Loss of the Intellectual Disability and Autism Gene Cc2d1a and Its Homolog Cc2d1b Differentially Affect Spatial Memory, Anxiety, and Hyperactivity

Marta Zamarbide¹, Adam W. Oaks¹, Heather L. Pond¹, Julia S. Adelman¹ and M. Chiara Manzini¹,²*

¹GW Institute for Neurosciences, Department of Pharmacology and Physiology, The George Washington University School of Medicine and Health Sciences, Washington, DC, United States, ²Autism and Neurodevelopmental Disorders Institute, The George Washington University, Washington, DC, United States

Hundreds of genes are mutated in non-syndromic intellectual disability (ID) and autism spectrum disorder (ASD), with each gene often involved in only a handful of cases. Such heterogeneity can be daunting, but rare recessive loss of function (LOF) mutations can be a good starting point to provide insight into the mechanisms of neurodevelopmental disease. Biallelic LOF mutations in the signaling scaffold CC2D1A cause a rare form of autosomal recessive ID, sometimes associated with ASD and seizures. In parallel, we recently reported that Cc2d1a-deficient mice present with cognitive and social deficits, hyperactivity and anxiety. In Drosophila, loss of the only ortholog of Cc2d1a, lgd, is embryonically lethal, while in vertebrates, Cc2d1a has a homolog Cc2d1b which appears to be compensating, indicating that Cc2d1a and Cc2d1b have a redundant function in humans and mice. Here, we generate an allelic series of Cc2d1a and Cc2d1b LOF to determine the relative role of these genes during behavioral development. We generated Cc2d1b knockout (KO), Cc2d1a/1b double heterozygous and double KO mice, then performed behavioral studies to analyze learning and memory, social interactions, anxiety, and hyperactivity. We found that Cc2d1a and Cc2d1b have partially overlapping roles. Overall, loss of Cc2d1b is less severe than loss of Cc2d1a, only leading to cognitive deficits, while Cc2d1a/1b double heterozygous animals are similar to Cc2d1a-deficient mice. These results will help us better understand the deficits in individuals with CC2D1A mutations, suggesting that recessive CC2D1B mutations and trans-heterozygous CC2D1A and CC2D1B mutations could also contribute to the genetics of ID.

Keywords: intellectual disability, learning, social function, anxiety, hyperactivity, rare diseases, mouse models

INTRODUCTION

Autosomal recessive loss of function (LOF) of the signaling scaffold Coiled-coil and C2 Domain containing 1A (CC2D1A) causes a spectrum of neurodevelopmental conditions including fully penetrant intellectual disability (ID), and variably penetrant autism spectrum disorder (ASD), seizures, and aggressive behavior (Basel-Vanagaite et al., 2006; Manzini et al., 2014;
In *Drosophila*, where only one CC2D1 homolog, lethal giant discs *lgd*, is present, removal of *lgd* is lethal during the larval stage (Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Expression of either human CC2D1A or CC2D1B can rescue the phenotypes observed in *Drosophila* (Drusenheimer et al., 2015), suggesting that CC2D1A and CC2D1B act redundantly. Despite wide expression of CC2D1A and its binding to multiple proteins involved in the immune response (Chang et al., 2011; Chen et al., 2012), CC2D1A LOF in humans appears to only affect the brain, leading to a spectrum of behavioral deficits. While this indicates that CC2D1B is not fully able to compensate in the brain leading to the human presentation, it is unclear whether CC2D1B itself could have a role in neurodevelopmental disorders.

Studies on the genetic causes of ID and ASD, in particular, are identifying a large contribution of *de novo* and hypomorphic mutations to these diseases (Sanders et al., 2012; Lim et al., 2013; Yu et al., 2013; Musante and Ropers, 2014). Many of the mutated genes would have greater impact on development if completely lost, leading to multi-system disorders and/or brain malformations, while the heterozygous and hypomorphic mutations found in ASD/ID affect neurons more mildly, leading to a grossly normal brain, but with cognitive and social deficits (Yu et al., 2013). We wondered whether a similar mechanism is at play in patients with CC2D1A LOF mutations, where CC2D1B can only partially compensate. If this was the case, removal of both CC2D1 genes would be incompatible with embryogenesis, indicating that these proteins together have a critical developmental role. Nothing is known about the role of CC2D1B in brain development. By comparing how individual loss of each gene affects cognitive, social, and affective function we have studied the relative role of CC2D1A and CC2D1B in the brain and defined whether CC2D1B should also be considered as a candidate gene for ID.

Mice deficient for *Cc2d1a* develop normally *in utero*, but die soon after birth because of breathing and swallowing deficits (Zhao et al., 2011; Al-Tawashi et al., 2012; Oaks et al., 2017). By conditionally removing *Cc2d1a* in the forebrain, we have previously shown that *Cc2d1a* LOF recapitulates features of ID and ASD in adult animals (Oaks et al., 2017). *Cc2d1a* conditional knockout (1a-cKO) mice show learning and memory deficits, social deficits, hyperactivity, anxiety, and repetitive behaviors (Oaks et al., 2017).

To define how CC2D1B compensates for loss of CC2D1A and contributes to these phenotypes, we generated a *Cc2d1b* knockout (1b-KO) line and developed an allelic series of *Cc2d1a* and *Cc2d1b* LOF, including *Cc2d1a*/*Cc2d1b* double heterozygous (1a/1b-dHET) and double KO (1a/1b-KO) animals. Removal of both CC2D1 proteins causes early embryonic lethality, showing that CC2D1 function has an essential developmental role as in *Drosophila*. 1b-KO and 1a/1b-dHET animals are viable and fertile, indicating that these proteins together have a critical developmental role. Nothing is known about the role of CC2D1B in brain development. By comparing how individual loss of each gene affects cognitive, social, and affective function we have studied the relative role of CC2D1A and CC2D1B in the brain and defined whether CC2D1B should also be considered as a candidate gene for ID.

**MATERIALS AND METHODS**

**Animals**

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee of The George Washington University. A *Cc2d1b* null mouse line (1b-KO) was generated by the Knockout Mouse Project Repository (Project ID CDS 34981) at the University of California Davis, with the allele *Cc2d1b*tm1a[KOMP]Wtsi. *Cc2d1b* null mice carry an *engrailed* 2 splice acceptor (En2SA) gene-trap allele with bicistronic expression of β-galactosidase as well as a neomycin resistance cassette, flanked by FRT (flipase recombinase target) recombination sites, in the genomic region between exons 2 and 3 of *Cc2d1b* (**Figure 1A**). *Cc2d1a*/*Cc2d1b* double heterozygous (1a/1b-dHET) mice were generated by crossing *Cc2d1a* heterozygotes (1a-HET) with *Cc2d1b* heterozygotes (1b-HET). 1a-HET mice were bred from a *Cc2d1a* null mouse line (KO) generated by the Knockout Mouse Project Repository (Project Design ID 49663) at the University of California as was previously described by Oaks et al. (2017). All lines are maintained on a C57Bl/6 background. For genotyping, polymerase chain reaction (PCR) amplifications were performed on 1 µL of proteinase K (New England Biolabs, Ipswich, MA, United States) digested tail DNA samples. PCR reactions (50 µL) consisted of GoTaq Flexi buffer (Promega, Madison WI, United States), 100 µM dNTPs, 50 µM each of forward and reverse primers (sequence available upon request), 1 mM MgCl2, and 1.25 U GoTaq Flexi DNA polymerase (Promega, Madison WI, United States), and were run with optimized reaction profiles determined for each genotype. A 25-µL aliquot from each reaction was analyzed by gel electrophoresis on a 1.0% agarose gel for the presence of the desired band.

**Histological Preparation and Microscopy**

To prepare tissue for histological analysis, deeply anesthetized mice were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed in PFA. Cryosections from adult mouse brains were prepared by mounting in Neg-50 (Thermo Fisher Scientific, Waltham, MA, United States) and cut at 40 µm on a Cryostar NX50 cryostat (Thermo Fisher Scientific, Waltham, MA, United States), then stained with Hematoxylin and Eosin (H&E, VWR International, Radnor, PA, United States) to visualize tissue architecture. Imaging of H&E stained sections
was performed on a Leica M165 FC stereo microscope (Leica Microsystems, Buffalo Grove, IL, United States).

**Behavioral Tests**

A standardized battery of behavioral testing was applied to each cohort of animals, 1b-KO and 1a/1b-dHET male and female mice, at 3–4 months of age. As both 1b-KOs and 1a/1b-dHETs were generated from the same 1a/1b-dHET, breeding the wild-type (WT) controls were littermates shared by both cohorts and all behavioral tests were performed at the same time for WT, 1b-KOs, and 1a/1b-dHETs. Behavioral tests were performed in the Manzini lab behavioral suite in the George Washington University Animal Research Facility following a 60 min period of acclimatization. Initial characterization to analyze any neurological abnormalities including the analysis of basic motor and somatosensory function was performed on a subset of the behavioral cohort as described by Rogers et al. (1997): righting reflex, wire hang, gait analysis, tail pinch, and visual reach. Cognitive and social function and other behaviors were tested in the open field test, novel object recognition test (NORT) (Bevins and Besheer, 2006), Morris water maze (MWM) (Vorhees and Williams, 2006), and 3-chamber social interaction test (Nadler et al., 2004). Behavioral analysis was performed via automated animal tracking using ANY-maze (Stoelting, Wood Dale, IL, United States).

**Righting Reflex**

Coordination, motor strength, and vestibular function were tested by placing each mouse on its back and timing its ability to return to an upright position.

**Wire Hang**

Motor strength was tested by timing the latency to fall to a mouse cage containing bedding while the mouse was hanging from a wire cage-top not higher than 18 cm.

**Gait Analysis**

Motor coordination and strength were assessed by painting the paws of each mouse with red non-toxic tempera paint and making them walk through a narrow tunnel over white paper. Abnormalities of paw placement and stride length were noted or indicated as normal.

**Tail Pinch**

The ability of each mouse to respond to mild pain was tested by pinching the tip of the tail with fine, ethanol-cleaned forceps. Reactions were categorized as either response or no response.

**Visual Reach**

Vision was tested by measuring the latency to the first attempt to reach for a nearby wire cage-top while the mouse was being held by the base of the tail at a height of 18 cm over an open cage.
Open Field Test

The open field test was performed in an unfamiliar 50 cm × 50 cm plastic box (Stoelting, Wood Dale, IL, United States). Animals were placed in the center of the arena and ambulatory activity was monitored by digital video for 15 min. The arena was divided into two areas, an outer zone and a center zone (25 cm × 25 cm; 25% of total area). Total distance traveled and time spent in each area was measured.

Novel Object Recognition Test

The NORT (Bevins and Besheer, 2006; Oaks et al., 2017) was performed in the same apparatus described for the open field test. The test consisted of three different phases: habituation, training, and test. The habituation phase lasted for 30 min while the animals were exposed to the box and then returned to the home cage while the box was cleaned. During the training phase, the animal was placed in the same box with two identical objects located in opposite corners, at a distance of 5 cm from the walls. To assess short-term memory, the animal was returned to the home cage during an interval of 15 min. During the test, a familiar object, identical to those used in the habituation phase, was placed in one corner, while in the opposite corner an unfamiliar object was placed. Exploration activity was monitored for 10 min at each phase, with exploration defined as time spent actively observing or touching the object from within a radius of 5 cm. Cumulative time spent with each object was measured by video analysis using ANY-maze to determine the location of the animal’s nose relative to the objects in the enclosure. Preference for the novel object was defined as the ratio of the time spent with the novel object to the time spent with the familiar object. Animals that did not interact with the object and stopped in a corner of the cage were removed from the analysis.

Morris Water Maze

The MWM (Vorhees and Williams, 2006; Oaks et al., 2017) apparatus was a 120 cm × 120 cm round metal tub (Stoelting, Wood Dale, IL, United States) where distinct visual cues were placed at the cardinal points. White non-toxic paint was added to the water to make the surface opaque for the hidden trials and it was maintained at 24°C. Each trial consisted of four independent drops, one at each cardinal point around the tub, with the mouse facing the wall of the tub. Each drop lasted 60 s, or until the mouse found the platform, whichever occurred first. Each animal completed two trials (four drops each) with a visible platform, five trials with a platform hidden under the water surface, and two reversal trials where the location of the hidden platform (HP) was changed. The sequence of nine trials was performed over 9 days, with one trial per day. A 60-s probe trial was also performed the day after the HP series was completed, by removing the platform from the water before proceeding to the reversal phase on the following day.

Three-Chamber Social Interaction Test

The social interaction test (Nadler et al., 2004; Kaidanovich-Beilin et al., 2011) was performed in a clear rectangular acrylic box (60 cm × 40 cm) divided into three chambers (40 cm × 20 cm) with small openings (10 cm × 5 cm) in the adjoining walls (Everything Plastic, Philadelphia, PA, United States). The test consisted of two phases, the habituation phase and the sociability phase. During the habituation phase, empty inverted wire cups (10 cm in diameter) were placed in the center of the chambers at the ends. Each mouse was placed in the center chamber of the apparatus and allowed to explore the different chambers for 5 min. During the second phase, an unfamiliar mouse of the same sex as the tested mouse was placed under the wire cup in one of the side chambers. The experimental mice were allowed to explore for 10 min during the sociability phase. Total time spent in the Object (containing empty cup) and Mouse (with unfamiliar mouse under the cup) chambers was used to determine the social preference of each mouse tested, while the time sniffing within a 2-cm radius of the mouse-containing cup were recorded as measures of social approach and social interaction.

RESULTS

CC2D1A and CC2D1B Have Partially Redundant Function in Development

Loss of CC2D1A in humans causes a variable spectrum of ID, ASD, and seizures and the removal of Cc2d1a in the murine forebrain leads to several cognitive, social, and affective behavioral phenotypes (Manzini et al., 2014; Oaks et al., 2017). As no human mutations in CC2D1B have been identified to date, we asked whether loss of Cc2d1b in the mouse would lead to similar phenotypes as loss of Cc2d1a. A Cc2d1b-deficient line (1b-KO) had been generated from the Knockout Mouse Project (KOMP) as a gene-trap allele inserted in intron 2 of Cc2d1b (Figure 1A). We obtained heterozygous animals and bred them to homozygosity, finding that 1b-KO mice are born in Mendelian ratios (Figure 1B). Differently from Cc2d1a KO (1a-KO) pups, which die shortly after birth (Zhao et al., 2011; Al-Tawashi et al., 2012; Drusenheimer et al., 2015; Oaks et al., 2017), 1b-KO mice are viable, fertile, and indistinguishable from WT littermates (Figure 1C). Basic behavioral functions were tested in adult WT and 1b-KO males and females: coordination (righting reflex), strength (wire hang), locomotion (stride and gait), pain sensitivity (tail pinch), and vision (visual reflex). No differences were observed in basic sensory and motor function (Table 1). We confirmed via western blot analysis of cortical protein lysates that CC2D1B was completely absent in these animals and that CC2D1A was expressed at normal levels (Figure 1D). Cryosections generated from the adult brain of 1b-KO animals and stained using hematoxylin and eosin (H&E) showed no differences in brain size and organization from WT littermates (Figure 1E). In summary, loss of Cc2d1b does not affect respiratory function and deglutition in the infant as observed in 1a-KOs, and 1b-KO adult mice are indistinguishable from WT littermates.

CC2D1A and CC2D1B contain very similar protein domains and are thought to have redundant functions in endocytic traffic and gene transcription (Hadjighassem et al., 2009; Usami et al., 2012; Drusenheimer et al., 2015). Because CC2D1B LOF did not result in postnatal lethality, we wondered whether
the two proteins would only be partially redundant. To test this hypothesis, we crossed 1b-KOs and 1a-KOs to generate Cc2d1a/Cc2d1b double heterozygous (1a/1b-dHET) and double KO mice (1a/1b-KO). As 1aKO pups die soon after birth (Zhao et al., 2011; Al-Tawashi et al., 2012; Oaks et al., 2017), we did not expect 1a/1b-KO animals to survive and we genotyped litters at postnatal day (P)0, collecting tissue from both live and dead pups. However, while dead 1a-KO and 1a-KO/1b-HET were found in the expected ratios, 1a/1b-dKO pups were never retrieved (Figure 2A), suggesting that double knockouts may die earlier during embryonic development. Examination of prenatal litters only identified 1a/1b-dKO tissue mid-gestation at E11.5, but the embryo was almost entirely absent, leaving only a hypomorphic and largely empty yolk sac (Figure 2B). These results indicate that removal of both CC2D1 proteins leads to early embryonic lethality.

1a/1b-dHETs were viable, fertile, and indistinguishable from WT littersmates with normal gross brain anatomy (Figure 2C) and normal basic motor and sensory function (Table 1). We tested the expression levels of CC2D1A and CC2D1B in 1a/1b-dHET mice and found that as expected, only a half dose of each CC2D1 protein was present (Figure 2D). Thus, combined CC2D1 function is necessary for embryonic morphogenesis, but 1b-KO or 1a/1b-dHET animals develop normally, indicating that CC2D1A and CC2D1B have similar functions as it pertains to gross anatomical development and survival.

**TABLE 1** | Analysis of basic motor and sensory function in 1b-KO and 1a/1b-dHET mice.

| Genotype    | Sex | Weight (g) | Righting reflex (s) | Wire hang (s) | Stride and gait | Tail pinch<sup>a</sup> | Visual reflex<sup>b</sup> |
|-------------|-----|------------|---------------------|---------------|----------------|------------------------|--------------------------|
| WT          | M   | 28.39 ± 0.94 | <1 s               | 56.5 ± 2.35   | Normal         | 6/9                    | 9/9                      |
| 1b-KO       | M   | 27.49 ± 0.67 | <1 s               | 52.21 ± 5.05  | Normal         | 7/10                   | 10/10                    |
| 1a/1b-dHET  | M   | 28.32 ± 0.85 | <1 s               | 53.61 ± 3.24  | Normal         | 6/8                    | 8/8                      |
| WT          | F   | 21.06 ± 0.53 | <1 s               | 58.7 ± 1.09   | Normal         | 7/10                   | 10/10                    |
| 1b-KO       | F   | 21.95 ± 0.83 | <1 s               | 55.87 ± 3.71  | Normal         | 6/8                    | 8/8                      |
| 1a/1b-dHET  | F   | 21.81 ± 0.88 | <1 s               | 58.2 ± 1.59   | Normal         | 7/10                   | 10/10                    |

<sup>a</sup>Animals responding to tail pinch. <sup>b</sup>Animals responding to visual stimulus.
Both CC2D1A and CC2D1B Are Important for Cognitive Function

We have previously found that loss of Cc2d1a leads to a constellation of behavioral deficits: cognitive and social impairment, anxiety, hyperactivity, and repetitive behaviors (Oaks et al., 2017). We generated a cohort of 1b-KO and 1a/1b-HET male and female mice for behavioral analysis by crossing 1a/1b-HETs, so that we could compare behavioral performance in both lines at the same time. In the short-term memory version of the NORT (Bevins and Besheer, 2006) mice are placed in an arena with two identical objects that they are free to explore. After being removed back to their cages for 15 min, they are put in the arena where one of the now known objects has been substituted for a familiar object after a 15 min interval. (Figure 3A). In this test, WT male and female mice spend roughly four times longer exploring the novel object, while 1b-KOs and 1a/1b-dHETs show no difference (Figures 3B,C) (Males: WT, T2/T1 = 1.21 ± 0.32, T4/T3 = 3.90 ± 0.75, n = 10, p = 0.004**; 1b-KO, T2/T1 = 1.05 ± 0.24, T4/T3 = 1.60 ± 0.46, n = 11, p = 0.309; 1a/1b-dHET, T2/T1 = 1.08 ± 0.23, T4/T3 = 1.62 ± 0.46, n = 12, p = 0.307. Females: WT, T2/T1 = 1.20 ± 0.25, T4/T3 = 4.39 ± 1.40, n = 10, p = 0.038 *; 1b-KO, T2/T1 = 0.84 ± 0.16, T4/T3 = 0.93 ± 0.24, n = 10, p = 0.757; 1a/1b-dHET, T2/T1 = 1.34 ± 0.48, T4/T3 = 1.46 ± 0.28, n = 10, p = 0.824). This deficit was not due to reduced interest in the objects, as animals spent similar amounts of time in exploratory behaviors, with 1a/1b-dHET males showing significantly more exploration (Figure 3D T1+T2 – Males: WT, t = 26.97 ± 5.75s, n = 10; 1b-KO, t = 23.17 ± 3.65s, n = 11, p = 0.999; 1a/1b-dHET, t = 66.25 ± 15.93s, n = 12, p = 0.167; Females: WT, t = 40.56 ± 5.19s, n = 10; 1b-KO, t = 71.67 ± 17.47s, n = 10, p = 0.423; 1a/1b-dHET, t = 54.30 ± 10.56s, n = 10, p = 0.960. Figure 3E T3+T4 – Males: WT, t = 21.93 ± 5.54s; 1b-KO, t = 17.91 ± 3.57s, p = 0.999; 1a/1b-dHET, t = 50.83 ± 16.0s, p = 0.640. Females: WT, t = 15.39 ± 2.12s; 1b-KO, t = 68.38 ± 26.04s, p = 0.090; 1a/1b-dHET, t = 31.86 ± 10.61s, p = 0.959. Figure 3F SUM T1,2,3,4 – Males: WT, t = 48.90 ± 9.35s; 1b-KO, t = 41.08 ± 6.20s, p = 0.942; 1a/1b-dHET, t = 116.1 ± 28.24s, p = 0.033 *; Females: WT, t = 55.95 ± 6.62s, 1b-KO; t = 140.1 ± 42.64s, p = 0.073; 1a/1b-dHET, t = 86.16 ± 20.52s, p = 0.660).

To further assess cognitive function, the 1b-KO mice were tested using the MWM paradigm which probes spatial memory acquisition, retention, and flexibility, by testing the ability of a mouse to learn, remember, and relearn the location of a platform hidden under opaque water (Morris, 1984). After the mice are trained using a visible platform to escape from the water, the platform is hidden under the surface in a different location and the animals undergo training on five consecutive days to learn...
the location of the platform. On the following day, memory retention is tested by removing the platform and measuring the amount of time the mouse spends in the area where the platform was previously located (probe trial). Finally, the position of the platform is changed and the animal must display flexibility by learning a new location (reversal). 1a-cKO animals show a delay in initial acquisition of the location of the HP, but after they learn, they can retain the memory in the probe trial, and learn a new location in the reversal (Oaks et al., 2017). Both 1b-KO and 1a/1b-dHET males and females presented deficits in this test (Figure 4). 1b-KO males and females and 1a/1b-dHET males were delayed in the HP acquisition showing significant differences in day 2 or 3 of the test (HP2 and HP3 in Figures 4B,F) (Males HP3: WT, \( t = 6.82 \pm 0.69s, n = 11; 1b-KO, t = 10.97 \pm 1.85s, n = 10, p = 0.042 \); 1a/1b-dHET, \( t = 11.99 \pm 1.28s, n = 13, p = 0.0027 ** \). Females HP2: WT, \( t = 12.30 \pm 1.32s, n = 13; 1b-KO, t = 19.62 \pm 1.74s, n = 10, p = 0.0025 **; 1a/1b-dHET, t = 14.66 \pm 1.64s, n = 11, p = 0.247\). 1a/1b-dHET males and females were also affected in the probe trial where they spent less time in the platform quadrant during the first 15 s of the 60-s trial (Figures 4D,H) (Probe 15 s – Males: WT, \( t = 9.51 \pm 0.83s, n = 11; 1b-KO, t = 6.13 \pm 0.50s, n = 10, p = 0.0029 **; 1a/1b-dHET, t = 5.48 \pm 0.80s, n = 13, p = 0.0021 **. Females: WT, \( t = 7.18 \pm 0.80s, n = 13; 1b-KO, t = 5.77 \pm 0.65s, n = 10, p = 0.203; 1a/1b-dHET, t = 4.43 \pm 0.82s, n = 11, p = 0.022 * \). Finally, 1b-KO males, but not females, were affected throughout the 60-s probe trial and spent less time exploring the correct quadrant in the probe trial testing memory retention (Figure 4D) (Probe 60 s – Males: WT, \( t = 25.40 \pm 1.78s, n = 11; 1b-KO, t = 19.58 \pm 1.30s, n = 10, p = 0.018 *; 1a/1b-dHET, t = 22.74 \pm 2.63s, n = 13, p = 0.428. females: WT, \( t = 21.19 \pm 1.85s, n = 13; 1b-KO, t = 20.57 \pm 1.54s, n = 10, p = 0.809; 1a/1b-dHET, t = 18.43 \pm 2.62s, n = 11, p = 0.389\).

Animals heterozygous for loss of Cc2dlα or Cc2dlβ alone showed normal behavioral performance (Supplementary Figures 1, 2 and Supplementary Table 1). In summary, loss of CC2DL1 leads to cognitive deficits in both memory acquisition and retention. In general, males appear more severely affected than females in both 1bKO and 1a/1bHET lines, suggesting that CC2DL1A and CC2DL1B have overlapping roles in cognitive function.

**Only CC2DL1A Is Involved in Anxiety and Hyperactivity**

1a-cKO animals showed increased mobility and reduced entry into the center of the open field arena, indicating hyperactivity and anxiety (Oaks et al., 2017). In addition, removal of Cc2dlα in the forebrain also leads to ulcerative dermatitis due to obsessive grooming and social interaction deficits (Oaks et al., 2017). 1b-KO males and females performed similarly to WT littermates in the open field test and showed no signs of hyperactivity or anxiety (Figure 5) (Distance – Males: WT, \( d = 25.16 \pm 2.29m, n = 11; 1b-KO, d = 29.63 \pm 1.96m, n = 11, p = 0.498; Females: WT, d = 34.65 \pm 1.36m, n = 13; 1b-KO, d = 42.37 \pm 3.28m, n = 11, p = 0.097. Time in center – Males: WT, \( t = 78.13 \pm 5.23s, n = 11; 1b-KO, t = 83.17 \pm 14.26s, n = 11, p = 0.988; Females: WT, \( t = 77.45 \pm 11.78s, n = 10; 1b-KO, t = 87.75 \pm 17.65s, n = 10, p = 0.969). Interestingly, 1a/1b-dHETs showed increased locomotion and avoidance of open spaces, as previously observed for the 1a-cKOs, but only in males, similar to the exploration in the NORT where increased

**FIGURE 4 | CC2DL1B Is involved in spatial memory formation and retention with mild male-specificity. Hippocampus-dependent spatial memory was assessed in 1bKO and 1a/1bHET mice via the Morris Water Maze test. Spatial learning was measured as latency to escape in three different stages, visible platform (VP), hidden platform (HP), or the reversal (RV) of the HP position. No deficits were shown by males (A) or females (B) of any genotype in identifying the platform in the VP trial. (B) Both 1bKO and 1a/1bHET males showed a delay in learning the location of the HP and a similar deficit was present in 1bKO females (F, C, G). No differences were found in the RV during the test. (D, H) Spatial memory retention was measured between the HP and RV trials by the time spent swimming in the quadrant where the platform was previously located. Significant spatial memory impairment was found in the 1bKO male mice compared to WT both during the first 15 s and at the end of the trial after 60 s, while female 1bKO mice showed no deficit. 1a/1b-dHET males and females spent less time looking for the platform during the first 15 s, but subsequently recovered. Two-way ANOVA with repeated measures was used for analysis of the HP phase. Multiple t-tests with equal variance were used for individual timepoints and probe analysis *p < 0.05, **p < 0.01.**
exploratory behavior was only observed in 1a/1b-dHET males (Figures 5A, B) (Distance – Males: WT, d = 25.16 ± 2.29m, n = 11; 1a/1b-dHET, d = 35.85 ± 2.94m, n = 13, p = 0.0076 **; Females: WT, d = 34.65 ± 1.36m, n = 13; 1a/1b-dHET, d = 35.35 ± 2.51m, n = 11, p = 0.999. Time in center – Males: WT, t = 78.13 ± 5.23s, n = 11; 1a/1b-dHET, t = 41.32 ± 3.71s, n = 13, p = 0.0198 *; Females: WT, t = 77.45 ± 11.78s, n = 10; 1b-KO, t = 121.90 ± 15.19s, n = 11, p = 0.1225). No ulcerative dermatitis or obsessive grooming was observed in any of these mouse lines.

Finally, all mice were tested in the social approach version of the three-chambered test. In this test, the mouse is placed in an apparatus with three communicating chambers. In the left chamber, there is a novel mouse of the same sex under a wire cup, while in the right chamber there is an empty wire cup. Mice spend more time exploring and sniffing the stranger mouse than the object and this is considered a social action (Nadler et al., 2004; Kaidanovich-Beilin et al., 2011). The 1a-cKO showed no preference for the conspecific both as in the time spent around the mouse enclosure and the time spent sniffing the stranger mouse (Oaks et al., 2017). 1a/1b-dHET males and females and 1bKO females showed no difference from WT littermates (Supplementary Figure 3). The deficit in 1b-KO males was primarily due to a subset of animals showing preference for the object (Supplementary Figure 3). All genotypes showed significantly increased time spent sniffing the stranger mouse, indicating that once in the chamber the 1b-KO animals interact with the other animal (Figures 5E, H) (Males: WT, time sniffing mouse (tsm) = 66.47 ± 7.44s, time sniffing object (tso) = 36.61 ± 7.51s, n = 11, p = 0.0105 *; 1b-KO, tsm = 56.04 ± 10.78s, tso = 21.47 ± 5.78s, n = 13, p = 0.009 **; 1a/1b-dHET, tsm = 58.40 ± 8.65s, tso = 31.11 ± 8.95s, n = 10, p = 0.042 *). Females: WT, tsm = 60.11 ± 10.60s, tso = 31.15 ± 7.71s, n = 8, p = 0.044 *; 1b-KO, tsm = 96.68 ± 13.00s, tso = 29.93 ± 5.55s, n = 8, p = 0.0033 **; 1a/1b-dHET, tsm = 55.80 ± 5.66s, tso = 18.26 ± 4.02s, n = 9, p = 0.00005 **].

In conclusion, 1b-KO and 1a/1b-dHET animals show only partially overlapping behavioral profiles in anxiety, hyperactivity, and sociability. 1b-KO mice of either sex do not appear anxious or hyperactive and only males show a mild sociability deficit in the three-chamber test. 1a/1b-dHET males are more similar to 1a-cKO mice, with increased locomotion and decreased time in the center of the open field. These results show that CC2D1A and CC2D1B only have partially redundant roles in cognitive and social function. Each of the Cc2d1 genes contributes to aspects of learning and memory and sociability, but Cc2d1a appears to be more critical for hyperactivity and anxiety. Interestingly, both
lines display sexually dimorphic phenotypes with males being mildly more affected than females.

**DISCUSSION**

Cognitive development is controlled by a multitude of mechanisms regulating synaptic transmission and neuronal function. Hundreds of genes have been found mutated in patients with ID and ASD and the generation of mouse models has deepened our understanding of how each gene contributes to disease and behavior (Nestler and Hyman, 2010; Ey et al., 2011; Kazdoba et al., 2015). Mutations in the gene encoding CC2D1A cause a rare form of ID and ASD in humans, and this protein is emerging as a critical regulator of intracellular signaling with roles in cognitive function (Basel-Vanagaite et al., 2006; Manzini et al., 2014), immunity (Zhao et al., 2010; Chang et al., 2011) and cancer (Yamada et al., 2015). Removal of the only CC2D1 homolog in Drosophila, 

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\text{lgd, causes early lethality and severe deficits in morphogenesis, and both human proteins can rescue lgd LOF phenotypes, suggesting that the vertebrate CC2D1 proteins have redundant functions (Drusenheimer et al., 2015). In fact, deficits in lgd mutant flies are more severe than in 1a-KO and 1b-KO mice (Drusenheimer et al., 2015). We hypothesized that the neuropsychiatric phenotypes observed in humans carrying CC2D1A LOF mutations are likely due to the inability of CC2D1B to fully substitute for CC2D1A.}
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Initial evidence to support our hypothesis was provided by the fact that 1a-KO mice are anatomically normal but die soon after birth due to breathing and swallowing deficits (Zhao et al., 2011; Al-Tawashi et al., 2012; Chen et al., 2012; Oaks et al., 2017), while 1b-KOs are viable and fertile (Drusenheimer et al., 2015). No respiratory deficits have been reported in humans with CC2D1A mutations and these findings indicated that CC2D1A cause a rare form of ID and ASD in humans, and this protein is emerging as a critical regulator of intracellular signaling with roles in cognitive function (Basel-Vanagaite et al., 2006; Manzini et al., 2014), immunity (Zhao et al., 2010; Chang et al., 2011) and cancer (Yamada et al., 2015). Removal of the only CC2D1 homolog in Drosophila, 

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are both present in heterozygosity. While complete loss of both CC2D1 genes is embryonic lethal, haploinsufficiency of both CC2D1A and CC2D1B may lead to ID and ASD as CC2D1A LOF does. In the Genome Aggregation Database browser, which collects allele frequency data from more than 100,000 individuals in different populations, there are 43 likely gene disrupting (stop codon, frameshift, or splice site) alleles for CC2D1A and 89 for CC2D1B. These variants alone or in combination may further contribute to the genetic burden of ID.

AUTHOR CONTRIBUTIONS
MZ and MM designed the study and wrote the manuscript. MZ, AO, HP, and JA conducted the experiments and analyzed the data.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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