied systematically among 48 patterns that regulated the sequence of start and goal arms, the turn angles and the relationship between the start and goal arms across trials.

Delayed match-to-sample testing

Beginning on P33, subjects were handled 5 min a day for the week prior to testing. On the initial testing day (P40), subjects had no prior exposure to the testing room, the water maze or the platform. All rats were assessed on an initial acclimation trial and were found to be capable of navigating the maze and mounting the platform. Subsequent testing of animals consisted of four daily sessions per week (four sessions per week, one session per day, one sample and one test trial per session). Each session consisted of a forced-choice sample trial and a test trial in which all arms were open (see Chrobak *et al*. 2008 for additional details). During the sample trial, all corridors were blocked at the intersection of the arm and central hub, except for the start and goal arms. Each rat swam out of the start arm, navigated to the only open corridor (the goal arm) and mounted the escape platform. The subject was removed immediately after mounting the platform, gently dried with a towel and returned to the home cage. Subjects took ∼4–20 seconds to complete the sample trial. This study employed a 10-min delay, so the test trial was administered 10 min after the sample. A new start arm was used during the test trial, but the goal location remained the same. (The start arm was changed to insure navigation based on spatial memory rather than memory of turn angle). During the test trial, all maze arms were open. Subjects were tested once a day each day of a 5-day work week, over a period of 12 weeks, using a different start and goal arm each day. Sequences of start arms and goal locations were varied systematically among 48 patterns. This regulated the sequence of the start and goal arms and the relationship between them, across trials. The goal location was restricted to arms 90° (two arms) or more away from the prior (i.e. yesterday's) goal location.

At the end of testing, all subjects were transcardially perfused for assessment of brain tissue and analysis of experimental treatment (Sham or *Dyx1c1* RNAi), such that the behavioral data could be analysed as a function of treatment. Importantly, before post-mortem analyses, all behavioral assessments were performed blind to treatment.

Control trials

To assess the possibility that subjects might use intramaze cues (i.e. visual, olfactory or somatosensory cues) to find the platform, we examined the performance on periodic 'control' trials. During these trials, no forced-choice sample trial was given and rats had to seek and find the platform in a random new location. These trials provided a measure of 'chance' performance and consistently revealed a range of mean errors at 4.4–4.7 errors per day for both groups for all control sample trials. There were no differences between the Control Trial performance of *Dyx1c1* RNAi and Sham animals.

Dependent measures

Dependent measures assessed included the number of incorrect arm entries (errors) during the test trial, mean latency per arm choice during the sample and test trials (total latency to reach the platform divided by the number of arms entered during the trial) and the type of the first error made during the test trial (when an error was made). First error types were divided into three groups: prior goal errors (in which the subject's first entry was into the prior day's goal arm), adjacent arm errors (in which the subject's first entry was into one of the two arms adjacent to the goal arm) and other errors (which describes random entry into an arm that was not the prior goal nor an adjacent arm).

Histological analysis

Upon the completion of testing, subjects were weighed, deeply anesthetized with ketamine/xylazine (100 and 15 mg/kg, respectively) and transcardially perfused with phosphate buffered saline followed by chilled 4% paraformaldehyde. Heads were removed and brains were extracted, bottled in paraformaldehyde and shipped to Glenn D. Rosen at Beth Israel Deaconess Medical Center for histological preparation. Brains were placed into a 30% sucrose buffer prior to being cut in the coronal plane at 40 μm section thickness. A 1-in-10 series of sections were mounted and stained with Thionin for Nissl substance, while an adjacent series of free-floating sections were mounted and screened using fluorescence microscopy for the presence of GFP or RFP. Another series of sections was immunohistochemically processed for visualization of RFP or GFP (Chemicon, 1:200) using ABC protocols. Light microscopic analysis was used to visualize the disposition of transfected cells and identify dysplasia in RNAi-treated and Sham subjects.

Data analyses

Multivariate analyses of variance (ANOVA) were used to analyze both error and latency data for order and trend. The pattern of error types was analyzed using a χ^2 analysis on the frequency distribution of prior, adjacent and other errors. All reported *P*-values are two-tailed. All statistical analyses were conducted using SPSS or Microsoft Excel.

Results

Histology

Fluorescence microscopy and immunohistochemical staining were used to confirm the presence or absence of GFP and/or RFP. Analysis revealed 33 experimental (RFP-negative and/or GFP-positive) and 28 control (RFP-positive and/or GFP-negative) subjects. Further histological examination revealed five categories of cortical characterization: (1) no visible malformations (RNAi treated $n = 18$, Sham $n = 23$), which describes any subject whose brain tissue was free of the gross malformations defined in the other categories, (2) injection site ectopia (RNAi treated $n = 10$, Sham $n = 5$), resulting from the injection puncture wound, forming an ectopic collection of cells in Layer 1, (3) non-injection site ectopia (RNAi treated $n = 2$, Sham $n = 0$), an ectopic collection of neurons in areas of Layer 1 that were distal to the injection site, (4) unmigrated neurons (RNAi treated $n = 12$, Sham $n = 0$), in which collections of neurons failed to migrate to their target layers and instead formed heterotopic pockets in the white matter near the border of the VZ and (5) hippocampal dysplasia (RNAi treated $n = 5$, Sham $n = 0$) or unmigrated neocortical neurons that primarily disrupted the dentate gyrus (Fig. 2). The various malformations varied in size and number and some subjects had multiple types of disruption. It is also worth noting that, other than focal injection site ectopia ($n = 5$), Sham animals did not display any cortical disruption. Note that in addition to being analyzed as a function of treatment (*Dyx1c1* RNAi vs. Sham), all behavioral results were analyzed as a function of these histological subgroups.

Errors to find the platform (test trials)

Analyses of the overall errors revealed an overall significant effect of Treatment, with *Dyx1c1* RNAi-treated subjects ($n = 33$) showing impaired acquisition and performance of the delayed match-to-sample radial water maze task as compared to Shams [$F_{(1,59)} = 7.826$, $P = 0.007$] (Fig. 3). We also found a significant effect of 2-week block (referring to 2-week blocks of testing) [$F_{(1,59)} > 20$, $P < 0.001$], with improved performance (fewer errors) over testing. There was no 2-week block × Treatment interaction [$F_{(1,59)} < 1$, not significant (NS)], indicating learning for both groups. Yet these data show that rats that received RNAi targeted against

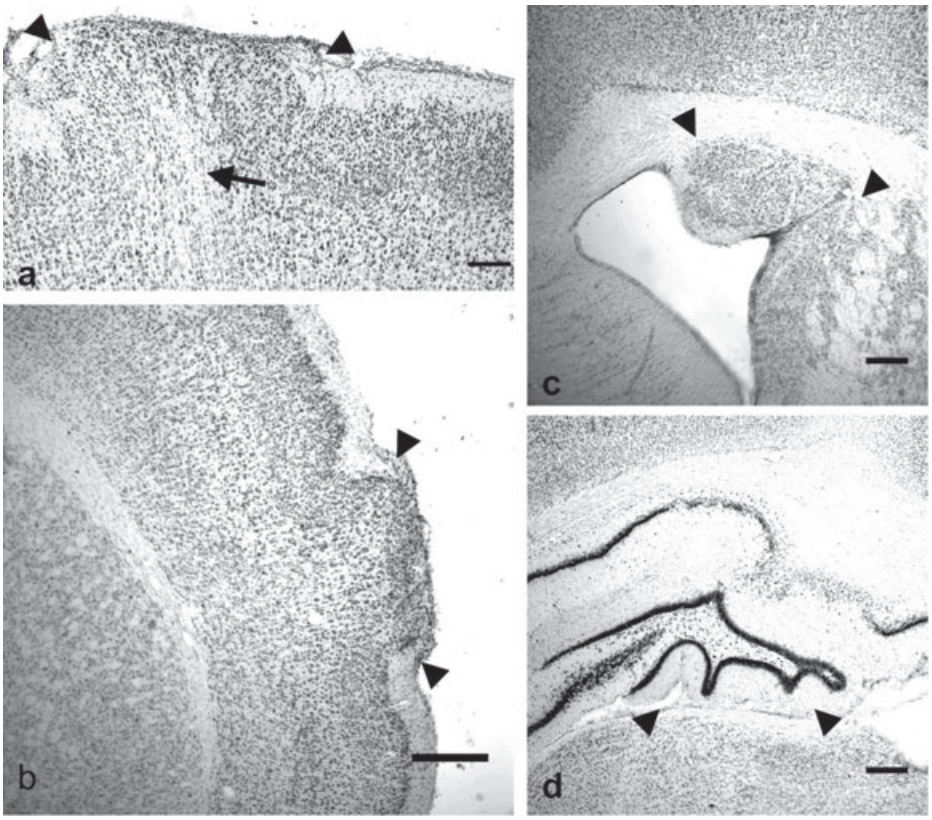

Figure 2: Histology of *Dyx1c1* RNAi subjects. Histology revealed four categories of gross cortical disruption. (a) Injection site ectopia (RNAi treated $n = 10$, Sham $n = 5$), resulting from the injection puncture wound, forming an ectopic collection of cells in Layer 1 (black arrowheads) and a characteristic cell-free streak in the subjacent layers (black arrow). Scale bar = 150 μm. (b) Non-injection site ectopia (RNAi treated $n = 2$, Sham $n = 0$), characterized by an ectopic collection of neurons in areas of Layer 1 that were distal to the injection site (black arrowheads). (Note the absence of the cell-free streak associated with the injection site). Scale bar = 150 μm. (c) Unmigrated neurons (RNAi treated $n = 12$, Sham $n = 0$), which describes the collections of neurons that failed to migrate to their target layers and instead formed heterotopic pockets in the white matter, near the border of the VZ (black arrowheads). Scale bar = 250 μm. (d) Hippocampal dysplasia (RNAi treated $n = 5$, Sham $n = 0$), which primarily affects the dentate gyrus (black arrowheads). Scale bar = 250 μm.

Dyx1c1 exhibited significant and sustained impairments across all 12 weeks of testing as compared to Shams.

Distribution of first error types

We divided the type of first errors made into three categories (for each test trial in which an error was made). Interestingly, in spite of the fact that the *Dyx1c1* RNAi-treated subjects made more errors than Shams throughout testing, the distribution of first error types for this group did not differ from Shams (Fig. 4). That is, the distribution of the types of first errors during the last 4-week block of testing indicated no Treatment difference ($\chi^2 = 0.93$, $df(2)$, NS). Recall that a prior goal error indicates proactive interference from the previous testing day, while an adjacent arm error indicates that there is a weakened representation of the current goal location. In fact, both Shams and *Dyx1c1* RNAi-treated rats showed more than 50% of initial errors in the last block of testing being made to the prior goal or to an adjacent arm. This finding indicates that initial errors in all subjects, regardless of treatment, were mainly due to proactive interference or weakened goal representation (rather than no memory of the goal at all, which was measured by 'other' errors). Thus, while the spatial working memory system was apparently impaired or weakened in the *Dyx1c1* RNAi-treated rats, they nonetheless performed the task using a similar strategy to Shams.

Latencies to mount the platform

An ANOVA was used to analyze the average latency per arm choice during the sample and test trials (Table 1). Results showed a significant 2-week block effect [$F_{(1,59)} > 10$, $P = 0.001$] indicating that rats swam and entered arms more rapidly over the 12 weeks of testing. There was not a significant Treatment effect [$F_{(1,59)} = 1.845$, NS], nor Block × Treatment interaction [$F's_{(1,59)} < 1$, NS]. This finding shows that the *Dyx1c1* RNAi-treated rats performed the

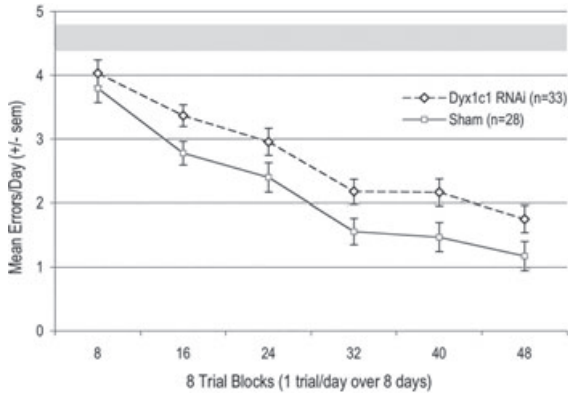

Figure 3: Mean errors made by Sham and *Dyx1c1* RNAi subjects over six 8-trial blocks (12 weeks/48 days) of testing. An overall Treatment effect on mean errors ($P < 0.01$) with no Treatment × Block interaction indicated that rats that received *Dyx1c1* RNAi made significantly more errors than Sham-treated rats. The shaded gray bar represents the span of average errors made by Sham and *Dyx1c1* RNAi animals (4.4–4.7 errors) on the weekly control trials which measured chance performance on the task.

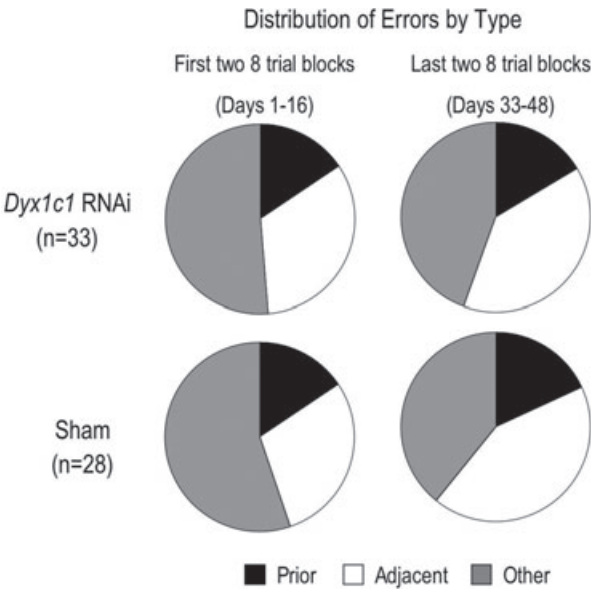

Figure 4: Distribution of error types for the first error made in the first and last 8-trial blocks of testing. We divided the first errors into three categories for each test trial in which an error was made (prior, adjacent and other). A χ^2 on the distribution of the type of first error between the first 2 weeks and last 2 weeks of testing showed no Treatment effect. For each group, more than 50% of the errors being made at the end of testing were either to the prior goal or to an adjacent arm, indicating that errors were mainly due to proactive interference or weakened goal representation, rather than no memory of the goal at all (which was measured by the percent of Other errors). Thus, while the spatial working memory system was apparently impaired in the *Dyx1c1* RNAi-treated rats, they performed the task in a similar manner (i.e. using a similar strategy) to Shams.

motor component of the task (i.e. swimming/entering arms) comparably to Shams.

Anatomical subgroups

The subjects were divided into three groups to determine the effect of the anatomical phenotype on performance. An ANOVA was used to compare the average errors made by Shams ($n = 28$), *Dyx1c1* RNAi animals with no visible malformations ($n = 18$) and *Dyx1c1* RNAi animals with gross malformations (i.e. ectopias or heterotopias; all animals with gross malformations were pooled into one group because of the small number of animals within each individual anatomy subgroup) ($n = 15$). A significant overall difference in the number of errors made among the three groups was detected [$F_{(1,58)} = 4.742$, $P = 0.012$] (Fig. 5). There was a significant Block effect [$F_{(1,58)} = 52.903$, $P < 0.001$] indicating significant improvement among all three groups over the 12 weeks of testing. There was not a significant Block × Group interaction [$F_{(2,58)} < 1$, NS]. Interestingly, when compared separately, the *Dyx1c1* RNAi animals with gross malformations did not differ significantly from Shams in the average number of errors made across 12 weeks of testing [$F_{(1,44)} = 2.476$, $P = 0.123$]. However, the *Dyx1c1* RNAi animals with no visible malformations did differ significantly from Shams, consistently making more errors across 12 weeks of testing [$F_{(1,44)} = 8.536$, $P = 0.005$]. The *Dyx1c1* animals with no visible malformations did not differ from Shams on any of the other behavioral measures.

Discussion

Here we report that *in utero* disruption of *Dyx1c1* is associated with impaired learning and memory on a delayed match-to-sample radial water maze task. Moreover, impairments were present even after 12 weeks of testing, indicating that the disruptions to higher order working memory induced by RNAi of *Dyx1c1* were persistent.

These findings are complementary to a recent study by Marino *et al*. (2007) which revealed a link between memory performance and *DYX1C1*. Marino's group studied associations between *DYX1C1* and several dyslexic phenotypes in a large, family-based study of Italian dyslexics. Results of the study revealed a significant association between specific variants [single nucleotide polymorphisms (SNPs)] within the *DYX1C1* gene and performance on the single letter backward task (a measure of auditory short-term memory). Dyslexic individuals who had a copy of the *DYX1C1* gene that contained the variants of interest made significantly more errors on the task than those without the variants.

A recent study by Dahdouh *et al*. (2009) found an association between *DYX1C1* variants and short-term memory in female dyslexics. This finding is interesting in light of a recent report that the DYX1C1 protein interacts with α and β estrogen receptors, which are present in the brain during development (Massinen *et al*. 2009). In this study, only

Table 1: Average latency per arm choice (in seconds) during the test trial

	Eight-trial blocks (1 trial/day over 8 days)					
	8	16	24	32	40	48
Dyx1c1 RNAi ($n = 33$)	7.4 (1.9)	6.2 (1.4)	6.5 (2.3)	6.4 (2.4)	5.8 (1.8)	5.6 (1.9)
Sham ($n = 28$)	6.6 (1.9)	6.3 (1.9)	6.0 (1.8)	5.9 (2.2)	5.2 (1.1)	4.8 (1.0)

Means ± SEM. All data are reported in seconds. Mean latencies as a function of the number of arms entered during the test trial. No significant differences.

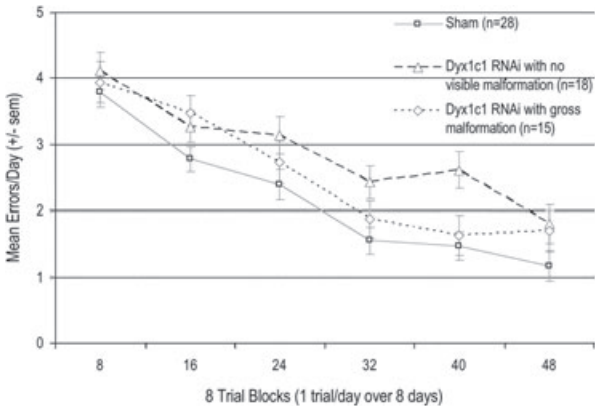

Figure 5: Mean errors per day as a function of anatomical phenotype. A repeated measures ANOVA reveals a significant group effect on mean errors ($P = 0.012$) among Shams ($n = 28$), *Dyx1c1* RNAi animals with no visible malformations ($n = 18$) and *Dyx1c1* RNAi animals with gross malformations ($n = 15$). When compared separately, a repeated measures ANOVA revealed a significant difference between the *Dyx1c1* RNAi animals with no visible malformations and Shams ($P = 0.005$), with the *Dyx1c1* RNAi animals with no visible malformations consistently making more errors than Shams. There was not a significant difference in performance between the *Dyx1c1* RNAi animals with gross malformations and Shams ($P = 0.123$). This result suggests that knockdown of *Dyx1c1* may lead to formation of subtle malformations that consequently result in memory deficits.

males were used for testing based on the higher incidence rates of dyslexia in males, as well as previous research which indicates that the functional deficits in male rodents are more robust than those in females following early disruption of neuronal migration (Fitch *et al*. 1997; Flannery *et al*. 2000; Katusic *et al*. 2001; Peiffer *et al*. 2002, 2004b; Rutter *et al*. 2004). Given the emerging evidence of sex-specific effects of *DYX1C1*, future studies will assess the effects of early interference with candidate dyslexia susceptibility genes in female rodents in addition to males.

The current results are interesting in view of the fact that phonological deficits associated with developmental dyslexia consistently occur in parallel with verbal and non-verbal short-term memory deficits (Smith-Spark & Fisk 2007). For example, when tested on tasks such as word-list recall (which is thought to tap into phonological short-term memory), dyslexic individuals show significant deficits as compared to age-matched controls (Baddeley & Hitch 1974). Language-impaired individuals also show deficits in higher order processing of new and stored phonological information, which is required for sentence processing. These deficits are typically characterized as 'verbal working memory deficits' (Archibald & Gathercole 2006; Gathercole *et al*. 2006; Smith-Spark & Fisk 2007).

Additionally, deficits in visuospatial short-term memory have occasionally been reported in dyslexics. For example, dyslexic individuals show impairment on tasks such as the Corsi block span task and the visual patterns test – both of which test pattern sequence recall abilities (Gathercole *et al*. 2006; Smith-Spark & Fisk 2007). Thus, a potential core deficit in central executive working memory systems in developmental dyslexia has recently been characterized (Smith-Spark & Fisk 2007). Such a cross-modal processing deficit would account for the wide range of working memory impairments observed in developmental dyslexia.

Concomitant evidence from animal models suggests that RAP deficits (as seen in cortically disrupted animals) are aggravated when difficulty and complexity of the task are increased (Fitch *et al*. 2008a,b; Peiffer *et al*. 2004a). Data from these studies suggest evidence of higher order learning and memory deficits in cortically disrupted animals, prompting questions about a possible relationship between cortical dysgenesis and other cognitive deficits associated with developmental dyslexia (i.e. working memory; Smith-Spark & Fisk 2007). In fact, deficits in working memory have been reliably replicated in the same animal models that showed difficulties in RAP (Boehm *et al*. 1996; Fitch *et al*. 2008a,b; Hoplight *et al*. 2001; Threlkeld *et al*. 2007; Waters *et al*. 1997). The fact that deficits in both RAP and also learning and memory can be reliably elicited from rodent models employing different forms of cortical disruption (e.g. microgyria, ectopia and hypoxic-ischemic injury) suggests that cortical disruption during vulnerable periods early in development appears to cause robust long-term impairments across an array of processing modalities (Fitch *et al*. 2008a,b; Threlkeld *et al*. 2007).

Higher order working memory disorders have previously been demonstrated in other models of early cortical disruption. For example, BXSB/MpJ mice – a strain in which 40–60% of mice exhibit spontaneously occurring molecular layer ectopias – have shown deficits in performance on a delayed match-to-sample Morris water maze, inverted Lashley III maze and two versions of the Hebb–Williams maze (all of which emphasize working memory demands; Boehm *et al*. 1996; Hoplight *et al*. 2001; Waters *et al*. 1997). Our present findings are consistent with these reports, all of

which suggest that early disruption of cortical development may lead to robust memory deficits.

It is worth noting, however, that when using the delayed match-to-sample Morris water maze, the inverted Lashley III maze and the Hebb–Williams maze performance of ectopic and non-ectopic BXSB/MpJ mice was found to converge rather quickly. That is, although the ectopic mice initially performed worse than their non-ectopic littermates, their performances converged within 1–2 weeks of testing (Boehm *et al*. 1996; Hoplight *et al*. 2001). This is in contrast to the current results, which demonstrate persistent working memory deficits even after 12 weeks of testing. Thus, the current results provide support for the use of the delayed match-to-sample radial arm water maze as a more demanding working memory task capable of eliciting sustained working memory deficits.

The neurophysiological underpinnings of the behavioral deficits induced by the knockdown of *Dyx1c1* remain unknown. The observation that roughly half of the *Dyx1c1* RNAi-transfected animals exhibit a lack of gross, visible malformations while the others exhibit large disruptions that can be observed with the naked eye is a paradox. Although there are not visible malformations in the brains of some of the *Dyx1c1*-treated animals, this does not imply normal cortex. The behavioral data suggest that there is some disruption of functional connectivity in the brains of the RNAi transfected animals lacking gross malformations (Fig. 5). This suggestion parallels overwhelming evidence of functional activation differences between dyslexic and typical samples during language processing tasks, even though anatomic anomalies are not always observed in these same dyslexic samples (Beneventi *et al*. 2010; Maisog *et al*. 2008; Wolf *et al*. 2010; also see Webster *et al*. 2008). Differential patterns of activation under fMRI indicate that subtle differences in circuitry or regional specialization are present, even though current methods of *in vivo* anatomic quantification cannot detect them. Previous studies have demonstrated that, in addition to creating gross malformations such as ectopias and heterotopias, knockdown of *Dyx1c1* can lead to subtle disruption of neuronal migration, causing individual neurons to migrate to Layer 2 instead of reaching their appropriate target destination in Layer 3 (Rosen *et al*. 2007; Wang *et al*. 2006). Thus, it is possible that such subtle laminar disruption exists in the *Dyx1c1* RNAi animals with no visible malformations, which could account for the significant impairment in this specific subgroup of animals. Future studies will further characterize the subtle disruption that exists in the *Dyx1c1* RNAi brains at the microscopic and electrophysiological level.

Finally, hippocampal malformations were observed in five *Dyx1c1* RNAi animals. In a previous study assessing the behavioral impacts of *Dyx1c1* RNAi, hippocampal malformations were specifically linked to a robust spatial learning impairment (Threlkeld *et al*. 2007). In the current study effects seen for this subgroup were no more significant than those for the other *Dyx1c1* RNAi animals without hippocampal malformations. This suggests that the radial arm maze taps into a circuit that is different than hippocampal-dependent spatial navigation abilities. However, the relative contribution of hippocampal anomalies to these behavioral effects cannot be discounted.

The results of the current study have significant implications for our understanding of the role of the *Dyx1c1* gene in the development of dyslexia. As previously mentioned, working memory deficits (both verbal and non-verbal) have been associated with dyslexia (Jeffries & Everatt 2004; Smith-Spark & Fisk 2007). Working memory is implicated in the storage of relevant representations that allow grapheme-to-phoneme conversion and phoneme assembly, both of which are necessary for reading. In addition to the previously mentioned study by Marino *et al*. (2007), studies by Wigg *et al*. (2004) and Smith *et al*. (2005) showed associations between phonological and memory impairment and variants within the coding region of the *Dyx1c1* gene in dyslexic and language-impaired populations, using the Nonword Repetition task which measures phonological memory abilities. Thus, *DYX1C1* variants are associated with both phonological and working memory deficits in dyslexic populations. Animal studies have provided a parallel to clinical work and have similarly shown that *Dyx1c1* knockdown is associated with both RAP and working memory deficits (Threlkeld *et al*. 2007). The convergent data on neuronal migration abnormalities in dyslexics, the discovered role of *Dyx1c1* in neuronal migration and the apparent links between *DYX1C1*/*Dyx1c1* and a range of dyslexic phenotypes in both human and animal studies (respectively), all provide support for a potential role of *DYX1C1* in the etiology of dyslexia.

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