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Archival Report

Male-Specific cAMP Signaling in the Hippocampus Controls Spatial Memory Deficits in a Mouse Model of Autism and Intellectual Disability

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ABSTRACT

BACKGROUND: The prevalence of neurodevelopmental disorders is biased toward male individuals, with male-to-female ratios of 2:1 in intellectual disability and 4:1 in autism spectrum disorder. However, the molecular mechanisms of such bias remain unknown. While characterizing a mouse model for loss of the signaling scaffold coiled-coil and C2 domain-containing protein 1A (CC2D1A), which is mutated in intellectual disability and autism spectrum disorder, we identified biochemical and behavioral differences between male and female mice, and explored whether CC2D1A controls male-specific intracellular signaling.

METHODS: CC2D1A is known to regulate phosphodiesterase 4D (PDE4D), which regulates cyclic adenosine monophosphate (cAMP) signaling. We tested for activation of PDE4D and downstream signaling molecules in the hippocampus of *Cc2d1a*-deficient mice. We then performed behavioral studies in female mice to analyze learning and memory, and then targeted PDE4D activation with a PDE4D inhibitor to define how changes in cAMP levels affect behavior in male and female mice.

RESULTS: We found that in *Cc2d1a*-deficient male mice PDE4D is hyperactive, leading to a reduction in cAMP response element binding protein signaling, but this molecular deficit is not present in female mice. *Cc2d1a*-deficient male mice show a deficit in spatial memory, which is not present in *Cc2d1a*-deficient female mice. Restoring PDE4D activity using an inhibitor rescues cognitive deficits in male mice but has no effect on female mice.

CONCLUSIONS: Our findings show that CC2D1A regulates cAMP intracellular signaling in a male-specific manner in the hippocampus, leading to male-specific cognitive deficits. We propose that male-specific signaling mechanisms are involved in establishing sex bias in neurodevelopmental disorders.

Keywords: Autism, cAMP, Intellectual disability, Intracellular signaling, Learning and memory, Sex bias

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Developmental disabilities are more prevalent in males, with male-to-female ratios ranging between 1.5:1 and 2:1 in intellectual disability (ID) and between 4:1 and 8:1 in autism spectrum disorder (ASD) (1,2). However, the molecular mechanisms underlying sex bias in neurodevelopmental disorders remain unknown. The male and female brains in normal subjects show developmental sex differences beyond sex-specific behaviors such as mating and aggression (3). Noninvasive imaging studies in humans have shown that male and female brains have different patterns of connection (4), and comparison of activation of brain areas involved in social cognition in children with ASD found deficits only in affected boys, and not in girls (5). Lesion studies testing learning and memory in rodents showed that certain brain regions result in different deficits in males and females (6,7). In addition, males and females have been shown to use different cellular and molecular strategies to encode information for the same behaviors during memory acquisition (8,9). Intracellular signaling differs in males and females and is a candidate for differentially controlling behavioral outputs. Activity of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) downstream of protein kinase A (PKA) and mitogen-activated protein kinases, mitogen-activated protein kinase (MAPK) and extracellular signal–related kinase (ERK), is differentially regulated by estrogen in the hippocampus of males and females throughout development (10,11).

Here, we introduce the signaling scaffold coiled-coil and C2 domain-containing protein 1A (CC2D1A) as a regulator of sex-specific intracellular signaling in the male hippocampus, controlling spatial memory acquisition. Biallelic mutations in *CC2D1A* cause a spectrum of neurodevelopmental conditions in humans with fully penetrant ID and variably penetrant ASD, attention-deficit/hyperactivity disorder, seizures, and aggressive behavior (12–15). While global *Cc2d1a* knockout (KO)

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pups die at birth owing to respiratory deficits, we found that conditional removal of this gene in the cortex and hippocampus leads to cognitive and social deficits, hyperactivity, and anxiety in male mice (16). CC2D1A was first described as a signaling and transcriptional regulator (17,18), and it has the structure of a scaffold with multiple protein-binding domains. In vitro studies in mouse embryonic fibroblasts and hippocampal neurons from *Cc2d1a* global KO mice showed that it binds to phosphodiesterase 4D (PDE4D), an enzyme involved in cAMP degradation, and regulates PDE4D activity and subcellular distribution (19,20). When cultured neurons lacking *Cc2d1a* are treated with forskolin to increase cAMP levels and activate CREB, hyperactive PDE4D reduces the effect of this drug, leading to reduced PKA and CREB activation (19). We wondered whether PDE4D and CREB function was perturbed in the *Cc2d1a*-deficient hippocampus and whether these changes caused the spatial cognitive deficits observed in the mice. During our studies, we found that only *Cc2d1a*-deficient male mice show disruptions in PDE4D signaling, which correlate with male-specific spatial memory deficits. We then used a PDE4D inhibitor to rescue these deficits, confirming that restoring signaling directly affects behavior. Female mice do not show these molecular and behavioral impairments. Our results show that CC2D1A regulates cAMP signaling in a sex-specific manner in male mice, revealing a novel mechanism controlling male-specific intracellular signaling and behavior.

METHODS AND MATERIALS

Animals

All animal care and use was in accordance with institutional guidance and approved by the Institutional Animal Care and Use Committee of George Washington University. The *Cc2d1a* conditional KO (cKO) mouse line was generated by crossing *Cc2d1a*-flx mice (16) with a CaMKIIa-cre mouse line driving Cre recombinase expression under the *CaMKIIa* promoter (Stock 005359; Jackson Laboratory, Bar Harbor, ME) (21). Global *Cc2d1a* and *Cc2d1b* KO mice were obtained from the Knockout Mouse Project and were previously described (16,22). All animals are fully backcrossed on a C57BL/6J background for at least six generations. As the *CaMKIIa*-cre transgene can be occasionally activated in male germ cells, leading to germline transmission (23), crosses were conducted between homozygous *Cc2d1a*-flx male mice and double heterozygous *Cc2d1a*-flx/*CaMKIIa*-cre female mice. For genotyping, polymerase chain reaction amplifications were performed on 1 μL of proteinase K (New England Biolabs, Ipswich, MA) digested tail DNA samples. Primer sequences are available upon request.

Behavioral Tests

A standardized battery of behavioral testing was used for cKO animals at 3 to 4 months of age. Behavioral tests were performed in the Manzini lab behavior analysis suite in the George Washington University Animal Research Facility following a 60-minute period of acclimatization. Initial characterization to test for of basic motor and somatosensory function was performed as described by Rogers *et al.* (24): righting reflex, wire hang, gait analysis, tail pinch, and visual reach. Spatial memory testing was performed in the Morris water maze (MWM) (25). Results for the male cKO mice were published in Oaks *et al.* (16), and the female mice presented in Figure 1 are littermates that were tested concurrently. A new behavioral cohort was generated for the PDE4D treatment. Behavioral analysis was performed via automated animal tracking using ANY-maze (Stoelting Co., Wood Dale, IL).

Morris Water Maze. The MWM (16,25) apparatus was a 120 × 120 cm round metal tub (Stoelting Co.), on which distinct visual cues were placed at the cardinal points. The surface of the water was made opaque by adding white nontoxic paint, and the temperature was maintained at 24°C. Each daily test consisted of four independent trials, one at each cardinal point around the tub, with the mouse being placed in the water facing the wall of the tub. Each trial lasted until the mouse found the platform or up to 60 seconds, whichever occurred first. Each animal completed two daily tests with a visible platform to learn that a platform is available to escape from the water, then performed five tests with a platform hidden under the water surface to memorize the platform location based on the cardinal cues on the walls of the apparatus. After the hidden platform (HP) trials, a 60-second probe trial was performed by removing the platform and measuring the time spent swimming over the correct platform location. Finally, two reversal tests were completed after changing the location of the platform to test for cognitive flexibility and the ability to learn a new platform location. Mice were considered nonperformers and removed from the analysis when they refused to swim and floated in the water for 60 seconds. Only animals that completed all trials were included.

Signaling Analysis via Western Blot and Enzyme-Linked Immunosorbent Assay

Protein lysates were prepared from fresh, frozen mouse hippocampal or cortical tissue that was homogenized in a buffer containing Tris-HCl (50 mM), NaCl (100 mM), ethylenediaminetetraacetic acid (5 mM), magnesium chloride (2 mM), and a protease and phosphatase inhibitor mixture (Sigma-Aldrich, St. Louis, MO), and extracted for 30 minutes with Triton X-100 (1%) and sodium dodecyl sulfate (0.1%). Lysates were cleared by centrifugation at 15,000*g* for 20 minutes at 4°C then combined with one volume of Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% beta-mercaptoethanol and denatured by heating at 95°C for 5 minutes. Protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4% to 12% Bis-Tris gels (Thermo Fisher Scientific, Waltham, MA) and transferred to Immobilon-FL (Millipore, Billerica, MA) polyvinylidine fluoride membranes. Immunoblots were probed with primary antibodies at optimized concentrations listed in Supplemental Table S1. Primary antibodies were then labeled with infrared fluorophore-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE) and imaged on an Odyssey Imager (LI-COR Biosciences).

cAMP levels were measured using the Cyclic AMP Complete Enzyme-Linked Immunosorbent Assay kit (ab133051; AbCam, Cambridge, MA) following instructions from the manufacturer. Briefly, flash-frozen hippocampal or cortical tissue was extracted in 0.1-M HCl and after HCl neutralization

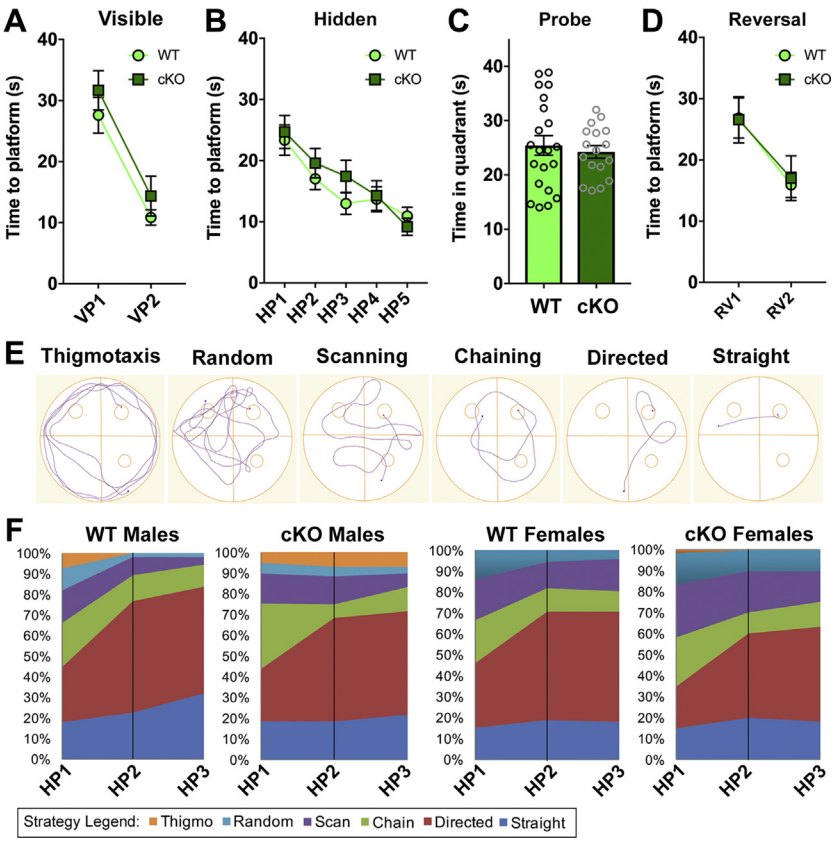

Figure 1. *Cc2d1a* conditional knockout (cKO) female mice do not show a deficit in spatial memory. **(A–D)** Hippocampus-dependent spatial memory was assessed in cKO female mice via the Morris water maze test. Spatial learning was measured as latency to reach the platform in two different stages: **(A)** visible platform (VP) and **(B)** hidden platform (HP). Spatial memory retention was measured in the **(C)** probe trial and flexibility in **(D)** the reversal (RV) trial. Differently from cKO male mice, who show a delay in the HP trial in Oaks *et al.* (16), cKO female mice showed no deficit (Supplemental Table S3). **(E)** Six different learning strategies were assayed using criteria from Garthe *et al.* (31). **(F)** Average use of different strategies is broken down for male and female mice during the first three HP trials, showing that cKO male mice maintain motor strategies such as thigmotaxis, scanning, and random search longer than female mice. WT, wild-type.

was mixed with anti-cAMP antibody and alkaline phosphatase for colorimetric detection in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific).

Protein Immunoprecipitation

Adult mouse forebrain tissue was homogenized by lysis buffer (100 mM NaCl, 20 mM Tris-Cl [pH 7.4], 1 mM ethylenediamine tetraacetate, protease inhibitors 1:100) and centrifuged at 14,000 rpm for 10 minutes at 4°C. One milligram of supernatant was incubated overnight at 4°C under rotary agitation with anti-CC2D1A (5 μg; AbCam) or normal mouse immunoglobulin G (5 μg) (Calbiochem, Burlington, MA) to detect nonspecific binding. Then, immunoprecipitated proteins were collected with 20 μL of precleared unconjugated agarose beads (Vector Laboratories, Inc., Burlingame, CA). Finally, the beads were collected, washed three times with wash buffer (150 mM NaCl, 20 mM Tris-Cl [pH 7.4], protease inhibitors 1:100), resuspended with Laemmli sample buffer 2x (Bio-Rad), boiled for 5 minutes at 95°C, and separated by electrophoresis on 4% to 12% Bis-Tris gels (Thermo Fisher Scientific).

Drug Treatments

Animals were treated with 3 μg/kg of GEBR-7b (Millipore, Burlington, MA) via intraperitoneal injection for 14 days before behavioral testing. Control animals were injected with saline only. Drug injections were continued every night during behavioral testing until the animals were sacrificed for tissue collection for Western blot analysis and enzyme-linked immunosorbent assay, as described above.

RESULTS

Cc2d1a cKO Mice Show Spatial Memory Deficits Only in Males

Forebrain-specific *Cc2d1a* cKO mice were generated to circumvent early postnatal respiratory failure identified in global KO lines (20,26–28) by removing *Cc2d1a* in the hippocampus and cortex via Cre recombinase expressed under the *CaMKIIa* promoter as previously shown (16,21). *Cc2d1a* cKO animals are viable and fertile and show normal somatosensory and motor function in male and female mice (Supplemental Table S2). Control homozygous *Cc2d1a*-flx mice are listed as wild-type (WT) and $Cc2d1a^{CaMKIIa\text{-}cre}$ mice are abbreviated as cKOs.

We had previously reported that *Cc2d1a* cKO male mice showed a deficit in spatial memory shown by a delay in memorizing the location of platform hidden under opaque water in the MWM (16). After an initial trial with a visible platform to learn that a platform is available to escape the water, each mouse is tested in multiple trials with an HP. The platform is then removed to test for permanence of the spatial memory (probe) and moved to a different location (reversal trial) to assess cognitive flexibility (25,29). *Cc2d1a* cKO male mice had showed a defect in learning the HP location in days 2 and 3 of

the 5-day HP test, but had normal performance in the probe and reversal trial test (Supplemental Table S3) (16). cKO female mice (n = 21 WT, 17 cKO) showed equal performance to control animals in all phases of the test and demonstrated normal spatial memory (Figure 1A–D). While sex has been shown not to affect performance in the MWM test in C57BL/6 mice (30), studies in rats showed that males and females can use different strategies to acquire the position of the HP, shifting from place-based strategies using visual cues to motor-based search strategies such as scanning the length of tub (8). To confirm that female mice were not just using a different strategy to identify the location of the platform, we compared learning strategies during the first 3 days of the HP test with our published male cKO cohort, using criteria outlined by Garthe *et al.* (31). Thigmotaxis (swimming close to the sides of the tub), random path, and scanning are considered motor strategies in which the animal does not appear to use visual cues to locate the platform. Circling around the tub at the distance of the platform (chaining) or swimming toward the platform with (directed) or without errors (straight) indicate that the animal is using a place-based strategy. WT male and female mice showed no differences in using motor-based strategies (thigmotaxis, random search, or scanning) and place-based strategies (chaining, directed search, or straight line) (Figure 1E). cKO male mice were slower in transitioning to place-based strategies in days 2 and 3 (Figure 1F, G). The primary factor driving the difference in performance in cKO male mice was the latency from drop location to the platform (Supplemental Table S3).

CC2D1A Controls Signaling Downstream of PDE4D in a Male-Specific Manner

As spatial memory deficits have been linked to reduced CREB activation in the hippocampus (32) and CC2D1A is known to regulate CREB activation in hippocampal neurons by increasing PDE4D activity (20), we asked whether this pathway was altered in the adult hippocampus of *Cc2d1a*-deficient mice (schematic in Figure 2A). We collected hippocampal tissue from 5-month-old male and female WT and cKO mice. We tested for PDE4D, PKA, and CREB phosphorylation via Western blot by determining the ratio of phosphorylated to total protein in hippocampal protein lysates. We also separately measured tissue levels of cAMP using enzyme-linked immunosorbent assay to confirm that PDE4D activity was altered. We found that, in fact, PDE4D phosphorylation was doubled in male *Cc2d1a*-deficient hippocampi (2.05 ± 0.13 of WT; p = .0001; n = 5 WT, 5 cKO) (Figure 2B). cAMP levels were almost halved (WT = 51.83 ± 6.88 pmol/mg, n = 4; cKO = 27.74 ± 1.92 pmol/mg, n = 4; p = .011) (Figure 2C). Finally, phosphorylation of Thr-197 in PKA (0.48 ± 0.13 of WT, p = .019; n = 4 WT, 4 cKO) (Figure 2D) and Ser-133 in CREB (0.70 ± 0.09 of WT, p = .046; n = 5 WT, 5 cKO) (Figure 2E) were also decreased. These findings indicate that in the adult hippocampus lacking *Cc2d1a* PDE4D activity is increased leading to baseline reduction in cAMP levels and PKA and CREB phosphorylation, which could underlie the cognitive deficits found in behavioral experiments.

When we tested the same biochemical changes in female mice, we found that there was no difference between WT and cKO animals (Figure 2F–H). Owing to the high variability in CREB activity in female control hippocampi, we increased the number of animals tested to confirm that there was no difference and saw no change (CREB: cKO = 0.93 ± 0.11 of WT; p = .69; n = 10 WT, 14 cKO) (Figure 1I). We tested for similar pathway alterations in cortical lysates in which CC2D1A is also removed and found that PDE4D phosphorylation and cAMP levels were comparable to WT in cKO male and female mice (Supplemental Figure S1A–D). The amount of CC2D1A protein was measured in the cortex and hippocampus of WT animals and we found no differences in baseline CC2D1A levels between male and female mice (Supplemental Figure S1E). We also confirmed that CC2D1A binds to PDE4D in the male brain as previously shown (Figure 3A) (19). We then wondered whether compensation could be present through *Cc2d1b*, which is the only homolog of *Cc2d1a* in vertebrates, and has been shown to have a redundant and overlapping function (22,28). We tested the hippocampus in both *Cc2d1b* KO and *Cc2d1a/Cc2d1b* double heterozygote mice. We found that PDED4D phosphorylation was not altered in *Cc2d1b* KO

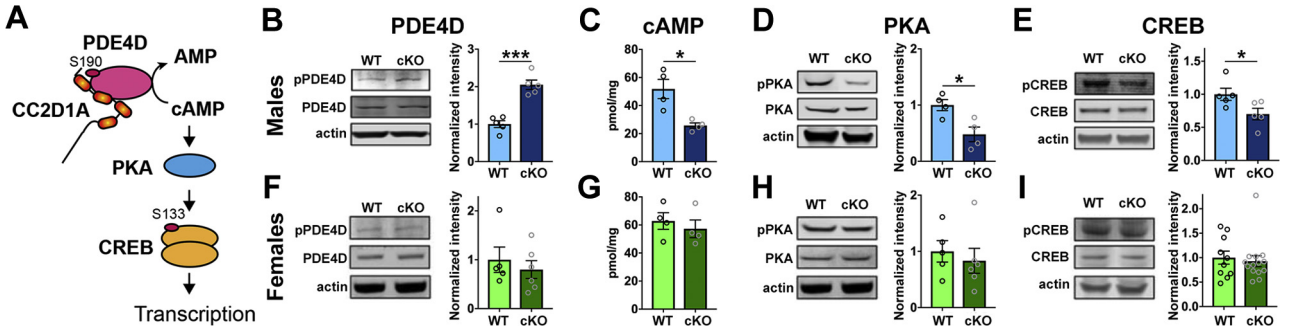

Figure 2. Loss of *Cc2d1a* causes male-specific disruption of phosphodiesterase 4D (PDE4D) and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) activity in the hippocampus. **(A)** Schematic of coiled-coil and C2 domain-containing protein 1A (CC2D1A) as a signaling scaffold for PDE4D and protein kinase A (PKA) upstream of CREB. **(B–E)** Conditional removal of *Cc2d1a* in conditional knockout (cKO) male mice causes **(B)** increased phosphorylation and activity of PDE4D, leading to **(C)** reduced levels of cAMP, and decreased phosphorylation of both **(D)** PKA and **(E)** CREB in hippocampal lysates. **(F–I)** No changes in **(F)** PDE4D, **(G)** cAMP, **(H)** PKA, or **(I)** CREB are found in the female hippocampus. Results expressed as mean ± SEM. Data averages and statistical information are reported in the Results. Two-tailed t test with equal variance: $^{*}p < .05$, $^{***}p < .001$. pCREB, phosphorylated CREB; pPDE4D, phosphorylated PDE4D; pPKA, phosphorylated PKA; WT, wild-type.

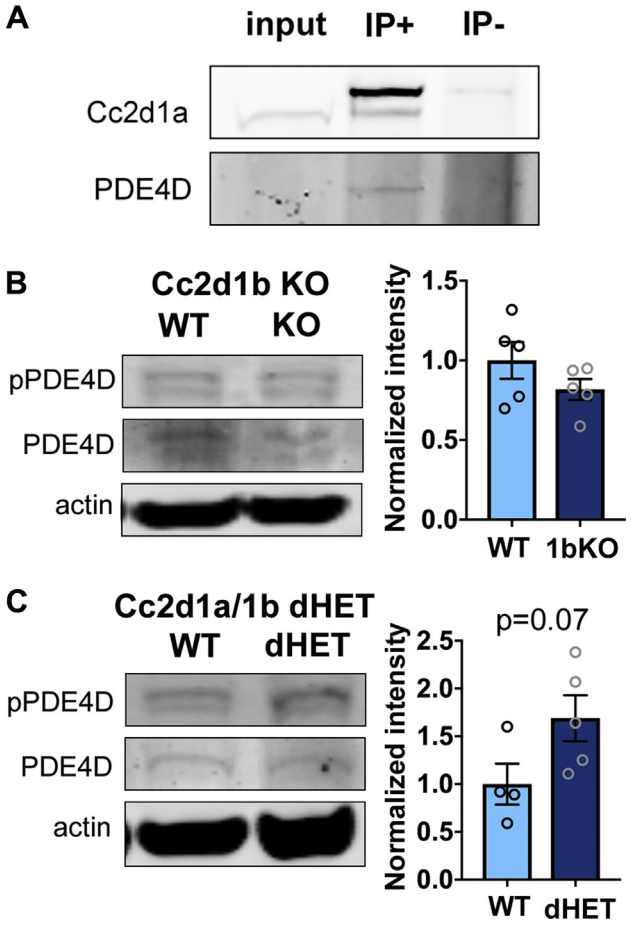

Figure 3. Regulation of phosphodiesterase 4D (PDE4D) phosphorylation is specific to coiled-coil and C2 domain-containing protein 1A (CC2D1A). **(A)** PDE4D immunoprecipitates with CC2D1A in brain lysates (IP+). **(B)** PDE4D phosphorylation is not changed in the *Cc2d1b* knockout (1bKO) hippocampus but **(C)** shows a trend toward being increased when expression of both *Cc2d1a* and *Cc2d1b* is halved in the *Cc2d1a/1b* double heterozygote (dHET) animals. IP, immunoprecipitation; pPDE4D, phosphorylated PDE4D; WT, wild-type.

animals (Figure 3B) and that it showed a trend toward being increased in the double heterozygote animals (Figure 3C), suggesting that CC2D1A specifically controls PDE4D activity. We also confirmed that CC2D1B expression levels are not altered in *Cc2d1a* cKO animals and that there is no difference in CC2D1A removal by CaMKIIa-cre–mediated recombination in the male and female cortex and hippocampus (Supplemental Figure S1F–I). In summary, we found that loss of *Cc2d1a* causes male-specific activation of PDE4D, leading to reduced cAMP levels only in the hippocampus.

Adult Restoration of PDE4D Activity Rescues Spatial Memory Deficits in *Cc2d1a* cKO Male Mice

CREB activity is critical for creating and storing spatial memories (32–34), and its activation levels must be carefully controlled (35). Upstream of CREB, PDE4D is also a key regulator of cognitive function (36). We asked whether inhibiting PDE4D would reduce cAMP degradation to restore control levels of cAMP and CREB activity, thus rescuing spatial memory deficits in male cKOs (Figure 4A). PDE4 inhibitors have been studied in dementia and Alzheimer's disease (AD) to slow cognitive decline by enhancing CREB function (37,38). However, the most commonly used drug, rolipram, targets multiple PDE4s and leads to severe side effects, including nausea and emesis, which cause poor tolerability (37). To inhibit PDE4D in the cKO hippocampus, we used a specific inhibitor GEBR-7b, which is selective for PDE4D and better tolerated (39). GEBR-7b treatment had proven effective in improving spatial memory in both WT mice and a mouse model of AD (39,40). We chose a concentration of 3 μg/kg of GEBR-7b, which would be able to restore normal cAMP levels without completely inhibiting PDE4D activity (Figure 4). A cohort of adult (2-month-old) control and cKO male and female mice were treated for 14 days via intraperitoneal injections. Treatment was effective at restoring levels of cAMP as tested by enzyme-linked immunosorbent assay (WT saline = 47.2 ± 3.0 pmol/mg, n = 4; cKO saline = 29.3 ± 2.7 pmol/mg, n = 4, p = .014 to WT; WT GEBR = 46.4 ± 3.0 pmol/mg, n = 4; cKO GEBR = 48.0 ± 4.9 pmol/mg, n = 3) (Figure 4B) and levels of CREB phosphorylation as tested by Western blot (cKO saline = 0.47 ± 0.08 of WT saline, p = .048, n = 7 WT, 6 cKO; WT GEBR = 1.19 ± 0.26, n = 6; cKO GEBR = 0.82 ± 0.07, n = 7) (Figure 4C).

After treatment, the mice were tested in an abbreviated MWM protocol consisting of the visible, hidden, and probe tests, as neither male nor female cKO mice had shown a difference in the reversal test. In this cohort, cKO male mice again showed a delay in acquiring the position of the hidden platform when compared with control mice in day 2, and the deficit was completely rescued by GEBR-7b treatment with an additional improvement for the cKO in day 1 (Figure 5A–C) (HP1 [Figure 5B]: WT saline = 28.1 ± 3.3 seconds, n = 14; cKO saline = 33.2 ± 4.3 seconds, n = 11; WT GEBR = 28.2 ± 3.8 seconds, n = 14; cKO GEBR = 18.3 ± 2.0 seconds, n = 14; p values to cKO saline: WT saline, p = .4; WT GEBR, p = .4; cKO GEBR, $p < .0001$; HP2 [Figure 5C]: WT saline = 9.6 ± 1.3 seconds, cKO saline = 22.1 ± 4.5 seconds, WT GEBR = 13.2 ± 2.0 seconds, cKO GEBR = 8.8 ± 1.3 seconds; p values to cKO saline: WT saline, p = .0005; WT GEBR, p = .025; cKO GEBR, p = .0002). Following spatial learning, treated and untreated male mice performed equally in the probe trial (Figure 5D) and showed no differences in swim speeds, showing that treatment did not change motor function (not shown). Female mice performed equally with and without GEBR-7b treatment (Figure 5E–H). Overall our results show that CC2D1A establishes male-specific CREB signaling through PDE4D that controls spatial memory acquisition.

DISCUSSION

In this study, we demonstrated that the ID/ASD gene *CC2D1A* controls male-specific CREB signaling in the hippocampus, leading to male-specific spatial memory deficits in mice. We found that loss of CC2D1A in male mice increased hippocampal PDE4D phosphorylation, leading to a reduction of cAMP levels and of CREB phosphorylation, which can be rescued by PDE4D inhibition in cKO male mice. cKO female mice, however, do not show any of the signaling deficits observed in male mice and are not impaired in HP acquisition

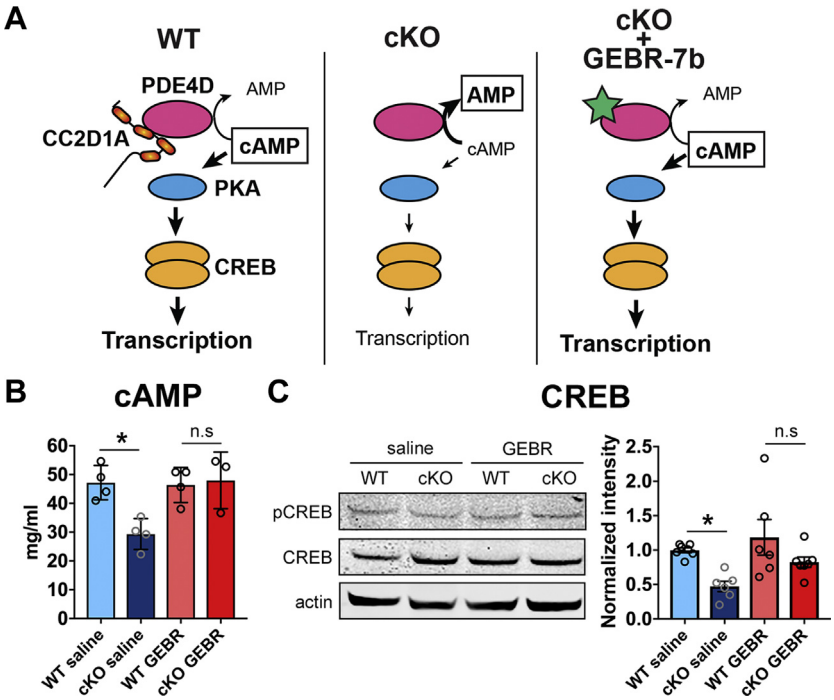

Figure 4. GEBR-7b treatment restores cyclic adenosine monophosphate (cAMP) levels and cAMP response element binding protein (CREB) phosphorylation in *Cc2d1a* conditional knockout (cKO) male mice. **(A)** Schematic of expected action of GEBR-7b (green star). When coiled-coil and C2 domain-containing protein 1A (CC2D1A) is absent, hyperactivity of phosphodiesterase 4D (PDE4D) in cKO male mice increases cAMP degradation (center panel). GEBR-7b is expected to restore cAMP levels and CREB-mediated transcription (right panel). **(B)** Fourteen-day daily treatment with 3 μg/kg of GEBR-7b (GEBR) restored cAMP to wild-type (WT) levels in the hippocampus of cKO male mice when compared with animals treated with saline. **(C)** CREB phosphorylation is also increased in GEBR-7b–treated cKO male mice. One-way analysis of variance with Tukey's multiple comparison test: $^{*}p < .05$. n.s., not significant; pCREB, phosphorylated CREB.

in the MWM. Our findings identify a novel molecular mechanism for sex-specific signaling regulation controlled by a single gene mutated in ID and ASD.

Multiple hypotheses have been put forth to explain the male bias in neurodevelopmental disorders (41,42). Increased levels of steroid hormones, including cortisol and testosterone, and their precursors, have been found elevated in the amniotic fluid of pregnancies that resulted in individuals with ASD (43), suggesting a role for sex hormones and other steroids in the etiology of the disorder in boys. On the other hand, women require more severe rare genetic mutations and more familial risk factors than men to become affected (44,45), supporting the hypothesis that females are protected. A study by Grissom *et al.* (46), in a mouse model of 16p11.2 deletion syndrome,

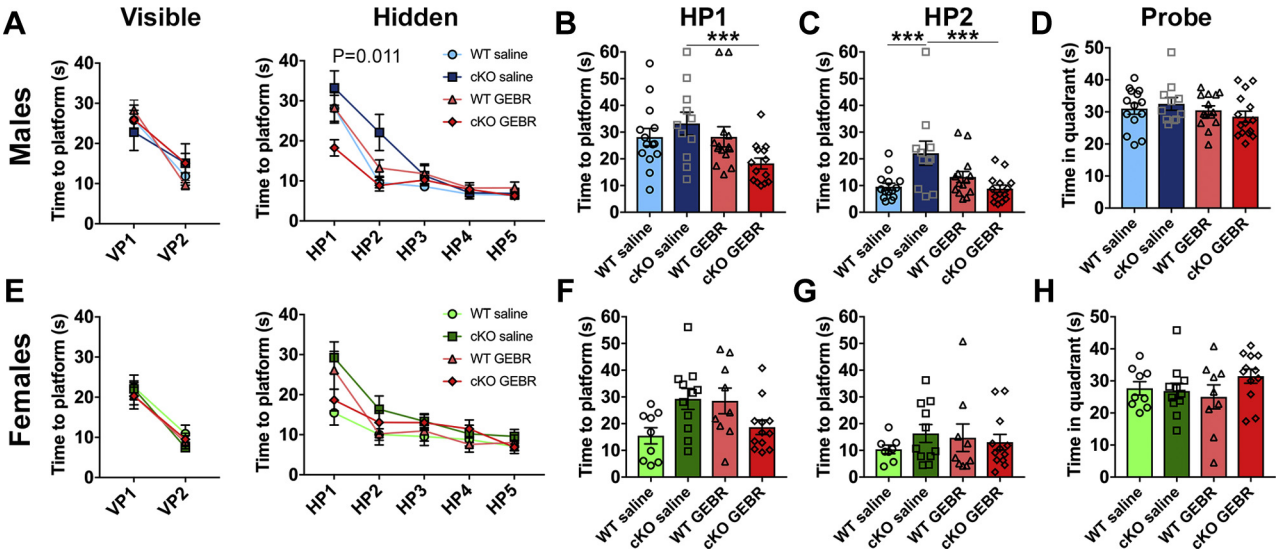

Figure 5. GEBR-7b treatment rescues spatial memory deficit in *Cc2d1a* conditional knockout (cKO) male mice and has no effect on female mice. **(A)** The learning delay in the hidden platform (HP) trial of the Morris water maze found in male *Cc2d1a* cKO animals is corrected by 3 μg/kg GEBR-7b treatment for 14 days before testing. Two-way analysis of variance effect of treatment in HP test: $p = .011$. **(B, C)** Significant improvement following GEBR-7b treatment is found in both HP1 and HP2 testing days via one-way analysis of variance for individual days. $^{***}p < .001$. **(D)** No significant change is found in the probe test. **(E–H)** GEBR-7b treatment has no effect on female mice. VP, visible platform; WT, wild-type.

found male-specific hyperactivity in MAPK3/ERK1 in the striatum correlating with male-specific striatal learning deficits, suggesting a link between sex-specific signaling and behavior. Our findings in *Cc2d1a* cKO mice, together with those from the 16p11.2 hemideletion model (46), indicate that sex-specific disruptions in intracellular signaling may be involved in establishing male-specific behavioral phenotypes. While these findings do not yet define whether males are susceptible or females are protected, they show that in inbred genetic models recapitulating human mutations equally in male and female mice, male mice develop sex-specific signaling deficits. Defining how these signaling differences are established will not only provide insight into the mechanisms of sex bias in the pathogenesis of neurodevelopmental disorders, but also likely identify sex-specific targets for therapy development.

We find that PDE4D inhibition can rescue cognitive deficits in a model of neurodevelopmental disorders. PDE4 proteins have been under investigation as a target for the improvement of cognitive function in neurodegenerative disorders for some time, as CREB activity is disrupted in multiple models of AD (37,38,47). It will be important to determine whether PDE4D function is disrupted in other models of neurodevelopmental disease, and whether PDE4D inhibition could be beneficial in ID. It should also be noted that AD and dementia show a sex bias that is opposite the bias observed in ID and ASD, with females comprising two thirds of individuals affected by AD (48). Studies in primary hippocampal neurons showed that CREB is primarily controlled by estrogen receptor activation in conjunction with metabotropic glutamate receptors in the neurons of female rats, and not in males (10). Sex differences in the regulation of PDE4D and CREB signaling at different ends of the human life span may need to considered in developing therapies as similar mechanisms may be involved in cognitive development and dementia.

To date, *CC2D1A* mutations have been identified in 34 individuals (21 males and 13 females) with fully penetrant developmental delay and ID in males and females, and partially penetrant reports of ASD, attention-deficit/hyperactivity disorder, aggressive behavior, and seizures, primarily in males (12–15). As most of the individuals with *CC2D1A* mutations reside in the Middle East and are not currently accessible to follow-up, we do not know whether there are significant differences in the behavioral presentation and severity between male and female individuals. Intriguing sex-specific differences in *CC2D1A* expression were found in major depressive disorder. Conditional *Cc2d1a* removal in serotoninergic neurons in the dorsal raphe demonstrated that CC2D1A also has a role in regulating serotonin receptor function and depression-like behaviors (49). *CC2D1A* levels were found reduced in the prefrontal cortex (50) and increased in the cingulate cortex (51) in male individuals, but not female individuals, affected by major depressive disorder, suggesting that male-specific impairment in CC2D1A function in humans could be present. *CC2D1A* expression was elevated in the blood of individuals with ASD (52), but the number of female individuals in the sample was too small to explore sex specificity of this change, and more extensive follow-up will be necessary. PDE4D expression is also regulated by both serotonin and norepinephrine reuptake inhibitors (53,54), suggesting a shared role for CC2D1A and PDE4D in cognition and depression. CC2D1A has also been studied in regulation of subcellular and endosomal regulation of signaling proteins (20,55,56), and future investigations should focus on whether this function is regulated by sex hormones and/or involved in sex hormone function in conjunction with neuronal activity and memory formation. Defining how these mechanisms control cognitive and affective function throughout life may provide insight into sex bias in disease susceptibility.

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ARTICLE INFORMATION

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