Male mice lacking *Magel2* in hypothalamic POMC neurons that innervate the medial amygdala exhibit increased stress-induced anxiety-like behavior and display reduced spatial reward learning.

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Abstract

Prader-Willi syndrome (PWS) results from a lack of expression in several paternally inherited, imprinted contiguous genes. Among the genes inactivated in PWS, the *Magel2* gene is considered a significant contributor to the etiology of the syndrome. The loss of the *Magel2* gene causes abnormalities in growth and fertility and increased adiposity with altered metabolism in adulthood, which aligns with some of the pathologies observed in PWS. Here we investigate the role of the *Magel2* gene, particularly in hypothalamic POMC neurons innervating the medial amygdala (MeA), in the behavioral phenotypes associated with Prader-Willi Syndrome (PWS). Both male and female mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons display no alterations in anxiety-like behavior during the open filed test, light/dark test, and elevated plus maze test in the absence of exposure to acute stress. However, male mice with a *Magel2* gene deletion in these specific neurons exhibit increased stress-induced anxiety-like behavior and reduce motivation/spatial learning, while female mice do not show these behavioral changes. Our results suggest that the *Magel2* gene in ARC^{Pomc} neurons, especially in males, is involved in stress-induced anxiety-like behavior and spatial learning deficits, offering new insights into the neurological basis of PWS beyond the commonly recognized hyperphagia and obesity. With the recent approval of a novel treatment for hyperphagia in PWS by the FDA, elucidating the cellular mechanisms by which MAGEL2 in ARC^{Pomc} neurons innervating the MeA regulates emotional behaviors may facilitate the development of new therapeutic strategies for addressing mental illness in individuals with PWS.

Keywords: stress, melanocortin, hypothalamus, anxiety, memory, obesity

Introduction

Prader-Willi Syndrome (PWS) is a neurogenetic disorder that results from the loss of several paternally inherited, imprinted contiguous genes in the human 15q11-q13 and mouse 7C regions [1; 2; 3; 4; 5]. One of the defining characteristics of PWS is an insatiable appetite for food, which can cause substantial weight gain in children during the early stages of development [6]. In addition to hyperphagia and obesity, individuals with PWS are also highly susceptible to mental health disorders [7]. Key phenotypic PWS behaviors include temper outbursts, anxiety, obsessive-compulsive behaviors, and social cognition deficits [7; 8]. These behaviors have a significant impact on the daily functioning and quality of life for individuals with PWS and their families [7]. Hence, it is necessary to determine which imprinted gene is responsible for these mental disorders in PWS.

Among the genes inactivated in PWS, the *Magel2* gene is highly expressed in the hypothalamus [9; 10; 11; 12]. Amongst hypothalamic neurons, pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (ARC) play a critical role in controlling energy balance and mood disorders [13]. Intriguingly, *Magel2*-deficient mice have fewer ARC^{Pomc} neurons [14] and lower levels of α-melanocyte-stimulating hormone (α-MSH) derived from POMC [15]. ARC^{Pomc} axonal projections to the paraventricular and dorsomedial hypothalamus are significantly reduced by *Magel2* deletion [11]. Furthermore, the basal spontaneous activity of ARC^{Pomc} neurons is lower in *Magel2*-null mice than in controls [16]. These prior studies support that MAGEL2 is critical for developing and maintaining ARC^{Pomc} neuronal circuits. In our prior studies [17; 18], we showed that optogenetic stimulation of the ARC^{Pomc} neurons -> the medial amygdala (MeA) pathway reduced acute food intake. More importantly, we also found that deleting the *Magel2* gene exclusively in ARC^{Pomc} neurons that project to the MeA inhibited the onset of diet-induced obesity (DIO) in both male and female mice during a high-fat diet (HFD) feeding, which seems to be linked to an increase in physical activity [18]. This finding contradicts the expectation that the loss of function of the PWS imprinted genes leads to the development of obesity, as observed in individuals with PWS. However, mice deficient in the paternal allele of *Magel2* exhibited no significant difference in body weight compared to the control group when fed a normal chow and did not develop DIO when maintained on HFD [19]. Hence, these prior findings suggest that the *Magel2* gene is unlikely to play a major role in regulating feeding behavior in PWS.

Acute emotional stressors, such as restraint and forced swim, trigger the expression of *c-fos* mRNA, a marker of neuronal activity, in most ARC^{Pomc} neurons as well as in the MeA [20]. The increased activity of ARC^{Pomc} neurons induces anxiety-like behavior [20]. Given that anxiety is a prominent phenotypic behavior in PWS [7], it is conceivable that the *Magel2* gene in ARC^{Pomc} neurons, which project to the MeA, might be involved in the onset of anxiety rather than regulating feeding behavior in individuals with PWS. Hence, the objective of this study was to investigate the behavioral

implications of the dysfunction of the *Magel2* gene in ARC^{POMC} neurons that innervate the MeA and to provide new and valuable insights into the role of central MAGEL2 in the regulation of emotional behaviors.

Materials and Methods

Ethics statement

All mouse care and experimental procedures were approved by the Institutional Animal Care Research Advisory Committee of the Albert Einstein College of Medicine and were performed following the guidelines described in the NIH guide for the care and use of laboratory animals. Stereotaxic surgery and viral injections were performed under isoflurane anesthesia.

Animals

Rosa26-floxed-stop Cas9-eGFP (Rosa26-$Cas9^f$, stock #026175) was purchased from the Jackson Laboratory. Mice were housed in cages at a controlled temperature (22 °C) with a constant humidity (40-60%) and a 12:12h light-dark cycle. Mice were fed a standard chow diet with *ad libitum* access to water.

Viral injection

Retrograde AAV-nPE-Cre-WPRE viruses were made at Applied Biological Materials, Inc (ABM). To knock down the *Magel2* gene in ARC^{POMC} neurons innervating the MeA, retrograde AAV-PGK-loxp-tdTomato-loxp-U6-mouse *Magel2* sgRNA viruses were also generated at ABM. The sgRNAs target the consensus coding sequence 52264.1 region of mouse *Magel2* (NM_013779.2). The sequences of *Magel2* sgRNA were the following: (1) sgRNA1: cgcagctaagtacgaatctg, (2) sgRNA2: gtagggcggctatggactgc, and (3) sgRNA3: atggtccaggctccaccgct.

6 and 7-week-old mice (males, ~ 20 g and females, ~18 g) will be anesthetized deeply with 3% isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments). A deep level of anesthesia will be maintained throughout the surgical procedure. Under isoflurane anesthesia (2%), a total of 300 nl (150+150 nl from each) of retrograde AAV-PGK-loxp-tdTomato-loxp-U6-mouse *Magel2* sgRNA viruses (titer, 1 X 10^{12} GC/ml) along with retrograde AAV-nPE-Cre-WPRE (titer, 1.2 X 10^{13} GC/ml) were bilaterally injected into the MeA of Rosa26-$Cas9^f$ mice (AP, -1.58 mm; ML, ± 2 mm; DV, -5 mm). A 2.5 ul Hamilton syringe, having a 33G needle, was used to inject a volume of 50 nl viruses every 5 min. The Hamilton syringe tip was left in place for 10 min after delivering viruses in order to prevent the backflow of the viral solution up the needle track.

Immunostaining

Mice were anesthetized with isoflurane (3%) and transcardially perfused with 4% paraformaldehyde. brain samples were post-fixed in 4% paraformaldehyde overnight in a cold room and then in 30% sucrose the following day. Tissues were sectioned using a cryostat at 20 μm. The sections were incubated with 0.3% Triton X-100 at room temperature (RT) for 30 min, and then blocked in PBS buffer containing 5% donkey serum, 2% bovine serum albumin and 0.15% Triton X-100 for 1hr at RT and then incubated with chicken anti-GFP (1:500, 600-901-2155, Rockland), rabbit anti-POMC (1:1000, H-029-30, Phoenix biotech), rabbit anti-RFP (1:500, 600-401-379, Rockland) and rabbit anti-MAGEL2 (1:2000, a gift from Dr. Tacer) antibodies for overnight at a cold room, and then sections were washed three times in PBS and incubated with Alexa 488 anti-chicken IgG (1:1000, 703-545-155, Jackson immunoresearch), Alexa 594 anti-rabbit IgG (1:1000, 711-585-152, Jackson immunoresearch) for 3hr at RT. After washing, the sections were stained with DAPI and mounted using VECTASHIELD medium. Images were acquired using a Leica SP8 confocal microscope.

Animal behavior tests

Open field exploration test: Mice were placed in the center of a chamber measured 40 cm (length) x 40 cm (width) x 40 cm (height) (Maze Engineers) and allowed to explore the chamber for 5 min freely. The center region was designated as 20 x 20 cm^2. The EthoVision XT video tracking system (Nodulus) was used to record the session and analyze the behavior, movement, and activity of animals. We wiped the chamber with 95% ethanol before subsequent tests to remove any scent clues [21]. The total distance traveled and time spent in the center and outer zones of the chamber were measured.

Elevated plus maze: Mice were placed in the central area of the maze consisting of four arms in the shape of a plus sign elevated approximately 1 m from the ground (Maze Engineers). Mice were allowed to explore the maze for 5 min freely. The session was recorded, and the number of arm entries and the amount of time spent in the open and closed arms were analyzed with the EthoVision XT video tracking system.

Light-dark box test. The box consists of two chambers, one light and one dark (Maze Engineers). Mice were placed in the center of light chamber and allowed to explore the chambers for 5 min freely. Time spent in each compartment and crossings from one compartment to the other were measured with the video tracking system.

Conditioned place preference test: The experiment involved four phases: habituation phase, pre-conditioning test, conditioning phase, post-conditioning test. Five days of free access to all environments allows the mice to habituate to the apparatus (Maze Engineers). The last day of habituation trial data was used as pre-conditioning test data. For conditioning phase, the compartments were divided into compartments with or without food and mice introduced into one of two compartments, with restricted

access for 30 min. After four days of conditioning phase, preference tests were performed. Mice introduced into the center compartment with free access to all compartments for 15 min without food. The result of the preference test was calculated using time spent in each compartment during the pre-conditioning and post-conditioning test. The CPP score was determined by subtracting the time spent in the food-paired chamber during habituation from the time spent in the same chamber on the test day.

For restraint stress, mice were restrained for 30 min in a 50 ml conical tube with the holes (0.5 cm in diameter) at the front and back for air flowing. Mice were only able to move their head and limb but not their body, and unable to access food and water during the restraint. After the restraint was completed, mice were performed behavior tests immediately.

Measurement of plasma corticosterone and norepinephrine levels

Blood samples were collected from the retroorbital plexus using heparinized capillary tube. Whole blood samples were centrifuged at 2,000 X g for 10 min, and the plasma was separated and stored at –20 °C until use. Plasma corticosterone and norepinephrine levels were determined using two-site sandwich ELISA kits (corticosterone: Cayman, 501320; norepinephrine: MyBioSource, MBS2600834) following the manufacturer's protocols.

Statistics

All statistical results were presented as mean±SEM. Statistical analyses were performed using Graphpad Prism 10.0. A two-tailed *t*-test were used for two-group comparisons. Data were considered significantly different when the probability value is less than 0.05.

Results

We sought to determine whether knockdown of *Magel2* expression in ARC^{Pomc} neurons that innervate the MeA causes an increase in anxiety-like behavior. To achieve this, we used a retrograde adeno-associated virus (AAVrg) containing Cre recombinase under the control of neuronal *Pomc* enhancers (nPEs) to genetically eliminate the *Magel2* gene, as we have done previously [22; 23]. We first validated our experimental approaches by injecting AAVrg-nPEs-Cre into the MeA of Rosa26-floxed-stop CRISPR-associated protein 9 (Cas9)-enhanced green fluorescent protein (eGFP) knock-in mice (Rosa26-$Cas9^f$) (Fig. 1A). Three-four weeks post viral infections, double immunostaining with anti-GFP and anti-POMC antibodies revealed that a subset of ARC^{Pomc} neurons co-expressed GFP (Fig. 1B). This finding suggests that these ARC^{Pomc} neurons are the ones projecting to the MeA, further confirming our previous studies [17; 18]. In this experimental setup, we injected AAVrg-DIO-tdTomato-U6-*Magel2* sgRNA along with AAVrg-nPEs-Cre into the MeA of Rosa26-$Cas9^f$ mice to specifically knock down the

expression of the *Magel2* gene in ARC^{Pomc} neurons that project to the MeA (Fig. 1 C-G). As shown in Fig. 1D, our results revealed that all cells expressing tdTomato were also positive for GFP, indicating that this viral strategy could effectively knock down the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons using the CRISPR-Cas9 system. Quantification of MAGEL2-positive cells within the ARC revealed a significant decrease in the number of MAGEL2-positive neurons (Fig. 1E-G), consistent with our prior study [18].

We then explored the impact of *Magel2* gene deletion in MeA-innervating ARC^{Pomc} neurons on animal behavior, with a focus on anxiety-like behavior (Fig. 2A) as anxiety is very common in individuals with PWS [7; 24]. We first examined if the loss of function of the *Magel2* gene in these neurons influences body weight and found that there were no significant differences in body weight between the groups (Fig, 2B), which further confirms our prior study [18]. We then performed a series of experiments in male mice. Initially, we conducted an open field test, a common method for evaluating anxiety-like behavior [21; 25]. Behavioral elements of anxiety include decreased total locomotor activity, lower distance traveled and lower percentage of time spent in the center region, and higher percentage of time spent in the periphery zone. We observed no significant differences in the total distance traveled or the duration spent in the center zone between the control and experimental groups (Fig. 2C), which is consistent with the prior findings with *Magel2* knockout (ko) mice [26; 27; 28]. Furthermore, we conducted a light/dark (LD) box test to evaluate changes in the willingness to explore the illuminated, unprotected area, as well as an elevated plus maze (EPM) test to investigate the inherent tendency of mice to explore novel environments by offering a choice between open, unprotected maze arms and enclosed, protected arms [29]. Mice deficient in the *Magel2* gene within MeA-projecting ARC^{Pomc} neurons did not demonstrate any differences in these assessments compared to the control group (Fig. 2D and E) as described in *Magel2* ko mice [27]. These findings suggest no significant differences in anxiety-like behavior between the two male groups, which is not consistent with the commonly recognized anxiety associated with PWS [24].

Individuals with PWS are highly stress-sensitive [30]. We thus examined if acute stress such as restraint affects anxiety-like behavior in male mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons. In the open field test, we found that, although both the control and experimental mice exhibited similar distance traveled, the mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons spent significantly less time in the center zone compared to that of the control group following short-term restraint stress (Fig. 2F). Furthermore, the results of the LD box test demonstrated that the experimental mice required a significantly longer duration to make their initial entry into the dark area compared to the control group (Fig. 2G). However, we observed that acute stress did not affect the EPM test (Fig. 2H). Our findings suggest that the loss of the *Magel2* gene may influence stress-induced anxiety-like behavior

in male mice. This observed difference in stress-induced anxiety-like behavior could be attributed to elevated levels of stress hormones including corticosterone and norepinephrine. However, our measurements of these hormones in plasma revealed no significant differences in their levels (Fig. 2I).

We also conducted same behavioral assessments on female mice (Fig. 3A). Similar to male mice, female mice lacking the *Magel2* gene in ARC^{Pomc} neurons innervating the MeA exhibited no difference in body weight compared to the control mice (Fig. 3B). Consistent with findings in *Magel2* ko female mice [28], the results from the open field test, LD box test, and EPM test in the absence and presence of acute restraint stress exhibited no significant differences between the control and experimental mice (Fig. 3C-H). In addition, there were no differences in the levels of plasma stress hormones (Fig. 3I).

Male mice lacking the *Magel2* gene showed reduced learning capabilities, especially in spatial learning [28]. We performed a conditioned place preference (CPP) test, which depends on the animal's capacity to learn and remember spatial associations between environmental cues and the rewarding effects of the paired stimulus. During the conditioning phase, Emmental cheese, a palatable food, was associated with spatial information. On the day of testing, the food was removed, and the duration spent in the compartment previously paired with the food was measured (Fig. 4A). Results revealed that male mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons spent significantly less time in the compartment paired with food compared to the controls, while no significant difference was observed in the total distance traveled between the groups (Fig. 4B). Interestingly, this difference was absent between the groups following exposure to restraint stress (Fig. 4C). In contrast to male mice, we found no significant differences between the female control and experimental groups with and without exposure to restraint stress (Fig. 4D-F). In other words, the time spent in the food-paired compartment was similar across these groups (Fig. 4E and F). Hence, these results suggest that the *Magel2* gene in ARC^{Pomc} neurons may play a role in short-term memory and spatial learning that was associated with food reward in male mice.

Discussion

PWS is a complex neurodevelopmental disorder characterized by a range of behavioral changes, including not only hyperphagia, but also anxiety, temper outbursts, and obsessive-compulsive behavior [7; 24]. The cellular mechanisms underlying the behavioral changes in PWS has been studied in animal models such as *Magel*2 ko mice [26; 27; 28]. In this study, we specifically deleted the *Magel2* gene in ARC^{Pomc} neurons that innervate the MeA, given that the MeA is a critical structure involved in emotional behaviors, particularly anxiety-like behavior [31]. We found that both male and female mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons did not display any significant alterations in open field maze, LD box, and EPM assessments in the absence of restraint stress. In contrast, the male mutant

mice exhibited increased anxiety-like behaviors such as a reduced time spent in the center zone and a latency to first entry to the dark box, compared to their control littermates following exposure to restraint stress. Moreover, the loss of function of the *Magel2* gene in these specific ARC^{Pomc} neurons resulted in decreased spatial learning that was associated with food reward in male mice. Contrary to the male mutant mice, the female mutant mice did not show any behavioral changes with and without exposure to restraint stress. Given that individuals with PWS are highly sensitive to stress, which can lead to behavioral changes [30], our present study may offer a novel perspective on the involvement of the *Magel2* gene, among other imprinted genes associated with PWS, in anxiety-related behaviors within this population.

Hyperphagia is a primary characteristic of PWS, resulting in excessive weight gain and obesity. Intriguingly, the loss of function of the *Magel2* gene did not replicate this characteristic feature in two distinct *Magel2* ko mouse models, namely the LacZ knock-in allele and *Magel2* deletion [10; 19; 27; 32]. Although the male *Magel2* ko mice with the LacZ knock-in allele showed a slight increase in body weight on a standard chow diet [19], the *Magel2* ko mice did not develop DIO compared to their control littermates when fed a high-fat diet (HFD). Instead the *Magel2* ko mice demonstrated a reduction in body weight relative to the control mice during HFD feeding [19]. *Magel2* ko mice with *Magel2* deletion also showed normal weight, temperature, motor abilities, and pain sensitivity [28]. These two distinct ko mice displayed a reduction in food intake compared to the controls [10; 19], which seems to be partially attributed to changes in circadian output [10]. In our prior study [18], deleting the *Magel2* gene in ARC^{Pomc} neurons that project to the MeA caused a reduction in body weight in both male and female mice compared to the control littermates on HFD feeding. This was associated with increased locomotor activity particularly in the dark phase. Additionally, these mutant mice did not exhibit any differences in food intake, glucose tolerance, and insulin sensitivity [18]. Hence, these prior studies suggest that the *Magel2* gene, among the imprinted genes associated with PWS, might not be crucial in the development of hyperphagia and obesity.

Despite the *Magel2* gene not affecting hyperphagia, the *Magel2* ko mice displayed altered behavioral phenotypes, such as a lack of preference for social novelty, a deficit in social interaction and recognition, and a reduced learning ability in a sex-dependent manner [26; 27; 28]. Interestingly, although anxiety is the most prevalent behavioral change in PWS [24], *Magel2* ko mice did not exhibit anxiety-related behavior in the open field maze [26; 28] and elevated plus maze assessments [27]. Contrary to these findings, it has been also demonstrated that both male and female *Magel2* ko mice spent more time in the open arms of the elevated plus maze compared to the control group, indicating changes in their exploratory behavior [26]. In this study, we found that deleting the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons also did not affect anxiety-like behavior in both male and female mice in the absence of

acute stress exposure, aligning with prior findings [27; 28]. According to the foundation for Prader-Willi research [30], individuals with PWS are highly susceptible to stress. Under conditions of short-term exposure to restraint stress, our male mutant mice exhibited reduced time spent in the central area during the open field test and demonstrated increased latency to first entry into the dark zone during the light-dark box test, indicating an elevation in anxiety-like behavior, whereas the female mutant mice did not display any alterations in anxiety-like behavior. In the Pavlovian fear-conditioning test, *Magel2* ko mice exhibited significantly higher absolute freezing rates following the tone compared to wild-type controls, suggesting increased anxiety [27]. Hence, our current findings, along with previous ones, support the idea that the loss of function of the *Magel2* gene may play a role in anxiety-like behavior triggered by acute stress and fear.

Although the cellular mechanisms underlying stress-induced anxiety-like behavior in mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons remain to be determined, it has been shown that acute emotional stressors such as restraint and forced swim led to increased c-fos mRNA expression mainly in ARC^{Pomc} neurons rather than another important feeding-related neurons, agouti-related protein-expressing neurons in the ARC [20]. Both stressors resulted in a reduction of food intake and an increase in anxiety-like behavior, effects that were entirely inhibited by the administration of a melanocortin receptor type 4 (MC4R) antagonist. [20]. Furthermore, MC4R-expressing neurons in the MeA were activated by acute stress exposure and inhibiting MC4Rs locally in the MeA abolished stress-induced anxiety-like behavior [33]. We previously showed that activation of the ARC^{Pomc} ->MeA pathway reduced food intake through MC4Rs [17]. It is possible that mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons may be more susceptible to stress compared to the control groups.

Besides exhibiting stress-induced anxiety-like behavior, male mice deficient in the *Magel2* gene also demonstrated a significant impairment in object recognition memory and a decrease in spatial learning abilities, whereas female mutant mice did not show these deficits [28]. In the CPP test, the animal utilizes spatial cues, such as walls with black stripes in our experimental setup, to establish a memory of the compartment associated with palatable food. Subsequently, the animal demonstrates a preference for this location based on the learned association. Our results revealed that male, but not female, mice lacking the *Magel2* gene in MeA-innervating ARC^{Pomc} neurons spent considerably less time in the compartment associated with food compared to the control mice, aligning with the previous findings [28]. The findings suggest that the *Magel2* gene, particularly within the ARC^{Pomc} neurons, may play a crucial role in short-term memory or spatial learning abilities.

The FDA has recently approved VYKAT™ XR (diazoxide choline, Soleno Therapeutics) as a treatment for hyperphagia in PWS. As MAGEL2 in ARC^{Pomc} neurons appears to be a key player in stress-induced anxiety-like behavior, understanding the cellular mechanisms of how MAGEL2 in

ARC^{POMC} neurons innervating the MeA controls mental disorders will open up new therapeutic strategies for the treatment of mental illness in individuals with PWS.

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Author contributions: SB carried out viral injection, immunostaining, behavioral tests, and ELISA assay. Y.-H. designed research, performed viral injection immunocytochemistry, analyzed the data, and wrote the manuscript.

Competing interests: There are no competing interests.

Figure legends

Figure 1. Selective deletion of *Magel2* in MeA-projecting ARC^{Pomc} neurons.

(A) Schematic illustration of the experimental condition. AAVrg-nPE-Cre viruses were injected into the MeA of Rosa26-Cas9^{f} mice.

(B) Images of confocal fluorescence microscopy showing co-expression of GFP and POMC in a subset of ARC^{Pomc} neurons within the ARC of Rosa26-Cas9^{f} mice injected with AAVrg-nPE-Cre. Scale bar, 30μm

(C) Schematic illustration of the experimental condition. A mixture of AAVrg-*Magel2* sgRNA viruses and AAVrg-nPE-Cre viruses was injected into the MeA of Rosa26-Cas9^{f} mice.

(D) Images of confocal fluorescence microscopy showing co-expression of GFP and tdTomato in a subset of ARC^{Pomc} neurons. TdTomato- and GFP-positive neurons represent ARC^{Pomc} neurons coinfected with both viruses. Scale bar, 50μm

(E) Schematic illustration of experimental conditions. A mixture of AAVrg-nPE-Cre viruses and either AAV-control or AAVrg-*Magel2* sgRNA viruses was bilaterally injected into the MeA of Rosa26-Cas9^{f} mice.

(F and G) Images of confocal fluorescence microscopy showing the expression of MAGEL2-positive neurons in Rosa26-Cas9^{f} mice injected with AAVrg-nPE-Cre and AAVrg-control or AAVrg-Magel2 sgRNA into the MeA (F, scale bar, 100 μm). Quantitative analysis of MAGEL2-positive neurons in the ARC (n = 4 mice per group) revealed a significant decrease in the number of MAGEL2-positive cells. Unpaired t-test, * $P<0.05$. Data are presented as mean values ± SEM.

Figure 2. Loss of function of the *Magel2* gene in ARC^{Pomc} neurons increases stress-induced anxiety in male mice.

(A) A schematic illustration of the experimental condition.

(B) Graph showing no significant difference in body weight between the control (n= 6 mice) and experimental (n= 7 mice) groups following viral infections. Body weight was measured weekly at 9 a.m.

(C) Graphs showing the total distance traveled and the time spent in the inner area during the open field test (n = 5 mice vs. 6 mice).

(D) Graphs showing the time spent in the light chamber and the latency to the first entry into the dark chamber during the light-dark box test (n = 7 mice vs. 5 mice).

(E) Graphs showing the cumulative duration in the open and closed arms during the elevated plus maze test (n = 7 vs. 5 mice).

(F) Graphs showing the total distance traveled and the time spent in the inner area during the open field test following exposure to restraint stress (n = 6 mice vs. 6 mice, unpaired *t*-test, ** $P<0.01$).

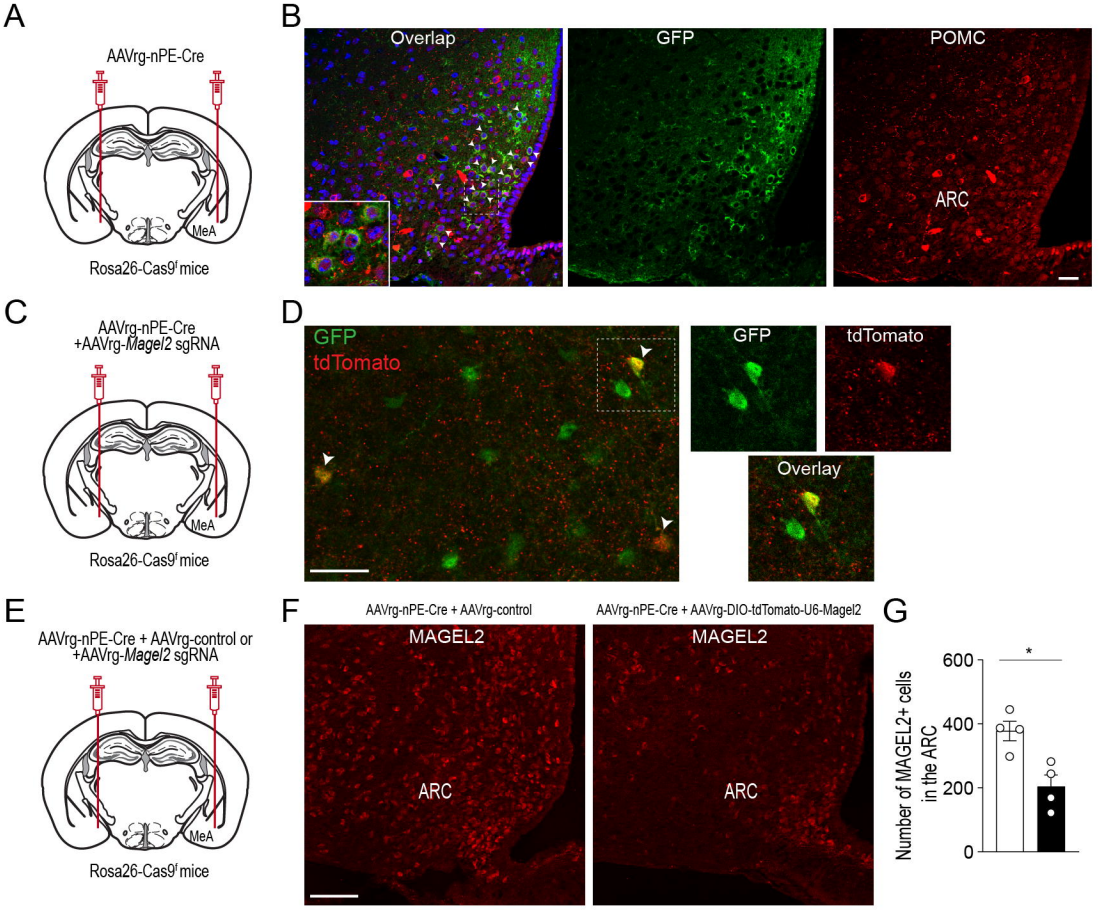
(G) Graphs showing the time spent in the light zone and the latency to the first entry into the dark chamber during the light-dark box test following exposure to restraint stress (n = 6 mice vs. 5 mice, unpaired t-test, ** $P<0.01$).
(H) Graphs showing the cumulative duration in the open and closed arms during the elevated plus maze test with restraint stress (n = 6 vs. 6 mice).
(I) Graphs showing plasma corticosterone (left) and norepinephrine (right) levels in the control (n=12 mice) and experimental (n= 10 mice) groups. Data are presented as mean values ± SEM.

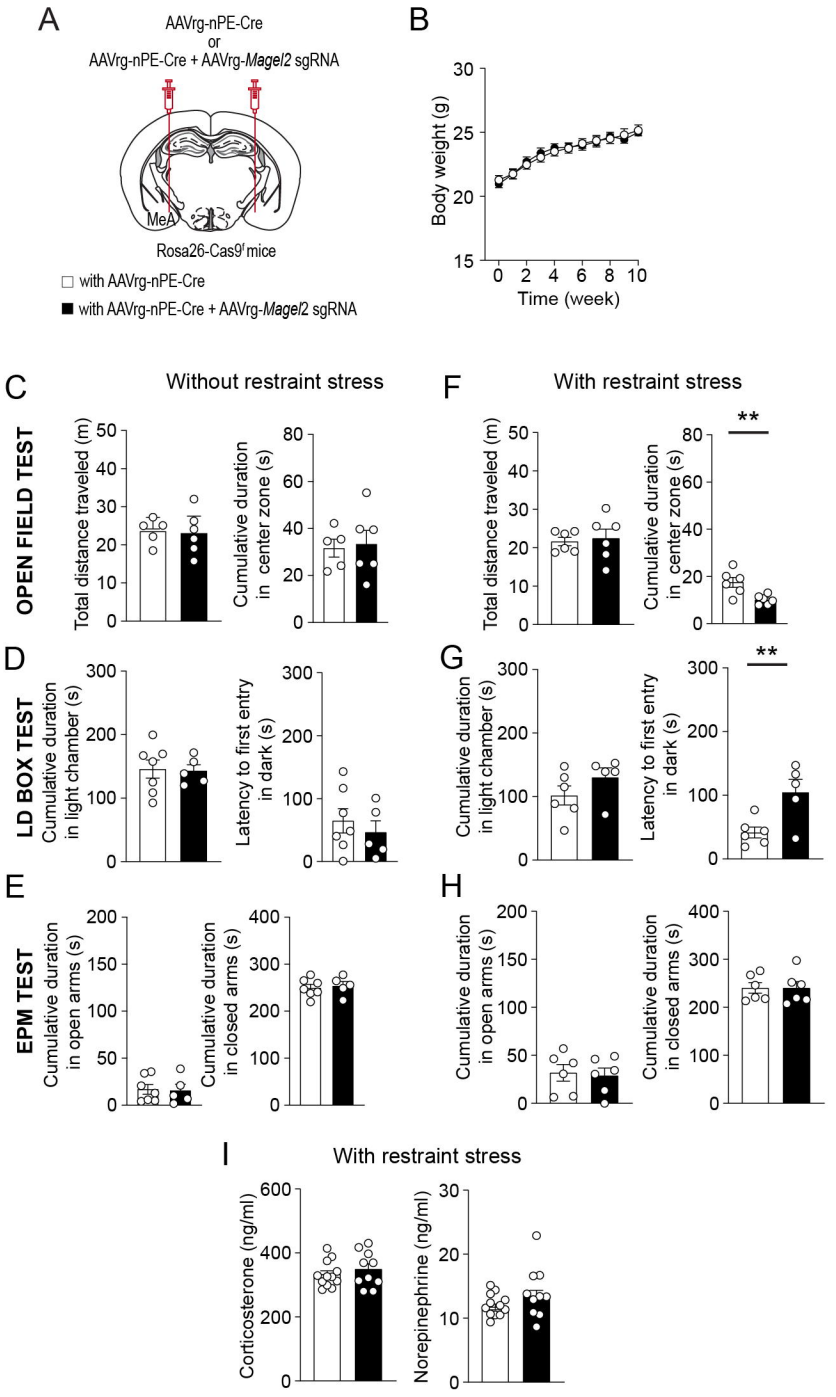
Figure 3. Deleting *Magel2* in ARC^{POMC} neurons dose not affect stress-induced anxiety in female mice.
(A) A schematic illustration of the experimental condition.
(B) Graph showing no difference in body weight between the control (n= 7 mice) and experimental (n= 8 mice) groups following viral infections. Body weight was measured weekly at 9 a.m.
(C, D, and E) Graphs showing the results of the open field test (C, n= 9 mice vs. 6 mice), the light-dark box test (D, n= 8 mice vs. 5 mice), and the elevated plus maze test (E, n= 7 vs. 6 mice) conducted on female control and experimental groups in the absence of restraint stress. No significant differences were observed between the groups.
(F, G, and H) Graphs showing the results of the open field test (F, n= 7 mice vs. 6 mice), the light-dark box test (G, n= 5 mice vs. 5 mice), the elevated plus maze test (H, n= 4 vs. 5 mice) performed on control and experimental groups following exposure to restraint stress. There were no significant differences between the groups.
(I) Graphs showing plasma corticosterone (left) and norepinephrine (right) levels in the control (n= 10 mice) and experimental (n= 8 mice) groups. Data are presented as mean values ± SEM.

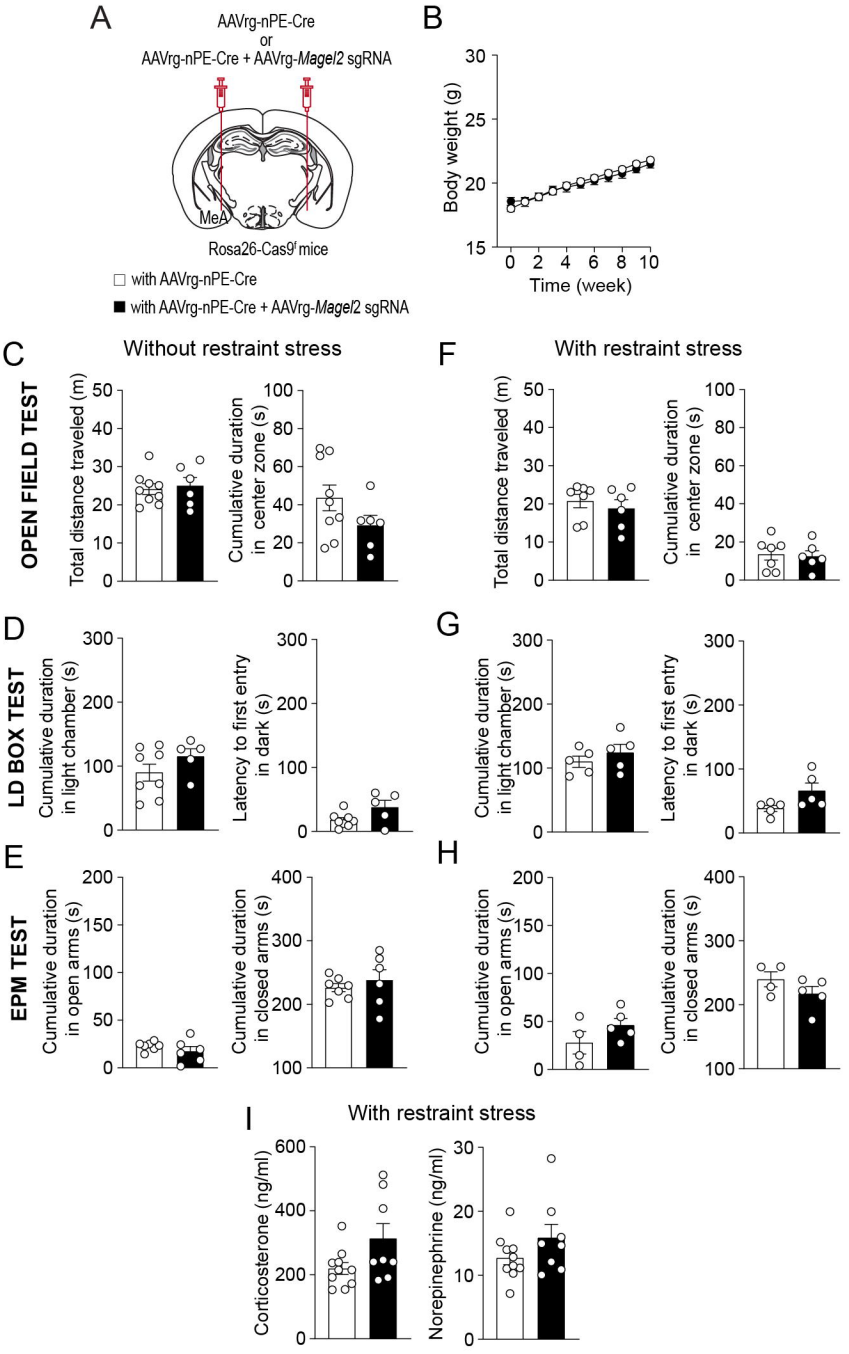
Figure 4. Knockdown of *Magel2* in ARC^{Pomc} neurons causes an impairment in spatial reward learning in male mice.
(A) Representative example of the travel paths of the control (top) and the experimental (bottom) groups during the CPP test in male mice. During the post-conditioning test, the control mice spent more time in the food-paired chamber than in the other chamber. In contrast, the experimental mice showed no preference for the food-paired chamber.
(B) Graphs showing the total distance (left) on the test day and the CPP score between the male control and experimental groups (n = 6 mice vs. 7 mice, unpaired t-test, ** $P<0.01$).
(C) Graphs showing the total distance (left) on the test day and the CPP score between the male control and experimental groups following exposure to restraint stress (n = 5 mice vs. 6 mice).
(D) Representative example of the travel paths of the control (top) and the experimental (bottom) groups during the CPP test in female mice.

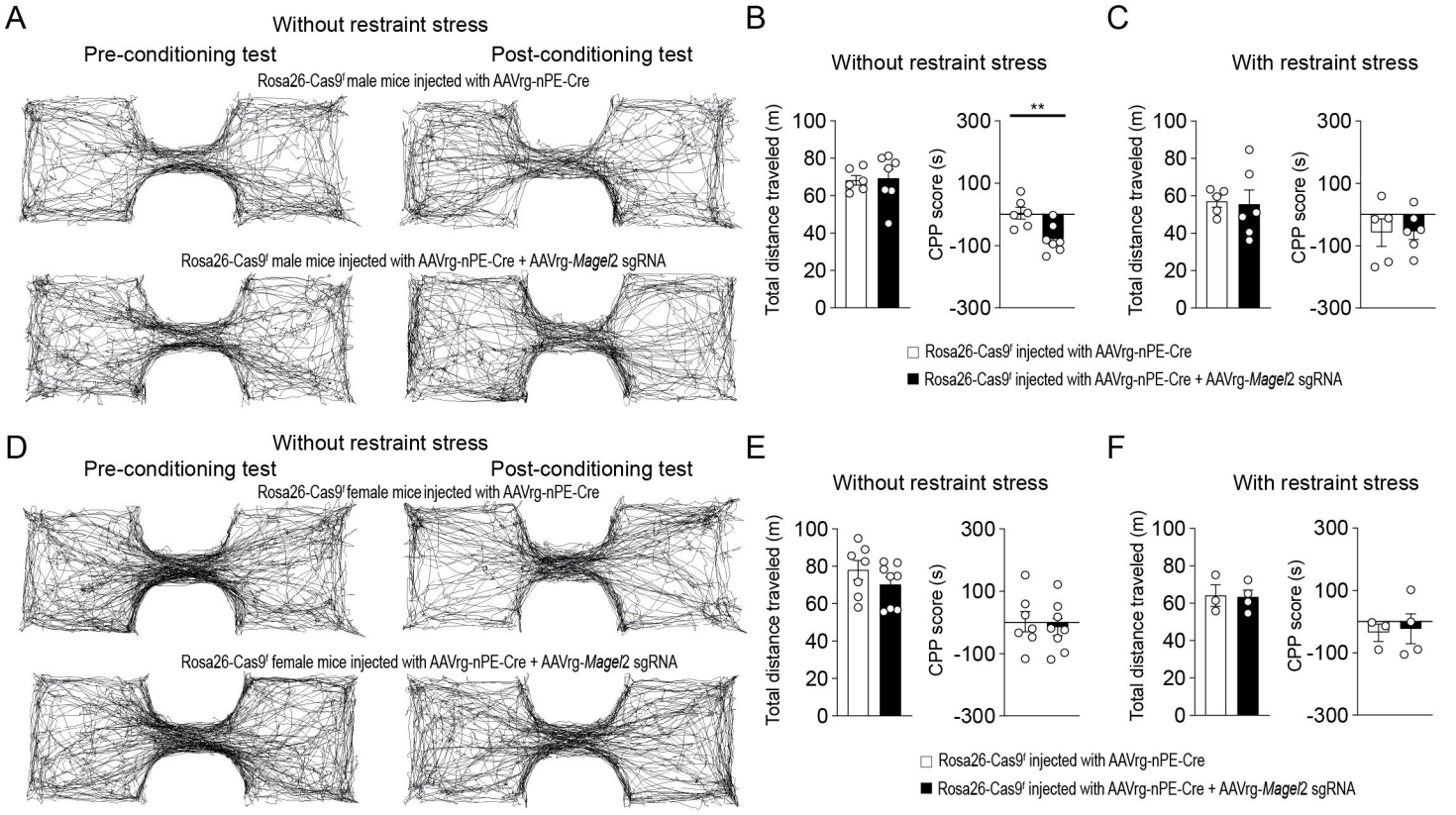
(E) Graphs showing the total distance and the time spent in the food-paired compartment in the conditioned place preference test between the female groups (n = 7 mice vs. 8 mice).

(F) Graphs showing the total distance and the time spent in the food-paired compartment in the conditioned place preference test between the female groups with restraint stress (n = 3 mice vs. 4 mice).


A
AAVrg-nPE-Cre
MeA
Rosa26-Cas9f mice
B
Overlap
GFP
POMC
ARC
C
AAVrg-nPE-Cre
+AAVrg-Magel2 sgRNA
MeA
Rosa26-Cas9f mice
D
GFP
tdTomato
GFP
tdTomato
Overlay
E
AAVrg-nPE-Cre + AAVrg-control or
+AAVrg-Magel2 sgRNA
MeA
Rosa26-Cas9f mice
F
AAVrg-nPE-Cre + AAVrg-control
AAVrg-nPE-Cre + AAVrg-DIO-tdTomato-U6-Magel2
MAGEL2
MAGEL2
ARC
ARC
G
Number of MAGEL2+ cells in the ARC
600
400
200
0
*


A
AAVrg-nPE-Cre
or
AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
MeA
Rosa26-Cas9f mice
with AAVrg-nPE-Cre
with AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
B
Body weight (g)
Time (week)
C
Without restraint stress
OPEN FIELD TEST
Total distance traveled (m)
Cumulative duration in center zone (s)
D
LD BOX TEST
Cumulative duration in light chamber (s)
Latency to first entry in dark (s)
E
EPM TEST
Cumulative duration in open arms (s)
Cumulative duration in closed arms (s)
F
With restraint stress
**
G
**
H
I
With restraint stress
Corticosterone (ng/ml)
Norepinephrine (ng/ml)


A
AAVrg-nPE-Cre
or
AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
MeA
Rosa26-Cas9f mice
with AAVrg-nPE-Cre
with AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
B
Body weight (g)
Time (week)
C
Without restraint stress
OPEN FIELD TEST
Total distance traveled (m)
Cumulative duration in center zone (s)
D
LD BOX TEST
Cumulative duration in light chamber (s)
Latency to first entry in dark (s)
E
EPM TEST
Cumulative duration in open arms (s)
Cumulative duration in closed arms (s)
F
With restraint stress
G
H
I
With restraint stress
Corticosterone (ng/ml)
Norepinephrine (ng/ml)


A
Without restraint stress
Pre-conditioning test
Post-conditioning test
Rosa26-Cas9f male mice injected with AAVrg-nPE-Cre
Rosa26-Cas9f male mice injected with AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
B
Without restraint stress
Total distance traveled (m)
CPP score (s)
**
C
With restraint stress
Rosa26-Cas9f injected with AAVrg-nPE-Cre
Rosa26-Cas9f injected with AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
D
Without restraint stress
Pre-conditioning test
Post-conditioning test
Rosa26-Cas9f female mice injected with AAVrg-nPE-Cre
Rosa26-Cas9f female mice injected with AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA
E
Without restraint stress
F
With restraint stress
Rosa26-Cas9f injected with AAVrg-nPE-Cre
Rosa26-Cas9f injected with AAVrg-nPE-Cre + AAVrg-Magel2 sgRNA