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Deficits in learning and memory in mice with a mutation of the candidate dyslexia susceptibility gene *Dyx1c1*

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Abstract

Dyslexia is a learning disability characterized by difficulty learning to read and write. The underlying biological and genetic etiology remains poorly understood. One candidate gene, dyslexia susceptibility 1 candidate 1 (*DYX1C1),* has been shown to be associated with deficits in short-term memory in dyslexic populations. The purpose of the current study was to examine the behavioral phenotype of a mouse model with a homozygous conditional (forebrain) knockout of the rodent homolog *Dyx1c1*. Twelve *Dyx1c1* conditional homozygous knockouts, 7 *Dyx1c1* conditional heterozygous knockouts and 6 wild-type controls were behaviorally assessed. Mice with the homozygous *Dyx1c1* knockout showed deficits on memory and learning, but not on auditory or motor tasks. These findings affirm existing evidence that *DYX1C1* may play an underlying role in the development of neural systems important to learning and memory, and disruption of this function could contribute to the learning deficits seen in individuals with dyslexia.

1. Introduction

Developmental dyslexia is a heritable learning disability defined by difficulties in learning to read and write that cannot be explained by factors such as intellectual impairment, lack of educational opportunity or other co-morbid neurological disorders (e.g., epilepsy, or primary sensory impairments (blindness, deafness)). Dyslexia can be deconstructed into underlying core components, known as “intermediate phenotypes”. These include deficits in phonological processing (Kovelman et al., 2012; Melby-Larvag, Lyster & Hulme, 2012; Peyrin et al., 2012), short-term and/or working memory (Beneventi et al., 2010; Gathercole et al., 2006; Menghini et al., 2010), visuospatial attention (Franceschini, 2012; Gabrieli &

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Norton, 2012), and rapid auditory processing (Cohen-Mimran & Sapir, 2007; Fitch & Szalkowski, 2012; Hamalainen, Salminen & Leppanen, 2013). Other behavioral deficits that have been specifically associated with dyslexia include naming speed, motor functioning and visual processing of motion (Denckla & Rudel, 1976; Liao et al., 2015; Capellini, Coppede & Valle, 2010; Olulade, Napeliello & Eden, 2013). Dyslexia is also encompassed by the term "specific developmental reading disability," although reading disability is generally regarded as a more inclusive term, capturing comprehension deficits that would not usually be classified as dyslexia (Snowling & Hulme, 2012). The symptomology for both dyslexia and reading disability is heterogeneous, and the biological mechanisms underlying associated intermediate phenotypes remain poorly understood.

What we do know of the biological and genetic etiology of dyslexia/reading disability indicates strong but complex genetic and environmental influences, with heritability estimates ranging from 40% to as high as 80% (Schumacher, Hoffmann, Schmal, Schulte-Korne, & Nothen, 2007). Not surprisingly, multiple genes have been implicated as contributing to this disorder, much as seen for other complex disorders such as autism and schizophrenia (Gelernter, 2015). Although the neurobiological mechanisms underlying dyslexia/reading disability are not yet fully understood, most of the risk genes identified to date appear to be involved in surprisingly similar biological mechanisms (i.e., neuronal migration and cilia function; Kere, 2014). These identified genetic and neurobiological mechanisms in turn contribute to establishing the complex neurocircuitry that may subserve abilities such as phonological and visual processing, as well as learning. Disruptions in this neurocircuitry could result in impairments that are associated disorders of language and reading functions.

The first candidate risk gene to be reported was dyslexia susceptibility 1 candidate 1 (*DYX1C1*), a gene identified in two Finnish families with a history of dyslexia (Nopola-Hemmi et al., 2000). *DYX1C1* was further supported as a candidate risk gene in 2003 (Taipale et al.,2003), although there have been inconsistent findings in clinical populations (Cope et al., 2005; Marino et al., 2005; and Meng et al., 2005). Importantly, some reports have shown that *DYX1C1* variants are specifically associated with core component features of dyslexia, including deficits in verbal short-term memory (Marino et al. 2007), short-term memory (Dahdouh, 2009), and orthographic choice tasks and non-word reading (Bates, 2009). These previous studies have provided evidence of *DYX1C1* variants being explicitly linked to memory deficits in some language-impaired populations.

With regard to the biological role of *DYX1C1,* this gene has been shown to be active in neuronal migration in the developing cortex, as well as more generalized cilia function (Wang et al., 2006; Tarkar et al., 2013). These appear to be recurrent biological “themes” in the etiology of dyslexia. For example, animal models using *in utero* RNA interference (RNAi) against the rat homolog *Dyx1c1* showed disruptions of neuronal migration in the developing neocortex, thus supporting a role for *DYX1C1* in neuronal migration (Wang et al., 2006). Migration anomalies in the neocortex have also been associated with dyslexia in clinical populations, as evidenced by cortical malformations indicative of early migration disturbances (Galaburda et al., 1985, Chang et al., 2005). Rodent models for these types of developmental cortical malformations have been used to evaluate rapid auditory processing

and working memory, since these are considered intermediate phenotypes of developmental dyslexia, and yet are accessible to rodent evaluations. Researchers found deficits in both rats with induced cortical malformations, and also rats with knockdown of *Dyx1c1*, in complex acoustic processing (Threlkeld *et al.* 2007; Threlkeld et al., 2009). Working memory abilities were also assessed in the *Dyx1c1* RNAi model, and significant impairments were noted for this core phenotype as well (Szalkowski et al., 2011). More recently, a mouse knockout model of *Dyx1c1* was generated that demonstrated cilia defects, as well as severe embryonic lethality of approximately two-thirds of homozygous mutants. Homozygous constitutive mutants that survived after birth developed severe hydrocephalus by postnatal day P16, and died by P21 (Tarkar et al., 2013). This mouse model obviously could not be used for extensive behavioral profiling, and a conditional forebrain *Dyx1c1* knockout model was developed.

The current study was designed to further examine the behavioral features of this conditional preparation, using male mice with both homozygous and heterozygous conditional (forebrain) knockout of rodent homolog *Dyx1c1.* A forebrain conditional knockout model was chosen in part based on evidence of malformations in neocortex as well as hippocampus in the RNAi *Dyx1c1* model (Rosen et al., 2007). These abnormalities in the neocortical and hippocampal regions resulted from disruption of neuronal migration, and have been observed in individuals with dyslexia (Galaburda et al., 1985). We hypothesized these differences in underlying neuroanatomy may contribute to deficits in learning and memory reported in individuals with dyslexia. A forebrain conditional was also selected to avoid the lethal hydrocephaly observed in the systemic KO. Adult male littermates (generated through het x het breedings) were assessed on various behavioral paradigms that have been validated in our lab as effectively tapping core functions implicated in language and reading impairments. These tasks include auditory processing, and working and reference memory. Based on previous research and clinical evidence, we hypothesized that animals with homozygous conditional knockout of *Dyx1c1* would show specific acoustic processing and/or memory impairments, while performance on other behavioral assessments (e.g., gross motor learning, pre-pulse inhibition, water escape) would not differ from matched wild-types.

2. METHODS

2.1 Subjects

Mice carrying the *loxP*– exon 2–4–*loxP* conditional allele of *Dyx1c1* (*Dyx1c1*^{flox}) were generated by the University of Connecticut Health Center Gene Targeting and Transgenic Facility, as described previously (Tarkar et al., 2013). Briefly, embryonic stem cells harboring a *loxP*-flanked allele of exons 2–4 of *Dyx1c1* were produced by electroporating mouse embryonic stem (ES) cells (129S6) with a targeting construct designed to replace exons 2–4 and flanking intronic sequence through homologous recombination. After PCR screening of the ES cell clones for correctly targeted colonies, a single positive colony was expanded, and chimeric mice were generated by embryo reaggregation. The animals were crossed with C57BL/6J mice and transmitted the targeted allele to the offspring through germ line. These mice were subsequently crossed with *129S4/SvJaeSor-*

Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$*/J* mice (The Jackson Laboratory) to remove the *PGK-Neo* cassette in the targeting construct and the offspring thus produced were used to generate *Dyx1c1*$^{flox/flox}$ mice colony. These mice were genotyped by PCR using three pairs of primers (PL452F-5'-CGAAGTTATTAGGTCCCTCG-3' and loxgtR 5'-TGAGCACCCTGCTTCTACCT-3'; loxwtF 5'-AAAACCAACCATCCAACCAA-3' and loxgtR 5'-TGAGCACCCTGCTTCTACCT-3'; FrtgtF 5'-TAGGGATTCACCGTCACACA-3' and FrtgtR 5'-AACCAAGTCCAAGGCCTTCT-3').

To generate the conditional forebrain knockout (*Dyx1c1*$^{flox/flox}$*/Emx-Cre*$^{+/+}$*)*, -- mice with a deletion of exons 2–4 only in the forebrain -- we crossed *Dyx1c1*$^{flox/flox}$ mice with Emx1-IRES-Cre knockin mice, B6.129-*Emx1*$^{tm1(cre)Krj}$/J (The Jackson Laboratory).). Emx1-IRES-Cre strain expresses Cre recombinase enzyme from the endogenous *Emx1* locus, and when crossed with loxP-site containing Dyx1c1 sequence, leads to recombination in approximately 88% of neocortical neurons as well as hippocampus. The heterozygous offspring (*Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/-}$*)* generated from this cross were then inbred to create the experimental forebrain conditional knockout (*Dyx1c1*$^{flox/flox}$*/Emx-Cre*$^{+/+}$*)* mice and the control (*Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/-}$*, Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/+}$*, Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/+}$ and *Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/-}$*)* mice. The knockout (KO) group was comprised of 12 animals with the genotype *Dyx1c1*$^{flox/flox}$*/Emx-Cre,*$^{+/+}$ while the heterozygous (HT) group was comprised of 6 subjects with either *Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/-}$ or *Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/+}$genotypes. Lastly, the wild type (WT) group consisted of 7 subjects with *Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/+}$*or Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/-}$ genotypes. Genotyping was performed by PCR for *Dyx1c1*$^{flox/flox}$ using the primers described above. The mice were genotyped for EMX-Cre using two pairs of primers (oIMR1084 5'-GCG GTC TGG CAG TAA AAA CTA TC-3'and oIMR085 5'-GTG AAA CAG CAT TGC TGT CAC TT-3'; oIMR4170 5'-AAG GTG TGG TTC CAG AAT CG-3' and oIMR4171 5'-CTC TCC ACC AGA AGG CTG AG-3').

All subjects were single-housed in standard mouse tubs (12 h/12 h light/dark cycle), with food and water *ad libitum,* and all behavioral testing occurred during the light cycle. At the start of testing, animals were between the ages of postnatal day (P) 61 - P75. Procedures were performed blind to Genotype (ascertained at weaning by tail-snip PCR), and in compliance with the National Institutes of Health and University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

2.2 Anatomy

Finally, it is important to note that in contrast to the systemic KO preparation that exhibited lethal hydrocephaly (Tarkar et al., 2013), the forebrain conditional preparation has been shown *not* to exhibit any gross neurologic abnormalities in the cortex or hippocampus (homozygous or heterozygous), nor to exhibit any gross behavioral anomalies. To confirm these and other prior characterizations of the conditional model, a western blot was performed to examine the *Dyx1c1* protein expression in the forebrain KO and the control conditions. Additionally, we included the constitutive KO and WT protein lysates as a control for the experiment. We further investigated the lamination of the cortex obtained from the forebrain knockout using the neocortical layer marker Ctip2 and Cux1. We measured the depth of each layer of the cortex marked by Ctip2 and Cux1, and we further

normalized the measurement with the total cortical depth to compare the thickness of the Ctip2 and Cux1 positive neuron-containing cortex.

2.3 Rotarod (P124 to P129)

All subjects were assessed at age P124 for sensorimotor ability and motor learning using the rotarod task. Subjects were placed on a rotating cylindrical drum that gradually accelerated from 4 to 40 rotations per minute across a span of 2 minutes. Four trials were administered per test day, across four consecutive days. For analysis, latency to fall from the rotating drum was measured and averaged across the four trials for each day.

2.4 Auditory Processing (P68 to P98)

All subjects were assessed for auditory processing ability using a modified pre-pulse inhibition paradigm (see Fitch et al., 2008 for review). Subjects were placed on individual load-cell platforms (Med Associates, St. Albans, VT) and presented with auditory stimuli generated using RPvdsEx on a Dell Pentium D PC and RZ6 multifunction processor (Tucker Davis Technologies, Alachua, FL). Sounds were amplified using a Niles SI-1260 Integration Amplifier (Niles Audio Corp., Carlsbad, CA) and delivered through powered Yamaha YHT-M100 speakers (Buena Park, CA). The acoustic startle reflex (ASR; a reflexive response elicited by an unexpected, intense stimulus) was recorded by an iMac 7.1 running Acknowledge 4.1, and obtained via the voltage output from each load cell platform through a linear amplifier (PHM-250U; Med Associates, St. Albans, VT) connected to a Biopac MP150 acquisition system (Biopac Systems, Goleta, CA). The modified pre-pulse inhibition paradigm measured differences in ASR to a loud startle-eliciting stimulus (SES; 105dB, 50 ms, broadband white noise burst (1kHz-10kHz)) when presented with or without a preceding acoustic cue. The ASR difference on cued versus uncued trials provided a measure of cue detection and/or discrimination. If the auditory cue was detected, a reduction (attenuation) in the ASR was expected relative to the ASR elicited when the auditory cue was not present (or not detected). This phenomenon was quantified using an “attenuation score” (ATT) that compared the average amplitude of the ASR from the cued trial to the average ASR of the uncued trial ([average cued ASR/average uncued ASR]*100).

2.4.1 Normal Single Tone P68—Prior to more complex auditory testing, all animals were tested on a Normal Single Tone (NST) to measure baseline pre-pulse inhibition and auditory ability (P68). This auditory PPI control task was used to establish whether subjects exhibited hearing deficits and/or impaired gross motor reflexes that could confound other auditory PPI tests, and provided an index of baseline auditory pre-pulse inhibition ability across test groups. Testing sessions consisted of 104 pseudorandomly presented cued and uncued trials at inter-trial intervals (ITI) of varying durations (16–24 s). The task comprised of a silent background and a simple single tone cue (50 ms, 75 dB, 8,000 Hz tone) presented 50 ms prior to the 50 ms, 105 dB. All subjects were able to perform this task and therefore were used for further behavioral evaluation.

2.4.2 Embedded Tone (P71 to P83)—First, the variable duration Embedded Tone (EBT) task was administered (with 300 sequential pseudorandom trials). This task assessed ability to detect a change in tone frequency relative to a standard background tone (cue was

a variable duration 5.6 kHz pure tone embedded in a 10.5 kHz background pure tone). On cued trials, the cue was presented 100 ms before the SES, while uncued trials used a "cue" of 0 ms. Two EBT tasks were used in this study – a long-duration EBT (0 ms to 100 ms), and a short-duration EBT (0 ms to 10 ms). A range of cue durations were used to evaluate specific thresholds for performance differences between the various genotypes, based on a hypothesis that subjects may perform comparably on longer durations yet may differ on shorter durations (which are more difficult to detect). Using a range of cue durations enables ascertainment of stimulus features that all animals can discriminate (ceiling), that no animals can discriminate (basement), as well as any group differences in the mid-range. Both EBT tasks were administered for five consecutive days, starting at P71 until P83.

2.4.3 Silent Gap (P86 to P98)—Next, a Silent Gap (SG) task was used to assess the ability to detect silent breaks in continuous white noise (P86 to P98). A session included 300 trials with a continuous 75 dB broadband white noise background. Cued and uncued trials occurred pseudorandomly. On cued trials, a silent gap of variable duration (0–100 ms) was presented 100 ms before the SES, with "0 ms" trials serving as the uncued condition. Subjects were tested on the Silent Gap task for five consecutive days, for each version of the task.

2.5 Water escape (P132)

Subjects were initially tested on a water escape task prior to the 4/8 arm radial water maze task, to evaluate the presence of any underlying impairments that might confound further maze testing (i.e., deficits in motivation, swimming, or visual acuity). Subjects were placed in the far end of an oval tub (103 cm x 55.5 cm) filled with room temperature water, and were given 45 seconds to swim to a visible escape platform (8.5 cm in diameter; 1 cm above water surface) located at the opposite end of the tub. Latencies to reach the visible platform were recorded for assessment. None of the subjects displayed any impairments on this task (the subject code was used to analyze this data by an investigator who was not conducting testing). Therefore, we proceeded to implement a water version of the 4/8 radial arm maze (adapted from Hyde, Hoplight & Denenberg, 1998).

2.6 4/8-arm radial water maze (P133 to P148)

This task was used to assess spatial reference and working memory ability simultaneously, using a standard 8 arm radial maze with 4 arms baited (i.e., containing a submerged goal platform), and 4 arms open but never baited with a platform. Configuration of goal arms were counterbalanced between subjects but remained fixed per subject across all test sessions. Additionally, high contrast extra-maze cues were present, and the locations of these remained static for the entire experiment.

The day prior to testing (Day 1), subjects were given a training session where all arms that would not contain a platform were blocked, forcing the animals to only enter arms containing a platform. Subjects were placed in the start arm and given 120 seconds to locate a platform. Every subject completed 4 training trials, and each time they found a platform, that platform was removed and the entrance to that arm was blocked. This ensured that the subject could no longer enter this arm for the remainder of the training session. If the subject

failed to find a platform in this window, they were guided to the nearest available goal. Once on the platform, subjects remained for 20 seconds and then were removed from the maze to their home cage (30 second inter-trial interval; ITI).

Testing began on Day 2 and continued for an additional 14 consecutive days. Here, instead of blocking the goal arm of the most recently located platform, the platform was simply removed during the 30 second ITI, but the arm remained open and unbaited for the remainder of the test session. Animals were required to locate all 4 platforms, and thus received 4 test trials per day. Test sessions were recorded using a Sony camera integrated with the SMART video-tracking program (Panlab, Barcelona, Spain). Latency and path distance were recorded and in addition, subjects were given a point for each completed trial (i.e., successful location of a platform in 120 sec), with a maximum of 4 points per test session. All scores were recorded and used for further analysis.

2.7 Novel Object Recognition (P162)

One day prior to testing, all subjects were habituated to the testing chamber (40 cm × 24 cm × 20 cm plexiglas tub with opaque walls) for 10 minutes. On testing day, each subject underwent a habituation phase of 5 minutes to reduce stress and the chance of a neophobic response, and to promote exploratory activity in the test phase. The subject was given a 1-minute resting period in their home cage, and was then exposed to two identical Lego configurations for 5 minutes. This constituted the “familiarization” session. Afterwards, the subjects were given a short delay period of 5 minutes (resting in their home cage). Then, they were introduced to the testing chamber containing a new object and one familiar object (different Lego configurations) for 3 minutes (“test” session). Anytime a mouse sniffed the object or touched the object while looking at it (i.e., when the distance between the nose and the object was less than 2 cm) this behavior was scored as exploratory. Total time spent exploring, and percent time spent with the familiar vs. novel object, were evaluated and analyzed.

2.8 Modified T-maze (P225 to P268)

Subjects were gradually food restricted to 80%–85% of their baseline body weight (3 weeks). During the last week before training, subjects were given a sample of the food reward in their home cage, to habituate them to its taste and eliminate hyponeophagia. Animals were then introduced to the modified T-maze (30 cm x 10 cm start arm; 30 cm x 10 cm goal arm). This configuration included curved arms, to eliminate visual or olfactory cues of the food reward at the end of the arm. The left and right arms were high contrast colors - - the left arm was black, and the right arm was white. Prior to rewarded alternation testing, animals were placed in the start arm with both arms opened and containing food wells (with food reward) for about 3 min. Any time an animal consumed the reward, a food well was replenished. This was performed four times, with intervals between exposures of at least 10 min.

During the training period, a subject was placed in the start arm with one of the goal arms blocked while the other arm remained opened, forcing the subject to enter the open arm. Multiple trials were administered in a daily session, with equal numbers of left and right

arms serving as the open arm. When the animal completed a forced trial by entering the open arm and consuming the reward, the blocked arm's door was removed, and the animal was placed back into the start arm. This process trained the animal to learn that once when it entered an arm and consumed the reward, there would no longer be a reward at that location, so they must visit a new arm for reward. Now the animal was placed again in the start arm facing away from the two goal arms, and was permitted to make a choice between the two opened arms. If the animal chose correctly, they were allowed time to consume the reward. If the subject chose incorrectly, they were removed (after the experimenter ensured the subject adequately explored the empty well).

Once the animals were habituated and trained, ten trials were given in a daily session. Each one of a squad of approximately 8 mice received a trial in succession before the first animal started its next trial. The identity of the sample goal arm for each trial was determined by random sequence. The maximum number of consecutive identical arms was three, as a precaution against development of temporary position habits or reinforced perservation. A percentage reflecting correct trials completed per animal was calculated and evaluated (Deacons & Rawlins, 2006).

2.9 Statistical analysis

All behavioral data was analyzed using a mixed factorial design. All subjects were used for analysis (WT, n = 7; HT, n = 6; KO, n = 12). Group differences in rotarod performance were analyzed using a repeated measures ANOVA with Genotype (3 levels: WT, HT and KO) as the between measure, and Day (4 levels) as the within measure. A one-way between subjects ANOVA was conducted to compare the effect of Genotype on average Attenuation Scores on all auditory processing tasks. This included: EBT 0-100 ms, EBT 0-10 ms, SG 0-300 ms and SG 0-100 ms. Total number of completed trials on the 4/8 radial arm maze were independently examined using a 3 x 14 repeated measures ANOVA, with Genotype (3 levels: WT, HT and KO) as the between measure, and Day (14 levels) as the within measure. Finally, a univariate ANOVA was performed to analyze group differences on total time of exploration on the novel object recognition task, as well as accuracy (% trials correct) on the modified T-maze task. All statistical analyses were conducted using SPSS 19 with an alpha criterion of 0.05, two-tailed.

3. Results

3.1 Anatomy

Western blot results showed that Dyx1c1 protein expression was reduced by 77% in the forebrain KO condition as compared to the control condition (Fig. 1). Since we know that Emx1-IRES-Cre strain expresses Cre recombinase enzyme from the endogenous *Emx1* locus that (when crossed with loxP-site containing Dyx1c1 sequence) leads to recombination in approximately 88% of neocortical neurons as well as hippocampus, the slightly higher Dyx1c1 expression observed in the forebrain knockout could be attributed to the other cell types in the neocortex and hippocampus (e.g. interneurons (20%)), as well as vasculature. We also investigated the motility of the cilia lining the lateral ventricles of the forebrain KO and the control using videomicroscopy, and found that the KO cilia were immotile --

consistent with the previously identified phenotype of ciliary immotility in the Dyx1c1 ubiquitous KO. Finally, we found no significant differences in the cortical lamination patterning, as evident from the Cux1 and Ctip2 staining in the forebrain conditional knockout compared to controls. We also found no observable anomalies in the cortex of the conditional forebrain knockout animals compared to controls (Fig. 2).

3.2 Rotarod

A repeated measures ANOVA examining average rotarod latency across 5 days of testing found no main effect of Genotype [$F(2,22) = .078$, N.S.], nor Genotype x Day interaction. These results indicate that all subjects had comparable sensorimotor/motor learning, at least as measured by this task. This is important in demonstrating that higher-order behavioral differences are unlikely to be confounded by underlying motor impairments in genetically engineered subjects (Fig. 3).

3.3 Auditory processing

All subjects were initially tested on a normal single tone (NST) task, to establish baseline hearing and PPI ability. None of the subjects showed impairments on NST, nor was there a main effect of Genotype [$F(2,22) = 2.196$, N.S.]. Therefore subjects were advanced to subsequent levels of more complex acoustic tasks. On the embedded tone (EBT) 0-100 ms task, we found no main effect of Genotype [$F(1,22) = .419$, N.S.]. Moreover, all subjects were able to discriminate the stimuli, based on cued/uncued amplitude comparisons. Subjects were then tested on a more difficult embedded tone task, using cue durations ranging from 0 to 10 ms (where 0 ms is the uncued condition). Again, we found no main effect of Genotype [$F(1,22) = 3.110$, N.S.], and all subjects showed significant discrimination of the cues (particularly longer gaps). Next, auditory processing ability was evaluated on a silent gap detection task. First, we administered the silent gap 0-300 ms task, and again saw no main effect of Genotype [$F(1,22) = .532$, N.S.]. Subjects overall performed well on the task, and therefore we advanced them to the more difficult version of the task with silent gap durations ranging from 0 to 100 ms. Overall performance was poor on the silent gap 0-100 ms task and there was again no main effect of Genotype [$F(1,22) = .591$, N.S.].

Finally, for each of the auditory tasks, we found that all 3 groups of animals showed significant discrimination of the cue, based on t-test comparisons between mean cued and uncued values within groups. The exception was on the hardest task (silent gap 0-100 ms), where *all* animals performed poorly (mean attenuation scores approximately 85%, and as high as 100% for the 2 ms cue). Comparing across groups, we found no significant effects of Genotype on any task, confirming that subjects performed comparably on all tasks (Fig. 4).

3.4 Water maze assessment

Prior to spatial water maze testing, a visible platform control task was conducted to assess for underlying impairments that could confound subsequent water maze performance (e.g., impairments in swimming ability, visual acuity, or motivation). A univariate ANOVA on latencies found no main effect of Genotype [$F(2,22)=.126$, N.S.], indicating that genetically

modified groups had no impairments on underlying aspects of the water task (e.g., swimming) (Fig. 5).

The 4/8 radial-arm water maze was then used to assess spatial working and reference memory ability. Animals were scored on errors and latencies, as well as their ability to complete this task (i.e., successfully locate a platform). Thus for every trial successfully completed, subjects received a point. We did find a main effect of Genotype on the number of trials successfully completed [$F(2,22)=7.518$, $p<.01$]. We also found a significant Day by Genotype interaction [$F(26,286)=4.887$, $p<.001$], with KOs continuing to drop in number of trials successfully completed as testing progressed (Fig. 6). Specifically, KOs performed comparably to WTs and HTs during the first few days of testing, with significant differences emerging by Day 5 [$F(2,22)=4.148$, $p<.05$].

3.5 Novel object recognition

Subjects were tested on a novel object recognition task, to assess both exploration and short-term memory abilities. We found a main effect of Genotype on total time of exploration, with KO animals exploring substantially less [$F(2,22) = 8.613$, $p<.01$]. As a result, KO animals did not meet criteria to evaluate novel object recognition ability (Fig. 7).

3.6 Modified T-maze

Lastly all subjects were evaluated on a modified T-maze involving a rewarded alternation task. However, 3 subjects were dropped from the analysis (1 WT, 1 HT and 1 KO) since they did not perform the task during the testing period. The percentage of correct responses as a function of total trials completed was calculated for each subject, and a univariate ANOVA did reveal significant group differences. There was a main effect of Genotype on this measure, [$F(2,19) = 5.453$, $p<.05$] with KOs performing the worst (Fig 8). Furthermore, a t-test was performed specifically comparing KOs versus WTs performance, which confirmed this significant difference between the two groups on accuracy of an alteration task using a T-maze [$t(15) = -2.229$, $p< .05$].

4. Discussion

The purpose of this study was to evaluate conditional forebrain knockout mice on motor learning, auditory processing, and working and reference memory tasks. Results showed that disrupting the function of *Dyx1c1 does* impair memory performance, but does *not* negatively impact motor learning and auditory processing abilities. These results further validate a role for *Dyx1c1* in learning and memory capabilities, and support prior animal model work with this gene. For example, research on the relative behavioral impact of embryonic transfection of RNAi for the dyslexia risk homologs *Kiaa0319* versus *Dyx1c1* in rats revealed: (1) deficits in the discrimination of rapidly changing acoustic stimuli but *not* working memory when *Kiaa0319* was knocked-down (Szalkowski et al., 2012); but (2) acoustic processing deficits for complex stimuli (e.g., FM sweeps) but not rapidly changing stimuli, coupled with *robust* deficits on a working memory task, when *Dyx1c1* was knocked-down (Threkeld et al., 2007; Szalkowski et al., 2011; Szalkowski et al., 2013). Unfortunately, the current study was not able to dissociate potential complex acoustic processing deficits in the

conditional *Dyx1c1* KO model, because we have found that mice are not capable of performing the same complex FM sweep and tone-pair discrimination tasks that can be effectively used in rats. Nonetheless, these cumulative results are intriguing in light of human evidence that has associated mutations in the *KIAA0319/DCDC2* region of chromosome 6 with anomalies in mismatch negativity (MMN) for acoustic phonologic stimuli (Czamara, 2011), while *DYX1C1* has been more closely associated with deficits in working memory and visual attention (Wigg et al., 2004; Marino et al. 2007; Dahdough et al., 2009; Bates et al., 2010; Lim et al., 2011; Mascheretti et al., 2013). Taken together, these findings suggest that different dyslexia risk genes may contribute more or less to different underlying intermediate phenotypes of dyslexia. If true, then early screening could provide indications for optimal intervention strategies on an individual basis.

Overall, our results add support to a putative role for *DYX1C1* in learning/memory components of language, based on the clear inability of conditional KO *Dyx1c1* mice to learn a radial arm maze. However, we acknowledge that the deficits seen in the current study could be interpreted as reflecting motivational or attentional problems. In fact, significant differences comparing the KO group to the WT and HT group emerged on day 5 of testing. Specifically, within the first few days of testing, all animals were learning the task and making a high number of errors. The KO animals continued to make a high number of errors, and then appeared to become unmotivated -- possibly due to the level of task difficulty. At this point, they simply stopped performing. At the same time, the WT and HT group continued to perform the task and make decreasing numbers of errors. Unfortunately, we could not quantify KO animals' errors during this later portion of testing, since they "timed out" and did not successfully complete the testing trials. Another interpretation may be that KO subjects performed poorly on this task because they did not attend to the paradigm and constraints, and therefore, did not successfully complete as many trials. This hypothesis is in fact consistent with a lack of initial exploration by KOs in the acclimation phase of the Novel Object task. In that paradigm, intact mice typically explore a novel object, and when presented with that same object and a similar but new one, attend more to the new as compared to the familiar object. In the current study, the KO mice did not attend to the initial novel object for an adequate duration to proceed with testing. This could reflect a lack of attention, which might also impact on the radial arm maze. (It is important to again emphasize that the failure to explore in the Novel Object task, and fewer trials successfully completed in the radial arm maze, are not likely a reflection of motor impairments in the KOs, given that our rotarod and visible platform water escape tasks showed no group differences). Importantly, a counter to this argument to the core "failure to perform" as an explanation for deficits is the significantly worse % correct scores seen on T-maze learning for KO mice. Here all the mice performed the task, yet KOs performed significantly more poorly than comparison wild-types.

Finally, in interpreting these findings, it is important to note that the conditional forebrain *Dyx1c1* KO model specifically targets gene function in the cortex and hippocampus, but *not* subcortical structures. Specifically, Emx1- Cre recombinase activity is reported in neurons of the neocortex and hippocampus, and in the glial cells of the pallium. This conditional KO was created to avoid the lethal hydrocephalous observed with a systemic KO. While some very early physiology data could be obtained from the systemic KO preparation, subjects did

not survive to an age that would allow behavioral testing. The forebrain conditional *Dyx1c1* preparation, in contrast, has no associated lethality. It would be interesting to assess the effects of a KO preparation that extended into some of the sub-cortical structures not affected here (for example the cochlear nucleus -- particularly with respect to our acoustic findings). This is an unfortunate limitation of the current model that may be overcome with future technologies.

In closing, the conditional forebrain *Dyx1c1* knockout mouse model examined here displayed learning and memory deficits, consistent with previous animal research using a *Dyx1c1* RNAi knockdown model, and with human findings linking *DYX1C1* to working memory performance. Future research, preferably using a larger sample size, will be needed to assess additional variables including effects in female mice, as well as relationships between aberrant behaviors and underlying changes in neural function and circuitry.

Acknowledgments

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Highlights

- A homozygous *Dyx1c1* conditional forebrain knockout mouse model was generated.
- Knockouts and their littermate controls were evaluated on various behavioral paradigms.
- Mice with the homozygous *Dyx1c1* knockout showed deficits in memory and learning.

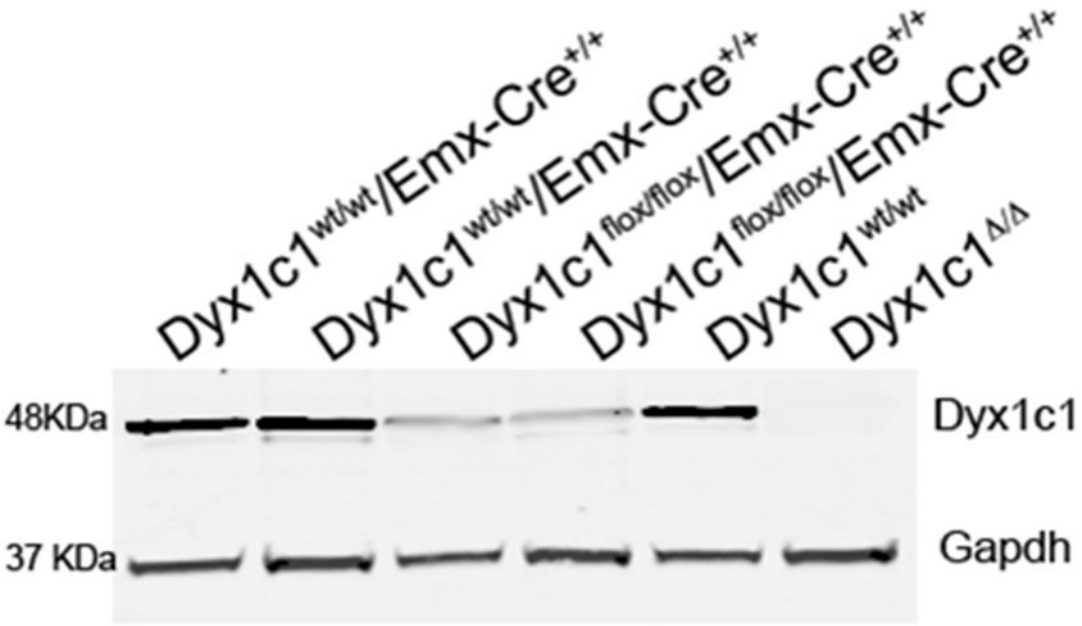

Figure 1.

This blot shows the Dyx1c1 protein expression in the forebrain KO and the control conditions. We have also included the constitutive KO and WT protein lysates as a control for the experiment. Dyx1c1 protein expression was reduced by 77% in the forebrain KO condition as compared to the control condition.

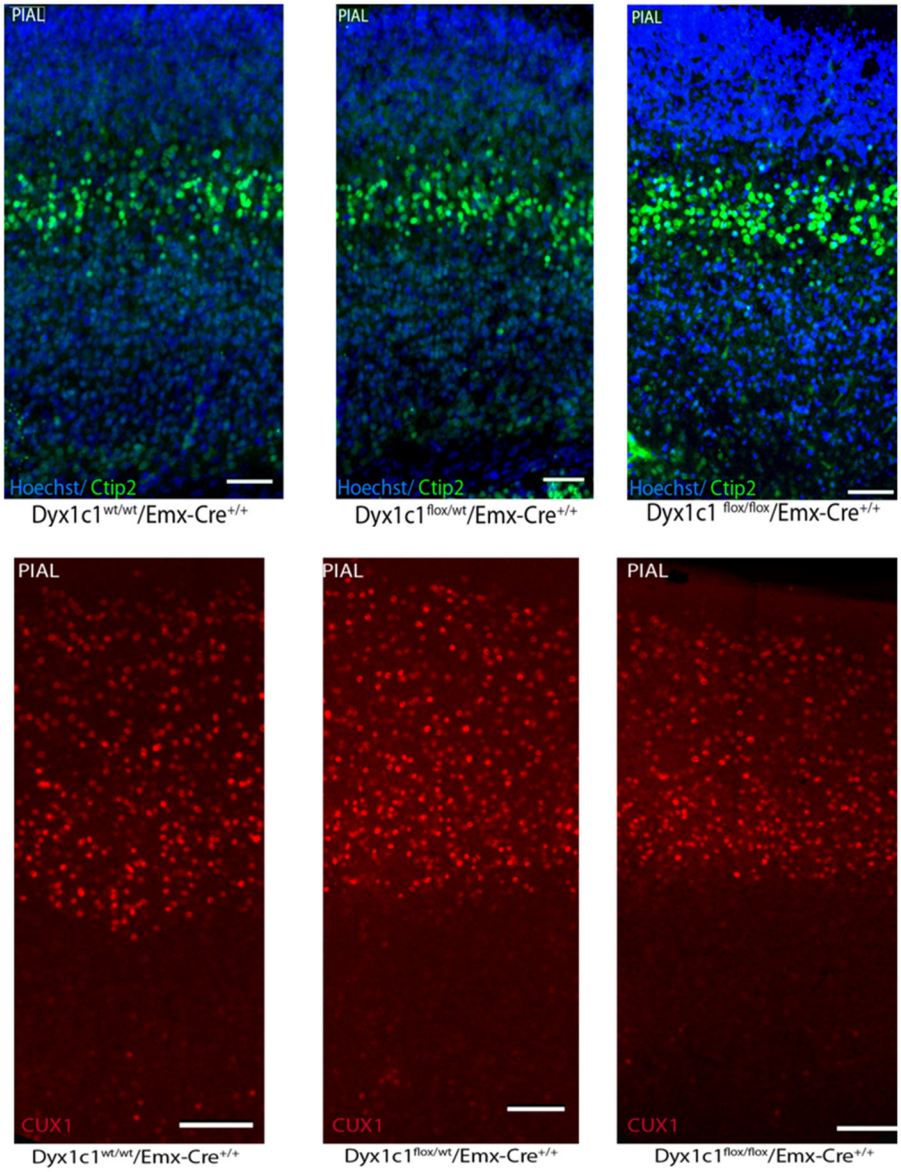

Figure 2.

There were no significant differences in the cortical lamination patterning as evident from the Cux1 and Ctip2 staining in the forebrain conditional knockout compared to the control. We found no observable anomalies in the cortex of the conditional forebrain knockout animals compared to the controls.

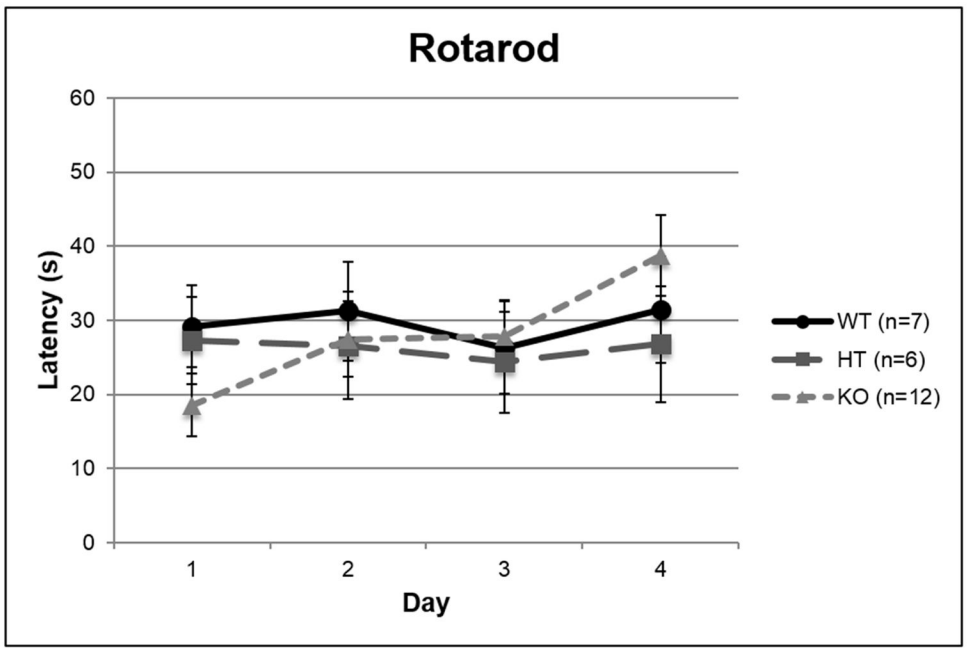

Figure 3.

Sensorimotor ability in *Dyx1c1* conditional knockout mice. No differences in sensorimotor performance between Genotype were observed on the rotarod task. Both groups were comparable in their latency to remain on the rotating cylinder across four days of testing.

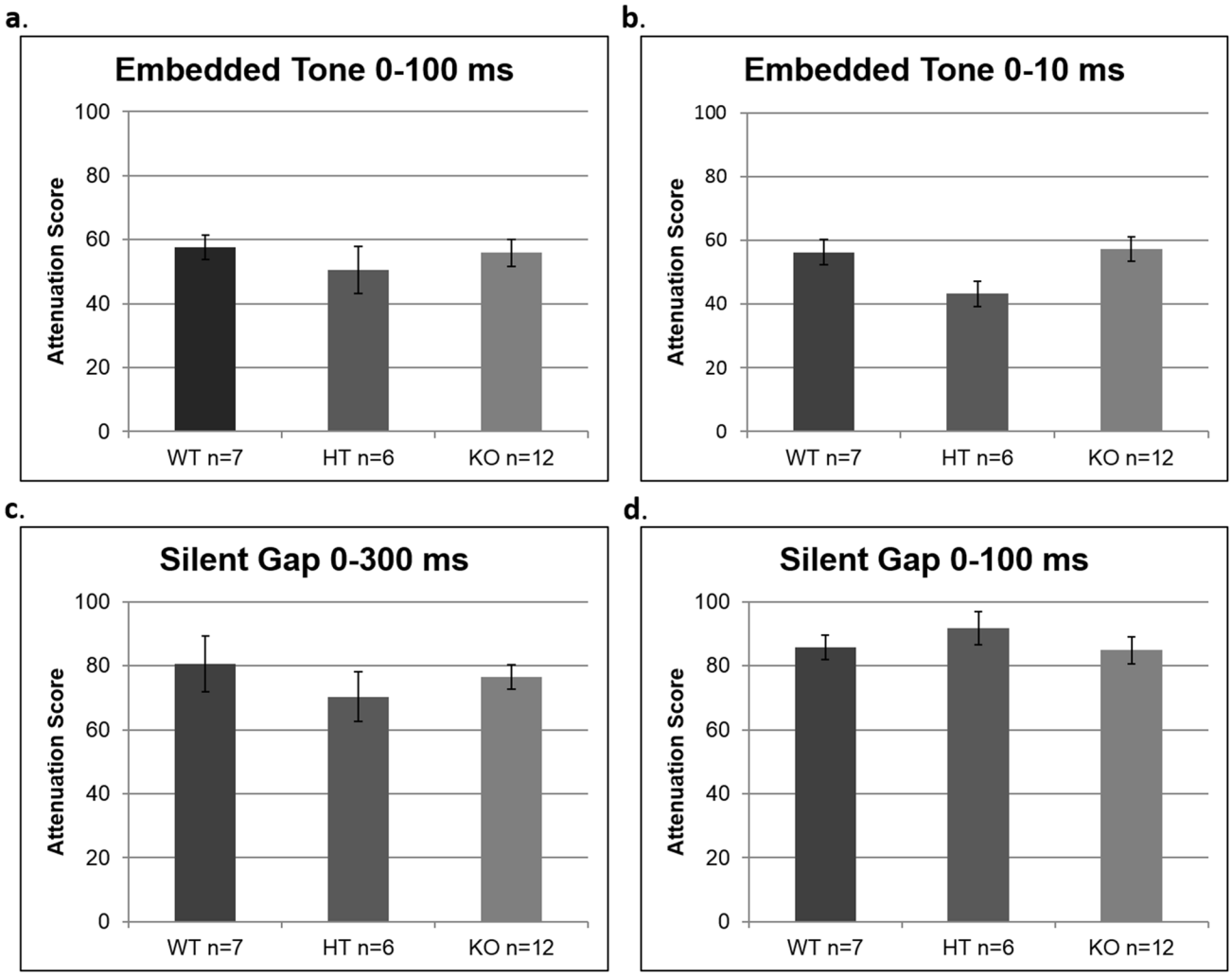

Figure 4.

Homozygous conditional *Dyx1c1* knockout mice displayed comparable auditory processing abilities on the following tasks (a.) Embedded Tone 0-100 ms, (b.) Embedded Tone 0-10 ms, (c.) Silent Gap 0-300 ms and (d.) Silent Gap 0-100ms.

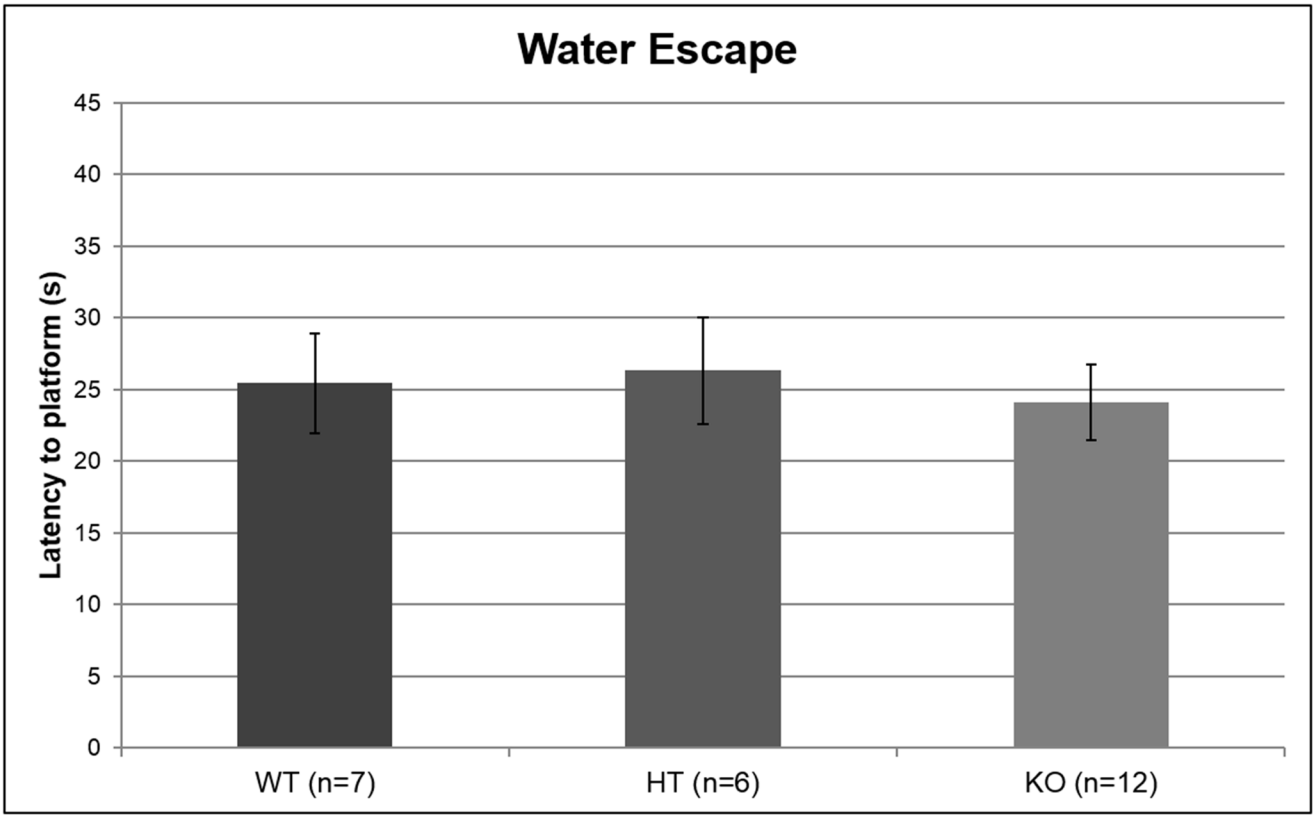

Figure 5.
Water escape performance. No significant differences between Genotypes on latency to swim to platform indicating no underlying motor or visual impairments.

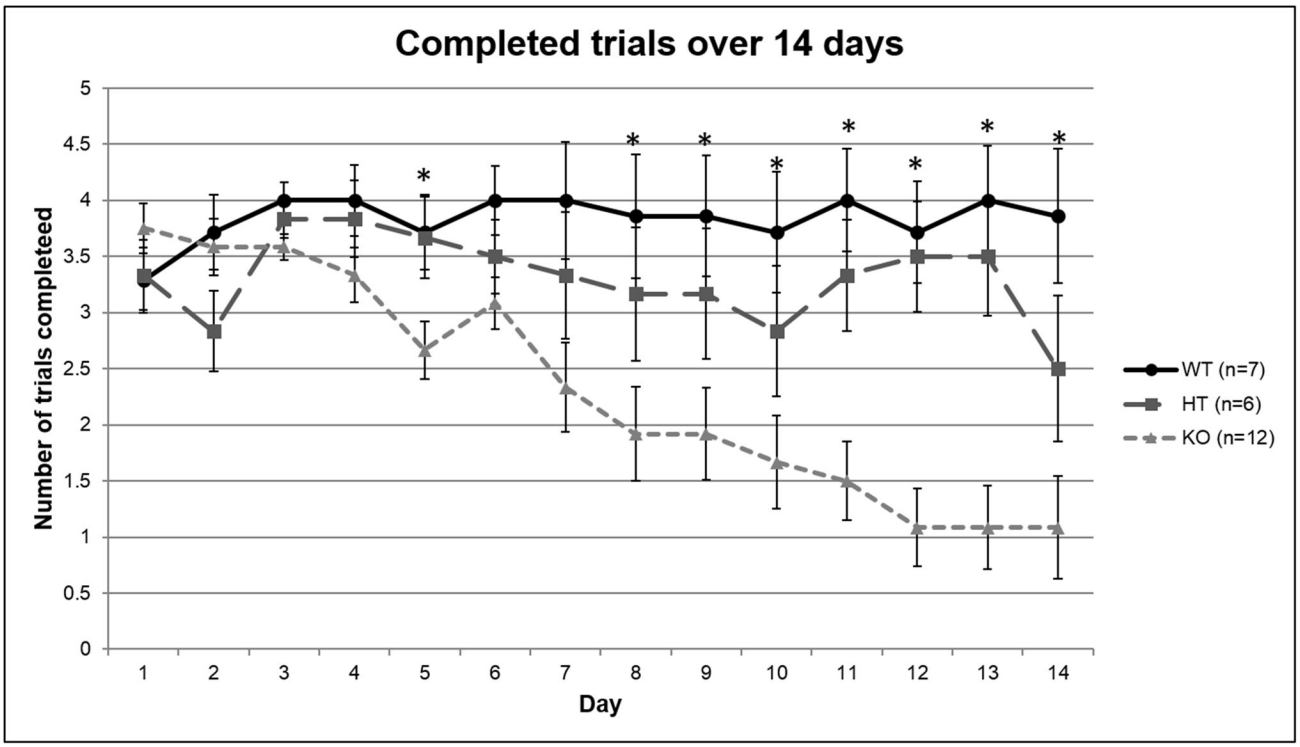

Figure 6.

General learning and memory impairment in mice with homozygous conditional *Dyx1c1* knockout mice on a 4/8 radial arm water maze. Analysis of total completed trials over 14 days reveal significant differences between Genotypes (*p <.05), as well as, significant Genotype x Day interaction (p<.001) revealing KOs drop in successfully completing trials as testing progresses.

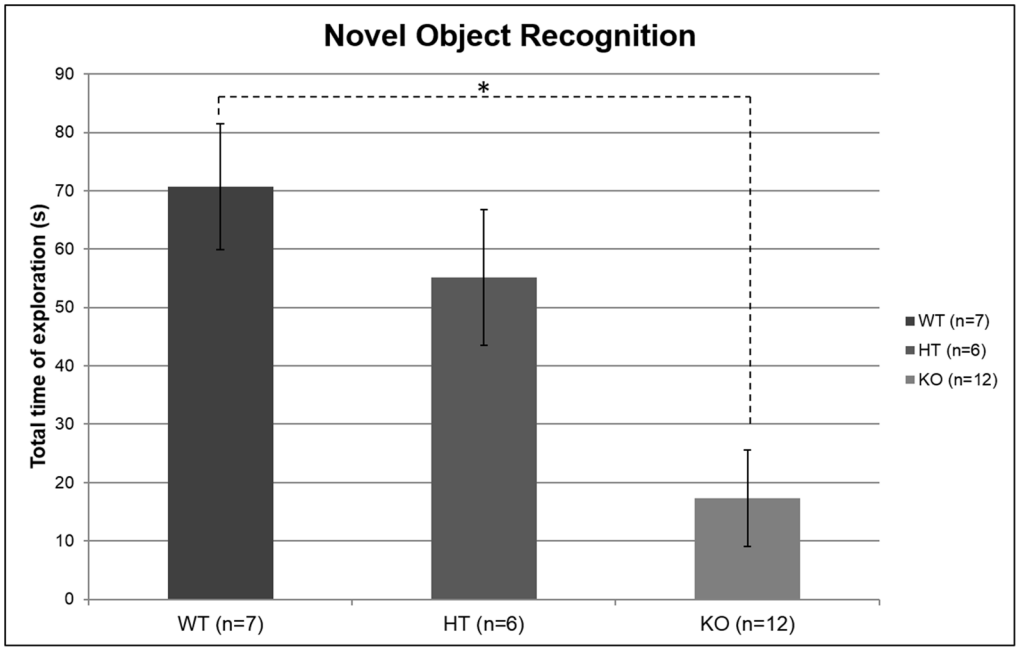

Figure 7.

Lack of exploration during the novel object recognition task in homozygous conditional *Dyx1c1* knockout mice (*p<.01).

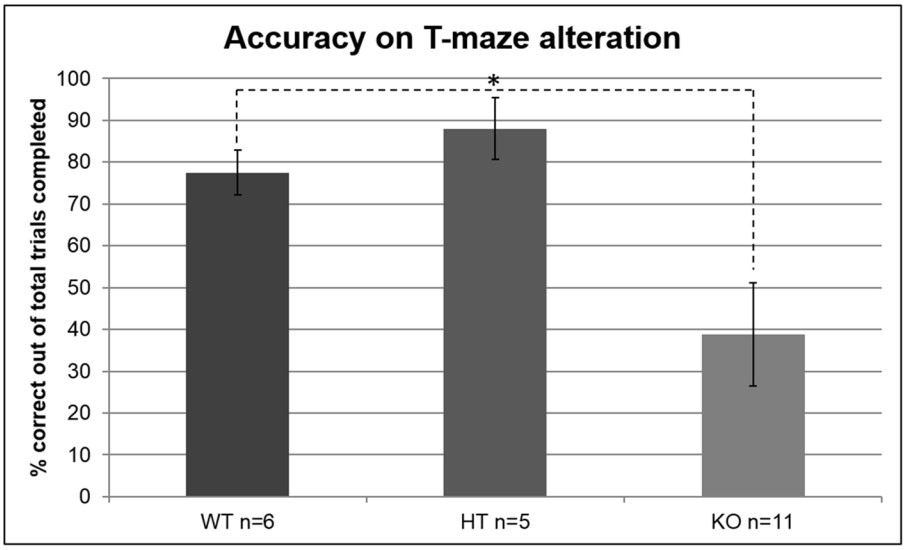

Figure 8.

Accuracy on an alteration T-maze task. Homozygous conditional *Dyx1c1* knockout mice exhibited significantly poorer performance on the T-maze (*p<.05) with accuracy around chance levels.