Developmental disruptions and behavioral impairments in rats following *in utero* RNAi of Dyx1c1

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Abstract

Developmental malformations of cortex have been shown to co-occur with language, learning, and other cognitive deficits in humans. Rodent models have repeatedly shown that animals with such developmental malformations have deficits related to auditory processing and learning. More specifically, freeze-lesion induced microgyria as well as molecular layer ectopias have been found to impair rapid auditory processing ability in rats and mice. In humans, deficits in rapid auditory processing appear to relate to later impairments of language.

Recently, genetic variants of four different genes involved in early brain development have been proposed to associate with an elevated incidence of developmental dyslexia in humans. Three of these, DYX1C1, DCDC2, and KIAA0319, have been shown by *in utero* RNAi to play a role in neuronal migration in developing neocortex. The present study assessed the effects of *in utero* RNAi of Dyx1c1 on auditory processing and spatial learning in rats. Results indicate that RNAi of Dyx1c1 is associated with cortical heterotopia and is suggestive of an overall processing deficit of complex auditory stimuli in both juvenile and adult periods ($p = .051$, one-tail). In contrast, adult data alone reveal a significant processing impairment among RNAi treated subjects compared to shams, indicating an inability for RNAi treated subjects to improve detection of complex auditory stimuli over time ($p = .022$, one-tail). Further, a subset of RNAi treated rats exhibited hippocampal heterotopia centered in CA1 (in addition to cortical malformations). Malformations of hippocampus were associated with robust spatial learning impairment in this sub-group ($p < .01$, two-tail). In conclusion, *in utero* RNAi of Dyx1c1 results in heterogeneous malformations that correspond to distinct behavioral impairments in auditory processing, and spatial learning.

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1. Introduction

Malformations of developing cortex are associated with language, learning, and other neurological deficits in humans [32,20–22,1,3]. We have previously reported that rodents with induced and spontaneous cortical malformations exhibit deficits in processing rapid changes in acoustic stimuli [24,8,34–37] that are similar to those seen in humans with language impairment [43,15]. That these defects in rapid auditory processing (RAP) can be produced in rodent models via different forms of cortical anomalies (microgyria, ectopia, perinatal hypoxic-ischemic injury) indicates that cortical disruption during an early period of developmental vulnerability may produce robust long-term impairments in auditory processing [45,27,28,34,38].

Over the past 3 years, genetic linkage experiments have provided evidence that variants of four different genes may associate with language-related developmental impairments and dyslexia [41,11,23,30,29,47,42]. Interestingly, all four may play roles in early brain development. Robo1, for example, is known to regulate axon guidance in the developing brain, and Dcdc2, a member

of the doublecortin family of genes, and Kiaa0319, have been recently shown to regulate neuronal migration in developing neocortex in rodent models [11,23,29,47]. DYX1C1 was the first reported candidate gene for dyslexia. The evidence was based on a study linking a chromosomal translocation that disrupted this gene to dyslexia in a single family, and on a linkage disequilibrium study of DYX1C1 variants in a population of Finnish dyslexics [42]. Since the first report, however, the genetic variants linked to dyslexia in the Finnish population have not been found to be linked to dyslexia in several other larger populations from the UK, US, and Italy [4,25,40]. Nevertheless, the possibility remains that some alleles of DYX1C1 increase dyslexia risk in some populations.

Studies evaluating key behavioral markers of developmental language or reading impairment reveal that deficiencies in detecting rapid changes in auditory stimuli predict later language outcome, including the emergence of dyslexia [5,44]. To further explore the possibility that developmental cortical disruption may lead to auditory processing and learning impairment, we employed a novel model to disrupt cortical neuronal migration via *in utero* RNAi in rats [2]. Dyx1c1 (a homolog to the human DYX1C1 gene) was selected as the RNAi target gene, based on its preliminary connection with dyslexia [42], and on its role in neuronal migration revealed in an RNAi screen for genes important for neuronal migration [46]. The current study sought to characterize the auditory and spatial processing abilities of rats intra-ventricularly transfected with Dyx1c1 RNAi.

2. Materials and methods

2.1. Transfection

Transfection of *in utero* RNAi of dyx1c1 was performed by JB at the University of Connecticut. In all Dyx1c1 treatments, plasmids encoding short hairpin (pU6DyxHPB) RNA (Dyx1c1 RNAi) and plasmids encoding eGFP (green fluorescent protein) were co-transfected into the ventricular zone (VZ) by *in utero* electroporation. Sham subjects received transfection only with plasmids (pCAGGS-RFP) encoding mRFP (red fluorescent protein). Transfection occurred around E14 in time–mated dams. Specifically, nine Wistar dams (Charles River Laboratory, Wilmington, MA) were anesthetized, the uterine horns were exposed and dyx1c1 RNAi plasmids (1.5 μg/μl), with GFP (0.4 μg/μl) (or RFP for shams, 0.4 μg/μl), were microinjected by pressure (General Valve picospritzer) through the uterine wall into one randomly chosen lateral ventricle of each embryo, using a pulled glass capillary (Drummond Scientific). Equal numbers of sham and Dyx1c1 RNAi injections were made, at roughly double the numbers needed since embryos were unable to be sexed at this age (only males were used in experimental assessments). Electroporation was achieved by discharge of a 500-mV capacitor charged to 50–100 V [2]. A pair of copper alloy plates (1 cm × .5 cm) pinching the head of each embryo through the uterus was the conduit for the voltage pulse. For each embryo, plasmid was injected in one hemisphere, with some diffusion of the solution into the opposite hemisphere expected. Voltage current was discharged through both sides of the brain, transfecting cortex in both hemispheres. Thirty-six male subjects were weaned on P21, and received right or left ear marking, and were housed into pairs. Treatment could not be identified until postmortem analysis, via confirmation of the presence of RFP or GFP labeling. Note that because equal numbers of RNAi treatment and sham injections were made, and only males were retained, the representation of sham and RNAi treated subjects was expected to be roughly evenly divided among the 36 males. [In fact, later histology confirmed Dyx1c1 treatment (*n* = 21), and sham (*n* = 15), for a total of 36.] Importantly, our research has consistently shown larger functional deficits in male as compared to female rodents following early neuromigrational disruption [17,34,36]. Thus, we uti-

lized male subjects in the current study to provide for optimal expression of any deficits associated with RNAi of Dyx1c1.

Subjects were reared under a 12-h light:12-h dark cycle with food and water available *ad lib*. Juvenile auditory testing began on P23. All procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, including adequate measures to minimize pain and discomfort. The Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut approved all procedures.

2.2. Behavioral testing: startle reduction

Juvenile auditory testing began on P23, comprising a startle response paradigm that has been discussed extensively elsewhere [35,36,45]. Briefly, the startle modification paradigm involves the presentation of an auditory cue prior to a startle-eliciting stimulus (SES). The SES elicits an acoustic startle reflex (ASR) – a ballistic large-motor "startle" response – but if the pre-SES auditory cue is detected, the intensity of the ASR is reduced. In this way, startle reduction (also called pre-pulse inhibition) can be used to assess stimulus discriminability [14]. For testing, subjects were placed on a load cell platform (Med Associates, Georgia, VT). The voltage of each platform was passed through a linear load cell amplifier (PHM-250-60) into a Biopac MP100WS acquisition system (Biopac Systems, Santa Barbra, CA) connected to two Macintosh computers, which recorded the amplitude of the subject's ASR. The maximum peak value was extracted from the 150 ms following the onset of the SES (i.e., the animals' response intensity for that trial, our dependent variable). Auditory stimuli were generated using a Pentium 4 Dell PC with custom programmed software and a Tucker Davis Technologies (RP2) real time processor. Stimulus files were played through a Marantz integrated amplifier connected to nine Cambridge Sound Works speakers, with sound levels calibrated by sound level meter [35]. Each pair of platforms had one speaker centered and mounted 30 cm above. Attenuated response scores (ATT) were calculated from the peak ASR using the formula ([mean cued response/mean uncued response] × 100), where absolute response scores indicate load-cell displacement for each subject's startle response on each trial. Thus, ATT scores (relative performance on a given condition, expressed as a percentage) represented a second dependent variable.

2.3. Single tone

Starting on P23, all subjects received 1 day of a single tone procedure [35]. The single tone test session was comprised of 104 trials (cued or uncued), presented in a pseudo-random manner. Uncued trials consisted of a silent background preceding a 105 dB, 50 ms SES. On cued trials, a 75 dB, 7 ms, 2300 Hz tone was presented 50 ms prior to the SES.

2.4. Long/short silent gap

A long silent gap detection procedure was utilized over P25-35 to assess gap thresholds over 4 days. The long-gap session (0–100 ms) comprised 300 trials consisting of the presentation of long silent gaps (2, 5, 10, 20, 30, 40, 50, 75, and 100 ms) embedded in 75 dB broadband white noise. Gaps of variable duration were presented 50 ms prior to a 105 dB burst of white noise, with a 0 ms "gap" as the uncued trial (see [37,19] for further details). A short silent gap procedure (0–10 ms; gap duration of 0, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ms) was also presented for 4 days to determine the shortest detectable gap for each group [19]. The parameters of this procedure were identical to that of the long silent gap, except that the gap durations were shifted from 0–100 to 0–10 ms.

2.5. Oddball

The oddball procedure included 104 trials (both cued and uncued) presented in a pseudo-random order across four sessions (one per day). Two oddball test sessions were administered in the juvenile period (P36–40) and two in adulthood (P60+). This procedure consisted of the repeated presentation of a standard 75 dB, high/low three-octave two-tone stimulus sequence (2300–1100 Hz, 7 ms duration) separated by a within-stimulus inter-stimulus interval (ISI) of 225, 125, or 100 ms (one ISI used per session, see [27] for further details). For purposes of analysis, note that the 125 and 100 ms ISI were classified as "short" in

comparison to a "long" (225 ms) duration ISI. Each sequence was delineated by a fixed between-stimulus ISI, which was always 200 ms greater than the variable duration within-stimulus ISI. Uncued trials consisted of 50 ms of silence after the last two-tone sequence, followed by a 105 dB, 50 ms SES. Cued trials involved the reversal of the standard high/low tone pair to a low/high (oddball) stimulus, followed by 50 ms of silence and then the SES.

2.6. Water escape and Morris water maze

The spatial learning test procedures employed were identical to those described in McClure et al. [27]. Briefly, all subjects used in the Morris water maze were first tested on a water escape task, to rule out motivational, motor, or visual impairments. The water escape task involved the use of a visible platform (4 in. in diameter) placed at one end of an oval tub (40.5 in. × 21.5 in.) filled with water (8 in.) at room temperature. Subjects were released in the opposite end of the tub from the platform, and the time taken to reach the platform was recorded. The following day, Morris water maze (MWM) testing began and was administered over a period of 5 days. Testing was conducted in a round 48 in. diameter tub with an 8 in. diameter submerged (invisible) platform, which was

consistently placed in the southeast (SE) quadrant. Fixed, extra-maze cues were abundant (computer, sink, door, table), while precaution was taken to eliminate intra-maze cues (tub and platform were painted black so the submerged platform blended into a consistent background) (see [13]). On each testing day, subjects underwent four trials, with each trial starting from a different randomly selected compass point (N, S, E, W). On day 1 trial one, each subject was placed on the platform for 10 s, removed from the platform and then released from one of the starting locations. The distance and latency to reach the platform for each trial were recorded using a computer tracing program and data recording computer.

2.7. Brain analysis

At the end of behavioral testing subjects were weighed, anesthetized with ketamine/xylazine (100 mg/kg/15 mg/kg), and transcardially perfused with phosphate buffered saline, followed by chilled 4% paraformaldehyde. Heads were removed, bottled in paraformaldehyde and shipped to GDR at Beth Israel Deaconess Medical Center for histological preparation. Identification of treatment conditions was performed visually postmortem using florescence
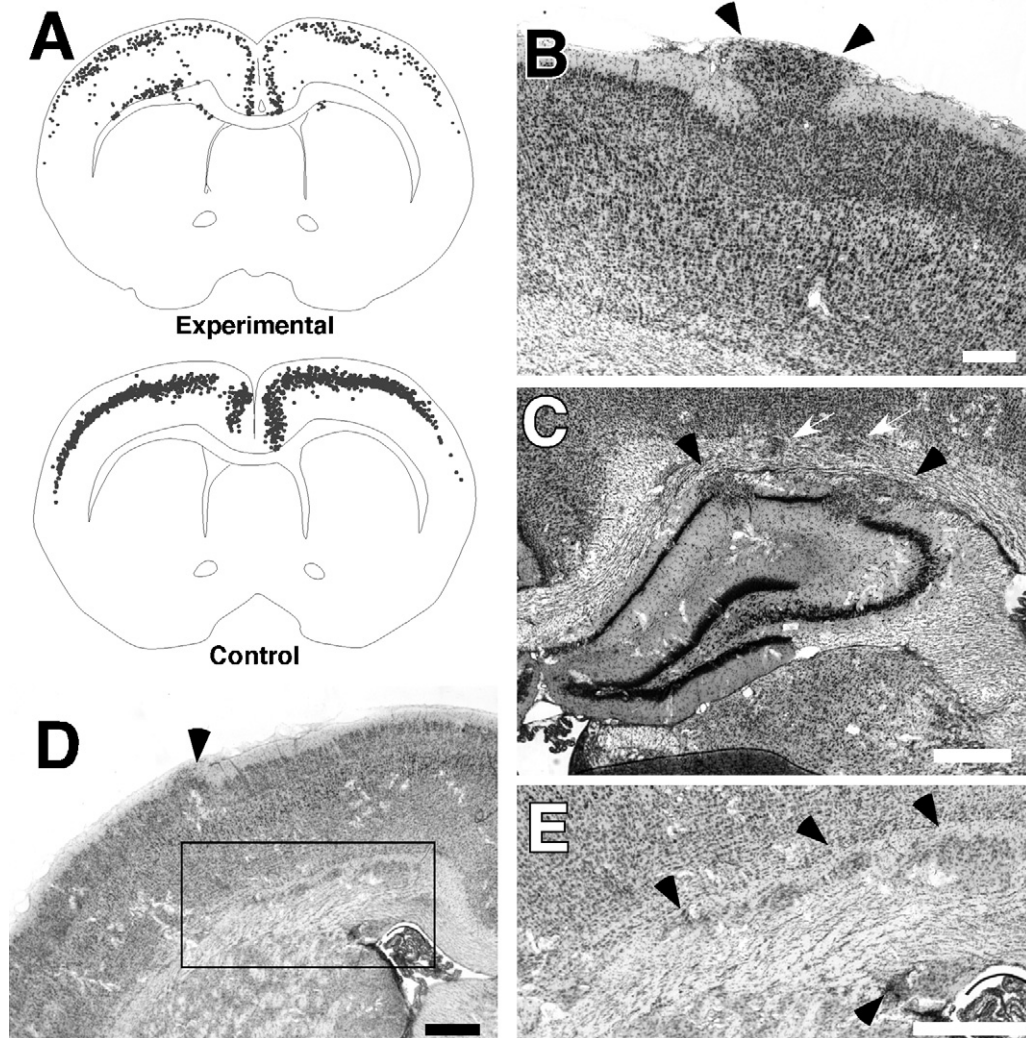

Fig. 1. Histologic characteristics of control and experimental brains. (A) Neurolucida tracings representing RFP (control) and GFP (experimental) expressing cell distributions. Notice the defuse distribution of cortical neurons in the Dyx1c1 RNAi case compared to a more laminar specific positioning of RFP labeled cells. (B) Example of a puncture wound ectopia resulting from the plasmid injection. (C) DAB labeling for GFP showing hippocampal and cortical heterotopia. (D) Enlarged section showing an injection puncture induced ectopia (dark arrow). The box outlines an area of band heterotopia in white matter. (E) Enlarged box taken from 'D' showing heterotopia immunohistochemically labeled for GFP.

microscopy and confirmed by immunohistochemical staining for RFP/GFP. Brains were either frozen or embedded in celloidin and serial sections were mounted and processed. Processing was completed with Nissl substance or immunohistochemistry with antibodies to GFP or RFP (Chemicon). Light microscopic analysis was used to visualize the disposition of transfected cells and identify dysplasia in GFP (RNAi treated) and RFP (sham) subjects.

3. Results

3.1. Histology

Postmortem analysis using florescence microscopy and confirmed by subsequent immunohistochemistry revealed 21 experimental (GFP-positive) and 15 control (RFP-positive) subjects. The results of the histologic examination of these brains is reported in detail elsewhere ([39] submitted), but are summarized here (Fig. 1). There were four categories of forebrain disruption, including: (1) *malformations due to trauma* (RNAi treated $n = 13$, sham $n = 13$), stemming from the injection puncture, typically resulting in layer one ectopia; (2) *non-traumatic ectopic malformations* (RNAi treated $n = 5$, sham $n = 0$), which were a second set of molecular layer ectopias distant to the injection site; (3) *unmigrated neurons* (RNAi treated $n = 21$, sham $n = 0$), representing cells that failed to reach their target layers and were located at the border of the white matter and neocortex; and (4) *hippocampal dysplasia* (RNAi treated $n = 5$, sham $n = 0$), which took the form of heterotopic collections of abnormally migrated pyramidal neurons in the stratum oriens and stratum radiatum of the hippocampus (see Fig. 1). Importantly, the *unmigrated neuron* condition, which incorporated all of the RNAi treated subjects, showed distinct deep layer cortical heterotopia with Nissl staining. Other than the localized injection site malformations, shams showed no evidence of cortical displacement as illustrated by appropriately positioned RFP-labeled neurons.

3.2. Auditory processing: single tone

Significant differences were seen between mean cued and uncued startle response (ASR) scores for all groups, as shown by paired samples *t*-test ($p < .05$), indicating significant discrimination of the single tone by all groups. Results from a univariate ANOVA with Treatment (2 levels) showed no main effect on ATT scores (ATT is calculated by dividing the mean cued response by the mean uncued response and multiplying by 100). Thus, results showed no performance difference on a basic auditory discrimination task, indicating that the *in utero* RNAi of Dyx1c1 treatment did not impact baseline hearing or the acoustic startle response.

3.3. Auditory processing: long/short silent gap

Analysis of mean ASR scores for cued and uncued trials showed that all groups were able to significantly detect the silent gaps (RNAi treated down to 4 ms, shams down to 3 ms). A repeated measures ANOVA was computed on the ATT scores for the 0–100 ms silent gap condition, using Treatment (2 levels) × Day (2 levels) × Gap (9 levels) as fixed factors. Results

showed no main effect of Treatment, thus indicating similar long gap detection performance for the two groups.

A repeated measures ANOVA was computed for the 0–10 ms silent gap condition using Treatment (2 levels) × Day (2 levels) × Gap (9 levels) as fixed factors. Again, results showed no main effect of Treatment, indicating similar performance between groups on the short silent gap task.

3.4. Oddball

An overall repeated measures ANOVA on ATT scores using Treatment (2 levels (sham/RNAi treatment)) × Age (2 levels (juvenile/adult)) × Oddball (2 levels (short/long)) showed a near-significant main effect of Treatment at a one-tail level [$F(1,34) = 2.816$, $p = .051$], suggesting that RNAi treated ($n = 21$) subjects performed worse overall then shams ($n = 15$) (Fig. 2).

Further analysis for the 2 ages using ATT scores and separate Treatment (2 levels (RNAi treatment/sham)) × Oddball (2 levels (long/short)) ANOVAs showed no main effect of Treatment nor interaction during the juvenile period (P35–40). However, among adults (P60+), a significant main effect of Treatment was seen [$F(1,34) = 4.387$, $p = .022$, one-tail], with RNAi treated subjects performing worse than shams.

3.5. Water escape and Morris water maze

For analysis of data for all water maze tasks, subjects were grouped into three Treatments. Since the role of the hippocampus in spatial and working memory is well documented, and given that a subset of RNAi treated animals had dorsal hippocampal heterotopias ($n = 5$), those subjects were analyzed as a separate group from RNAi treated rats with cortical heterotopia only ($n = 14$), and shams ($n = 15$). Note that due to time
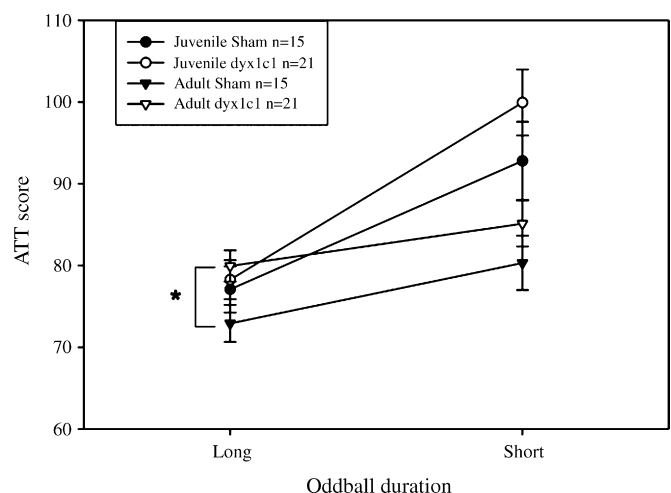

Fig. 2. Oddball combined juvenile and adult attenuation response scores. This graph illustrates a near significant effect of treatment on detection of the oddball stimulus between *in utero* RNAi of Dyx1c1 and sham subjects, between long/short ISI durations, across the juvenile and adult periods. The significant effect of treatment on adult long-duration oddball is shown next to the bracket, indicating worse detection of the stimulus in adulthood by RNAi treated subjects compared to shams (* indicates $p = .022$, one-tail).
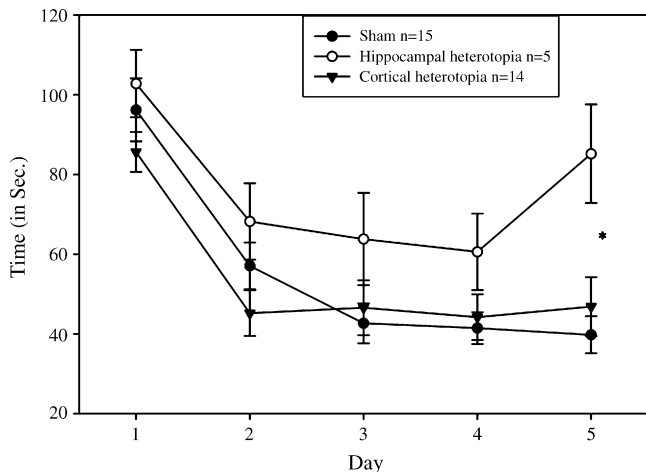
Fig. 3. Morris water maze latency to reach platform scores for three conditions (sham, hippocampal heterotopia, cortical heterotopia only). Graph shows significantly worse performance from hippocampal heterotopia subjects compared to sham and cortical heterotopia only groups (* indicates main effect, $p < .01$, two-tail).

constraints a maximum of 34 subjects could be tested on Morris Water Maze (MWM), thus two randomly selected subjects were dropped. Later histological assessment confirmed they were RNAi treated cortical heterotopia only subjects (hence the *n* for this group was 14, rather than 16 as seen for auditory testing). A univariate ANOVA performed on water escape latency scores showed no effect of Treatment [$F(2,32) = .409$, $p = $ns], indicating equivalent motor performance.

For the MWM, a 3 (Treatment) × 5 (Day) repeated measures ANOVA was conducted on mean latency to reach the platform across 4 trials. This analysis revealed a main effect of Treatment [$F(2,32) = 5.658$, $p < .01$], indicating that RNAi treated subjects with hippocampal heterotopia took significantly longer to reach the platform than sham or RNAi treated rats with only cortical disruption (Fig. 3). A 2 (Treatment (sham/hippocampal heterotopia)) × 5 (Day) repeated measures ANOVA similarly revealed a significant effect of Treatment [$F(1,18) = 6.637$, $p < .05$], indicating that RNAi treated rats with hippocampal heterotopia took longer to reach the platform as compared to sham subjects. Further, a 2 (Treatment) × 5 (Day) Repeated Measures ANOVA comparing RNAi treated subjects with cortical anomalies to RNAi treated rats with cortical and hippocampal heterotopia, revealed a significant effect of Treatment [$F(1,17) = 9.722$, $p < .01$], indicating that RNAi treated rats with hippocampal heterotopia took longer to reach the platform than similarly treated subjects with only cortical heterotopia. Comparable significant differences were found when measuring distance to reach the platform. Importantly, no MWM performance differences were found on any measure when comparing RNAi treated subjects with cortical heterotopia only to shams.

4. Discussion

The current study shows that *in utero* RNAi of Dyx1c1 leads to variable patterns of cortical and hippocampal disruption, presumably reflecting anomalies of neuronal migration. These two malformation types in turn associate with different forms of behavioral impairment.

4.1. Implications for auditory processing impairment

Since previous research evaluating auditory processing as a function of cortical malformations has shown that cortical disruption is an important factor in emergent deficits, all RNAi treated animals were analyzed together as a unified group for performance on auditory discrimination tasks [34–37,24]. Overall results, as well as results specifically on the adult oddball detection task, suggest impairment in the auditory processing ability of rats that received *in utero* RNAi of Dyx1c1, as well as failures to improve with experience relative to matched controls (supported by evidence that RNAi treated subjects showed much less improvement as compared to shams across periods of juvenile to adult oddball testing). Importantly, RNAi treatment and sham differences were seen only on the oddball task as opposed to the easier auditory discrimination tasks, which showed no differences between RNAi and sham conditions in the juvenile period at long (0–100 ms) or short (0–10 ms) gap durations. Combined results suggest that task difficulty is an important factor in eliciting an auditory processing impairment. Similarly, Fitch et al. [18] showed that rats with developmental cortical injury exhibited deficits in processing two-tone sequences of around 300 ms duration when tested in an operant conditioning paradigm, as opposed to a startle response paradigm where deficits were seen at intervals of 75, 50, and 10 ms ISI duration [35,8,18,17]. In both studies, no significant differences were seen for easier and/or longer duration tasks, however the temporal threshold for the emergence of processing deficits appears to increase with added task demand. Future studies evaluating auditory processing in rats with RNAi of genes known to mediate neuronal migration, especially those related to the incidence of dyslexia in humans, should continue to investigate the relative contributions of temporal (stimulus duration) and cognitive (task complexity) demands as they relate to deficits associated with neuromigrational disruption. In fact, it is possible that sensitivity to increased demand along these two axes may relate to different patterns of neuronal disruption.

4.2. Significance of hippocampal heterotopia and memory impairment

In accord with the above suggestion, Morris water maze results showed dissociation between RNAi rats with hippocampal and cortical malformations and those with cortical malformations only. The malformations of the hippocampus present in a subset of rodents with *in utero* RNAi of Dyx1c1 have been shown in other models of disruption to developing cortical neurons and have also been reported in humans [33,9]. For example, *in utero* exposure to methylazoxymethanol (MAM) on E15 results in the formation of deep layer cortical heterotopia in addition to gross hippocampal heterotopia, believed to be a result of the disruption of processes occurring during cortical formation [33,7]. In the present study, transfection of dyx1c1 RNAi plasmid was targeted at the population of neuronal progenitors at

the ventricular zone surface of neocortex, with the result of some malformations in untargeted areas (hippocampus). This may have occurred as a result of the formation of intercellular bridges developing between the hippocampus and neocortical ventricular zone (VZ) following disruption of neuronal progenitors at the VZ surface. Similar bridges and hippocampal dysplasia are known to occur following MAM treatment [33,7]. Regardless of the method of induction, *in utero* RNAi of Dyx1c1 affecting hippocampal morphology clearly produces a robust spatial learning impairment.

4.3. Implications for developmental dyslexia

The current study sought to bridge genetic, anatomical and behavioral variables associated with dyslexia through the use of a novel animal model. Given that deficits in complex auditory processing and learning, as well as verbal memory have been suggested to play a role in reading and language problems such as dyslexia [6,10,15–17,26,31,32,44], our current results suggest that genetically mediated anomalies in cortical neuronal migration could play a role in establishing some of these deficits. As human developmental research and animal models of learning impairment progress in parallel, investigators and clinicians will continue to gain an improved understanding of dyslexia's neurobehavioral components. In future studies, the relative influence of altered neuronal migration in specific brain regions may indeed be found to impact differentially on the composite behavioral deficits that characterize dyslexia and other language-based developmental impairments in humans (for review of dyslexia, see [12]).

5. Conclusion

In utero RNAi of Dyx1c1 disrupts neuronal migration and leads to impairments of processing complex changes in auditory stimuli. Further, different categories of malformations correspond to distinct behavioral impairments, such as spatial learning (in the case of the hippocampal heterotopia). Combined with previous research exploring the link between cortical developmental disruptions and later processing impairments, the current findings lend further support to the hypothesis that developmental cortical injury of various etiologies can result in auditory processing and other learning impairments.

Acknowledgement

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