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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 towards males with male:female ratios of 2:1 in intellectual disability (ID) and 4:1 in autism spectrum disorder (ASD). 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 1A* (CC2D1A), which is mutated in ID and ASD, we identified biochemical and behavioral differences between males and females, and explored whether CC2D1A controls male-specific intracellular signaling.

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

Results.—We found that in *Cc2d1a*-deficient males PDE4D is hyperactive leading to a reduction in CREB signaling, but this molecular deficit is not present in females. *Cc2d1a*-deficient males show a deficit in spatial memory which is not present in *Cc2d1a*-deficient females. Restoring PDE4D activity using an inhibitor rescues cognitive deficits in males, but has no effect on females.

Conclusions.—Our findings show that CC2D1A regulates cAMP intracellular signaling in a malespecific 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.

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Keywords

autism; intellectual disability; sex bias; intracellular signaling; learning and memory; cAMP

INTRODUCTION

Developmental disabilities are more prevalent in males with male:female ratios ranging between 1.5–2:1 in intellectual disability (ID) and 4–8:1 in autism spectrum disorders (ASD) (1; 2). However, the molecular mechanisms underlying sex bias in neurodevelopmental disorders remain unknown. The male and female brain show developmental sex differences in normal subjects beyond sex-specific behaviors such as mating and aggression (3). Non-invasive 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 cAMP response element binding protein (CREB) downstream of protein kinase A (PKA) and mitogen-activated protein (MAPK/ERK) kinases 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 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, ADHD, seizures, and aggressive behavior (12–15). While global *Cc2d1a* knock-out pups die at birth due 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 males (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 (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 males 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. Females do not show these molecular and behavioral impairments. Our results show that CC2D1A regulates cAMP

signaling in a sex-specific manner in males, 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 The George Washington University. The *Cc2d1a* conditional knock-out (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 Laboratories) (21). Global *Cc2d1a* and *Cc2d1b* knock-out (KO) mice were obtained from the Knock Out Mouse Project and were previously described (16; 22). All animals are fully backcrossed on a C57BL/6J background for at least 6 generations. Since the *CaMKIIa*-cre transgene can be occasionally activated in male germ cells leading to germline transmission (23), crosses were conducted between homozygous *Cc2d1a*-flx males and double heterozygous *Cc2d1a*-flx/*CaMKIIa*-cre females. For genotyping, polymerase chain reaction (PCR) amplifications were performed on 1µL 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 3–4 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 min period of acclimatization. Initial characterization to test for of basic motor and somatosensory function was performed as described by Rogers (24): righting reflex, wire hang, gait analysis, tail pinch and visual reach. Spatial memory testing was performed in the Morris water maze (25). Results for the male cKO mice were published in Oaks et al (16), and the females presented in Fig. 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, Wood Dale IL).

Morris Water Maze—The Morris Water Maze (MWM) (16; 25) apparatus was a 120×120cm round metal tub (Stoelting, Wood Dale IL), where distinct visual cues were placed at the cardinal points. The surface of the water was made opaque by adding white non-toxic 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 s, 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 trials, a 60 s 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 s. Only animals that completed all trials were included.

Signaling analysis via Western blot and ELISA

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), protease and phosphatase inhibitor mixture (Sigma-Aldrich, St. Louis MO) and extracted for 30 min with Triton X-100 (1%) and sodium dodecyl sulfate (0.1%). Lysates were cleared by centrifugation at 15,000 $\times g$ for 20 min 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 min. Protein was separated by SDS-PAGE on 4–12% Bis-Tris gels (Thermo Scientific, Waltham MA) and transferred to Immobilon-FL (Millipore, Billerica MA) polyvinylidene fluoride membranes. Immunoblots were probed with primary antibodies at optimized concentrations listed in Supplemental Table 1. Primary antibodies were then labeled with infra-red fluorophore-conjugated secondary antibodies (LI-COR Biosciences, Lincoln NE) and imaged on an Odyssey Imager (LI-COR Biosciences, Lincoln NE).

Cyclic AMP (cAMP) levels were measured using the Cyclic AMP Complete ELISA Kit (AbCam, ab133051, Cambridge, MA) following instructions from the manufacturer. Briefly, flash frozen hippocampal or cortical tissue was extracted in 0.1M HCl and after HCl neutralization, mixed with anti-cAMP antibody and alkaline phosphatase for colorimetric detection in a Varioskan LUX multimode microplate reader (ThermoFisher Scientific, Waltham, MA).

Protein immunoprecipitation

Adult mouse forebrain tissue was homogenized by lysis buffer (100 mM NaCl, 20 mM Tris-Cl (pH 7.4), 1 mM EDTA, protease inhibitors 1:100) and centrifuged at 14,000 $\times rpm$ for 10 min at 4°C. 1mg of supernatant was incubated overnight at 4°C under rotary agitation with anti-CC2D1A (5 μg , AbCam) or normal mouse IgG (5 μg , Calbiochem) to detect non-specific binding. Then, immunoprecipitated proteins were collected with 20 μL of precleared unconjugated agarose beads (Vector Laboratories, Inc.). Finally, the beads were collected, washed 3 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, Hercules CA), boiled for 5 min at 95°C, and separated by electrophoresis on 4–12% Bis-Tris gels (Thermo Scientific, Waltham MA).

Drug treatments

Animals were treated with 3 $\mu g/kg$ of GEBR-7b (Millipore, Burlington, MA) via intraperitoneal injection for 14 days prior to 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 ELISA, 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 males and females (Suppl. Table 2). Control homozygous *Cc2d1a*-LoxP mice are listed as WT and *Cc2d1a*^{CaMKIIa-cre} mice are abbreviated as cKOs.

We had previously reported that *Cc2d1a* cKO males showed a deficit in spatial memory shown by a delay in memorizing the location of platform hidden under opaque water in the Morris Water Maze (MWM) (16). After an initial trial with a visible platform (VP) to learn that a platform is available to escape the water, each mouse is tested in multiple trials with a hidden platform (HP). The platform is then removed to test for permanence of the spatial memory (probe) and moved to a different location (reversal trial, RV) to assess cognitive flexibility (25; 29). *Cc2d1a* cKO males had showed a defect in learning the HP location in days 2 and 3 of the 5-day hidden platform test, but had normal performance in the probe and RV test (Suppl. Table 3) (16). cKO females (N=21 WT, 17 cKO) showed equal performance to control animals in all phases of the test and demonstrated normal spatial memory (Fig.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 hidden platform shifting from place-based strategies using visual cues, to motor-based search strategies such as scanning the length of tub (8). To confirm that females were not just using a different strategy to identify the location of the platform we compared learning strategies during the first three 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 where 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 males and females showed no differences in using motor- (thigmotaxis, random search or scanning), and place-based strategies (chaining, directed search, or straight line) (Fig.1E). cKO males were slower in transitioning to place-based strategies in day 2 and 3 (Fig.1F–G). The primary factor driving the difference in performance in cKO males was the latency from drop location to the platform (Suppl. Table 3).

CC2D1A controls signaling downstream of PDE4D in a male-specific manner

Since 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 Fig.2A). We collected hippocampal tissue from 5 months 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 enzymelinked immunosorbent assay (ELISA) 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, $p=0.0001$ ***, $N=5$ WT; 5 cKO, Fig. 2B). cAMP levels were almost halved (WT= 51.83 ± 6.88 pmol/mg, $N=4$; cKO= 27.74 ± 1.92 pmol/mg, $N=4$, $p=0.011$ *, Fig. 2C). Finally, phosphorylation of Thr-197 in PKA (0.48 ± 0.13 of WT, $p=0.019$ *, $N=4$ WT; 4 cKO, Fig. 2D) and Ser-133 in CREB (0.70 ± 0.09 of WT, $p=0.046$ *, $N=5$ WT; 5cKO, Fig. 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 females, we found that there was no difference between WT and cKO animals (Fig. 2F-H). Due 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, $p=0.69$, $N=10$ WT; 14 cKO, Fig. 1I). We tested for similar pathway alterations in cortical lysates where CC2D1A is also removed and found that PDE4D phosphorylation and cAMP levels were comparable to WT in cKO males and females (Suppl. Fig. 1A-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 males and females (Suppl. Fig. 1E). We also confirmed that CC2D1A binds to PDE4D in the male brain as previously shown (Fig. 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* knock-out (KO) and *Cc2d1a/Cc2d1b* double heterozygote (dHET) mice. We found that PDE4D phosphorylation was not altered in *Cc2d1b* KO animals (Fig. 3B) and that it showed a trend towards being increased in the dHET animals (Fig. 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 CaMKII- α mediated recombination in the male and female cortex and hippocampus (Suppl. Fig. 1F-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 males

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 this rescuing spatial memory deficits in male cKOs (Fig. 4A). PDE4 inhibitors have been studied in dementia and Alzheimer's Disease 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 Alzheimer's Disease (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 (Fig. 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 ELISA (WT saline=47.2±3.0 pmol/mg, N=4, cKO saline=29.3±2.7 pmol/mg, N=4, p=0.014 to WT; WT GEBR=46.4±3.0 pmol/mg, N=4, cKO GEBR=48.0±4.9 pmol/mg, N=3. Fig.4B) and levels of CREB phosphorylation as tested by Western blot (cKO saline=0.47±0.08 of WT saline, p=0.048 *, N=7 WT, 6 cKO; WT GEBR=1.19±0.26, N=6; cKO GEBR=0.82±0.07, N=7. Fig.4C).

After treatment, the mice were tested in an abbreviated MWM protocol consisting of the visible, hidden, and probe tests, since neither male nor female cKO mice had shown a difference in the reversal test. In this cohort, cKO males again showed a delay in acquiring the position of the hidden platform when compared to controls in day 2 and the deficit was completely rescued by GEBR-7b treatment with an additional improvement for the cKO in day 1 (Fig. 5A-C. **HP1** Fig. 5B: WT saline=28.1±3.3s, N=14; cKO saline=33.2±4.3s, N=11; WT GEBR=28.2±3.8s, N=14; cKO GEBR=18.3±2.0, N=14, p values to cKO saline: WT saline p=0.4 WT GEBR p=0.4, cKO GEBR p<0.0001 ***; **HP2** Fig. 5C: WT saline=9.6±1.3s; cKO saline=22.1±4.5s; WT GEBR=13.2±2.0s; cKO GEBR=8.8±1.3; p values to cKO saline: WT saline p=0.0005 ***, WT GEBR p=0.025 *, cKO GEBR p=0.0002 ***). Following spatial learning treated and untreated males performed equally in the probe trial (Fig.5D), and showed no differences in swim speeds showing that treatment did not change motor function (not shown). Females performed equally with and without GEBR-7b treatment (Fig.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 males increased hippocampal PDE4D phosphorylation leading to a reduction of cAMP levels and of CREB phosphorylation which can be rescued by PDE4D inhibition in cKO males. cKO female mice, however, do not show any of the signaling deficits observed in males and are not impaired in hidden platform acquisition 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 males. On the other hand, females require more severe rare genetic mutations and more familial risk factors than males to become affected (44; 45), supporting the hypothesis that females are protected. A study by Grissom et al., in a mouse model of 16p11.2 deletion syndrome found male specific hyperactivity in MAPK3/ERK1

kinase in the striatum correlating with male-specific striatal learning deficits (46), 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 males and females, males 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 will 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, since CREB activity is disrupted in multiple models of Alzheimer's disease (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 which is opposite than 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 female neurons, 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 be 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, ADHD, aggressive behavior, and seizures, primarily in males (12–15). Since 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 males and females. Intriguing sex-specific differences in *CC2D1A* expression were found in Major Depressive Disorder (MDD). Conditional *Cc2d1a* removal in serotonergic 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 males, but not females affected by MDD, 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 females in the sample was too small to explore sexspecificity 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Cc2d1a* cKO females do not show a deficit in spatial memory.

A-D. Hippocampusdependent spatial memory was assessed in cKO females via the Morris Water Maze test. Spatial learning was measured as latency to reach the platform in two different stages, visible platform (VP, **A.**) and hidden platform (HP, **B.**). Spatial memory retention was measured in the Probe trial (**C.**) and flexibility in the reversal (RV, **D.**) trial. Differently from cKO males which show a delay in the HP trial in Oaks et al. (16), cKO females showed no deficit (Suppl. Table 3). **E.** Six different learning strategies were assayed using criteria from Garthe et al (31). **F.** Average use of different strategies is broken down for males and females during the first three HP trials showing that cKO males maintain motor strategies such as thigmotaxis, scanning and random search longer than females.

Figure 2. Loss of *Cc2d1a* causes male-specific disruption of PDE4D and CREB activity in the hippocampus.

A. Schematic of CC2D1A as a signaling scaffold for PDE4D and PKA upstream of CREB.

B-E. Conditional removal of *Cc2d1a* in cKO male mice causes increased phosphorylation and activity of PDE4D (**B.**), leading to reduced levels of cAMP (**C.**), and decreased phosphorylation of both PKA (**D.**) and CREB (**E.**) in hippocampal lysates. **F-I.** No changes in PDE4D (**F.**), cAMP (**G.**), PKA (**H.**) or CREB (**I.**) are found in the female hippocampus.

Results expressed as mean $\pm$ SEM. Data averages and statistical information are reported in the Results. Two-tailed t-test with equal variance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3. Regulation of PDE4D phosphorylation is specific to CC2D1A.

A. PDE4D immunoprecipitates with CC2D1A in brain lysates (IP+). **B-C.** PDE4D phosphorylation is not changed in the Cc2d1b KO hippocampus (**B.**) but shows a trend towards being increased when expression of both Cc2d1a and Cc2d1b is halved in the Cc2d1a/1b dHET animals (**C.**).

Figure 4. GEHR-7b treatment restores cAMP levels and CREB phosphorylation in *Cc2d1a* cKO males.

A. Schematic of expected action of GEHR-7b (green star). When CC2D1A is absent hyperactivity of PDE4D in cKO males increases cAMP degradation (center panel). GEHR-7b is expected to restore cAMP levels and CREB-mediated transcription (right panel). **B.** 14-day daily treatment with 3µg/kg of GEHR-7b (GEHR) restored cAMP to WT levels in the hippocampus of cKO males when compared to animals treated with saline. **C.** CREB phosphorylation is also increased in GEHR-7b treated cKO males. One-way ANOVA with Tukey's multiple comparison test, * $p < 0.05$.

Figure 5. GEBR-7b treatment rescues spatial memory deficit in *Cc2d1a* cKO males and has no effect on females.

A. The learning delay in the hidden platform (HP) trial of the MWM found in male *Cc2d1a* cKO animals is corrected by 3μg/kg GEBR-7b treatment for 14 days before testing. Two way ANOVA effect of treatment in hidden platform test $p=0.011$. **B-C.** Significant improvement following GEBR-7b treatment are found in both HP1 and HP2 testing days via one way ANOVA for individual days. *** $p<0.001$ **D.** No significant change is found in the Probe test. **E-H.** GEBR-7b treatment has no effect on female mice.