HHS Public Access

Author manuscript

FASEB J. Author manuscript; available in PMC 2022 November 19.

Published in final edited form as:

FASEB J. 2021 November ; 35(11): e21910. doi:10.1096/fj.202100733RR.

CTRP4 ablation impairs associative learning and memory

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Abstract

C1q/TNF-related protein (CTRP) family comprises fifteen highly conserved secretory proteins with diverse central and peripheral functions. In zebrafish, mouse, and human, CTRP4 is most highly expressed in the brain. We previously showed that CTRP4 is a metabolically responsive regulator of food intake and energy balance, and mice lacking CTRP4 exhibit sexually dimorphic changes in ingestive behaviors and systemic metabolism. Recent single-cell RNA sequencing also revealed *Ctrp4/C1qtnf4* expression in diverse neuronal cell types across distinct anatomical brain regions, hinting at additional roles in the central nervous system not previously characterized. To uncover additional central functions of CTRP4, we subjected *Ctrp4* knockout (KO) mice to a battery of behavioral tests. Relative to wild-type (WT) littermates, loss of CTRP4 does not alter exploratory, anxiety-, or depressive-like behaviors, motor function and balance, sensorimotor gating, novel object recognition, and spatial memory. While pain-sensing mechanisms in response to thermal stress and mild shock are intact, both male and female *Ctrp4* KO mice have increased sensitivity to pain induced by higher-level shock, suggesting altered nociceptive function. Importantly, CTRP4 deficiency impairs hippocampal-dependent associative learning and memory as assessed by trace fear conditioning paradigm. This deficit is sex-dependent, affects only female mice, and is associated with altered expression of learning and memory genes (*Arc, c-fos*, and *Pde4d*) in the hippocampus and cortex. Altogether, our behavioral and gene expression analyses have uncovered novel aspects of the CTRP4 function and provided a physiological context to further investigate its mechanism of action in the central and peripheral nervous system.

Keywords

C1QTNF4; cortex; hippocampus; learning and memory; mouse behavior; secreted hormone

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AUTHOR CONTRIBUTIONS

Dylan C. Sarver, Chantelle E. Terrillion, and G. William Wong contributed to the experimental design; Dylan C. Sarver, Cheng Xu, Yi Cheng, and Chantelle E. Terrillion performed the experiments; Dylan C. Sarver, Cheng Xu, Yi Cheng, Chantelle E. Terrillion, and G. William Wong analyzed and interpreted the data; Dylan C. Sarver and G. William Wong wrote the paper.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

1 | INTRODUCTION

Since the discovery of immune complement C1q,[1,2] over 30 additional secretory proteins that comprise the vertebrate C1q family have been characterized in humans, mice, and zebrafish.[3-5] The key feature that unites all family members is the presence of a globular C1q domain located at the C-terminus of each protein.[3] These secretory proteins have important and diverse biological functions, ranging from immune response,[6] to blood pressure control and platelet hemostasis,[7] metabolism,[8] cardiovascular function,[9] myoblast fusion,[10] and nervous system functions.[11]

Among the C1q family members are the fifteen proteins referred to as the C1q/TNF-related proteins (CTRP1-15), which we have identified and characterized as part of an ongoing effort to understand how secreted hormones facilitate inter-organ communications to maintain metabolic homeostasis.[12-19] Recent studies by us and others have highlighted the salutary roles of CTRPs in the peripheral tissues, most notably related to metabolic and cardiovascular functions.[20-28] Since the unexpected role of complement C1q in synaptic pruning was described,[29,30] the central nervous system (CNS) functions for several brain-enriched C1q family members have also been uncovered. These include the important roles of cerebellins (Cbln1-4) in hippocampus, cerebellum, and midbrain,[31-35] as well as the functions of C1ql3/CTRP13[36] and C1ql1/CTRP14[37,38] in cerebellum and forebrain.

The distinguishing feature of CTRP4 (encoded by *C1QTNF4* gene) is that it is the only vertebrate C1q family member with two tandem globular C1q domains connected by a short linker.[12,39] In divergent vertebrate species such as human, mouse, and zebrafish, *CTRP4* expression is predominantly detected in the CNS.[39] One other tissue with a relatively high expression of *CTRP4* transcript in humans is the adipose tissue.[39] We first demonstrated its in vivo role in reducing food intake and altering energy balance via central delivery of recombinant CTRP4 into the lateral ventricle.[39] Subsequently, we and others have further confirmed and extended its CNS role in food intake and energy metabolism using overexpression[40] and knockout[41] mouse models. Interestingly, anti-inflammatory properties of CTRP4 have recently been noted in humans and rodents. Transgenic mice with elevated plasma CTRP4 have significantly reduced gastrointestinal inflammation and disease severity index when subjected to dextran sulfate sodium-induced colitis.[42] Conversely, its deficiency in *Ctrp4* KO mice results in heightened inflammation and mortality induced by endotoxin (LPS) shock or sepsis.[43] Exome sequencing of individuals with a severe form of the autoimmune disease systemic lupus erythematosus (SLE) also identified a rare variant of *CTRP4/C1QTNF4* (H198Q) which encodes a protein that can reduce TNF-α-mediated NF-κB activation and cell death in vitro.[44] Whether this rare variant, also identified in a separate cohort of SLE patients,[45] is causally linked to SLE remains to be established. In other brain inflammatory condition due to infection, such as herpes simplex encephalitis, expression of CTRP4 was shown to be upregulated.[46] More recently, nucleolin (a soluble intracellular protein) was suggested as a potential cell surface docking protein that interacts with CTRP4 on monocytes, B-cells (to a much lesser extent), and dead cells.[47] Collectively, these studies demonstrated the potential central and peripheral functions of CTRP4.

Aside from the few published studies highlighted, the contribution of CTRP4 to other aspects of physiology remains largely unknown and unexplored. Recent single-cell RNA sequencing has provided an unprecedented and comprehensive view of *Ctrp4* expression in different brain regions of the mouse at remarkable cellular resolution.[48] We hypothesized that since *Ctrp4* is expressed predominantly in the brain[39] in a variety of neuronal cell types that spread across many different anatomical regions,[48] CTRP4 may likely have additional CNS roles beyond regulating neuropeptide gene expression in the hypothalamus to control food intake. To test this hypothesis and explore additional functions of CTRP4 in the brain as hinted by its widespread expression, we subjected the *Ctrp4* KO mice to a battery of behavioral tests. We reasoned that any behavioral deficits seen in the *Ctrp4* KO mice would provide a clue to its role in the CNS. We discovered that CTRP4 deficiency impairs associative learning and memory in a sex-dependent manner, and this deficit correlates with altered learning- and memory-associated gene expression in the hippocampus and cortex. We also showed that loss of CTRP4 modestly enhances sensitivity to shock, highlighting a potential nociceptive function. Given how little is known about CTRP4 biology, the novel CNS role we uncovered will provide a context for future studies to interrogate its mechanisms of action and cellular targets within the brain.

2 | MATERIALS AND METHODS

2.1 | Mouse model

The CTRP4 (c1qtnf4$^{tm1a(KOMP)Wtsi}$) knockout (KO) mouse strain used was previously published.[41] The CTRP4-null mice were generated and maintained on a C57BL/6N genetic background. Genotyping primers for wild-type (WT) allele were forward 5′-CTCAGGGCCTTTGGAAGGGCA-3′ and reverse 5′-GGGAG GGCTTATCACAGC ACC-3′. The size of the WT band was 333 bp. Genotyping primers for the *Ctrp4* KO allele were forward 5′-GGTAA ACTGGCTCGGATTAGGG-3′ and reverse 5′-TTGACTGTAGCGGCTGATGTTG-3′. The size of the KO band was 211 bp. The genotyping PCR parameters were as follows: 94°C for 5 min, followed by 10 cycles of (94°C for 15 s, 65°C for 30 s, 72°C for 1 min), then 35 cycles of (94°C for 15 s, 55°C for 30 s, 72°C for 30 s), and lastly 72°C for 5 min. All mice were generated by intercrossing *Ctrp4* heterozygous (+/−) mice. *Ctrp4* KO (−/−) and WT (+/+) littermate controls were housed in polycarbonate cages on a 12-h light–dark photocycle with ad libitum access to water and food. Mice were fed a standard chow (Envigo; 2018SX). At termination of the study, mice were fasted for 2 h and euthanized. Brain tissues were dissected, snap-frozen in liquid nitrogen, and kept at −80°C until analysis. All mouse experiments (protocol # MO19M481) and mouse behavioral tests (protocol # SP20M311) were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine. All animal experiments were conducted in accordance with the National Institute of Health guidelines and followed the standards established by the Animal Welfare Acts.

2.2 | Tissue collection

Cortex and hippocampus were immediately dissected from euthanized mice, flash-frozen in liquid nitrogen and kept at −80°C until analysis.

2.3 | Quantitative real-time PCR analysis

Total RNA was isolated from micro-dissected brain regions using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Purified RNA was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-rad). Real-time quantitative PCR analysis was performed on a CFX Connect Real-Time System (Bio-rad) using SsoAdvanced Universal SYBR Green Supermix (Bio-rad) per manufactuer's instructions. Data were normalized to *18S* rRNA and expressed as relative mRNA levels using the ΔΔCt method.[49] Fold change data were log transformed to ensure normal distribution and statistics were performed. Real-time qPCR primers used in this study are included in Table S1.

2.4 | Open field

Locomotor activity was assessed over 30 min in a 40 cm × 40 cm activity chamber with infrared beams (San Diego Instruments Inc., San Diego, CA, USA). Horizontal activity, as well as time spent in the center or periphery of the chamber was automatically recorded. All behavioral tests described in this study were conducted by experimenters blinded to genotypes, and all tests were performed during the active (dark cycle) phase of the mouse circadian cycle.

2.5 | Elevated plus maze

Anxiety related behavior was evaluated using the elevated plus maze test. Mice were placed in the center of a 54 cm high maze consisting of two open and two closed (30.5 cm long) arms for 5 min. Distance travelled and time spent in the open and closed arms was automatically recorded using Anymaze Tracking Software (Stoelting, Co., Wood Dale, IL, USA).

2.6 | Y-maze spontaneous alternation

Working memory was assessed in the Y-maze spontaneous alternation task. Mice were placed at the end of one arm of a Y-maze consisting of three 38 cm long arms (San Diego Instruments Inc., San Diego, CA, USA) and allowed to explore the maze for five minutes. Distance travelled and entries into the arms were automatically recorded using Anymaze Tracking Software (Stoelting, Co., Wood Dale, IL, USA). The percent alternation was calculated using the equation: % Alternation = (Correct alternations/(Total arm entries – 2)) × 100.

2.7 | Y-maze spatial recognition

Spatial memory was assessed using the Y-maze spatial recognition test. This test consists of a “Training” phase and a “Test” phase. During the “Training” phase, one arm of a Y-maze consisting of three 38 cm long arms (San Diego Instruments) was blocked off. Mice were placed at the end of one of two open arms and allowed to explore for five minutes. After a 30-min inter-trial interval, the “Test” phase began. The blockade was removed, and mice were allowed to explore all three arms of the maze for five minutes. Distance travelled and time spent in each arm was automatically recorded using Anymaze Tracking Software (Stoelting, Co., Wood Dale, IL, USA). Data from the first two minutes of the “Test” phase were used to evaluate percent time spent in the novel arm.

2.8 | Novel object recognition test

The novel object recognition test (NORT) was used to evaluate short term memory. Briefly, mice were allowed to habituate to an empty 20 cm × 20 cm box for 10 min. Twenty-four hours following habituation, mice were placed in the box with two identical objects for a 10-min training period. Thirty minutes following the training period, one object was replaced with a novel object, and the mouse was allowed to explore the objects for five minutes. Time spent sniffing the novel and familiar objects was measured using CleverSys Topscan tracking software (Cleverysy Inc., Reston, VA, USA).

2.9 | Rotarod

Motor coordination and learning was evaluated using the accelerating rotarod test. Mice were placed on the rotarod (Columbus Instruments, Columbus, OH, USA) with a starting speed of 4 rpm, with an acceleration of 6 rpm/minute. The time at which each mouse dropped from the rotating rotarod was recorded. Each mouse was given three trials per day with a 2-min inter-trial interval, over three days.

2.10 | Hotplate

Mice were placed on an 11 cm × 11 cm black anodized aluminum plate heated to 52°C (IITC Life Science, Woodland Hills, CA). The latency for each mouse to withdraw one of the hind limbs from the plate was recorded, and the mouse was returned to the home cage. If a mouse did not withdraw the hind limb after 30 s, it was removed from the hotplate and placed back in the home cage.

2.11 | Hargreaves plantar test

Nociception was evaluated using a Plantar Analgesia Meter Model 390G (IITC Life Science, Woodland Hills, CA) as previously described.[50] Mice were placed on a glass platform heated to 32°C and habituated to the platform for 30 min prior to testing. A focused thermal heat stimulus was delivered from a light source to the plantar surface of the hind paw for up to 30 s. A full leg raises at the site where the heat stimulus was directed was considered a reaction to the thermal stimulus. The average of six trials was used as the latency for each mouse, alternating paws between trials.

2.12 | Morris water maze

The Morris water maze test (MWM) was based on the protocol previously described.[51] The maze consists of a circular stainless-steel tank 4 m in diameter filled with room temperature water made opaque with white tempera paint for the training, probe, and reversal trials. During the hidden platform training phase, a 10 cm platform was placed in a fixed location in the maze, with the top of the platform hidden beneath 1 cm of water. Mice were placed into the maze around the perimeter in one of four start positions used in a semi-random fashion throughout six trials each day for three days. Mice were allowed to search for the platform for 60 s and after finding the platform remained there for 10 s. Escape latency was recorded for each trial. 24 h following the hidden platform training phase, mice were tested in one 60-s probe trial. Latency to cross into the platform area, number of crossings in the platform area, and time spent in the platform quadrant were measured using Anymaze

tracking software (Stoelting Co., Wood Dale, IL, USA). During the reversal phase, the hidden platform was placed in a different location of the MWM than that used during the hidden platform training phase. Like the hidden platform training phase, mice were placed into the maze around the perimeter in one of four start positions used in a semi-random fashion throughout six trials each day for two days. Mice were allowed to search for the platform for 60 s and after finding the platform remained there for 10 s. Escape latency was recorded for each trial.

2.13 | Trace fear conditioning

Trace fear conditioning was conducted as previously described.[52] Briefly, trace fear conditioning consisted of a habituation day, a training day, and a test day over three consecutive days. On the habituation day, mice were exposed to the shock box (Med Associates, Inc., Fairfax, VT, USA) for ten minutes. On the training day, mice were placed in the shock box and give a two-minute habituation, after which a 20-s tone (80 db, 2000 Hz) was delivered. Twenty seconds following the termination of the tone, a scrambled two-second 0.5 mA shock was delivered. The tone-shock pairing was repeated three additional times. On the test day, mice were placed in the shock box for three minutes to measure freezing in response to the context. Mice were then placed in a separate context and freezing in response to the 20-s tone was measured. Freezing behavior was automatically scored using Med Associates, Inc. Video Freeze Software (Med Associates, Inc., Fairfax, VT, USA).

2.14 | Shock Sensitivity

Sensitivity to shock was evaluated using the method previously described.[53] Mice were placed in a shock box (Coulbourn Instruments, Whitehall, PA) and allowed to habituate for two minutes. Following habituation mice were exposed to a 1 s shock which increased in increments of 0.05 mA every 30 s from 0.1 to 0.5 mA. The shock level at which each mouse first flinched, vocalized, and jumped was recorded.

2.15 | Prepulse inhibition PPI

PPI was assessed as described.[51] The experimental session consisted of a 5-min acclimatization period to a 70-dB background noise (continuous throughout the session), followed by the presentation of 10 40-ms 120-dB white noise stimuli at a 20-s inter-stimulus interval (the habituation session). Upon the completion of the habituation session, each mouse was left in the enclosure for 5 min without presentations of any startle stimuli. Immediately after, the pre-pulse inhibition (PPI) session was begun. During each PPI session, a mouse was exposed to the following types of trials: pulse-alone trial (a 120-dB, 100-ms, broadband burst); the omission of stimuli (no-stimulus trial); and five pre-pulse–pulse combinations (pre-pulse–pulse trials) consisting of a 20-ms broadband burst used as a pre-pulse and presented 80 ms before the pulse using one of the five pre-pulse intensities: 74; 78; 82; 86 and 90 dB. Each session consisted of six presentations of each type of the trial presented in a pseudorandom order. PPI was assessed as the percentage scores of PPI (%PPI): 100 × (mean startle amplitude on pulse-alone trials – mean startle amplitude on prepulse–pulse trials/ mean startle amplitude on pulse-alone trials) for each animal

separately. The percentage of PPI for each animal was used as the dependent variable in statistical analysis.

2.16 | Tail-suspension test

The tail-suspension test was performed as previously described.[54] Each mouse was suspended individually by its tail in a styrofoam box (28 cm × 30 cm × 25 cm) in a quiet room. The tip of the mouse tail was fixed on the top part inside the styrofoam box using adhesive tape. The duration of the test was 6 min, and the immobility time of the tail-suspended mice was measured during the last 4 min of the test. The immobility time was determined using a stopwatch by an observer blinded to the genotype.

2.17 | Statistical analyses

All results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with Prism 8 software (GraphPad Software, San Diego, CA). All real-time PCR data were log2 transformed to ensure linear distribution. Data were analyzed with two-tailed Student's *t*-tests or by repeated measures ANOVA. For two-way ANOVA, we performed Bonferroni post hoc tests. $p < .05$ was considered statistically significant. All data are presented as mean ± SEM (standard error of the mean).

3 | RESULTS

3.1 | Cell-level expression patterns of *Ctrp4* across distinct anatomical regions of the mouse brain

Our previous study indicated predominant and widespread expression of *Ctrp4* transcript in the mouse brain.[39] Recently single-cell RNA sequencing, however, has afforded a unique opportunity to re-examine *Ctrp4* transcript expression across different mouse brain regions at single-cell resolution.[48] In the mouse central and peripheral nervous system, there are a total of 23 distinct anatomical regions that express the *Ctrp4* transcript (Figure 1). Within these anatomical regions, there are a total of 32 functional cell types and 175 single-cell clusters that express *Ctrp4* (Figure 1). The top six anatomical regions with the highest expression level of *Ctrp4* are the spinal cord, midbrain, hindbrain, hypothalamus, cerebral cortex, and hippocampus; together, they account for >73% of the total *Ctrp4* transcript in the brain (Figure 1B). Within different brain regions, the *Ctrp4* transcript can be found in a variety of functional cell types that include excitatory, inhibitory, dopaminergic, glutamatergic, cholinergic, peptidergic, and nitregic neurons (Figure 1). The expression patterns of *Ctrp4* within the central and peripheral nervous system suggest potentially undiscovered CNS role of CTRP4 in regulating physiology and behavior.

3.2 | Loss of CTRP4 does not alter exploratory, anxiety- or depressive-like behaviors

We first assessed the impact of CTRP4 deficiency on general exploratory behaviors. In an open field test for anxiety-like behavior, *Ctrp4* KO mice of either sex spent similar amount of time as the WT littermates in the center and periphery of the open field, as well as having similar numbers of rearing activity (Figure 2A-H). In the elevated plus maze that also measures anxiety-like behavior, *Ctrp4* KO mice of either sex also spent a similar proportion of time exploring the open arms when compared to the WT littermates (Figure 2I-L). In

the Y-maze spontaneous alternation test, a measure of working memory, the number of arm entries and % correct alternation between the three arms were similar between genotypes of either sex (Figure 2M-R). Finally, in the tail suspension test, which measures potential depressive-like behavior, we also did not observe a genotype effect between WT and KO male and female mice, as indicated by the comparable percent time spent in the state of immobility (Figure 2S-T). These data, together, indicate that CTRP4 is dispensable for general exploratory, as well as anxiety- and depressive-like behaviors.

3.3 | CTRP4-null mice have normal motor function and prepulse inhibition

Since *Ctrp4* transcript is expressed in the cerebellum that regulates motor function (Figure 1B), we assessed neuromuscular function with the rotarod test. Over a 3-day test period, both WT and *Ctrp4* KO mice of either sex gradually improved their ability to stay on the rotarod for longer periods of time (ie, increased latency to drop), indicating that CTRP4 is not required for motor coordination and balance (Figure 3A,B). Next, we examined whether CTRP4 deficiency results in neuropsychiatric-like behaviors. Humans with neuropsychiatric disorders (eg, schizophrenia) and rodents with schizophrenia-like phenotypes are known to have reduced prepulse inhibition of acoustic startle response.[55,56] In response to different prepulse intensities, both WT and *Ctrp4* KO mice of either sex showed a similar degree of inhibition to subsequent acoustic startle response (Figure 3C,D), indicating that CTRP4 is not required for sensorimotor gating.

3.4 | CTRP4 deficiency enhances sensitivity to pain induced by shock

Given *Ctrp4* transcript expression in the spinal cord and dorsal root ganglia (Figure 1), we examined the potential contribution of CTRP4 to somatosensory function, focusing on sensitivity to pain elicited by thermal heat or shock. In both hotplate and Hargreaves tests, the latency to lift the hind legs in response to thermal stress was not significantly different between genotypes of either sex (Figure 4A-D), indicating that CTRP4 is dispensable for pain sensation induced by heat. We next tested whether another pain modality, such as that induced by shock, is different between genotypes. We observed the low-level shock intensity needed to elicit a flinch or vocalization was not different between genotypes of either sex (Figure 4E,F,I,J), indicating that *Ctrp4* KO mice have intact shock sensing mechanisms. Greater shock will elicit a jump in mice. We observed that the shock intensity needed to elicit the first hop was significantly lower in both male and female *Ctrp4* KO mice relative to WT littermates (Figure 4H,L), suggesting enhanced sensitivity to pain induced by higher shock level in the CTRP4-null animals. The shock intensity needed to elicit a full jump (where all four feet left the ground), however, was also lower in *Ctrp4* KO mice relative to WT littermates but fell short of being significant (Figure 4G,K).

3.5 | Loss of CTRP4 impairs associative learning and memory in female mice

The prominent expression of *Ctrp4* transcript in the cortex and hippocampus (Figure 1) led us to next ask if CTRP4 plays a role in learning and memory. We first assessed spatial memory using two different tests, the Y-maze spatial recognition test and Morris water maze. In the former, the total distance traveled and the percent of time spent in familiar and novel arms were not significantly different between genotypes of either sex (Figure 5A-D). In the novel object recognition test, a measure of short-term memory, the percent

preference for the novel object was not significantly different between WT and KO mice of either sex (Figure 5E,F). And in the more complex Morris water maze, the latency time to find the hidden platform was also comparable between WT and *Ctrp4* KO female mice (Figure 5G,H). Male mice, however, were not assessed. These results indicate that CTRP4 is dispensable for spatial learning and memory. We next performed the trace fear conditioning test to evaluate complex associative learning and memory in these animals. During training and the subsequent context and cue tests, male *Ctrp4* KO mice performed just as well as the WT littermates (Figure 6A-C), indicating no deficit in associative learning and memory. *Ctrp4* KO female mice, however, did not learn as well during the training session (Figure 6D), as indicated by reduced freezing in response to paired tone/shock. Although *Ctrp4* KO female mice performed similarly well as the WT littermates during subsequent context tests (Figure 6E), they froze significantly less in response to cue tests (Figure 6F), indicating impaired associative memory.

3.6 | CTRP4 deficiency alters learning and memory gene expression in the hippocampus and cortex

Associative learning and memory involve multiple brain regions, including the hippocampus and cortex. Consequently, we performed targeted expression analyses on genes known to be important for learning and memory[57] (Table S1). Because a deficit in associative learning and memory was only seen in female *Ctrp4* KO mice, we therefore focused our gene expression analyses in female mice. As expected, the expression of *Ctrp4* transcript in the hippocampus and cortex was completely abolished in the CTRP4-null mice (Figure 7A,D). Of the 44 selected genes analyzed, *Arc* (Activity regulated cytoskeleton associated protein) expression was significantly reduced in both hippocampus and cortex (Figure 7B,E). In addition, *Pde4d* (Phosphodiesterase 4D) expression was significantly upregulated in the hippocampus (Figure 7C), and *c-fos* (Fos proto-oncogene) expression was significantly downregulated in the cortex (Figure 7F), of *Ctrp4* KO female mice relative to WT controls. The average expression values ($\log_2$ fold change) of all the 44 genes analyzed by real-time PCR were shown on a heatmap (Figure 7G). Although the expression of most genes fell short of being significant (Figure S1), there were many more genes whose expression tended to be altered in the hippocampus than in the cortex of *Ctrp4* KO female mice relative to WT controls (Figure 7C). Some of these genes whose expression trended to be upregulated in the *Ctrp4* KO hippocampus include *Fkbp1a (fkbp12*; +59%, $p = .06$), *Paip2* (+57%, $p = .06$), and *Hdac1* (+65%, $p = .08$). Combined, these data suggest a possible link between altered gene expression in the hippocampus and cortex and the observed deficit in associative learning and memory in *Ctrp4* KO female mice.

4 | DISCUSSION

Despite widespread expression of *Ctrp4* transcript in many different neuronal cell types across distinct anatomical brain regions, a battery of behavioral tests indicated that CTRP4 is dispensable for CNS functions governing general exploratory behaviors, motor function and balance, sensorimotor gating, as well as anxiety- and depressive-like behaviors. Loss of CTRP4, however, enhances pain sensitivity elicited by shock in both male and female mice lacking CTRP4, implying altered nociceptive function; this effect is modest since

CTRP4 deficiency lowers the shock intensity needed to elicit the first hop. The shock intensity needed to induce the first jump (all four legs are off the ground), however, was not significantly different between genotypes of either sex, although it trended to be lower in *Ctrp4* KO female mice relative to WT controls. Given the expression of *Ctrp4* transcript in dorsal root ganglia (DRG) and our behavioral data, CTRP4 likely has a role in nociception, but that additional confirmation using different pain modality paradigms (eg, acute or chronic pain stimuli induced by chemical or mechanical damage to nerve cells) are needed to fully establish if and what forms of nociception are regulated by CTRP4.

While spatial learning and memory was preserved in the *Ctrp4* KO mice, associative learning and memory was impaired in female, but not male, *Ctrp4* KO mice. Sexual dimorphism in learning and memory is not entirely unexpected, as this has been documented in humans and animal models.[58-60] The underlying mechanisms accounting for sex differences in learning and memory are complex and not well understood, and likely involve sex hormones. Although estrogen is generally thought to account for sexual dimorphism in memory formation,[61,62] there is no simple relationship between female sex hormone and memory formation.[60] It should be noted that sex differences in the activation of synaptic kinases (eg, CaMKKα, CaMKKβ, ERK), transcription factors (eg, CREB, Nf-κB), and gene transcription (eg, *BDNF, GAA1, SRp20*) have also been documented[60]; it is unclear, however, whether any of these molecules are differentially altered in *Ctrp4* KO female relative to male mice. At least at the transcript level, we observed no differences in the expression of *Camk4, Creb*, and *Bdnf* between WT and *Ctrp4* KO female mice. Additional studies are needed to clarify the sex differences in associative learning and memory in CTRP4 deficient animals.

Associative learning and memory, as assessed by the trace fear conditioning paradigm, is dependent on multiple brain regions, including the hippocampus, amygdala, and cortex.[63-66] Altered expression of genes in these brain regions can affect learning and memory. Indeed, when we examined 44 select genes known to play roles in learning and memory in mice (based on gain- or loss-of-function mouse models),[57] the expression of *Arc* and *c-fos* were significantly downregulated in the hippocampus and/or cortex of *Ctrp4* KO female mice relative to WT littermates. Arc/Arg3.1 and c-fos are known to play important roles in learning and memory,[67,68] and mice lacking Arc or c-Fos in the CNS have a marked deficit in long-term memory.[69,70] Thus, reduced expression of *Arc* and *c-fos* in the hippocampus and cortex may contribute to the deficit in associative learning and memory as seen in the *Ctrp4* KO female mice. In addition to impaired associative learning and memory (as judged by trace fear conditioning paradigm), loss of Arc also impaired novel object recognition and spatial memory (as indicated by the Morris water maze test).[69] In contrast, reduced expression of *Arc* in the hippocampus and cortex of *Ctrp4* KO female mice appears to adversely affect associative learning and memory but has no impact on short-term memory for object recognition and complex spatial (Morris water maze) memory. The difference may be attributable to the complete absence of *Arc* in the *Arc* KO mice versus 30%–40% reduction in *Arc* expression seen in the *Ctrp4* KO female mice.

Interestingly, we also observed increased expression of *Pde4d* in the hippocampus of the *Ctrp4* KO female mice. *Pde4d* encodes phosphodiesterase 4D, an enzyme that

hydrolyzes cAMP and plays an important role in modulating cAMP/PKA/CREB pathway in the hippocampus critical for learning and memory.[71] Increased neuronal cAMP level in the hippocampus via modulation of a G-protein coupled receptor enhances hippocampal synaptic plasticity and improves short and long-term memory for contextual fear conditioning.[72] Similarly, increasing cellular cAMP level by pharmacologic inhibition of Pde4d[73,74] or genetic inactivation of *Pde4d*[75] enhances memory. Thus, elevated *Pde4d* expression in the hippocampus of *Ctrp4* KO female mice would presumably reduce hippocampal cAMP level and signaling, thereby contributing to impaired associative learning and memory seen in these animals. Complete deletion of *Pde4d* gene or miRNA-mediated downregulation of *Pde4d* have also been shown to improve short-term memory of object recognition and long-term spatial memory (Morris water maze)[75]; neither of these two types of memory was affected in *Ctrp4* KO female mice with an elevated hippocampal expression of *Pde4d*. Since *Pde4d* is expressed in multiple brain regions (including hippocampus and cortex), the complete loss of *Pde4d* or its downregulation by miRNA would likely affect multiple neuronal cell types throughout the brain. Thus, in these loss-of-function mouse models, both short-term and long-term memory are improved, whereas the modest (~65%) upregulation of *Pde4d* seen in the hippocampus (but not cortex) of *Ctrp4* KO female mice may not be sufficient to negatively impact short-term memory and spatial memory.

Our expression analyses also uncovered multiple genes that trended to be upregulated in the hippocampus of *Ctrp4* KO female mice relative to WT controls; among these are *Fkbp1a/fkbp12, Paip2a*, and *Hdac1*. The potential relevance of these upregulated genes is highlighted by enhanced hippocampal-dependent memory in KO mouse models lacking FK506-binding protein 1a (Fkbp1a) or Poly(A) binding protein interacting protein 2 (Paip2a).[76,77] Similarly, pharmacologic inhibition of class I histone deacetylase (Hdac1-3, −8) also improves hippocampal-dependent memory in contextual fear conditioning.[78] Thus, the combined trended upregulation of *Fkbp1a, Paip2a*, and *Hdac1* in the hippocampus of *Ctrp4* KO female mice could also potentially contribute to impaired associative learning and memory seen in these animals.

Several limitations of the study are noted. Our behavioral tests were not exhaustive, even though we had assessed many of the mouse behaviors commonly used to evaluate altered brain function and memory deficit. Additional behavioral tests, in particular those related to somatosensory functions, may reveal a previously unrecognized role of CTRP4 in the central or peripheral nervous system. Although CTRP4 deficiency altered learning and memory gene expression in the hippocampus and cortex, we do not know the mechanism that links CTRP4 deficiency to impaired associative memory in a sex-dependent manner. To date, no plasma membrane receptor has been identified for CTRP4. Although nucleolin, an intracellular protein, was recently shown to be a docking protein that can bind CTRP4, such interaction has only been demonstrated in monocytes, B-cells, and dead cells.[47] The relevance of this finding to CTRP4 action in the brain is presently unclear.

In summary, our behavioral and gene expression analyses have highlighted a novel and sexually dimorphic CNS role of CTRP4 in regulating hippocampal-dependent memory. Given the dearth of knowledge concerning the biological function of this highly conserved

secretory protein, our study provides important and valuable information needed to further investigate its mechanism of action in the central and peripheral nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (DK084171 to GWW).

Funding information

HHS | NIH | National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Grant/Award Number: DK084171

Abbreviations:

Arc	activity regulated cytoskeleton associated protein
Camk4	calcium/calmodulin dependent protein kinase IV
cAMP	cyclic adenosine monophosphate
c-fos	Fos proto-oncogene
CNS	central nervous system
CREB	cAMP response element binding protein
CTRP	C1q/TNF-related protein
Fkbp1a	FK506-binding protein 1a
Hdac1	histone deacetylase 1
KO	knockout
Paip2	Poly(A) binding protein interacting protein 2
PKA	protein kinase A
WT	wildtype

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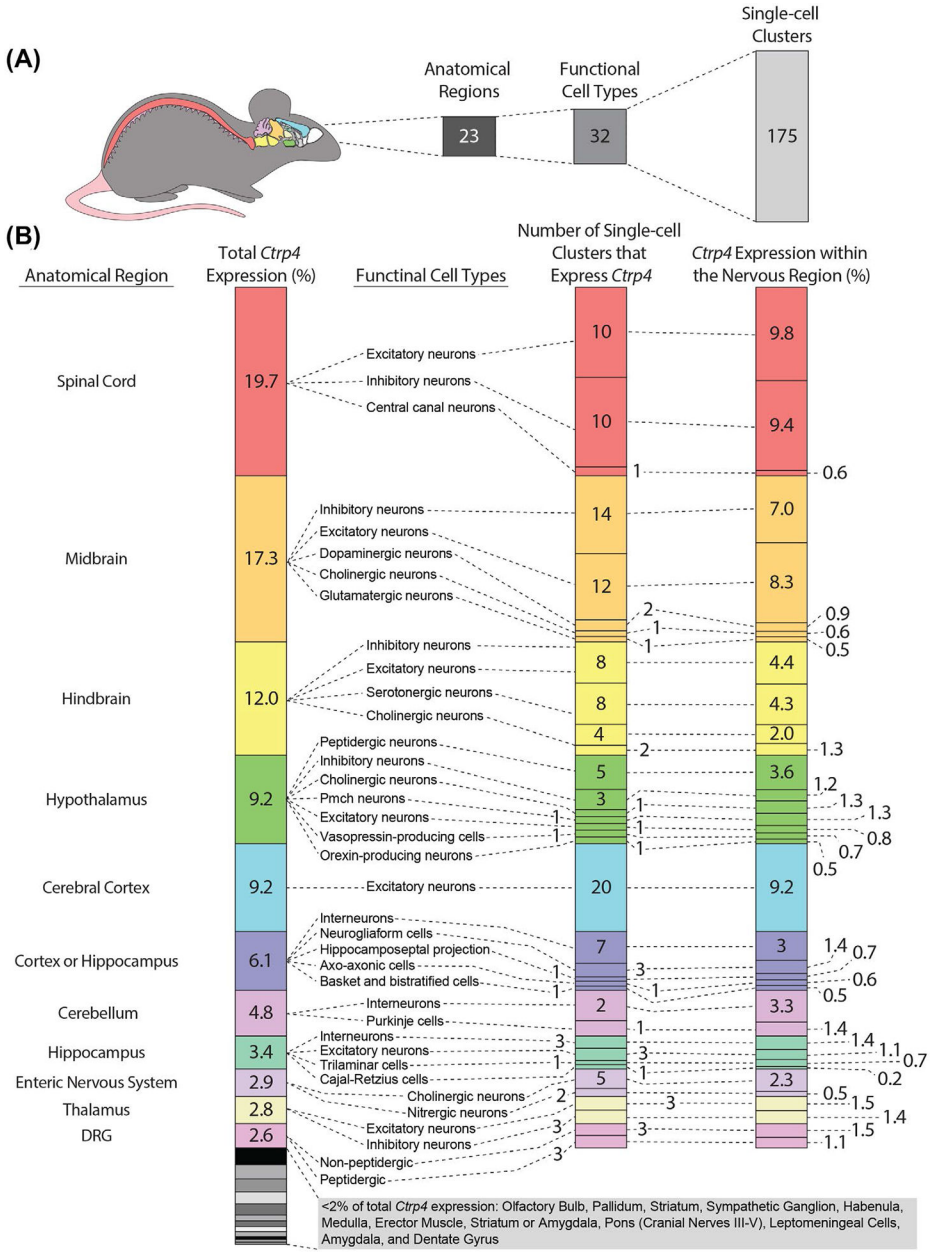

FIGURE 1.

Mouse *Ctrp4* transcript is widely expressed in the central and peripheral nervous system. (A) Summary of unique mouse anatomical regions, functional cell types, and single-cell clusters that express *Ctrp4* based on single-cell RNA sequencing data generated by the Linnarsson lab; Details of methods and cell type clustering as performed by the Linnarsson's lab can be found in the published work.[48] The data can be accessed through the mousebrain.org website. (B) Data organized by total *Ctrp4* transcript expression per anatomical region. Expression values for each single-cell cluster as defined are obtained from mousebrain.org.[48] The cumulative *Ctrp4* expression of all single-cell clusters expressing *Ctrp4* is considered the total possible *Ctrp4* and represents 100% of the expression. Single-cell clusters expressing *Ctrp4* were organized by their anatomical

location. The *Ctrp4* expression levels of each single-cell cluster within a particular anatomical region were summed and the percent of the total possible *Ctrp4* expressed is indicated. The data are organized from anatomical regions expressing the most *Ctrp4* to those expressing the least. For example, the spinal cord is the region of the nervous system that expressed the greatest amount of *Ctrp4* mRNA. We then grouped the single cell populations by functional cell type (excitatory neuron, cholinergic interneuron, etc) and summed the amount of *Ctrp4* expressed by each functional cell type. This allows us to see within a particular anatomical region: (1) which functional cell types are expressing *Ctrp4* and (2) which of those are expressing the most and least *Ctrp4*. The next column shows the number of single-cell clusters that express *Ctrp4* of that particular functional cell type (ie how many different single cell clusters are excitatory neurons that express *Ctrp4* within that anatomical region). The final column shows the percent total *Ctrp4* expression in a particular group of functional cell types located within a given anatomical region

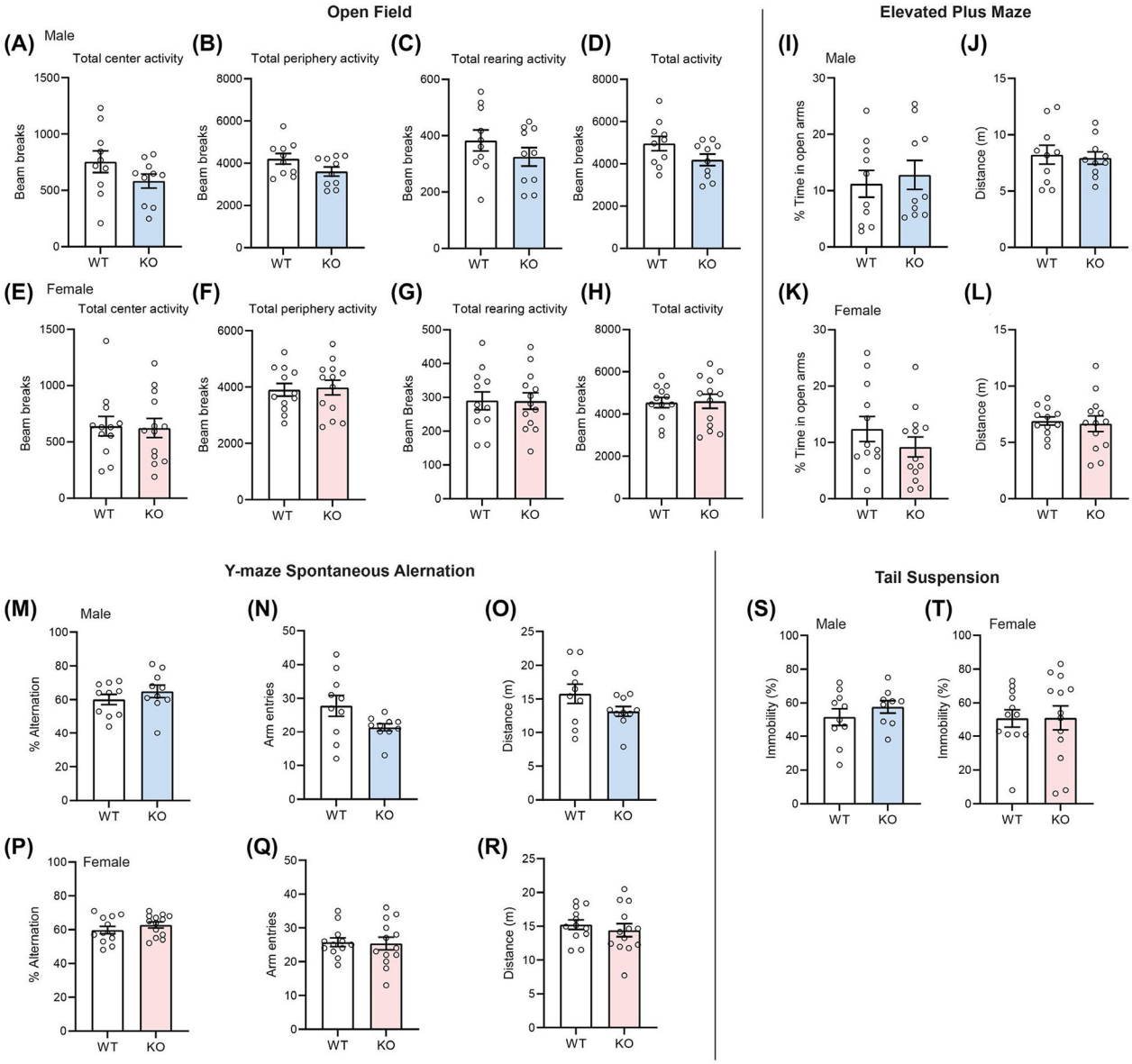

FIGURE 2.

Loss of CTRP4 does not affect exploratory, anxiety or depressive-like behaviors. (A–H) Open field tests for male (A–D) and female (E–H) wild-type (WT) and *Ctrp4* knockout (KO) mice. The amount of total activity spent at the center and periphery of the open field, as well as rearing activity, were not significantly different between genotypes of either sex. (I–L) Elevated plus maze tests of male (I,J) and female (K,L) mice. The percent of time spent in the open arms and the total distance travelled were not significantly different between genotypes of either sex. (M–R) Y-maze spontaneous alternation tests of male (M–O) and female (P–R) mice. The percent alternation, arm entries, and total distance travelled were not significantly different between genotypes of either sex. (S,T) Tail suspension tests for depressive-like behavior of male (U) and female (V) mice. The % of time spent in the state of immobility was not significantly different between genotypes of either sex. All data

are presented as mean ± SEM Male mice (WT, $n = 10$; KO, $n = 10$); Female mice (WT, $n = 12$; KO, $n = 13$)

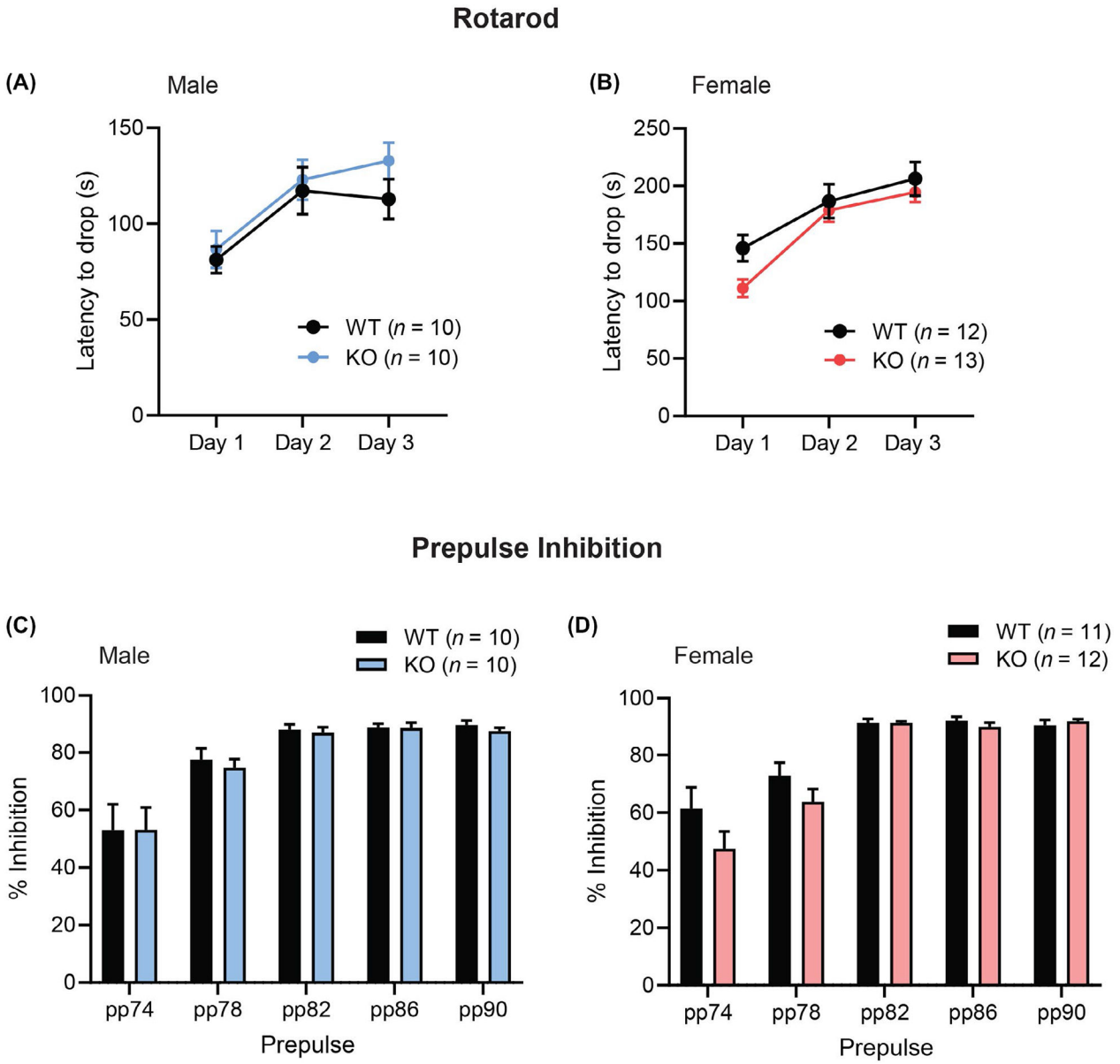

FIGURE 3.

CTRP4 deficiency does not affect motor coordination and balance, as well as prepulse inhibition of acoustic startle response. (A,B) Rotarod tests of wild-type (WT) and *Ctrp4* knockout (KO) male and female mice. Latency to drop from the rotatod over a three-day period of testing was not significantly different between genotypes of either sex. (C,D) Prepulse inhibition of acoustic startle response in male (C) and female (D) mice. Percent of inhibition in acoustic startle response following different prepulse (74–90 decibels) were not different between genotypes of either sex. All data are presented as mean ± SEM

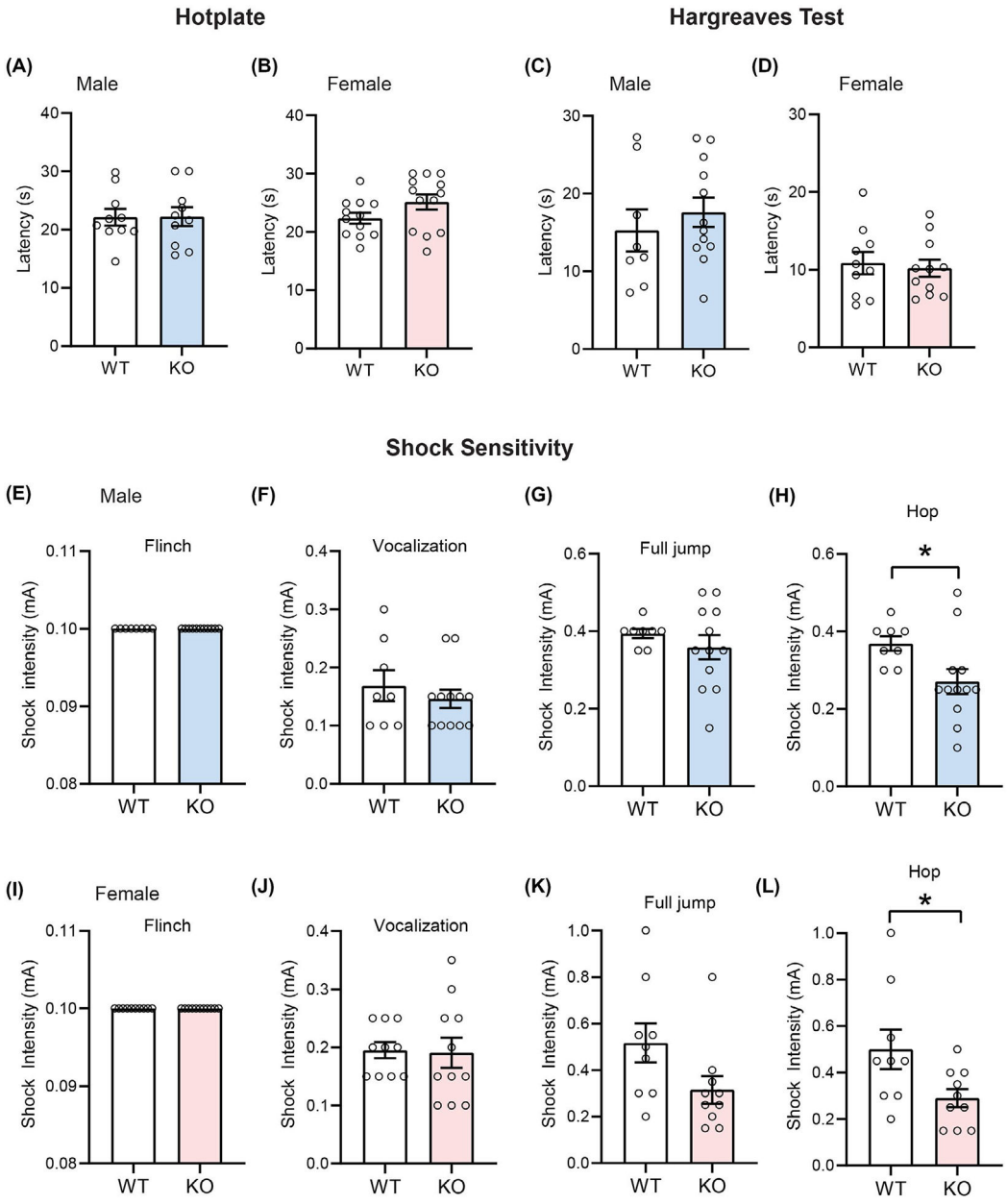

FIGURE 4.

Loss of CTRP4 modestly enhances pain sensitivity to shock. (A,B) Hotplate tests of wild-type (WT) and *Ctrp4* knockout (KO) male and female mice. Latency time to withdraw one of the hind limbs from the plate was not significantly different between genotypes of either sex. (C,D) Hargreaves tests of male (C) and female (D) mice. The latency time for a full leg raise at the site of thermal stimulus was not significantly different between genotypes of either sex. (E–L) Shock sensitivity tests of male (E–H) and female (I–L) mice. The shock intensity (mA) at which mice flinched, vocalized, jump, and hop are indicated. Relative to WT littermates, *Ctrp4* KO male and female mice required a lower shock intensity to elicit the first full jump. All data are presented as mean ± SEM $*p < .05$. Male mice (WT, n = 8–10; KO, n = 10–12); Female mice (WT, n = 8–12; KO, n = 10–13)

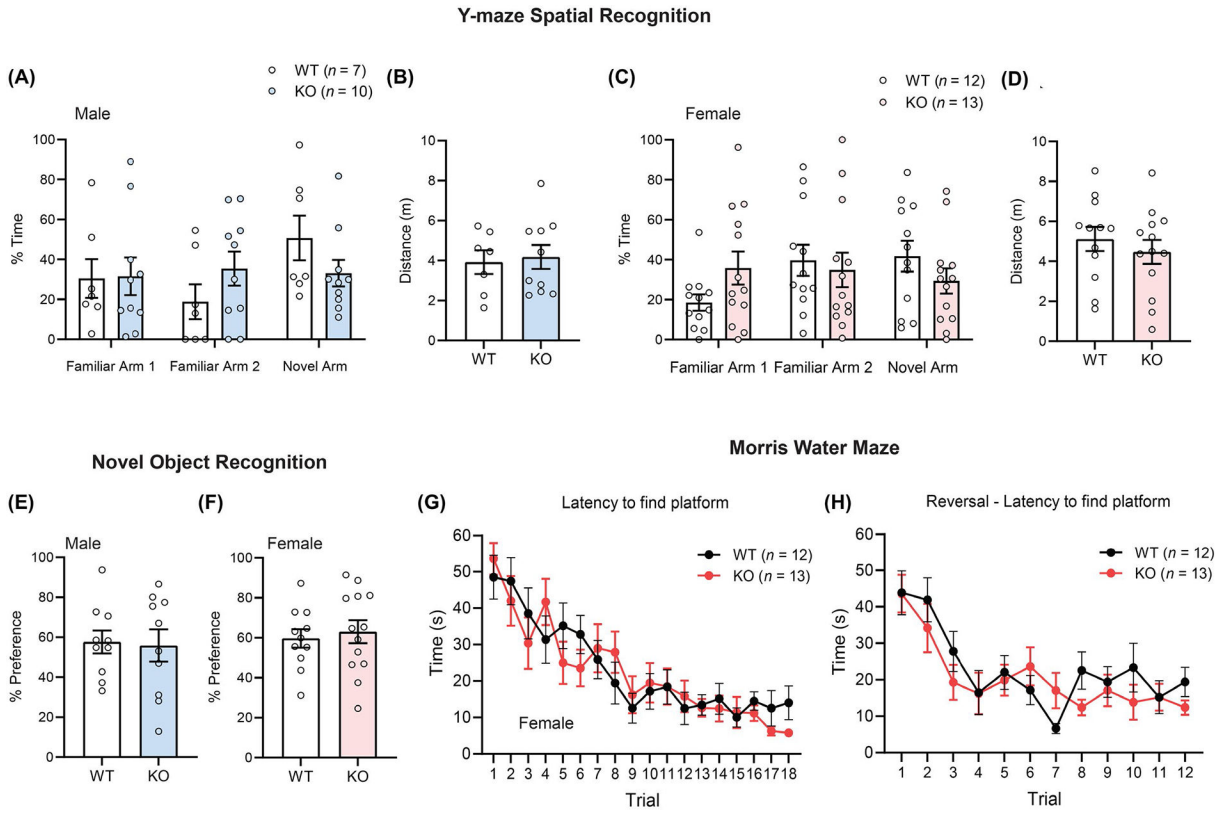

FIGURE 5.

CTRP4 deficiency does not impair short-term memory and long-term spatial memory. (A–D) Y-maze spatial recognition tests of male (A,B) and female (C,D) mice. Percent time spent in familiar and novel arm, as well as total distance travelled, were not significantly different between genotypes of either sex. (E,F) Novel object recognition tests of male (E) and female (F) mice. The percent preference for novel object was not significantly different between genotypes of either sex. (G,H) Morris water maze tests of WT and *Ctrp4* KO female mice. The latency time to discover the platform was not significantly different between genotypes

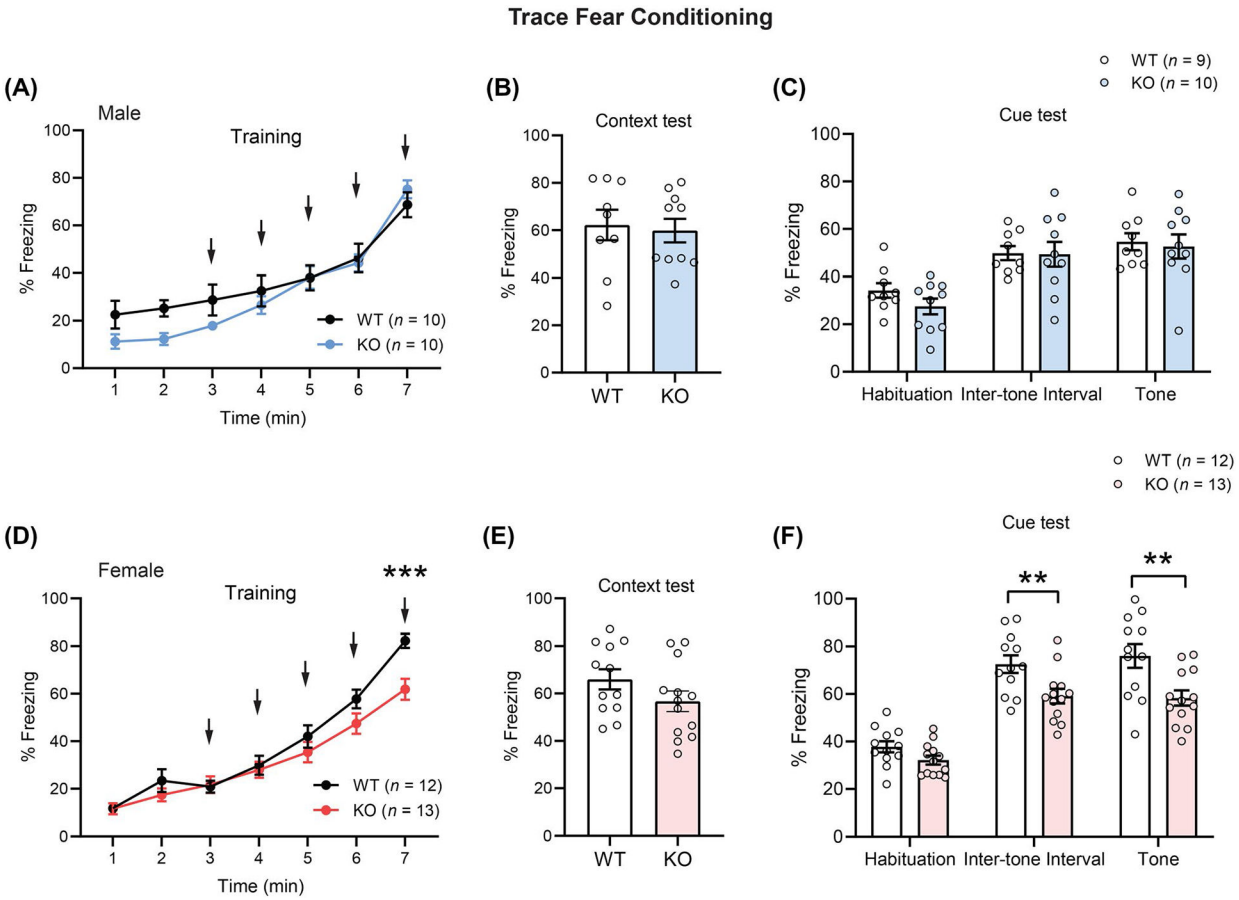

FIGURE 6.

Loss of CTRP4 impairs associative learning and memory. (A–F) Trace fear conditioning tests of male (A–C) and female (D–F) mice. Percent freezing during training, as well as context and cue tests, were not significantly different between WT and KO male mice. *Ctrp4* KO female mice, however, had reduced freezing during training and in the cue tests. Arrows point to time when shock was administered. All data are presented as mean ± SEM $^{**}p < .01$; $^{***}p < .001$

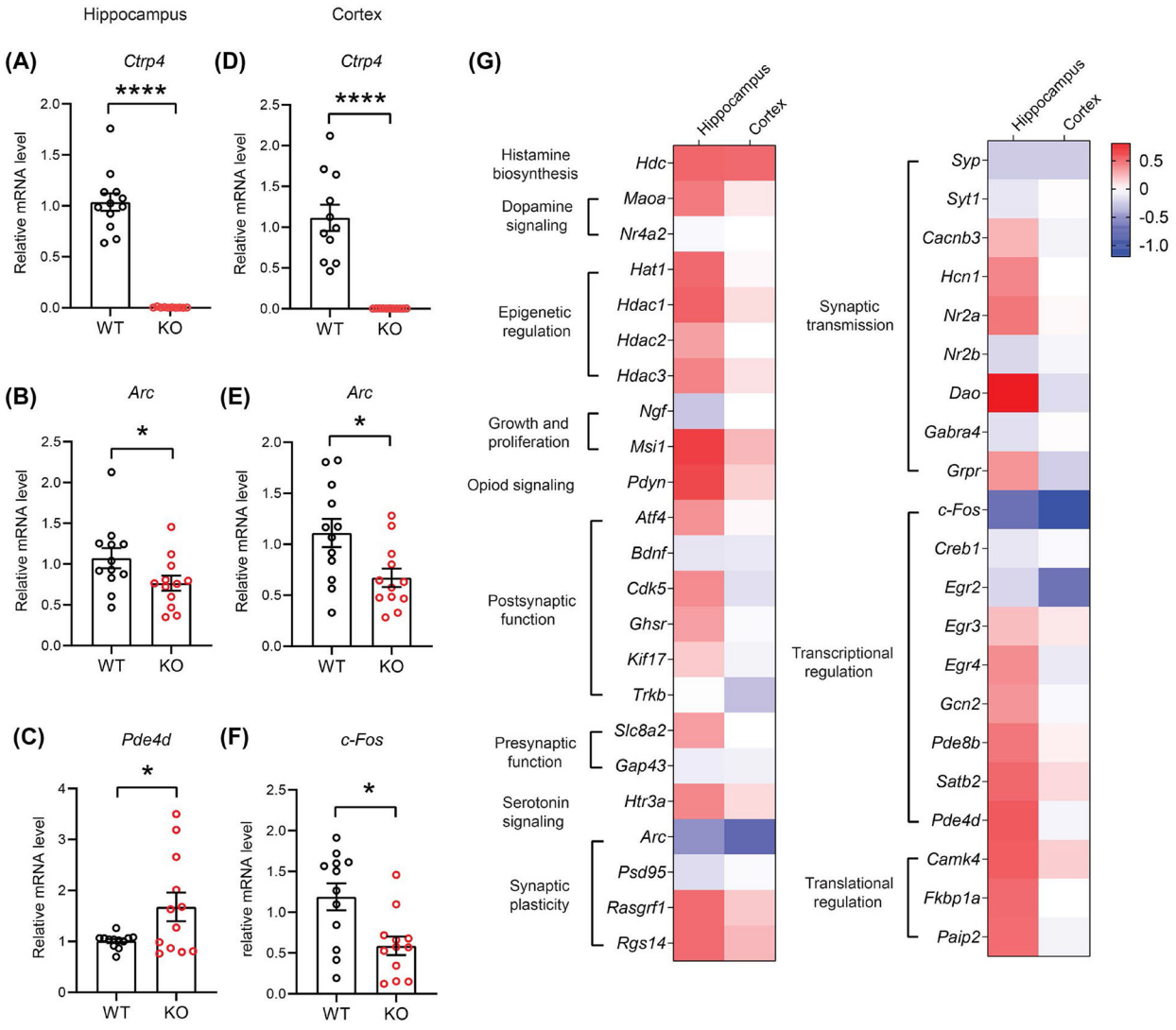

FIGURE 7.

Altered expression of learning and memory genes in the hippocampus and cortex of WT and *Ctrp4* KO female mice. (A–C) Altered expression of *Ctrp4, Arc*, and *Pde4d* in the hippocampus of wild-type (WT) and *Ctrp4* knockout (KO) female mice. (D–F) Altered expression of *Ctrp4, Arc*, and *c-fos* in the cortex of WT and KO female mice. All data are presented as mean ± SEM $*p < .05$; $***p < .001$; $****p < .0001$. (G) Heatmap of average Log_2(fold change) expression data in hippocampus and cortex of WT and KO female mice based on real-time qPCR analyses. WT, $n = 12$; KO, $n = 12$