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Aplnr knockout mice display sex-specific changes in conditioned fear

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Abstract

The G-protein-coupled receptor APLNR and its ligands apelin and ELABELA/TODDLER/apela comprise the apelinergic system, a signaling pathway that is critical during development and physiological homeostasis. Targeted regulation of the receptor has been proposed to treat several important diseases including heart failure, pulmonary arterial hypertension and metabolic syndrome. The apelinergic system is widely expressed within the central nervous system (CNS). However, the role of this system in the CNS has not been completely elucidated.

Utilizing an *Aplnr* knockout mouse model, we report here results from tests of sensory ability, locomotion, reward preference, social preference, learning and memory, and anxiety. We find that knockout of *Aplnr* leads to significant effects on acoustic startle response and sex-specific effects on conditioned fear responses without significant changes in baseline anxiety. In particular, male *Aplnr* knockout mice display enhanced context- and cue-dependent fear responses. Our results complement previous reports that exogenous Apelin administration reduced conditioned fear and freezing responses in rodent models, and future studies will explore the therapeutic benefit of APLNR-targeted drugs in rodent models of PTSD.

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CRediT authorship contribution statement

Lucas T. Laudermilk: Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Kathryn M. Harper:** Investigation, Data curation, Writing - original draft, Writing - review & editing. **Sheryl S. Moy:** Investigation, Data curation, Supervision, Methodology. **Scott Runyon:** Funding acquisition. **Bin Zhou:** Resources. **Beverly Koller:** Investigation, Data curation. **Rangan Maitra:** Funding acquisition, Supervision, Project administration, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2020.113059.

Keywords

Apelin; receptor; behavior; conditioned fear; PTSD

1. Introduction

Aplnr codes for a class A G-protein-coupled receptor, APLNR, initially characterized in human brain tissue [1]. APLNR [2,3] and its cognate ligands [4] are well conserved between humans and rodent populations, which suggests an important role for APLNR-mediated signaling. The apelinergic system has demonstrated roles in cardiovascular function [5–9] and metabolic phenotypes [10–14], and efforts are underway to produce drugs that target APLNR in cardiometabolic and other disease contexts [15,16].

Among the peripheral tissues of adult mice, *Aplnr* expression is largely restricted to endothelial cells, with highest expression in sprouting vasculature [17–20]. In the central nervous system (CNS), however, APLNR is more widely expressed [21,22]. *Aplnr* mRNA expression has been noted in whole mouse brain [23,24] with strong localization to elements of the hypothalamic pituitary adrenal (HPA) axis [25]. Allen Mouse Brain Atlas in situ hybridization data suggest somewhat wider expression of *Aplnr*, including expression in the hippocampal formation ([26], © 2004 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: https://mouse.brain-map.org/). Despite this relatively broad CNS expression pattern, there is a paucity of behavioral characterization among rodent models lacking *Aplnr* expression. Difficulty in breeding *Aplnr* knockout animals is likely a contributing factor to this, as mice lacking functional APLNR suffer from abnormal embryonic development and significant lethality starting on embryonic (E) day E10.5 through E12.5 [27–29].

Despite a relative lack of broad behavioral characterization of *Aplnr* knockout models, there is mounting evidence in the literature that suggests a role for the apelinergic system in stress responses and the hypothalamic pituitary adrenal (HPA) axis. Studies utilizing *Aplnr* knockout mice revealed regulatory roles of APLNR in response to acute stressors, including LPS challenge, insulin-induced hypoglycemia, and forced swim test [30], and administration of apelin was shown to reduce the severity of conditioned fear and stress phenotypes in rats ([31], [32]). Intracerebroventricular injections of Apelin-13 in rats led to increased c-fos in the paraventricular nucleus of the hypothalamus (PVN), indicating neuronal activity, and increased the release of the stress hormone ACTH [33,34]. Apelin-13 administration also increased release of the stress hormones ACTH and AVP from hypothalamic explants *in vitro* [34], and *Aplnr* mRNA expression levels are elevated in the PVN following acute and chronic stress [35].

Much remains to be learned about the function of APLNR in the CNS, including further exploration of its role in stress and potential impacts on other behavioral phenotypes. This work is important for the general understanding of the apelinergic system in the CNS and for the potential of targeting this receptor for appropriate centrally mediated indications. In this study, we utilize *Aplnr* knockout mice in tests related to anxiety, activity, motor and sensory ability, social preference, learning and memory, and sucrose preference. Our results showed

that deficiency of *Aplnr* expression had significant, sex-dependent effects on fear-based responses in a conditioned fear test. Male KO mice had higher levels of freezing than WT in both context and cue-dependent paradigms. Histological analysis in male and female *Aplnr* reporter mice revealed differential expression of *Aplnr* in the dentate gyrus, a component of the hippocampus believed to play a role in retrieval, extinction, and establishment of contextualized fear (Bernier et al. 2017). We also observe a small but significant impact of *Aplnr* knockout on startle amplitude in an acoustic startle test. Together, our results lend further support to the role of *Aplnr* in conditioned fear and suggest therapeutic potential of APLNR-targeting drugs in PTSD and other psychiatric indications.

2. Methods

2.1. Mice

2.1.1. Knockout Mice—*Aplnr* knockout mice C57BL/6 N-Aplnr$^{tm1.1(KOMP)Vlcg}$/Mmucd (MMRRC Stock number: 046631-UCD) were obtained through the trans-NIH Knock-Out Mouse Project (KOMP) Repository to establish a breeding colony. Subjects for behavioral testing were 10 Aplnr$^{tm1.1(KOMP)Vlcg/tm1.1(KOMP)Vlcg}$ (referred to throughout as knockout or KO) mice and 10 wild-type (WT) mice, on a C57BL/6 N background. Animals were maintained in the same genetic background throughout the experiments. Each genotype group was comprised of 5 males and 5 females. Testing began when mice were 10–12 weeks of age.

All behavioral experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory animals.

2.1.2. Fluorescent Reporter Mice—*Aplnr*-driven Cre recombinase mice [19] were obtained from Shanghai Biomodels (Shanghai, China) and crossed to tdTomato reporter mice ([36]; Jackson Labs stock number 7909) obtained from the Jackson Laboratory (Bar Harbor, ME). Mice heterozygous for both the *Aplnr*-driven Cre and tdTomato reporter alleles were used for histological analysis.

All reporter mouse experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory animals, and protocols were approved by the Mispro Biotech Services institutional animal care and use committee (IACUC).

2.2. Elevated plus maze

Mice were evaluated for behavioral phenotypes using published methods [37]. A single five-minute trial was conducted for each mouse on the plus maze, which had two walled arms and two open arms. The maze was elevated 50 cm from the floor, the arms were 30 cm long, and the walls of the closed arms were 20 cm in height. Mice were placed on the center section (8 cm × 8 cm) and allowed to freely explore the maze. Measures were taken of time on, and number of entries into, the open and closed arms by a human observer.

2.3. Marble-burying assay for exploratory digging

Mice were individually placed in a Plexiglas cage located in a sound-attenuating chamber with ceiling light and fan. The cage contained a 5 cm layer of corncob bedding and 20 black glass marbles (14 mm diameter) arranged in an equidistant 5 × 4 grid on top of the bedding. Measures were taken of buried marbles (covered 2/3 or more with bedding) after a 30-minute testing period by a human observer.

2.4. Buried food test for olfactory function

Several days before the olfactory test, an unfamiliar food (Froot Loops, Kellogg Co., Battle Creek, MI) was placed overnight in the home cages of the mice to avoid food neophobia on the day of testing, and observations of consumption were taken to ensure that the novel food was palatable.

Sixteen to twenty hours before the test, all food was removed from the home cage. On the day of the test, each mouse was placed in a large, clean tub cage (46 cm L × 23.5 cm W × 20 cm H) containing a 3 cm layer of paper chip bedding and allowed to explore for five minutes. The animal was removed from the cage, and one Froot Loop was buried in the cage bedding. The animal was then returned to the cage for a 15-minute olfactory test. Measures were taken of latency to find the buried food reward.

2.5. Hot plate test for thermal sensitivity

Individual mice were placed in a tall plastic cylinder located on a hot plate with a surface heated to 55 °C (IITC Life Science, Inc., Woodland Hills, CA), and measures were taken of latency to respond. Reactions to the heated surface, including hindpaw lick, vocalization, or jumping, led to immediate removal from the heated surface. The maximum test length was 30 sec, to avoid paw injury.

2.6. Open field test

Exploratory activity in a novel environment was assessed by a one-hour trial in an open field chamber (41 cm × 41 cm × 30 cm) crossed by a grid of photobeams (VersaMax system, AccuScan Instruments, Columbus, OH). Counts were taken of the number of photobeams broken during the trial in five-minute intervals, with separate measures for locomotor activity (total distance traveled), rearing movements, and time spent in the center region.

2.7. Wire hang test

Mice were placed on the lid of a metal cage, and the lid was gently shaken to induce grip onto the wire grid. The lid was flipped over and latency to fall was recorded. The maximum trial length was 60 s.

2.8. Rotarod

Mice were tested for motor coordination on an accelerating rotarod (Ugo Basile, Stoelting Co., Wood Dale, IL). The first test consisted of three trials, with 45 seconds between each trial. Two additional trials were conducted 48 hours later to evaluate consolidation of motor learning. Revolutions per minute (rpm) was set at an initial value of 3, with a progressive

increase to a maximum of 30 rpm across five minutes (the maximum trial length). Measures were taken for latency to fall from the top of the rotating barrel.

2.9. Social approach in a three-chamber choice test

This procedure consisted of three 10-minute phases in a rectangular, three-chambered box fabricated from clear Plexiglas. The first phase was a habituation period in which the mouse was allowed to freely explore the three chambers. The second phase consisted of a sociability assay in which the mice were given a choice between proximity to an unfamiliar, sex-matched C57BL/6 J adult mouse ("stranger 1"), versus being alone. The stranger mouse was placed in one of two side chambers, enclosed in a small Plexiglas cage drilled with holes which allowed nose contact. An identical empty Plexiglas cage was placed in the second side chamber. The final phase of the assay was a test of social novelty in which mice were given a choice between the already-investigated stranger 1, versus a new unfamiliar mouse ("stranger 2"). An automated image tracking system (Noldus Ethovision) provided measures of time spent in and entries into each side of the social test box.

2.10. Novel object recognition test (NORT)

A three trial NORT was conducted based on previously published methods [38]. The chambers used for the open field test (41 cm × 41 cm × 30 cm) were modified to include hole-board floors with 16 equidistant holes, and the space underneath the holes was covered by screen mesh. Objects (small vinyl animal head toys) were fixed in place over one hole and paired with a novel odor (either one oat Cheerio (General Mills, Golden Valley, MN) or one corn Kix (General Mills) placed underneath the screen mesh below the object).

On Day 1, mice were given a 1-hr acclimation session in the hole-board chambers without any objects present. The 3-trial NORT was conducted on Day 2. Mice were first given a 10-min habituation period in the hole-board chamber. Five min following habituation, mice were given the first of three object-interaction trials (10 minutes each), with each trial separated by 5 min intervals. In the first trial, mice were introduced to 2 identical novel objects, each paired with the same odor. In the second trial, mice were re-exposed to the same object/odor combinations (now familiar). In the third trial, one familiar object was replaced with a novel object, paired with a novel odor. Measures were taken of time spent in proximity to each object (within 6.5 cm) and number of entries into each object zone by an automated photo-beam system (Versamax, Accuscan). Preference for the unfamiliar object was used as an index of object recognition and memory. The object designated as familiar or unfamiliar and side of the chamber for presentation were counterbalanced across subjects.

2.11. Acoustic startle test

Mice were tested for acoustic startle reflex and prepulse inhibition using an SR-Lab Startle Response system (San Diego Instruments SR-Lab system).

Mice were placed into individual small Plexiglas cylinders within larger, sound-attenuating chambers. Each cylinder was seated upon a piezoelectric transducer, which quantified vibrations. Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter (San Diego Instruments). Each session began

with a five-minute habituation period, followed by 42 trials (7 of each type): no-stimulus trials, trials with the acoustic startle stimulus (40 msec; 120 dB) alone, and trials in which a prepulse stimulus (20 msec; either 74, 78, 82, 86, or 90 dB) occurred 100 ms before the onset of the startle stimulus. Measures were taken of the startle amplitude for each trial across a 65-msec sampling window, and an overall analysis was performed for each subject's data for levels of prepulse inhibition at each prepulse sound level (calculated as 100 - [(response amplitude for prepulse stimulus and startle stimulus together / response amplitude for startle stimulus alone) × 100]).

2.12. Conditioned fear

Mice were evaluated for conditioned fear using the Near-Infrared image tracking system from MED Associates (MED Associates, Burlington, VT), using published methods [39]. The conditioned response is immobility or freezing, a species-specific defense reaction to a threatening situation. The procedure had the following phases:

2.12.1. Training—On Day 1, mice were placed in the test chambers, contained in sound-attenuating boxes. The mice were allowed to explore the novel chambers for 2 minutes before presentation of a 30-sec tone (80 dB), which co-terminated with a 2-sec scrambled foot shock (0.4 mA). Mice received 2 additional shock-tone pairings, with 80 sec between each pairing, and were removed from the test chambers 80 sec following the third shock.

2.12.2. Context- and cue-dependent learning—On Day 2, mice were placed back into the original conditioning chamber for a test of contextual learning. Immobility was determined across a 5-min session. On Day 3, mice were evaluated for associative learning to the auditory cue in another 5-min session. The conditioning chambers were modified using a Plexiglas insert to change the wall and floor surface, and a novel odor (dilute vanilla flavoring) was added to the sound-attenuating box. Mice were placed in the modified chamber and allowed to explore. After 2 min, the acoustic stimulus (an 80 dB tone) was presented for a 3-min period. Levels of freezing before and during the stimulus were obtained by the image tracking system.

Second tests for context- and cue- dependent learning were conducted 2 weeks following the first tests.

2.13. Two-bottle choice test for sucrose preference

Before beginning the sucrose preference test, mice were given a 1-week acclimation to single housing and the presentation of water in two bottles. The following week, mice were presented with a free choice between water and a 1% sucrose solution. In the third week, mice had a free choice between water and 0.5% sucrose solution.

The amount of fluid in each bottle was measured daily, across four days of testing per week. To prevent effects of side preference, the position of the bottles was switched each day. An empty cage with two bottles was used to obtain control measures of non-drinking fluid decreases. The amount of fluid lost from the control bottles was averaged for each day and subtracted from all measurements of drinking as a correction factor.

2.14. Histology

Mice heterozygous for both the *Aplnr*-driven Cre [19] and tdTomato reporter [36] alleles described above were used for histological analysis. At 13 weeks of age, mice were dosed with 75 mg/kg tamoxifen intra-peritoneally once daily for 5 days. Seven days following the final tamoxifen injection, brains were harvested.

Immediately following harvest, tissues were placed in 10% neutral buffered formalin for 2 hours at 4 °C. Next, brains were placed in a PBS solution with 30% sucrose at 4 °C overnight for cryoprotection. Tissues were ultimately frozen in OCT, and 10 μm sections were taken and stained with DAPI. Fluorescent images were captured with a 10X objective using DAPI and Texas Red filters on an Olympus DP70 camera system mounted to an Olympus IX51 fluorescence microscope. Images were captured with identical settings and conditions across mice. Overlays of DAPI and Texas Red images were made using the Image J merge channels function.

2.15. Statistics

For each procedure, measures were taken by an observer blind to mouse genotype. Data were first analyzed using one-way or repeated measures Analysis of Variance (ANOVA), with the factor genotype. Separate analyses were conducted for male and female data for weight measures, due to significant body size differences. In addition, separate analyses were conducted for conditioned fear due to sex-dependent genotype effects. Fisher's protected least-significant difference (PLSD) tests were used for comparing group means only when a significant F value was determined. Within-genotype comparisons were conducted to determine side preference in the social approach test. For all comparisons, significance was set at $p < 0.05$.

3. Results

Mice underwent a battery of behavioral tests (Fig. 1), with testing order planned so that more stressful procedures (conditioned fear) and single housing (2-bottle choice tests) occurred at the end of the study. *Aplnr* knockout did not lead to overt changes in health or motor coordination. The WT and KO cohorts had similar weights across the testing regimen (Fig. 2A–B). In a wire-hang test for grip strength, all but one mouse was able was able to remain suspended from a cage lid for the maximum trial time of 60 sec (data not shown).

Anxiety-like behaviors were initially assessed through movement tracking in an elevated plus maze. Mice of each genotype spent similar percent time in the open arms of the plus maze and had comparable total numbers of entries (Table 1). WT and KO mice also displayed similar levels of locomotion and exploratory activity in an open field test (Fig. 2, C–D), with each genotype spending a comparable amount of time in the center region of the open field (Table 1, data summed across 1 hour). A marble burying assay was performed to additionally assess anxiety-like or compulsive behavior, and no significant difference was noted in the performance of WT and KO mice in this test (Table 1). Motor coordination and motor learning were assessed in an accelerating rotarod assay, and both genotypes displayed similar good proficiency (Fig. 2, E).

Mice were evaluated for the effects of *Aplnr* deficiency on social preference during a 3-chamber choice procedure. In a test for sociability (Supplemental Fig. 1A), both genotypes had similar robust preference for the side chamber containing the stranger mouse, versus an empty cage [within-genotype analyses following significant effect of side, $F(1,18) = 45.79$, $p < 0.0001$]. In a subsequent test for social novelty preference (Supplemental Fig. 1B), both WT and KO mice demonstrated a shift in preference to a newly-introduced stranger [significant effect of side, $F(1,18) = 14.08$, $p = 0.0015$]. As shown in Supplemental Fig. 1C, both genotypes made more entries into the side with the stranger mouse, versus the empty cage [effect of side, $F(1,18) = 16.42$, $p = 0.0007$]. This side preference for entries was not observed in the social novelty test (Supplemental Fig. 1D).

Novel object recognition tests were based on the one used by Spanos and colleagues (2012). Small objects were paired with a novel odor, and a three-trial test was performed. An initial pilot study with C57BL/6 mice showed generally low levels of object investigation (data not shown). The results from WT and *Aplnr* KO mice indicated comparable performance in the NORT (Supplemental Fig. 2), with neither group demonstrating strong novel object recognition.

The effect of *Aplnr* knockout on sensory function and reactivity to environmental stimuli was tested. Olfactory function was assessed through a buried food test, and no significant differences in latency to finding buried food were observed (Table 1). Similarly, *Aplnr* knockout mice displayed no significant impacts on thermal sensitivity, as assayed in latency to respond in a hot plate test (Table 1). An acoustic startle test was used to assess auditory function, reactivity to environmental stimuli, and sensorimotor gating. A repeated measures ANOVA revealed a significant genotype × decibel interaction for startle response amplitudes [$F(6,108) = 2.29$, $p = 0.0406$]. Examination of the data suggests the KO mice had lower startle amplitudes than WT, although these differences were only individually significant at one sound level (Fig. 3A). Both genotypes demonstrated similar, robust prepulse inhibition (Fig. 3B).

Two bottle choice tests were conducted to assess preference for sucrose over water, and no differences were observed between the genotypes (Supplemental Fig. 3). Notably, both groups demonstrated a preference for the sucrose solution, similar to a previous report with C57BL/6 J mice [40].

Mice were tested for conditioned fear, and an initial examination of the data revealed clearly divergent results between the male and female groups. This was confirmed by a repeated measures ANOVA for the first cue test, which revealed a highly significant main effect of sex [$F(1,16) = 9.65$, $p = 0.0068$] and sex × time interaction [$F(4,64) = 4.89$, $p = 0.0017$]. Therefore, data from the conditioned fear tests were analyzed separately for males and females.

In the male groups, the KO mice had higher levels of freezing than WT in the first context test [genotype × time interaction, $F(4,32) = 3.06$, $p = 0.0304$] (Fig. 4A). This genotype effect was even stronger in the second context test, conducted two weeks later [main effect of genotype, $F(1,8) = 6.05$, $p = 0.0394$; genotype × time interaction, $F(4,32) = 5.04$, $p =$

0.0029] (Fig. 4B). In contrast, the WT and KO female mice had similar levels of freezing in the first context test (Fig. 4C). In the second context test, a repeated measures ANOVA indicated a significant genotype × time interaction in the female groups [$F(4,32) = 2.74$, $p = 0.0458$]. However, post-hoc comparisons failed to reveal additional significant differences (Fig. 4D). Enhanced conditioned fear responses were also observed in the male KO mice during the tests for cue-dependent learning [first test, main effect of genotype, $F(1,8) = 8.53$, $p = 0.0193$; and second test, main effect of genotype, $F(1,8) = 6.68$, $p = 0.0324$] (Fig. 5A, B). These significant genotype differences were not found in the female groups (Fig. 5C, D).

Overall, there were no genotype effects on freezing during conditioned fear training. In the male groups, WT and KO mice had similar low levels of freezing at the beginning of the session, and progressive increases in freezing with each tone-shock presentation (Supplemental Fig. 4A). Activity was measured during training to determine responsivity to the unconditioned stimulus (the foot shock). Both male groups had higher levels of activity during the 2-s periods of shock, in comparison to either the preceding or following 2-s periods (Supplemental Fig. 4B). A repeated measures ANOVA for the activity data indicated a significant genotype × interval interaction in the male groups [$F(8,64) = 2.49$, $p = 0.0202$]. Post-hoc comparisons did not reveal any further significant differences, although a trend for lower activity in the KO group was observed during the first shock ($p = 0.0769$). In the female groups, levels of freezing were similar in both WT and KO mice (Supplemental Fig. 4C). However, the female KO mice had significantly higher activity than WT during the third shock presentation [$F(8,64) = 2.4$, $p = 0.0249$] (Supplemental Fig. 4D), indicating a sex-dependent change in response to the aversive stimulus.

In addition to levels of freezing, we evaluated general activity during the conditioned fear tests. As shown in Supplemental Fig. 5, the male KO mice had lower activity than WT in both context tests [Test 1, main effect of genotype, $F(1,8) = 7.6$, $p = 0.0248$; Test 2, main effect of genotype, $F(1,8) = 11.36$, $p = 0.0098$; genotype × time interaction, $F(4,32) = 3.33$, $p = 0.0218$]. No significant genotype differences were found in the female groups. A similar pattern was observed in the tests for cue- dependent learning (Supplemental Fig. 6). In the male groups, the KO mice had decreased activity, in comparison to WT, in both cue tests [main effect of genotype, Test 1, $F(1,8) = 9.13$, $p = 0.0165$; Test 2, $F(1,8) = 10.57$, $p = 0.0117$]. In contrast, the female KO and WT mice had comparable levels of activity in both tests.

As a follow-up to these sex-specific conditioned fear phenotypes, histological brain sections from male and female *Aplnr* reporter mice were examined for sites of differential expression. Briefly, a tamoxifen-inducible *Aplnr*-driven Cre strain [19] was crossed to a tdTomato reporter (Jackson Laboratory Strain 7909) and brain sections were taken from male and female mice. Differential *Aplnr* expression was noted in the dentate gyrus, with males (Fig. 6A, B) having lower expression than females (Fig. 6 C, D). Females also appeared to have generally higher expression in surrounding tissues in which both sexes show signal.

4. Discussion

Wildtype and *Aplnr* knockout mice were evaluated in tests related to anxiety, activity, motor and sensory ability, social preference, thermal sensitivity, learning and memory, and sucrose preference. Our results showed that genetic ablation of *Aplnr* did not lead to general changes in behavior or reward preference but did have a significant impact on acoustic startle response and significant, sex-dependent effects on fear-based responses in a conditioned fear test. Our results show a significant difference in freezing response between WT and KO males in both the context and cue-dependent tests, and these genotype differences in fear response were not observed in the female groups.

The differential outcomes in male and female KO mice could be related to sex differences in the neurocircuitry engaged during fear learning [41,42] or sex-specific *Aplnr* expression patterns, as described in the present report. The hippocampus is critical for forming the memory of environmental context, and the dentate gyrus, part of the hippocampal formation, is suggested to play a role in retrieval, extinction, and establishment of contextualized fear [43]. Our results demonstrate differential expression of *Aplnr* between male and female mice in the dentate gyrus.

Our results may indicate a potential benefit of APLNR-targeted therapeutics in post-traumatic stress disorder (PTSD), a condition for which effective pharmacological treatments are lacking [44] and perhaps other psychiatric indications. Rodent paradigms of conditioned fear have proven to be a useful model for PTSD, as PTSD is a memory disorder closely tied to fear conditioning and extinction [45]. Our findings indicate that deletion of *Aplnr* led to persistent increases in conditioned fear for both context and cue-based learning in male KO mice, without significant effects in females. In humans, women have a significantly higher incidence of PTSD due to a number of sex- and gender-specific factors, including levels of gonad hormones (Christiansen and Berke, 2020). Previous reports in rodent models suggest that the apelinergic system regulates serum levels of testosterone and luteinizing hormone [46,47], though potential impacts of this on development of conditioned fear responses among our mice will require further studies. Other reports have implicated the apelin receptor in behaviors related to stress, including conditioned fear in rats. Central administration of apelin-13 peptide before conditioned fear training led to significant decreases in freezing in a context test [31], an effect opposite to and supportive of the phenotype in our *Aplnr* KO mice. Similarly, apelin-13 has been shown to reverse stress-induced immobility in a forced swim test [32].

Differences in *Aplnr* expression level among structures of the brain that modulate stress responses and memory may begin to explain the sex-specific conditioned fear results noted in our results. Numerous studies demonstrate dose-dependent effects of targeting the apelinergic system [5,8,48–51], and differential responses with independent signaling pathways have been noted in response to high and low doses of apelin [52]. Loss of APLNR function may produce differential effects in groups of mice with differing levels of basal APLNR expression in relevant tissues. Notably, sex-specific results in stress response were previously reported in an LPS-induced acute stress model among *Aplnr* WT and KO mice, and those results show a significant impact among males that is not noted in females [30].

Additional studies may be required to delineate the underlying signaling pathways that lead to our differential conditioned fear responses. The apelinergic system has been shown to regulate phosphorylation of ERK1/2 [49,50,53–55] and to activate the PI3K/Akt pathway [53,56–59]. Differences in the phosphorylation of ERK in the hippocampus is suggested to underlie differential conditioned fear responses in male and female rats [60], and ERK2 phosphorylation in the hippocampus may impact sex differences in fear extinction among rats [61]. Similarly, the PI3K/Akt pathway is involved in the formation, retrieval, and extinction of contextual fear memory [62] and long-term retention of trace fear in rats [63]. The apelin receptor also has a role in the regulation of angiotensin II signaling, which has been implicated in learned fear responses. Activation of the angiotensin II receptor in the amygdala decreases fear responses in male C57BL/6 J mice [64]; further, treatment with angiotensin II signaling blockers has been proposed as a possible treatment for PTSD, based on effects on learned fear [65]. Our results may represent a downstream effect of one or more of these pathways, and future studies will more thoroughly explore these molecular phenotypes.

We report here a novel sex-specific impact of *Aplnr* knockout on conditioned fear and a significant impact on acoustic startle response. Our results lend additional support to the therapeutic promise of the apelinergic system in models of stress and PTSD, and future studies will explore the benefits of APLNR-targeting drugs in these paradigms and the pathways that underlie our sex-specific effects. PTSD is a complex, heterogeneous disorder, and no single animal model captures a complete set of clinically relevant phenotypes [66]. A more complete characterization of the role of *Aplnr* in PTSD may involve additional models that capture disease phenotypes not addressed in the present study, and future studies will explore the molecular phenotypes that underlie the behavioral phenotypes herein. Additional assessment of baseline anxiety measures after fear conditioning in *Aplnr* knockout mice may lend further insights into the role of the apelinergic system in anxiety disorders related to persistent effects of exposure to stressful and aversive events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Aplnr/APLNR	Apelin receptor
PTSD	Post Traumatic Stress Disorder
HPA	hypothalamic pituitary adrenal
LPS	Lipopolysaccharide
PVN	paraventricular nucleus of the hypothalamus
ACTH	Adrenocorticotropic hormone
AVP	Vasopressin/arginine vasopressin
NORT	Novel object recognition test
ASR	acoustic startle response
PPI	pre-pulse inhibition

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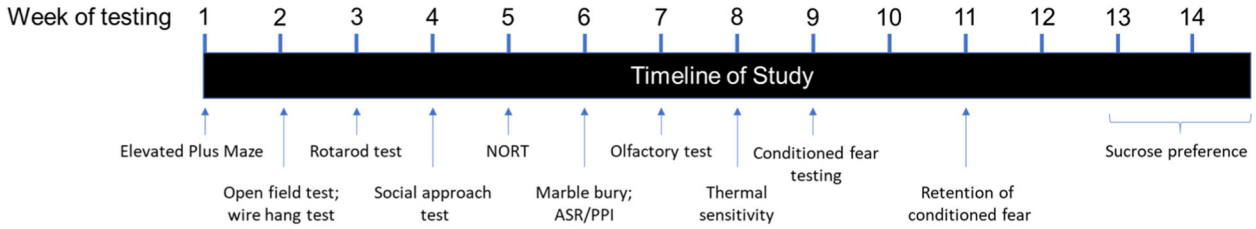

Fig. 1. Behavioral testing timeline.

Mice (10–12 weeks in age at the beginning of the study) underwent a battery of behavioral tests, with order planned so that more stressful procedures (conditioned fear) and single housing (2-bottle choice test for sucrose preference) occurred at the end of the study. NORT = novel object recognition test; ASR = acoustic startle response; PPI = pre-pulse inhibition.

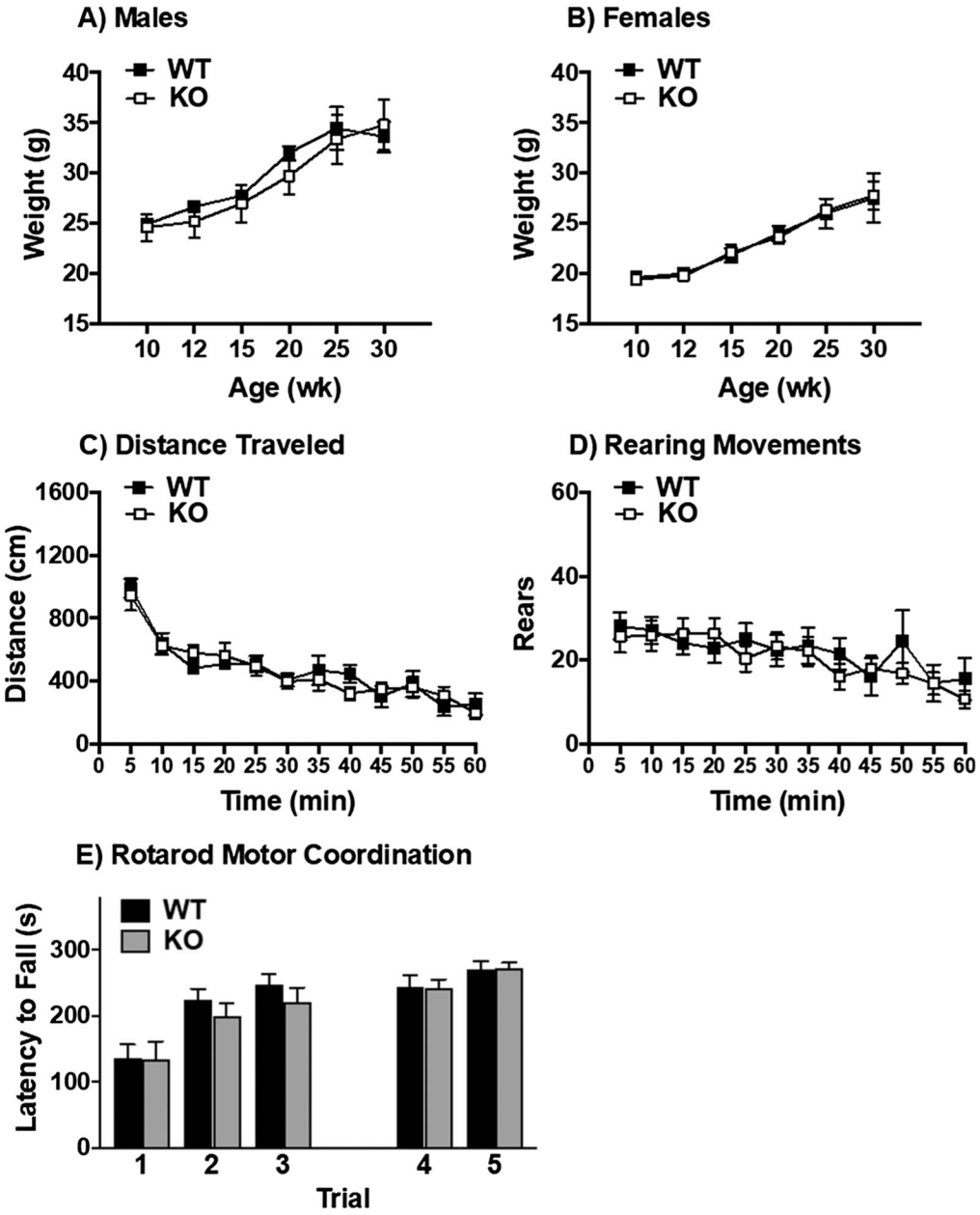

Fig. 2. No genotype differences in body weight, activity, or motor coordination between Aplnr wild-type and knockout mice across the behavior study.

Data are means ± SEM) for each group. A, B) Age is approximate number of weeks. C, D) Locomotor activity and rearing movements during a one-hour test in a novel open field. E) Latency to fall from an accelerating rotarod. Maximum trial length was 300 sec. Trials 4 and 5 were given 48 hours after the first three trials.

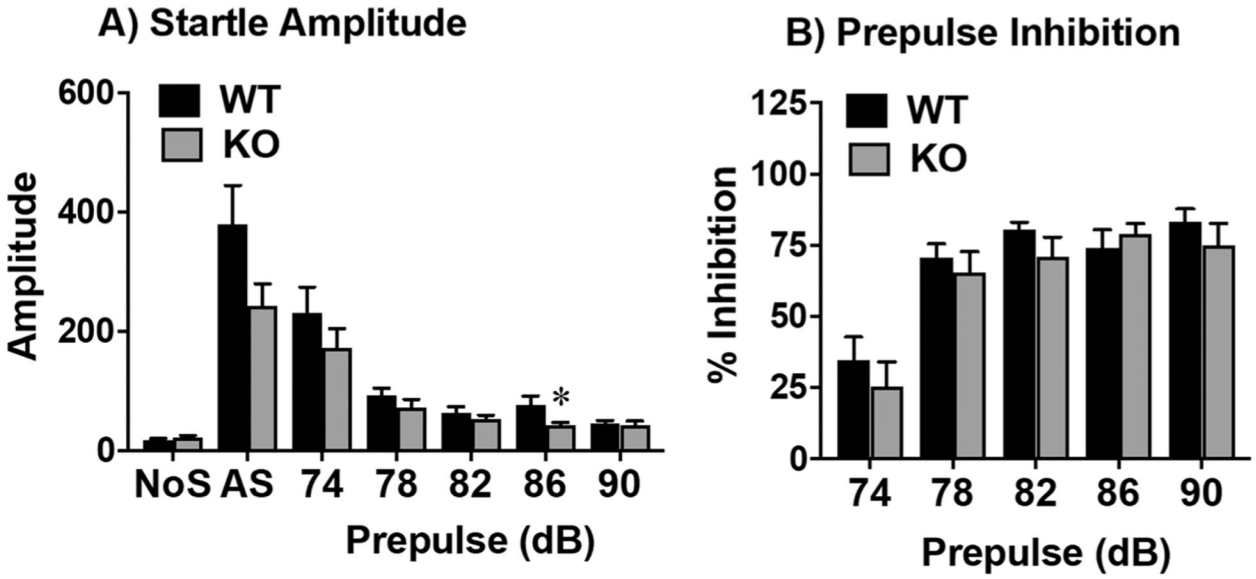

Fig. 3. Magnitude of acoustic startle responses and prepulse inhibition.

Data shown are means (+ SEM) for each group. Trials included no stimulus (No S) trials and acoustic startle stimulus (AS; 120 dB) alone trials. *$p < 0.05$.

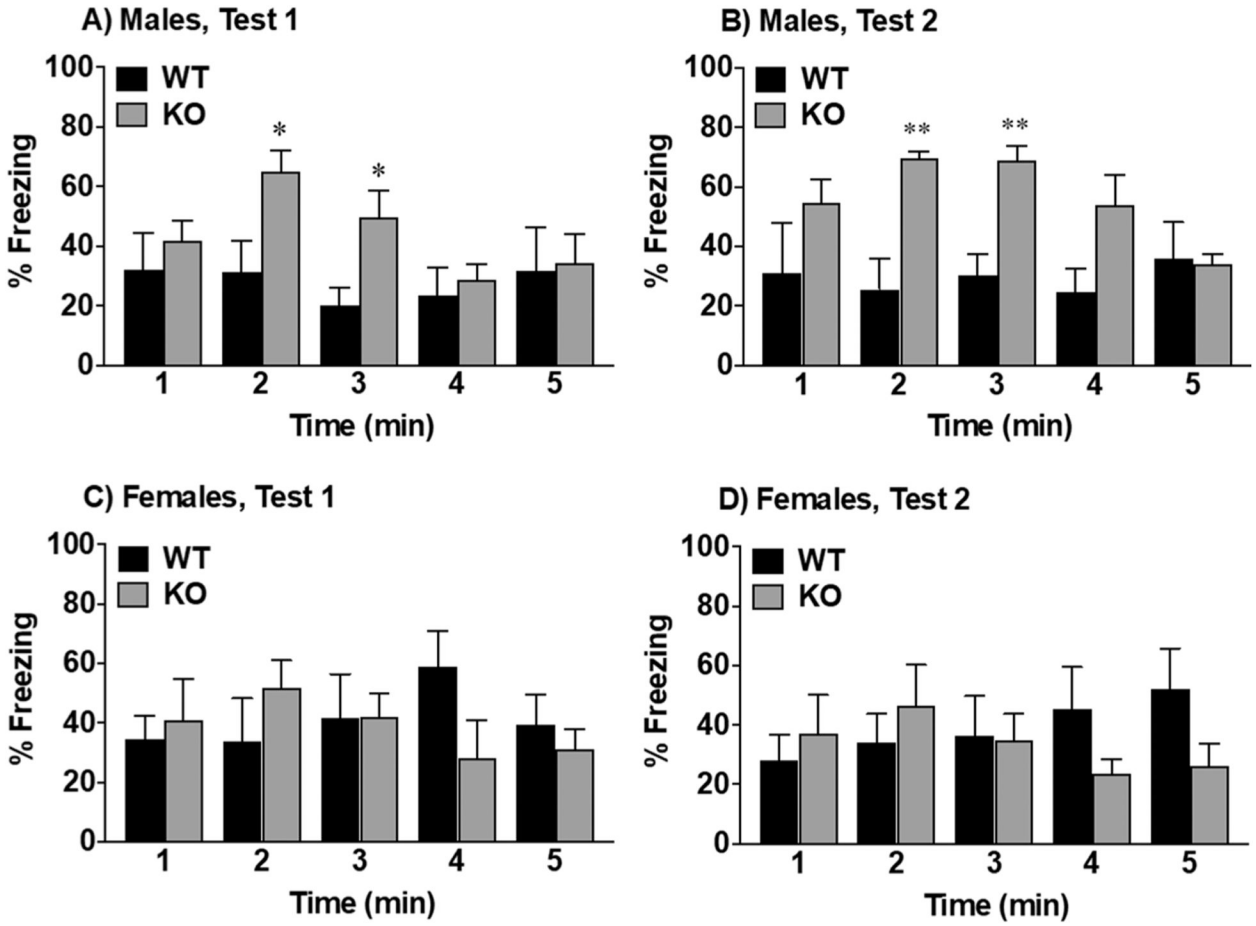

Fig. 4. Increased freezing responses in male Aplnr KO mice during tests for contextual learning.
Data are means (+ SEM) for a 5-min test. Test 1 was conducted 24 hr after training. The second test for contextual learning (Test 2) was conducted 2 weeks following Test 1. *$p < 0.05$; **$p < 0.01$.

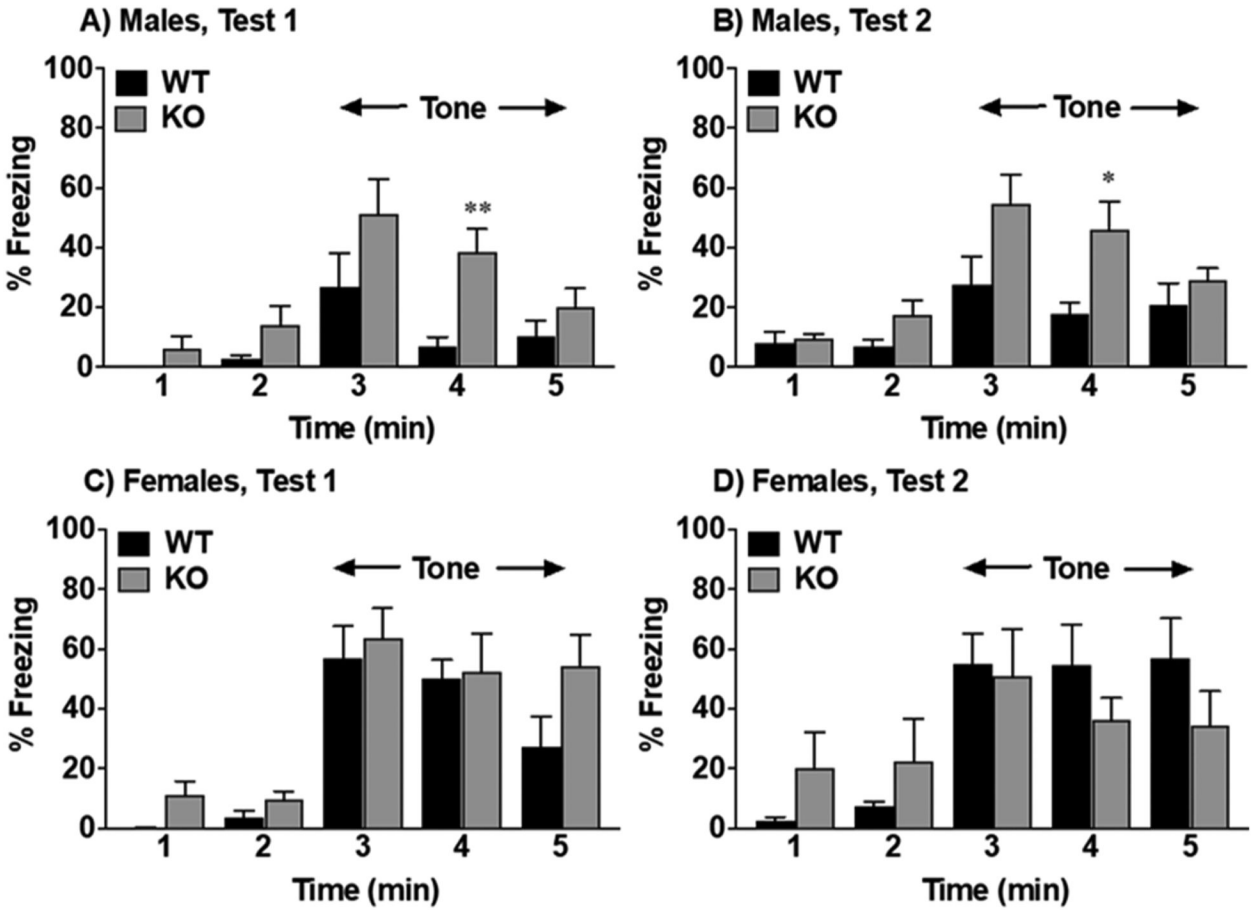

Fig. 5. Increased freezing responses in male *Aplnr* KO mice during tests for cue-dependent learning.

Data are means (+ SEM) for a 5-min test. Test 1 was conducted 48 hr after training. The second test for cue learning (Test 2) was conducted 2 weeks following Test 1. During each test, a 3-min, 80 decibel acoustic stimulus was presented 2 min after mice were placed in modified conditioned fear chambers. *$p < 0.05$; **$p < 0.01$.

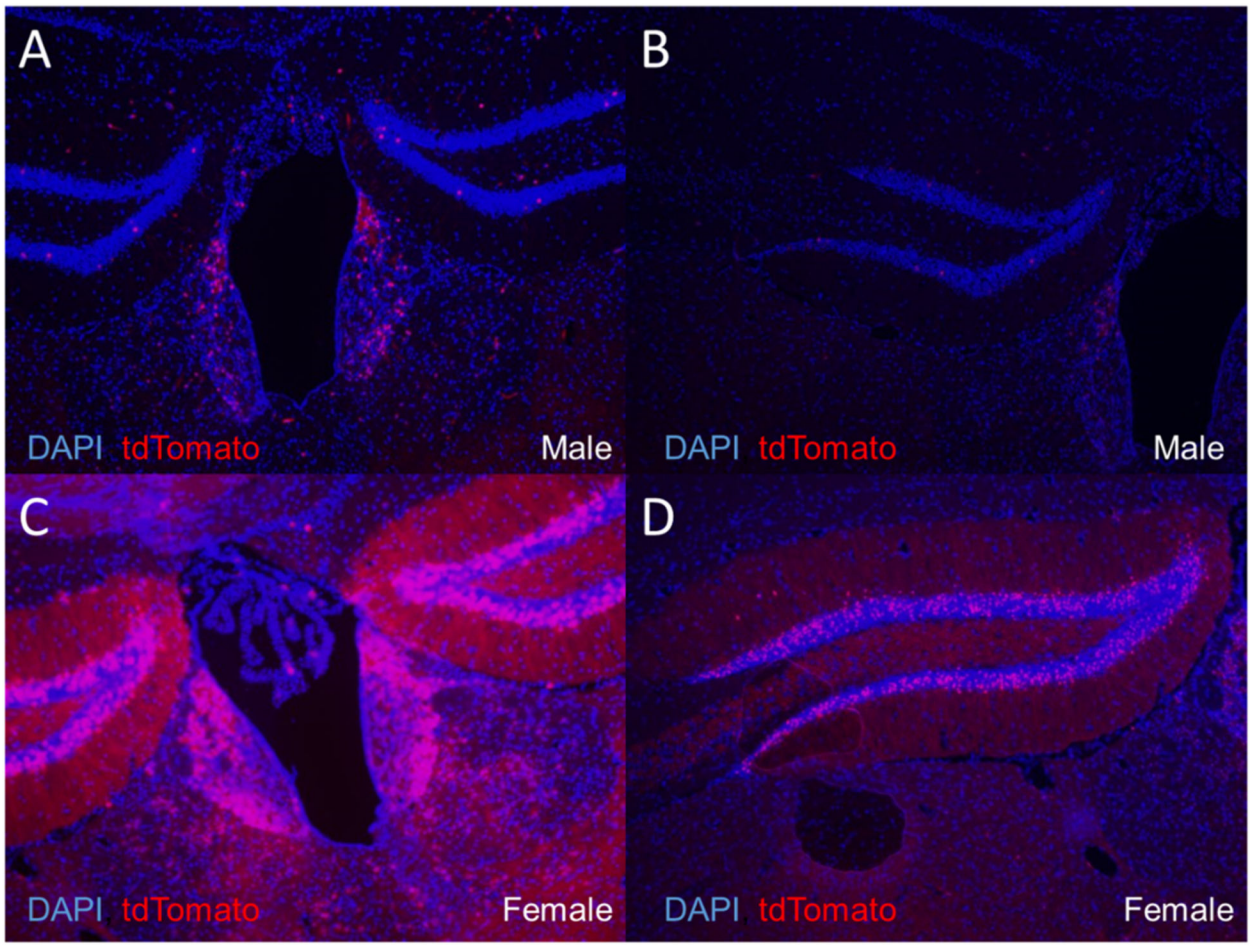

Fig. 6. Differential expression of Aplnr in the dentate gyrus between male and female mice.

Aplnr-driven Cre recombinase mice were crossed to a tdTomato cre reporter strain (Jackson Labs Strain 7909). A–B: dentate gyrus of male mice. C–D: dentate gyrus of female mice. Tissues were frozen in OCT, and 10 μm sections were taken and stained with DAPI. Fluorescent images were captured at 10X magnification using a Texas Red filter on an Olympus DP70 camera system mounted to an Olympus IX51 fluorescence microscope. Images were captured with identical settings and conditions across mice.

Table 1

Aplnr knockout animals do not significantly differ from wildtype in performance in the Elevated plus maze, open field test, marble-burying assay, olfactory test, or hot plate assay.

Test	Readout	WT	KO
Elevated plus maze (5 min)	Percent open arm time	19 ± 4	18 ± 3
	Percent open arm entries	32 ± 3	30 ± 3
	Total number of entries	20 ± 2	21 ± 2
Open field test (1 hour)	Total time in center region (s)	250 ± 49	203 ± 38
Marble-burying assay	Number buried in 30 min.	13 ± 1.2	15 ± 0.6
Olfactory test	Latency to find buried food (s)	38 ± 10	40 ± 9
	Subjects finding buried food	100%	100%
Hot plate assay	Latency to respond (s)	20 ± 2	17 ± 1