Gordon Holmes syndrome model mice exhibit alterations in microglia, age and sex-specific disruptions in cognitive and proprioceptive function

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Title: Gordon Holmes syndrome model mice exhibit alterations in microglia, age and sex-specific disruptions in cognitive and proprioceptive function

Abbreviated Title: Behavior disruption in Gordon Holmes syndrome mice

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Abstract

Gordon Holmes syndrome (GHS) is a neurological disorder associated with neuroendocrine, cognitive, and motor impairments with corresponding neurodegeneration. Mutations in the E3 ubiquitin ligase RNF216 are strongly linked to GHS. Previous studies show that deletion of Rnf216 in mice led to sex-specific neuroendocrine dysfunction due to disruptions in the hypothalamic-pituitary-gonadal axis. To address RNF216 action in cognitive and motor functions, we tested Rnf216 knockout (KO) mice in a battery of motor and learning tasks for a duration of one year. Although male and female KO mice did not demonstrate prominent motor phenotypes, KO females displayed abnormal limb clasping. KO mice also showed age-dependent strategy and associative learning impairments with sex-dependent alterations of microglia in the hippocampus and cortex. Additionally, KO males but not females had more negative resting membrane potentials in the CA1 hippocampus without any changes in miniature excitatory postsynaptic current (mEPSC) frequencies or amplitudes. Our findings show that constitutive deletion of Rnf216 alters microglia and neuronal excitability, which may provide insights into the etiology of sex-specific impairments in GHS.

Significance Statement

Gordon Holmes syndrome (GHS) is a rare neurological disorder associated with neuroendocrine, cognitive, and motor impairments with corresponding neurodegeneration. Although mutations in Rnf216 have been identified in male and female GHS individuals, it has yet to be tested if removal of Rnf216 causes cognitive and motor dysfunction. This study characterizes motor and learning behaviors in male and female GHS model mice. We find that deletion of Rnf216 does not disrupt motor function but does lead to sex-specific effects on proprioceptive and age-dependent changes in cognitive behaviors. Our study provides new insight into how disruptions in Rnf216 contribute to the emergence of sex-specific phenotypes in individuals with GHS.

Introduction:
Neurological disorders (NDs) are a large group of nervous system-altering diseases that affect a large fraction of the population and encompass a wide variety of signs and symptoms. Gordon Holmes syndrome (GHS) is one example of a rare ND where individuals exhibit neuroendocrine abnormalities and motor dysfunction (Holmes, 1908, 1910; Neuhauser and Opitz, 1975; Seminara et al., 2002; Gonzalez-Latapi et al., 2021). GHS patients often have cognitive decline with signs of dementia as evaluated through cognitive diagnostic tests (Wechsler Adult Intelligence Scale-Revised China, Mini-Mental State Examination, and Montreal Cognitive Assessment) (Margolin et al., 2013; Ganos et al., 2015; Alqwaifly and Bohlega, 2016; Mehmood et al., 2017; Calandra et al., 2019; Lieto et al., 2019; Chen et al., 2020).

Disruptions in neuroendocrine, motor, and memory functions have been observed in GHS individuals, which are correlated with white and gray matter abnormalities in hippocampal, cortical, and cerebellar regions. GHS individuals also exhibit thinning of the corpus callosum and gliosis (Margolin et al., 2013; Ganos et al., 2015; Alqwaifly and Bohlega, 2016; Mehmood et al., 2017; Calandra et al., 2019; Lieto et al., 2019; Chen et al., 2020). Although GHS was documented in the early 1900s (Holmes, 1908), causative gene mutations were only recently discovered (Margolin et al., 2013; Shi et al., 2014; Synofzik et al., 2014). Recessive mutations in CHIP/STUB1 and RNF216 along with recessive digenic mutations in RNF216 and OTUD4 have been identified in individuals diagnosed with GHS (Margolin et al., 2013; Shi et al., 2014; Alqwaifly and Bohlega, 2016; Hayer et al., 2017; Calandra et al., 2019; Chen et al., 2022). RNF216 mutations have also been reported in GHS-related syndromes that include Huntington-like disorder and 4H syndrome (Santens et al., 2015; Chen et al., 2020).

Functionally, CHIP/STUB1, RNF216, and OTUD4 encode for classes of protein enzymes that are responsible for adding and removing an abundant posttranslational tag called ubiquitin to other proteins. Ubiquitin is a 76-amino acid protein that is covalently attached to other proteins in the cell. This posttranslational modification regulates diverse biological processes that include signal transduction, protein degradation, cell cycle regulation, DNA repair, inflammation, and neural development (Hershko and Ciechanover, 1998; Mabb and Ehlers, 2010; Upadhyay et al., 2017; Mabb, 2021). This ATP-dependent reaction utilizes an enzymatic cascade consisting of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). These three types of enzymes act in concert to attach diverse configurations of ubiquitin to substrate proteins (Komander and Rape, 2012; Zheng and Shabek, 2017). Protein ubiquitination can also be reversed by deubiquitinases (DUBs), which remove a variety of ubiquitin configurations from substrates (Komander et al., 2009). CHIP/STUB1 and RNF216 are both classified as E3 enzymes.
enzymes whereas OTUD4 is characterized as a DUB. In GHS, missense mutations within the catalytic regions of RNF216 and CHIP/STUB1 decrease the ability of these E3s to ubiquitinate their targets (Shi et al., 2014; Husain et al., 2017). Cumulatively, these findings strongly imply that disruptions in the ubiquitination of substrates facilitated by RNF216 and CHIP/STUB1 most likely contribute to GHS pathogenesis.

In a previous study, constitutive Rnf216 KO mice were found to have sex-specific alterations in reproductive phenotypes that were accompanied with altered microglia in the preoptic area of the hypothalamus (George et al., 2022). Given that GHS is a rare disorder with reported clinical differences in males and females, comorbidity of motor and learning impairments, and limited longitudinal tracking for pathogenesis, we monitored motor and cognitive functions in Rnf216 male and female KO mice up to 1 year of age. Here, we found that KO females displayed abnormal limb clasping without any outstanding motor phenotypes in KO males and females. When evaluating cognitive function, Rnf216 constitutive KO mice exhibited spatial and associative learning impairments that were age dependent. These alterations in learning and memory were associated with sex-specific changes in reactive microglia in the hippocampus and cortex. Although male and female KO mice had no changes in CA1 hippocampal miniature excitatory postsynaptic current (mEPSC) frequencies or amplitudes, KO males had a more negative resting membrane potential. Our findings show that deletion of Rnf216 in mice leads to alterations in microglia and neuronal excitability, which may lead to impairments in select behavioral outputs.

Materials and Methods

Animals

Mice were kept in standard housing with littermates, provided with food and water ad libitum, and maintained on a 12:12 (light-dark) cycle. All behavioral tests were conducted in accordance with the National Institutes of Health Guidelines for the Use of Animals. Mice were treated in accordance with the Animal Welfare and Ethics Committee (AWERB) and experiments were performed under the appropriated project licenses with local and national ethical approval. All experimental mice used in this study were housed separately by sex and in groups of 4-5 with balanced WT and KO genotypes. Samples sizes for behavior and immunohistochemistry experiments were calculated using variance from previous
experiments to indicate power, which statistical analysis for significance was set at 95%. All behavioral
studies and isolation of body tissue for biochemical experiments were approved by the Animal Care and
Use Committee.

Generation of Rnf216 knockout mice

Embryonic stem cell clones were generated to target exons 4 to 5 of the Rnf216 gene on mouse chromosome 5, which prevents the production of all isoforms (International Knockout Mouse Consortium). ES cell clones were injected into blastocytes and implanted into pseudopregnant mice. These mice were crossed to heterozygous mice for Flp recombinase to excise out the LacZ/neomycin cassette to obtain one Rnf216 allele flanked by loxP (fl) sites to generate C57BL/6N-Rnf216<tm1c(EUCOMM)Wtsi>/Tcp (Canadian Mouse Mutant Repository at the Hospital for Sick Children, Toronto, CA). Homozygous floxed conditional male mice (Rnf216<fl/fl>) were crossed with homozygous CMV-CRE+/- female mice (The Jackson Laboratory, JAX stock #006054) to allow the CRE to excise out exons 4 to 5, creating a dysfunctional gene. To breed out the CRE, female offspring that were heterozygous for loxP and CRE (Rnf216<fl/fl>;CMV-CRE+/-) were bred with WT males to generate heterozygous Rnf216<+/-> mice. Male Rnf216<+/-> mice were then bred with WT females to generate Rnf216<+/-> mice. Rnf216<+/> male and female mice were then bred together to generate the experimental mice used for this study (Rnf216<+/> (WT), Rnf216<+/-> (HET), and Rnf216<-/-> (KO)).

Righting reflex

Mice were tested at ages P7 and P12 for developmental motor impairment. Briefly, mouse pups were separated from their mother and placed in a small paper box. One pup was removed from the box and placed in a supine position on a paper surface. Upon release of the mouse, the experimenter measured the time it took for the pup to return to a prone position (assessed by ensuring all 4 paws touched the ground surface). Pups were placed back in the box to rest for 1 minute and the procedure was repeated 1 additional time. Following the final time, the pup was immediately returned to its mother so as not to induce excessive stress.

Grip strength
Mice were tested in grip strength for muscular strength at ages P60 and P365. Briefly, we measured the peak force applied by the forelimbs of mice using a grip strength meter (Ugo Basile). Mice were acclimated to the testing room 30 minutes prior to performing the test. To measure forelimb strength, the mouse was gently lowered onto the grid with its two forepaws. The investigator gently pulled back on the tail of the mouse, ensuring that the torso of the mouse remained horizontal and peak force was measured. The mouse was placed back in its home cage for at least 5 minutes to rest. The procedure was repeated two additional times and the apparatus was cleaned with 70% ethanol between each mouse.

Rotarod

Mice aged P65 and P370 were tested for balance, coordination, physical condition, and motor planning using the Rotarod apparatus (Ugo Basile). Tests were performed for 5 consecutive days and mice were returned to standard housing daily after each day of testing. Briefly, mice were acclimated to the testing room for at least 30 minutes prior to performing the assay. Mice were placed horizontally on a rotating cylinder (rod) which was suspended 4-6 inches above the cage floor and spinning at 4 rpm. Thick bench paper was placed on the bottom of the rotarod to minimize stress if the mouse fell off. The rotarod speed was increased gradually (20 rpm/min for a maximum speed of 40 rpm) and the time (latency) it took for the mouse to fall off the apparatus was measured. Any mouse that fell off the apparatus was retrieved by the investigator and placed back in its home cage. For the first session (Day 1), mice were given three trials with a 60-second rest in between each trial.

Open field

Mice aged P62 and P367 were tested in the open field activity apparatus (Med Associates, Inc.), which uses an array of infrared (IR) photo beams to measure locomotion and motor activity in a square arena. The apparatus consisted of 3 sets of matching IR photo beams that projected across the open field along three axes: X, Y, and Z. The software, Activity Monitor 7, detected when and where the photo beams were disrupted by the presence of the mouse. The software also breaks the field arena into zones, or areas of interest, depending on the experiment; data analysis displayed the measurements accordingly. Briefly, mice were acclimated to the testing room for at least 30 minutes prior to performing the assay. Mice were placed in the novel open field chamber for a total time of 15 minutes. After the assay, mice were returned to standard housing.
Clasping

A separate cohort of mice was used to evaluate clasping to avoid any changes related to behavioral tasks. Mice from this cohort were tested for clasping at 3, 9, and 41 weeks of age. A portion of this cohort was used for brain harvesting and measurements of cerebellar weights. Mice were suspended in the air by their tail for 2 sessions, 30-seconds each. A score of 0 was defined as no clasping with both forelimbs and hindlimbs extended; a score of 1 was flexion of forelimbs without clasping of hind limbs; a score of 2 was assigned when forelimbs and hindlimbs were clasped.

Barnes maze

Mice aged P70 and P375 were first habituated to the Barnes maze 2 days prior to testing. On the first day of habituation, each mouse was allowed to explore the maze for ~30s and then gently directed to a random hole (not used during testing). The mouse stayed in the hole for 1.5 min and then was returned to their home cage. The maze was sprayed with 70% ethanol between each mouse. 1 day prior to testing, the mice were given a chocolate flavored pellet treat (Bio-Serv Supreme Mini-Treat) in their home cage. Each mouse was tested only once per day. Prior to testing sessions, mice were acclimated to the room 30 minutes. Before each session (including the testing session), the mice were held in an opaque cylinder in the middle of the maze before recording. Each session cutoff was set to 15 minutes. If the mouse did not find the exit hole during the 15 minutes, then the investigator guided the mouse into the hole. The learning task was a 10-day duration. On days 1-5 (Training), the exit hole contained a chocolate treat to associate entry into the exit box as a positive and motivating experience. Once the session was completed, the mouse was held in a holding cage until all mice in the home cage were tested. On days 6-10 (Learning), the chocolate flavored treat was given in the home cage after each mouse in the cage was done testing. During the reversal phase on days 11-16 (Reversal), the exit hole was rotated 180 degrees and the mice were still given a chocolate flavored treat in the home cage after testing. Mice were excluded from the study if they did not enter the correct exit hole within the 15-minute time limit by Day 7. The scoring and criteria for search strategy was performed as previously described (Wall et al., 2018).

Fear conditioning
Mice aged P90 and P395 were acclimated to the testing room for at least 30 minutes prior to performing the assay. On day 1 (Conditioning), each mouse was placed in the testing chamber that contained a sound-attenuating box and the mouse was allowed to explore for 2 minutes. Mice were then exposed to a 30-second tone (80 dB), followed by a 2-second scrambled foot shock (0.4 mA). Mice received 2 additional shock-tone pairings, 80 seconds between each pairing. Mice were then returned to a new, clean cage after testing. After all mice from one cage were tested, they were returned to the original home cage. On day 2 (context-dependent recall), mice were placed back in the original conditioning chamber for a test of contextual learning across a 5-6 minute session. There was no shock or tone. On day 3 (cue-dependent recall), mice were placed back in the original conditioning chamber with new inserts attached to the walls of the chamber to provide a different environment. There was an auditory cue similar to Day 1 without the shock in a 5-6 minute session.

**Western blotting**

Mouse tissue was thawed on ice and 300-500 µL of RIPA buffer was added. The tissue was then homogenized using sterile pestles. The samples were then centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was collected for protein quantification. The protein concentration of the soluble fraction was measured using the Pierce 660nM Protein Assay Kit (ThermoFisher). The samples underwent SDS-polyacrylamide gel electrophoresis and were transferred on a nitrocellulose membrane (BioRad) for 1hr at 70 mV. The blots were incubated overnight at 4°C with blocking buffer (Intercept (TBS) blocking buffer, LiCor). Membranes were then probed with the following primary antibodies prepared in a 1:1 ratio of TBST (1X TBS, 0.1% Tween-20) and blocking buffer solution containing a 1:1,000 dilution of 20% NaN₃: rabbit polyclonal anti-RNF216 (LifeSpan Biosciences (aa 100-150), 1:1,000) and mouse anti-β-Actin (Genetex, 1:3,000) then incubated overnight at 4°C. Membranes were washed 3 times in distilled water. The following secondary antibody dilutions were prepared in a 1:1 ratio of TBST (1X TBS, 0.1% Tween-20) and blocking buffer solution with 1:2,000 20% SDS: IRDye 680RD Goat anti-Mouse IgG (H+L) (Li-COR, 1:20,000) and IRDye 800CW Goat anti-Rabbit IgG (H+L) (Li-COR, 1:15,000) and were incubated for 1hr at room temperature. Membranes were washed 2 times in TBST and then 2 times with distilled water. Blots were imaged using the Odyssey CLx Imaging System (Li-COR) with a resolution of 169 µm, medium quality, and a 0 mm focus offset. Images were processed using the Gel Analysis tool in Image J using individual channels. Briefly, boxes were drawn around each band. Once the lanes were labeled and plotted, the area
of the peaks was selected and measured. For each blot, proteins of interest were normalized to the Beta-
ACTIN loading control.

Microglia staining, imaging, and analysis

Male and female WT and KO mouse brains were sectioned at 40 \( \mu \)m. Sections for each mouse were
selected based on stereological matching using the mouse brain stereotaxic coordinate reference atlas.
Samples were then blinded and processed as follows: Free-floating sections were rinsed with 3% hydrogen
peroxide 2x for 7 minutes each to remove any endogenous peroxidases, then washed 6x in 1X KPBS (in
mM) (1.6 NaCl, 0.4 K_2HPO_4, and 0.09 KH_2PO_4 dissolved in ddH_2O to make a 10x solution and diluted to 1x)
at room temperature. Sections were then incubated overnight at room temperature in 1X KPBS containing
1.0% Triton and 1:50K dilution of Goat anti-Iba1 (Novus Biologicals). Sections were then washed 10x with
1X KPBS before incubation in 1X KPBS containing 0.4% Triton and 1:600 dilution of Donkey anti-Goat
biotin-SP (Jackson ImmunoResearch) for 1 hour at room temperature. Sections were washed 5x in 1X KPBS
before being incubated in avidin-biotin-peroxidase complex (1:10, ABC Elite Kit, Vector Laboratories) for
one hour at room temperature. After rinsing sections 3x in 1X KPBS and 3x in 0.175M sodium acetate
buffer, Iba1 immunoreactivity was visualized with nickel sulfate enhanced 3,3’-diaminobenzidine (DAB)
solution (0.2mg/mL) containing 0.08% hydrogen peroxide in 0.175M sodium acetate buffer. Sections were
incubated in the DAB solution for 15 minutes before rinsing 3x with 0.175M sodium acetate buffer
followed by 3x in 1X KPBS. Sections were then mounted onto gelatin-subbed slides, air-dried, then
dehydrated in a graded series of alcohols, and cleared with xylenes. Slides were then coverslipped with
Permount mounting media. Brightfield images of the dorsal hippocampus and S1 cortex were acquired
with the researcher blinded to condition on a Keyence BZ-X700 (Keyence Corporation, Osaka, Japan)
microscope using a 20X Plan Apochromat 0.75 N.A. objective with a z-step size of 1 \( \mu \)m. For densitometry,
background subtraction and thresholding were first performed on the images using FIJI/ImageJ (National
Institutes of Health). Cell count analysis was performed using Cell Profiler 4.2.1 (Broad Institute)
(Carpenter et al., 2006). As the morphology of multiple microglia were analyzed from one mouse, the
hierarchical bootstrapping and permutations test were employed as they do not require the assumption
of sample independence (Saravanan et al., 2020). The area of microglia soma in S1 cortex and dorsal
hippocampus was measured on maximum intensity projection images by using the freehand selection
tool in FIJI ImageJ2 version 2.9.0/1.53t. Normal distribution scores for the mean were then analyzed by
hierarchical bootstrapping using the ClusterBootstrap package program (Deen and de Rooij, 2020) in R Software version 4.2.3 (Free Software Foundation). For quantification of microglia branching, microglia were traced using the Simple Neurite Tracer (SNT) plugin for FIJI ImageJ2. The intersections of skeletonized microglia were then measured using the Sholl analysis plugin in FIJI ImageJ2 with the concentric circle set to 1 μm increments. The area under the curve (AUC) for each microglia was then assessed using the ClusterBootstrap package program in R. The permutation testing was set at 1,000 random shufflings, which allowed us to determine the robustness of the estimated means. Data was visualized as a probability density function implemented in MS Excel 2023 version 16.75.2 (Microsoft). Representative microglia reconstructions were created from maximum intensity projection images using the SNT plugin tool fill option. Constructed images were exported and cropped using identical scales.

Electrophysiology

Male and female WT and Rnf216 KO (postnatal day 28) mice were sacrificed, and hippocampal slices were prepared for electrophysiological recordings. After decapitation, the brains were dissected out quickly and immediately immersed in oxygenated (95% O2 and 5% CO2) ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl 126, KCl 2.5, CaCl2 2.4, NaH2PO4 1.2, MgCl2 1.2, glucose 11.1, NaHCO3 21.4. Hippocampal transverse slices (300 μm thick) were cut by using a Leica VT1000S vibratome (Leica Microsystems, Wetzlar, Germany). The brain slices were transferred to a holding chamber filled with oxygenated aCSF and kept at room temperature (25°C) for 1 hour before recording. For recording, a single brain slice was transferred to the recording chamber and continuously perfused with oxygenated aCSF maintained at 30°C.

All recordings were performed using an Axopatch 700B amplifier and a Digidata 1440A data acquisition system (Molecular Devices). Whole-cell voltage clamp recordings of mEPSCs were performed on an Olympus upright microscope (BX51WIF) with a 40X water-immersion objective. The holding potential of all neurons was -70 mV. Recordings were filtered at 10 kHz and digitized at 10 kHz. The electrode internal solution contained the following (in mM) K-gluconate 120, KCl 10, MgCl2 2, HEPES 10, CaCl2 0.1, EGTA 1, MgATP 3, and NaGTP 0.5 (pH 7.2 with KOH). Only recording pipettes with a resistance of 2-3MΩ after being filled with internal solution were used. Synaptic blockers APV (25µM), picrotoxin (100 µM), and action potential blocker Tetrodotoxin (TTX 1 µM) were added in aCSF during the recordings. Series
resistance was monitored, and the cells were discarded when the series resistance increased > 15%. The liquid junction was –8 mv detected from the intracellular solution and subtracted from the membrane potential values in the final result. The mEPSCs were analyzed using MiniAnalysis with threshold events set at 5 pA. Electrophysiology recordings and data analysis were conducted with the experimenter blinded to genotype.

**Statistical Analysis**

Statistical analyses applied were the post hoc Student’s t-test, One-way ANOVA, chi-square test (for categorical limb clasping data), Pearson correlation, and two-way and three-way ANOVA with multiple comparisons. For k = 2, data were analyzed using Student’s t-test. When k > 2, data were analyzed for significant main effects of genotype, age and sex using ANOVA. When time (trials) was a factor or there were missing values, data were analyzed using a mixed model ANOVA. If no significant main effect of sex was observed, then data were collapsed to increase power. Sidak’s or Tukey’s tests were used for comparing group means only when a significant F value was determined. For all comparisons, significance was set at p < 0.05. Data presented in figures and tables are means (± SEM). See Table 1 for statistical reporting table.

**Code accessibility**

The code for microglia normal distribution scores by hierarchical bootstrapping is provided as Extended Data and is freely available online at https://github.com/mablab/Microglia-analysis. Code was executed on a MacBook Air running the macOS Ventura 13.4.1 with hardware specifications of 8 GB of RAM and a 1.6 GHz Dual-Core Intel Core i5 Processor.

**Results**

**No gross motor impairments in Rnf216 KO mice**

*Rnf216* knockout (KO) mice have reproductive impairments with KO males having reduced testicular weights and females displaying alterations in estrus cycling (George et al., 2022). In addition to these neuroendocrine abnormalities, GHS individuals with mutations predicted to be deleterious for *RNF216*
also exhibit ataxia and dementia with corresponding cortical and cerebellar atrophy (Margolin et al., 2013; Alqwaifly and Bohlega, 2016; Calandra et al., 2019). However, there are no studies that have established a role for Rnf216 in motor function and learning, which are core phenotypes that are disrupted in individuals with GHS (Holmes, 1908; Margolin et al., 2013). As expected, we found a significant decrease in RNF216 in the hippocampus, cortex, and cerebellum in male and female Rnf216 KO mice (Figure 1A).

To establish a role for Rnf216 deletion on GHS-related phenotypes, we devised a battery of behavior tests to evaluate motor and memory function in adult and middle-age KO mice (Figure 1B). Righting reflexes were found to be normal in P7 and P12 KO males and females suggesting that motor development was not grossly impaired in developing mice (Figure 1C). We next measured peak force using the grip strength assay but found no significant differences (Figures 1D and 1E, left), indicating that KO mice do not have deficiencies in neuromuscular strength. Based on these data, we sought to determine if KO mice had alterations in motor learning and coordination. We measured the total distance traveled and rearing movements in an open field test in adult and middle-age mice (Figures 1D and 1E, middle and right). Although there was no significant difference in the distance traveled in adult mice (Figure 1D, middle), there was a sex-dependent reduction in the number of rearing movements in adult female mice (Figure 1D, right). Middle-age KO mice had an increase in the distance traveled in the center of the open field (Figure 1E, middle) but there were no significant differences in the number of rearing movements between sex or genotype (Figure 1E, right). Next, we measured another metric of motor function using the accelerating rotarod but found no differences in the latency to fall or maximum speed in adult and middle-age KO mice (Figures 1F-G).

Because cerebellar ataxia mouse models are known to display abnormal limb-clasping reflexes (Lalonde and Strazielle, 2011; Kojic et al., 2018), we measured the limb-clasping reflex in adult and middle-age mice. Under normal conditions, adult mice splay out their forelimbs and hindlimbs away from their abdomen when held upside-down by their tail. However, limb reflex abnormalities result in the clasping of the forelimbs and hindlimbs when held upside-down. Limb-clasping was scored in WT and KO mice at different developmental ages that included weaning (3 weeks), young adult (9 weeks) and middle-age (41 weeks). Although there was no significant difference in KO males (Figure 2A), a small percentage (4.17%) of KO females had abnormal forelimb and hindlimb clasping that appeared to emerge as early as 9 weeks.
and increased at 41 weeks of age (11.54%) (Figure 2B). The onset of abnormal clasping in females could be related to reductions in cerebellar volume. However, we did not find any genotypic differences in cerebellar brain weights in 16- and 52-week-old male and female KO mice (Table 2). Taken together, these findings indicate that motor learning and coordination are intact in male and female Rnf216 KO mice up to 1 year of age. However, given the limb-clasping phenotype, female Rnf216 KO mice may have compromised proprioception.

**Middle-age Rnf216 KO mice reveal nuances in hippocampal-dependent learning and memory**

Because GHS individuals with RNF216 mutations exhibit dementia, we reasoned that Rnf216 KO mice would develop impairments in spatial learning and memory. To explore the role of RNF216 in hippocampal-dependent spatial learning, we used the Barnes maze task (Eales et al., 2014). Mice were tested for 16 consecutive days to evaluate training (Days 1-5), learning (days 6-10), and reversal learning (days 11-16) (Figure 3A). In both WT and KO mice, we found that the distance traveled significantly decreased during the training and learning phases (Days 1-10) and during the reversal phase (Days 11-16), indicating that all mice were able to learn the location of the exit hole. Evaluation of distance traveled in each phase did not reveal significant differences in sex or genotype (Figure 3B). Next, we measured the number of errors performed by each mouse during training, learning, and reversal but found no sex or genotype-specific differences (Figure 3C). We also calculated the quadrant bias ratio as a measure of the frequency of visitation to the quadrant containing the exit hole during the training/learning phases (Days 1-10) but also found no significant differences (Figure 3D, left). Finally, we calculated the perseverance ratio as a reflection of the frequency of visitation to the quadrant to the old location of the exit hole during the reversal phase but again found no significant differences (Figure 3D, right). The employment of specific strategies to evaluate efficiency can also be utilized in the Barnes maze task. As described previously, we divided navigation strategies into 3 different categories: spatial, serial, and random (Wall et al., 2018). When separated into these different phases (training, learning, and reversal), we found no sex- or genotype-specific differences in strategy selection across phases (Figures 3E-G).

Given the lack of phenotype in adult KO mice, we next measured learning phenotypes in middle-age mice (> 1 year). Although there were no genotypic differences in KO males or females in total distance traveled, quadrant bias ratio, or perseverance ratio (Figures 4A-D), there were sex-specific differences in the
number of errors selectively during reversal with a significant effect found between male and female KO mice (Figure 4C). Upon separating the assay into time blocks of phases, sex differences were not observed in strategy usage between groups (Figures 4E-4G, Table 1). However, there was a main effect of genotype on serial and random strategy usage (Figures 4F and 4G). Rnf216 KO mice had a significant increase in using the serial strategy across training, learning, and reversal phases with a reduction in the random search strategy during the training phase. These findings show that upon aging, Rnf216 KO mice selectively adopt alternative search strategies in a hippocampal-dependent spatial task.

In addition to testing hippocampal-dependent spatial memory, we evaluated another type of learning that included associative learning through fear conditioning. In our fear conditioning task, mice were given 3 pairs of a tone associated with a shock (Day 1). On day 2, mice were tested for context recall (same environment), and on Day 3, mice were tested for cue recall (different environment + tone). Although there were no differences in time spent freezing, number of freezing episodes, or length of bout during conditioning, context recall, or cue recall in adult male and female KO mice (Figure 5A-C), middle-age KO mice had an overall decrease in freezing time during context recall (Figure 5E).

We next compared Barnes maze strategy behavioral alterations between adult and middle-age mice. In both WT and KO mice, we observed significant shifts in correlations suggesting a transition in the dependence of strategy usage as mice age (Figures 6A and 6B). WT middle-age mice overall had reduced use of the serial strategy and adopted the inefficient random search strategy, which was confirmed by a strong significant correlation between spatial and serial strategy use in adult WT (Figure 6A, top) and significant correlations between spatial and random strategies in middle-age WT mice (Figure 6B, top). Although distinct patterns between adult and middle-age mice emerged in both genotypes, the patterns in middle-age KO mice were highly like that of adult WT mice, where the correlation between spatial and serial strategy use was strongest (Figure 6B, bottom). Cumulatively, these findings suggest that middle-age Rnf216 KO mice exhibit strategies that are more tuned to adult WT mice, which could reflect a developmental stunting of strategy formation. Overall, we conclude that middle-age KO mice have a breakdown in performance necessary to support the use of age-appropriate strategies. Moreover, middle-age Rnf216 KO mice begin to develop subtle fear learning impairments, which is concordant with the development of dementia in individuals with GHS (Margolin et al., 2013; Alqwaifly and Bohlega, 2016).
Rnf216 KO males display lower intrinsic excitability in hippocampal CA1 neurons

Due to the role of RNF216 in synaptic plasticity and receptor trafficking (Mabb et al., 2014; Wall et al., 2018; George et al., 2022) and our findings related to fear conditioning, we sought to determine if neurotransmission was altered in Rnf216 KO mice. We measured miniature excitatory postsynaptic currents (mEPSCs) in CA1 neurons of acute hippocampal slices (Figure 7A) in P28 mice. In both males and females, there were no differences in amplitude or frequency (Figures 7B-D); however, there were sex differences in rise and decay times (Figures 7C and 7D). Notably, Rnf216 KO males had a more negative resting membrane potential (Figure 7E) suggesting a decreased probability of firing action potentials in male KO mice. There were no significant differences observed in females.

Adult Rnf216 KO mice have altered microglia in the hippocampus and cortex

RNF216 has a function in innate inflammatory signaling (Chuang and Ulevitch, 2004; Fearns et al., 2006; Nakhai et al., 2009) and phenotypes related to neuroinflammation were previously observed in the preoptic area of the hypothalamus of adult Rnf216 KO male mice (George et al., 2022). We analyzed microglia density, area, soma size and morphology in the dorsal hippocampus and S1 cortex of adult Rnf216 WT and KO mice, by labeling microglia with the microglia-specific marker Iba1. Although there were no sex- or genotypic differences in Iba1 total cell area (Figure 8B, left), we observed a significant increase in the density of microglia selectively in Rnf216 KO female mice (Figure 8B, right). We further identified sex differences between WT male and female microglia soma size and a sex-specific reduction in male KO somas (Figure 8C). When evaluating microglia morphology using the Sholl-based method, sex differences in male and female microglia were observed with no genotypic differences. Overall, female WT and KO microglia at this age had an increase in the number of intersections compared to males (Figure 8D and 8E). Surprisingly, there were no significant differences between WT and KO male microglia (Figure 8D and 8E). When evaluating microglia in S1 cortex (Figure 8F), there were no differences in Iba1 area (Figure 8G, left). Like the hippocampus, KO females had a selective increase in microglia density (Figure 8G, right). Significant decreases in soma size were still observed in male KO mice but there were no sex differences in soma size (Figure 8H). Upon inspection of microglia morphology using Sholl analysis, there were significant changes between all groups (Figures 8I and 8J), with male KO mice exhibiting a dramatic reduction in the number of intersections (Figures 8I and 8J). Taken together, our findings indicate that
global deletion of Rnf216 leads to sex- and region-specific alterations in microglia characteristics in select brain regions. These alterations in microglia profiles over time may lead to proprioceptive phenotypes and increase susceptibility to cognitive disruptions observed in middle-age GHS model mice.

Discussion

In this study, we evaluated changes in motor and memory using Rnf216 constitutive KO mice over the time course of a year. We speculated that deletion of Rnf216 would result in motor and learning impairments that increase in severity upon age, similar to phenotypes observed in GHS patients (Margolin et al., 2013). In GHS, the onset of motor disturbances ranges in age from teens to middle adult age. Our 1-year-old mice are similar to middle-age adults, which is why we selectively chose this timepoint for our behavior analysis (Dutta and Sengupta, 2016). However, to our surprise, we were unable to detect motor impairments or other measures of behavior that would be indicative of ataxia in these mice, which is a core feature of GHS (Gonzalez-Latapi et al., 2021). This lack of phenotype was also coupled with our inability to detect decreases in cerebellar mass that is a distinguishing feature in all late-stage GHS patients (Seminara et al., 2002; Margolin et al., 2013; Alqwaifly and Bohlega, 2016; Calandra et al., 2019; Chen et al., 2022). Both male and female Rnf216 KO mice developed normal motor functions as demonstrated by righting reflexes and hindlimb clamping at 3 weeks of age. Deficits in the rotarod, open field, and grip strength tasks were also not observed, except for middle-age KO mice traveling an increased distance in the center of the open field. However, Rnf216 KO females did develop abnormal limb clamping beginning at 9 weeks of age, which affected a larger fraction of female KO mice at almost 1-year suggesting that females may begin to develop proprioceptive deficits.

Surprisingly, adult Rnf216 KO mice did not exhibit learning deficits in the Barnes Maze spatial task but middle-age KO mice exhibited a bias in usage of a serial strategy across all task phases. Increases in serial strategy use were also observed in a previous transgenic mouse model for the RNF216 substrate Arc (Mabb et al., 2014; Wall et al., 2018). Here, mutation of RNF216-dependent Arc ubiquitination sites led to selective reversal learning deficits in juvenile male constitutive Arc knock-in mice (ArcKR) (Wall et al., 2018). However, in contrast to our findings here, ArcKR mice had an increase in error number that was also correlated with enhanced perseverance ratios and increased serial strategy use in the reversal phase only. Reasons for these discrepancies could involve the age in which we conducted this task (ArcKR mice...
were tested at the juvenile stage whereas Rnf216 KO mice were tested in early and late adulthood). E3 ligase compensation for Arc ubiquitination, additional requirements of RNF216-dependent Arc ubiquitination sites, or involvement of other RNF216 substrates. When comparing strategies during aging, middle-age WT mice exhibited an increase in random over serial strategy search. However, Rnf216 KO mice exhibited serial strategy correlation profiles that were more similar to adult WT mice, suggesting that KO mice have delays in age-appropriate use of the random search strategy. These subtle differences in strategy use may be reflective of age-related changes in the output from the hippocampal system to associative areas such as the prefrontal cortex, which is selectively active during spatial navigation strategy and responsible for goal location and route planning (Poucet et al., 2004; Negrón-Oyarzo et al., 2018).

The fear conditioning task in our study had 2 components: context- and cue-dependent recall, both of which have overlapping and non-overlapping microcircuits underlying each type of recall. Although there were no differences seen during cue recall, middle-age Rnf216 KO mice displayed decreased freezing time only during context recall. The underlying circuitry of fear conditioning includes projections from ventral hippocampal CA1 to the basal amygdala that encode context-dependent recall (Kim and Cho, 2020). However, during cue-recall, some studies show that an auditory cue excites PV+ interneurons that indirectly disinhibit the basolateral amygdala (Wolff et al., 2014) while other studies posit that auditory stimuli evoke the pathway from the thalamus to the lateral amygdala (Bordi and LeDoux, 1994). From this, we gather that RNF216 may not directly impair whole brain regions, but rather influence transmission between distinct brain regions. This is consistent with GHS clinical cases that show neuropathologies with loss of neurons and atrophy in specific brain regions and hippocampal inclusion bodies, indicative of neurodegeneration. Because there are multiple brain regions affected in GHS individuals, it is possible that the connections between them become dysfunctional.

The unique feature of this study compared to the clinical literature that identified specific RNF216 mutations is that we used a constitutive KO model. Not all GHS patients have deletions, but rather point mutations that create dysfunctional RNF216 proteins (Margolin et al., 2013), which may account for the presence of a motor phenotype. However, male and female individuals with RNF216 mutations that were predicted to be deleterious still exhibit ataxia so using Rnf216 KO mice is deemed an appropriate disease
model. As an example, individuals with a E205fsX15+ C59X mutation or P606L mutation demonstrate severe limb, gait, appendicular, and truncal cerebellar ataxia with prominent cerebellar atrophy (Mehmood et al., 2017; Calandra et al., 2019). Homozygous variants predicted to be deleterious such as c.2251C>T and c.2149C→T found within the catalytic domain of RNF216 still display progressive ataxia with cerebellar atrophy (Margolin et al., 2013) indicating regardless of the type of mutation, ataxia is still present. Along with progressive ataxia, some individuals with heterozygous mutations of G13GfxX74 and C597X+E205DfsX15 that lie outside the catalytic domain, also display chorea which involves involuntary jerking movements, distinct from ataxia. The human RNF216 gene differs from mouse in that RNF216 has 5 predicted isoforms (TRIAD3A, TRIAD3B, TRIAD3C, TRIAD3D, and TRIAD3E) that are invariably spliced at the N-terminus (Chuang and Ulevitch, 2004) while mouse Rnf216 has only 3 predicted isoforms (TRIAD3A, TRIAD3B, and TRIAD3C) (Consortium, 2020). It is possible that although both humans and mice express TRIAD3A, TRIAD3B, and TRIAD3C, the additional human isoforms such as TRIAD3D/E (that are also differentially expressed in the brain) have a more dominant role in specific pathologies such as ataxia (Chuang and Ulevitch, 2004). Our Rnf216 KO mouse is predicted to remove all 3 mouse isoforms of RNF216, but it is possible that these mice still express truncated versions of different isoforms. Our inability to detect any measure of brain volume loss in this study and in previous work (George et al., 2022) may point to additional distinct differences in how RNF216 is utilized in rodents compared to humans. This could in part be resolved by studying human models that include patient-derived iPSC cells. Another possibility is that compound mutations in other genes in addition to RNF216 may contribute to a more severe phenotype. Due to identification of digenic recessive mutations in GHS individuals that contain OTUD4 and RNF216 (Margolin et al., 2013), other unidentified genes may be participating in this complex syndrome. Environmental factors may also drive some of the severe phenotypes in human patients, which could be further pursued in our mouse model.

Another layer of data uncovered in this study is that electrophysiological and microglia alterations in constitutive male and female Rnf216 KO mice were found to be region- and sex-specific. Functionally, microglia are involved in synaptic maturation through synaptic engulfment during postnatal development and are thought to be important for neuroprotection as a synaptic ‘stripper’ (Trapp et al., 2007; Paolicelli et al., 2011; Kettenmann et al., 2013). It is known that microglia profiles differ between males and females (Schwarz et al., 2012; Bordt et al., 2020). For example, as early as postnatal day 0, sex differences in microglia morphologies are observed in the CA1 hippocampus and the paraventricular nucleus of the
hypothesis (Schwarz et al., 2012). The pro-and anti-inflammatory cytokine profiles also differ between sex and region upon aging (Schwarz et al., 2012). Although males had lower resting membrane potentials (RMP) and reduced microgliosis soma size in the hippocampus, females had no change in RMP but an increased density of microglia. The sex differences we have reported with microglia in the hippocampus are concordant to those reported in young adult mice (Schwarz et al., 2012; Bordt et al., 2020). Upon examination of microglia branching using Sholl, there were no differences in the number of intersections between WT and KO males. However, KO females had an increase in the number of intersections around the soma compared to WT females. It is possible that the select microglia alterations in male mice might lead to reduced RMP in this region with the alternative interpretation that these are unrelated observations. Microglia express a myriad of receptors that are classically involved in neurotransmitter and cytokine signaling (Kettenmann et al., 2011) and also act as active sensors in the CNS for “on” and “off” receptor-mediated signaling (Hanisch and Kettenmann, 2007). Therefore, it is possible that the hyperpolarized state observed in early male hippocampal neurons disrupt microglia receptor-mediated signaling, preventing their appropriate function throughout development. Compared to the hippocampus, adult male KOs displayed altered microglia branching (decreased intersections via Sholl analysis) and decreased soma size in the cortex. The collective alterations in microglia in these different brain regions reflect their diversity in function, namely in the remodeling of neuronal circuits which may become more pronounced with age (Hanisch and Kettenmann, 2007). Cumulatively, these data indicate that removal of Rnf216 differentially alters microglia states in male and female KO mice.

One account for microglia differences could also be related to alterations in gonadotropin and steroid hormone release. Gonadal hormones are thought to play a role in microglia sex-differences (VanRyzin et al., 2018). Although we did not measure estradiol directly in these mice, previous work did not identify ovarian failure in KO female mice (George et al., 2022). However, our previous studies also demonstrated that male KO mice selectively have elevated baseline FSH levels and reductions in Inhibin B compared to WT with signs of increased neuroinflammation as evidenced by alterations in microglia area and increases in the pro-inflammatory cytokine IL-1β (George et al., 2022). This is consistent with our current work showing males, in contrast to female KO mice, display reduced microglia soma size and branching. Importantly, testosterone gives rise to sexual differentiation in the vertebrate nervous system (Morris et al., 2004) and estradiol which is aromatized from testosterone, has effects on hippocampal morphology (Lephart, 1996). Accordingly, there may be a lack of organizational effects in KO males during the perinatal
or puberty time point leading to gonadal failure and altered hormonal release. This may also explain male-specific deficits in RMP. It is important to note that the electrophysiological and microglia-related phenotypes precede any of the learning deficits that we have observed. These early changes may alter neural circuit development and changes in microglia status, which collectively may render KO mice susceptible to behavioral alterations upon aging.

Taken together, our findings suggest that RNF216 may have region and sex-specific effects on anatomical and behavioral outputs. These alterations may also be present in male and female GHS individuals, which has yet to be evaluated in the clinical setting. Our findings of microglia alterations also suggest that a neuroinflammatory environment supersedes any display of neurodegeneration or behavioral impairments. Currently, there are limited treatment options for GHS whose pathologies worsen over time. Our study points to possible targets for therapeutic intervention specific to activated or reactive microglia. Identifying mechanisms and substrates involved in RNF216 ubiquitination would help elucidate the cause of neuroinflammation and different aspects of behaviors. Likewise, restoring behavioral impairments in Rnf216 KO with these targets would help shed light on interventions in other disorders with similar dysfunctions.

References


Figure 1: Neuromuscular and motor function are unaffected in adult and middle-age Rnf216 KO mice. (A) Top, Representative western blots for RNF216 in male and female Rnf216+/− (WT) and Rnf216−/− (KO) mice. Bottom, RNF216 protein levels in WT and KO mice. RNF216 values were normalized to ACTIN. For hippocampus, t(14) = 3.583, **p = 0.0030; cortex, t(14) = 3.676, **p = 0.0025; and for cerebellum, t(14) = 4.337, ***p = 0.0007. Unpaired t-test. N = 4 per genotype/sex for each brain region. Data are represented as box and whisker plots. (B) Behavior battery timeline for adults (P60) (top) and middle-age (P365) mice (bottom). (C) No differences in righting reflex in KO mice. \( F_{\text{time}}(1, 96) = 210.8, ****p < 0.0001; F_{\text{Genotype}}(1, 96) = 1.943, P=0.1666; F_{\text{time*Genotype}}(1, 96) = 2.261, P=0.1359. \) 2-way ANOVA. \( N = 49 \) mice per genotype. Data are represented as a violin plot. (D) Left, Average peak force of grip strength for adult mice. \( t(40) = 1.684, p = 0.0999. \) Middle, Total distance traveled in adult mice in an open field. \( t(40) = 0.7746, p = 0.4431. \) Unpaired t-test. \( N = 20 \) for WT and \( N = 23 \) for KO. Right, Total rearing movements in adult in an open field. \( F_{\text{sex}}(1, 38) = 23.80, ****p < 0.0001; F_{\text{Genotype}}(1, 38) = 0.004722, p = 0.9456; F_{\text{sex*Genotype}}(1, 38) = 1.721, p = 0.1974. \) Post-hoc: WT-Males vs. WT-Females, ***p = 0.0007; WT-Males vs. KO-Females, **p = 0.0067; WT-Females vs. KO-Males, **p = 0.0078. 2-way ANOVA with Tukey multiple comparisons. \( N = 10-12 \) mice per sex/genotype. Data are represented as box and whisker plots. (E) Left, Average peak force of grip strength in middle-age mice \( t(31) = 0.7981, p = 0.4309. \) Middle, Total distance traveled in middle-age mice in an open field. \( t(31) = 2.081, *p = 0.0457. \) Unpaired t-test. \( N = 17 \) for WT and \( N = 16 \) for KO. Right, Total rearing movements in adult in an open field. \( F_{\text{sex}}(1, 29) = 0.5386, p = 0.4689; F_{\text{Genotype}}(1, 29) = 0.05047, *p = 0.8238; F_{\text{sex*Genotype}}(1, 29) = 1.199, p = 0.2826. \) 2-way ANOVA. \( N = 7-9 \) mice per genotype/sex. Data are represented as box and whisker plots. (F) No genotypic differences in adult male and female KO mice in latency to fall off the rotating rod (left), latency to fall off the rotating rod for each trial on Day 1 (middle), or maximum velocity (right). \( N = 20-22 \) per genotype. (G) No significant differences in middle-age male and female KO mice. \( N = 16-17 \) per genotype. Error bars measured as ± SEM in F-G.

Figure 2: Abnormal limb-clasping in Rnf216 female KO mice. (A) Pie charts of limb-clasping in WT- and KO-Males. Limb-clasping was scored based on the following parameters: 0- no clasping (black); 1- forelimb clasping only (teal); 2- forelimb and hindlimb clasping. (B) Pie charts of limb-clasping in WT- and KO-Females. Limb-clasping was scored based on the following parameters: 0- no clasping (black); 1- forelimb clasping only (purple); 2- forelimb and hindlimb clasping (striped purple). KO-Females began to show hind limb-clasping at 9- and 41-weeks. At 41-weeks for females, \( (\chi^2(1, N = 45) = 4.724, *p = 0.0297; \) Chi-square
For trend). For 3-weeks, $N = 35-36$ for WT and 26-32 for KO; for 9-weeks, $N = 22-31$ for WT and 18-24 for KO; and for 41-weeks, $N = 19-24$ for WT and 17-26 for KO mice per sex/genotype.

**Figure 3: Lack of spatial learning deficits in adult Rnf216 KO mice.** (A) Schematic of Barnes maze task to evaluate spatial and reversal learning. Learning phase consisted of training (days 1-5) followed by assessments of learning (days 6-10). During the reversal phase (Days 11-16), the exit hole was rotated 180°. (B) Adult (~P70) WT and KO mice show no difference in total distance traveled on the maze before finding the exit hole. (C) No difference in the number of errors during the training, learning, or reversal phase. (D) Left, No differences in quadrant bias ratio. Right, no difference in perseverance ratio in KO mice. (E) Top, No differences in spatial strategy during each day across phases. Bottom, No differences in spatial strategy consolidated for each phase. (F) Top, No differences in serial strategy during each day across phases. Bottom, No differences in serial strategy consolidated for each phase. (G) Top, No differences in random strategy during each day across phases. Bottom, No differences in random strategy consolidated for each phase. $N= 20$ for WT and $N= 21$ for KO. Error bars are represented as ± SEM on top graphs in E-G and as box and whisker plots on bottom graphs.

**Figure 4: Altered search strategies in middle-age Rnf216 KO mice.** (A) Schematic of Barnes maze task to evaluate spatial and reversal learning. Learning phase consisted of training (days 1-5) followed by assessments of learning (days 6-10). During the reversal phase (Days 11-16), the exit hole was rotated 180°. (B) Middle-age (~P375) WT and KO mice show no difference in total distance traveled on the maze before finding the exit hole. (C) No genotypic differences in the number of errors in both males and females during training, learning, and reversal phase. There were sex differences during the reversal phase. KO-Males have a higher number of errors than KO-Females. $F_{\text{Genotype}} (1, 27) = 0.09622, \ p = 0.7588; F_{\text{Sex}} (1, 27) = 16.42, ***p = 0.0004; F_{\text{Sex*Genotype}} (1, 27) = 0.04985, \ p = 0.8250.$ Post-hoc: WT-Males vs. KO-Females, *p = 0.0156; KO-Males vs. KO-Females, *p = 0.0239. 2-way ANOVA with Tukey multiple comparisons. (D) Left, No differences in quadrant bias ratio. Right, no difference in perseverance ratio. (E) Top, No differences in spatial strategy during each day across phases. Bottom, No differences in spatial strategy consolidated for each phase. (F) Top, differences in serial strategy during each day across phases. Training: $F_{\text{Time}} (2.770, 79.62) = 2.583, \ p = 0.0637; F_{\text{Genotype}} (1, 29) = 8.306, **p = 0.0074; F_{\text{Time*Genotype}} (4,115) = 1.121, \ p = 0.3502;$ Learning: $F_{\text{Time}} (3.436, 99.63) = 0.1508, \ p = 0.9463; F_{\text{Genotype}} (1, 29) = 8.537, **p = 0.0067;$
F_{Time*Genotype} (4,116) = 0.9341, P = 0.4468. Post-hoc: Day 7, \(*p = 0.0482\); Day 10, \(*p = 0.0309\); Reversal: F_{Time} (4.011, 116.3) = 1.960, P= 0.1070. Post-hoc: Day 14, **\(p = 0.0050\). 2-way ANOVA with Sidak’s multiple comparisons. Bottom, differences in serial strategy consolidated for each phase. Training: t(29) = 2.838, **\(p = 0.0082\); Learning: t(29) = 2.984, **\(p= 0.0057\); Reversal: t(29) = 2.648, *\(p= 0.0130\). Unpaired t-test. (G) Top, differences in random strategy during the training phase. F_{Time} (3.475, 99.90) = 11.99, ****\(P < 0.0001\); F_{Genotype} (1, 29) = 5.205, *\(P= 0.0300\); F_{Time*Genotype} (4,115) = 0.5664, P = 0.6875. Mixed-effects model. Bottom, differences in random strategy during the consolidated training phase. t(29) = 2.252, *\(p = 0.0320\). Unpaired t-test. N = 15 for WT and N = 16 for KO.

**Figure 5:** Decreased context recall in middle-age Rnf216 KO mice. (A) No differences in time spent freezing during conditioning on Day 1 in adult WT and KO mice or average number of freezing episodes (bottom left) or length of bout (bottom right). (B) No differences in time spent freezing during context recall on Day 2 in adult WT and KO mice or average number of freezing episodes (bottom left) or length of bout (bottom right). (C) No differences in time spent freezing during cue recall on Day 3 in adult WT and KO mice or average number of freezing episodes (bottom left) or length of bout (bottom right). N = 10-11 mice per sex/genotype. (D) No differences in time spent freezing during conditioning on Day 1 in aged WT and KO mice or average number of freezing episodes (bottom left) or length of bout (bottom right). (E) Middle-age KO mice spent less time freezing during context recall on Day 2. F_{Time} (3.852, 57.79) = 10.60, ****\(P<0.0001\); F_{Genotype} (1, 15) = 12.37, **\(P=0.0031\); F_{Time*Genotype} (3.648, 54.71) = 1.275, P=0.2918. 2-way ANOVA with Sidak’s multiple comparisons. There were no differences on average number of freezing episodes (bottom left) or length of bout (bottom right). (F) No differences in time spent freezing during cue recall on Day 3 in middle-age WT and KO mice or average number of freezing episodes (bottom left) or length of bout (bottom right). N = 7-9 mice per genotype/sex. Top, Error bars are represented as ± SEM. Bottom, Data are represented as box and whisker plots.

**Figure 6:** Middle-age Rnf216 KO mice exhibit search strategies that are more tuned to adult WT mice. (A) Pearson correlation in adult WT (top) and KO (bottom) mice using behavioral output parameters related strategy search in the Barnes maze. (B) Pearson correlation in middle-age WT (top) and KO (bottom) mice using behavioral output parameters related to open field, strategy search in the Barnes maze. *\(P < 0.05\), **\(P < 0.005\), ***\(P < 0.0005\), ****\(P < 0.00005\).
Figure 7: Decreased intrinsic excitability and microglial expression in the hippocampus of Rnf216 KO male mice. (A) Representative miniature excitatory postsynaptic potential (mEPSC) traces from WT and KO male and female mice. There were no differences in (B) amplitude or frequency. (C) Sex differences were observed in rise time. F_{Sex} (1, 94) = 13.45, ***P = 0.0004; F_{Genotype} (1, 94) = 0.04758, P = 0.8278; F_{Sex*Genotype} (1, 94) = 0.8068, P = 0.3714. Post-hoc: WT-Males vs WT-Females, *p = 0.0181; WT-Males vs. KO-Females, *p = 0.0262. 2-way ANOVA with Tukey multiple comparisons. (D) There were sex differences in decay time. F_{Sex} (1, 94) = 15.77, ***P = 0.0001; F_{Genotype} (1, 94) = 0.4680, P = 0.4956; F_{Sex*Genotype} (1, 94) = 3.839, P = 0.0530. Post-hoc: WT-Males vs. WT-Females, **p = 0.0011; KO-Male vs. WT-Female, *p = 0.0109. 2-way ANOVA with Tukey multiple comparisons. (E) More negative resting membrane potential in KO male mice. F_{Sex} (1, 94) = 2.363, P = 0.1276; F_{Genotype} (1, 94) = 8.877, **P = 0.0037; F_{Sex*Genotype} (1, 94) = 0.4267, P = 0.5152. Post-hoc: WT-Males vs. KO-Males, *p = 0.0258; KO-Males vs. WT-Females, *p = 0.0144. 2-way ANOVA with Tukey multiple comparisons. N = 4 mice per sex/genotype and n = 16 cells for WT-Females, 24 for KO-Females, 37 for WT-Males, and 43 for KO-Males.

Figure 8: Altered microglia in the hippocampus and cortex of adult Rnf216 KO mice. (A) Top, representative images of microglia stained with Iba1 in the hippocampus in adult (~P112) WT and KO males (left) and females (right) imaged at 20X magnification. Scale bar represents 100 μm, inset represents 10 μm. (B) Left, Area of Iba1. F_{Sex} (1, 20) = 2.087, P = 0.1641; F_{Genotype} (1, 20) = 0.9083, P = 0.3519; F_{Sex*Genotype} (1, 20) = 6.251, *p = 0.0212. 2-way ANOVA. Right, Density of Iba1 positive cells. There were significant increases in cell density but not area in KO-Females. F_{Sex} (1, 20) = 33.08, ****P < 0.0001; F_{Genotype} (1, 20) = 14.13, **P < 0.0012; F_{Sex*Genotype} (1, 20) = 16.69, ***P < 0.0006. Post-hoc: WT-Males vs KO-Females, ****p < 0.0001; KO-Males vs Female KO, ****p < 0.0001; WT-Females vs. KO-Females, ***p = 0.0001. 2-way ANOVA with Tukey’s multiple comparisons. N = 3 per sex/genotype with 1-2 sections per mouse represented in summary plots. (C) Distribution of soma size in WT and KO mice. Male KO mice have smaller soma size. Welch’s t values for WT-Male vs. KO-Male -0.472 (p = 0.033), WT-Male vs. WT-Female -4.620 (p < 0.001), KO-Male vs. KO-Female -12.116 (p < 0.001). N = 127-293 cells per genotype/sex. (D) Left, Sholl analysis of reconstructed microglia measuring number of intersections in proximity to the soma. Right, Area Under the Curve fittings using hierarchical bootstrapping for individual microglia. Female microglia had more intersections than male microglia. Welch’s t value for WT-Male vs. WT-Female 7.071 (p < 0.001) and KO-Male vs. KO-Female 6.752 (p < 0.001). N = 3 mice per genotype/sex, n = 30 reconstructed cells per.
group. Error bars are represented as ± SEM. (E) Example 3D reconstructions of microglia from each group. (F) Top, representative images of microglia stained with Iba1 in the cortex in adult (~P112) WT and KO males (left) and females (right) imaged at 20X magnification. Scale bar represents 100 µm, inset represents 10 µm. (G) Left, Area of Iba1. F
\text{Sex} (1, 8) = 0.6448, P=0.4452; F
\text{Genotype} (1, 8) = 4.515, P=0.0663; F
\text{Sex*Genotype} (1, 8) = 1.305, P=0.2863. 2-way ANOVA. Right, Density of Iba1 positive cells. There were significant increases in cell density but not area in KO females. F
\text{Sex} (1, 8) = 29.89, ***P=0.0006; F
\text{Genotype} (1, 8) = 26.11, ***P=0.0009; F
\text{Sex*Genotype} (1, 8) = 17.33, **P=0.0032. Post-hoc: WT-Males vs. KO-Females, ***p = 0.0003; KO-Males vs. KO-Females, ***p = 0.0006; WT-Females vs. KO-Females, ***p = 0.0008. 2-way ANOVA with Tukey's multiple comparisons. N = 3 per sex/genotype with 1 section per mouse represented in summary plots. (H) Distribution of soma size in WT and KO mice. Male KO mice have smaller soma size. Welch's t values for WT-Male vs. KO-Male 5.663 (p < 0.001) and KO-Male vs. KO-Female -7.267 (p < 0.001). N = 128-267 cells per genotype/sex. (I) Left, Sholl analysis of reconstructed microglia measuring number of intersections in proximity to the soma. Right, Area Under the Curve fittings using hierarchical bootstrapping for individual microglia. All groups were significantly different from one another with KO male microglia exhibiting the greatest reduction in intersection number. Welch's t value for WT-Male vs. KO-Male -4.330 (p < 0.001), WT-Female vs. KO-Female 2.029 (p = 0.04), WT-Male vs. WT-Female 3.267 (p = 0.002), KO-Male vs. KO-Female 8.398 (p < 0.001). N = 3 mice per genotype/sex, n = 30 reconstructed cells per group. Error bars are represented as ± SEM. (J) Example 3D reconstructions of microglia from each group.
Table 1: Statistical table results from experiments in this manuscript analyzed for sex differences and collapsed to genotype only.

Table 2: Top, Cerebellar weights in 16- and > 52-week mice. There were no significant differences. $N = 11$-25 mice per genotype. Cerebellar weights were normalized to brain weights. Error bars are $\pm$ SEM.
<table>
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<tr>
<th>Figure</th>
<th>Data structure</th>
<th>Type of test</th>
<th>Number of samples</th>
<th>Statistical report</th>
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<td>2-way ANOVA, Hippocampus</td>
<td>N for male KO = 4, N for male WT = 4, N for female KO = 4, N for female WT = 4</td>
<td>Sex F (1, 12) = 0.4458, P=0.5179. Genotype F (1, 12) = 8.506, *P=0.0129</td>
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<td>2-way ANOVA, Cortex</td>
<td>N for male WT = 4, N for male KO = 4, N for female WT = 4, N for female KO = 4</td>
<td>Sex F (1, 12) = 0.02658, P=0.8732. Genotype F (1, 12) = 14.08, **P=0.0028</td>
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<td>2-way ANOVA, Cerebellum</td>
<td>N for male WT = 4, N for male KO = 4, N for female WT = 4, N for female KO = 4</td>
<td>Sex F (1, 12) = 0.005076, P=0.9444. Genotype F (1, 12) = 34.84, ****P&lt;0.0001</td>
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<td>Figure 1e</td>
<td>normal, peak force</td>
<td>2-way ANOVA</td>
<td>N for male WT = 21, N for male KO = 22, N for female WT = 28, N for female KO = 27</td>
<td>Sex F (1, 12) = 0.3461, p = 0.5577. Genotype F (1, 12) = 0.05198, p = 0.8202. Sex*Genotype F (1, 12) = 3.724, p = 0.0567</td>
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<td>normal, distance</td>
<td>2-way ANOVA</td>
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<td>Sex F (1, 38) = 4.314, <em>p = 0.0446. Genotype F (1, 38) = 4.314, p = 0.0808. Sex</em>Genotype F (1, 38) = 1.691, p = 0.2013</td>
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<td>2-way ANOVA</td>
<td>N for male WT = 8, N for male KO = 7, N for female WT = 9, N for female KO = 9</td>
<td>Sex F (1, 29) = 11.14, **p = 0.0023. Genotype F (1, 29) = 1.163, p = 0.2897. Sex*Genotype F (1, 29) = 0.4416, p = 0.5116</td>
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<td>3-way ANOVA</td>
<td>Normal, day 1</td>
<td>3-way ANOVA</td>
<td>Normal, speed</td>
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<td>Time F (2.774, 105.4) = 37.32, ***P&lt;0.0001</td>
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<td>Time F (1.880, 71.44) = 5.504, ***P=0.0069</td>
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<td>Sex F (1, 38) = 11.15, **P=0.0019</td>
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<td>Genotype F (1, 38) = 0.5063, P=0.4811</td>
<td>Time x Sex F (4, 152) = 0.6667, P=0.6161</td>
<td>Genotype F (1, 38) = 0.5077, P=0.4805</td>
<td>Time x Genotype F (2, 76) = 0.6286, P=0.6428</td>
<td>Genotype F (1, 38) = 0.0506, P=0.9879</td>
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<td>Time x Sex x Genotype F (2, 76) = 0.3379, P=0.7143</td>
<td>Sex x GenotypeF (1, 38) = 0.04574, P=0.8318</td>
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<td>Time x x x Genotype F (4, 152) = 0.6596, P=0.6210</td>
<td><strong>Figure 1f</strong></td>
<td><strong>Figure 1g</strong></td>
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<td>Normal, day 1</td>
<td>3-way ANOVA</td>
<td>Normal, speed</td>
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<td>Time F (2.669, 77.39) = 11.20, ****P&lt;0.0001</td>
<td><strong>N for male WT = 8</strong></td>
<td>Time F (1.608, 46.62) = 2.867, P=0.0777</td>
<td>Time F (2.669, 77.39) = <strong>N for male WT = 8</strong></td>
<td>Time F (3.414, 99.00) = 0.6498, P=0.6043</td>
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<td>Genotype F (1, 29) = 0.01372, P=0.9076</td>
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<td>Chi-square test for trend</td>
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<th>2-way ANOVA</th>
<th>N for male WT = 10 N for male KO = 10 N for female WT = 10 N for female KO = 11</th>
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<th>Two-way ANOVA</th>
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<td>N for male WT = 10</td>
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<td>Sex F (1, 37) = 0.5789, p = 0.4516</td>
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<td>Normal, Training</td>
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N for male WT = 10  
N for male KO = 10  
N for female WT = 10  
N for female KO = 11  
  
Time F (3.616, 132.9) = 5.999, ***p = 0.0003  
Sex F (1, 37) = 0.008932, p = 0.9252  
Genotype F (1, 37) = 0.5855, p = 0.4490  
Time*Sex F (4, 147) = 1.278, p = 0.2812  
Time*Genotype F (4, 147) = 1.449, p = 0.2209  
Sex*Genotype F (1, 37) = 1.607, p = 0.2129  
Time*Sex*Genotype F (4, 147) = 0.6212, p = 0.6481  

Figure 3f, top  

| Normal, Learning          | 3-way ANOVA   |  
N for male WT = 10  
N for male KO = 10  
N for female WT = 10  
N for female KO = 11  
  
Time F (3.516, 130.1) = 1.419, p = 0.2358  
Sex F (1, 37) = 1.014, p = 0.3204  
Genotype F (1, 37) = 2.447, p = 0.1262  
Time*Sex F (4, 148) = 0.1663, p = 0.9552  
Time*Genotype F (4, 148) = 1.169, p = 0.3268  
Sex*Genotype F (1, 37) = 0.6726, p = 0.4174  
Time*Sex*Genotype F (4, 148) = 0.1262, p = 0.9728  

| Normal, Reversal          | 3-way ANOVA   |  
N for male WT = 10  
N for male KO = 10  
N for female WT = 10  
N for female KO = 11  
  
Time F (4.661, 172.5) = 0.7716, p = 0.5631  
Sex F (1, 37) = 0.1616, p = 0.6900  
Genotype F (1, 37) = 0.8228, p = 0.3702  
Time*Sex F (5, 185) = 0.2535, p = 0.9377  
Time*Genotype F (5, 185) = 1.107, p = 0.3584  
Sex*Genotype F (1, 37) = 4.646, *p = 0.0377  
Time*Sex*Genotype F (5, 185) = 0.3423, p = 0.8867  

Figure 3f, bottom
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<th>df</th>
<th>p-Value</th>
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<td>normal, training</td>
<td>Mixed-effects model</td>
<td>Time F (3.373, 124.0) = 15.79, ***p&lt;0.0001</td>
<td>(1, 37) = 0.6634, p = 0.4206</td>
<td>N for male WT = 10, N for male KO = 10, N for female WT = 10, N for female KO = 11</td>
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<td>Sex F (1, 37) = 0.07769, p = 0.7920</td>
<td>(1, 37) = 0.1038, p = 0.7491</td>
<td>Time*Sex F (4, 147) = 0.5468, p = 0.7017</td>
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<td>3-way ANOVA</td>
<td>Time F (3.279, 121.3) = 1.011, p = 0.3948</td>
<td>(1, 37) = 0.2329, p = 0.6322</td>
<td>Sex*Genotype F (1, 37) = 2.650, p = 0.1120</td>
<td>N for male WT = 10, N for male KO = 10, N for female WT = 10, N for female KO = 11</td>
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<tr>
<td>normal, reversal</td>
<td>3-way ANOVA</td>
<td>Time F (4.441, 164.3) = 4.504, **p = 0.0012</td>
<td>(1, 37) = 1.063, p = 0.2824</td>
<td>Sex*Genotype F (1, 37) = 2.650, p = 0.1120</td>
<td>N for male WT = 10, N for male KO = 10, N for female WT = 10, N for female KO = 11</td>
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<tr>
<td><strong>Figure 3g, bottom</strong></td>
<td></td>
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<tr>
<td>normal, training</td>
<td>Two-way ANOVA</td>
<td>N for male WT = 10, N for male KO = 10</td>
<td>Sex F (1, 37) = 0.7567, p = 0.3900</td>
<td>Genotype F (1, 37) = 0.2900, p = 0.6064</td>
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<tr>
<td>normal, learning</td>
<td>Two-way ANOVA</td>
<td>N for male WT = 10, N for male KO = 10</td>
<td>Sex F (1, 37) = 0.07769, p = 0.7820</td>
<td>Genotype F (1, 37) = 0.1038, p = 0.7491</td>
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</tr>
<tr>
<td>normal, reversal</td>
<td>Two-way ANOVA</td>
<td>N for male WT = 10, N for male KO = 10</td>
<td>Sex F (1, 37) = 0.2329, p = 0.6322</td>
<td>Genotype F (1, 37) = 2.119, p = 0.1539</td>
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</tbody>
</table>
| Figure 4b | normal, training | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 0.2161, p = 0.6458
Genotype F (1, 27) = 0.7201, p = 0.4036
Sex*Genotype F (1, 27) = 0.1749, p = 0.6791 |
| Figure 4b | normal, learning | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 6.217, *p = 0.0191
Genotype F (1, 27) = 0.3967, p = 0.5341
Sex*Genotype F (1, 27) = 0.005580, p = 0.9410 |
| Figure 4c | normal, training | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 12.76, **p = 0.0014
Genotype F (1, 27) = 3.819, p = 0.0611
Sex*Genotype F (1, 27) = 0.0004932, p = 0.9824
**p = 0.0018 |
| Figure 4c | normal, learning | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 7.576, *p = 0.0104
Genotype F (1, 27) = 0.02448, p = 0.8768
Sex*Genotype F (1, 27) = 0.0003824, p = 0.9845 |
| Figure 4d | normal, reversal | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 16.42, ***p = 0.0004
Genotype F (1, 27) = 0.09622, p = 0.7588
Sex*Genotype F (1, 27) = 0.04985, p = 0.8250
Tukey multiple comparisons:
Males WT vs. Females KO, *p = 0.0156
Males KO vs. Females KO, *p = 0.0239 |
| Figure 4d | normal | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 0.7561, p = 0.3922
Genotype F (1, 27) = 0.7561, p = 0.3922
Sex*Genotype F (1, 27) = 0.07468, p = 0.7867 |
| Figure 4d | normal | 2-way ANOVA | N for male WT = 8
N for male KO = 7
N for female WT = 7
N for female KO = 9 | Sex F (1, 27) = 1.094, p = 0.3048
Genotype F (1, 27) = 0.7384, p = 0.3839
Sex*Genotype F (1, 27) = 0.1619, p = 0.6906 |
| Figure 4e, top | normal, training | Mixed-effects model | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (3.346, 89.50) = 3.582, p = 0.0137  
Sex F (1, 27) = 0.0443, p = 0.8348  
Genotype F (1, 27) = 0.0128, p = 0.9105  
Time*Sex F (4, 107) = 1.088, p = 0.3662  
Time*Genotype F (4, 107) = 0.6137, p = 0.6537  
Sex*Genotype F (1, 27) = 3.396, p = 0.0764  
Time*Sex*Genotype F (4, 107) = 2.133, p = 0.0817 |
| Figure 4e, bottom | normal, training | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (3.908, 105.5) = 2.622, p = 0.0401  
Sex F (1, 27) = 3.849, p = 0.0662  
Genotype F (1, 27) = 3.603, p = 0.0684  
Time*Sex F (5, 135) = 0.5075, p = 0.7702  
Time*Genotype F (5, 135) = 1.994, p = 0.0835  
Sex*Genotype F (1, 27) = 0.3262, p = 0.5726  
Time*Sex*Genotype F (5, 135) = 1.334, p = 0.2534 |
| Figure 4f, top | normal, training | Mixed-effects model | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (1.164, 31.43) = 0.4812, p = 0.5212  
Sex F (1, 27) = 0.1388, p = 0.7124  
Genotype F (1, 27) = 4.718, p = 0.0388  
Time*Sex F (4, 108) = 0.6351, p = 0.6385  
Time*Genotype F (4, 108) = 0.5682, p = 0.6747  
Sex*Genotype F (1, 27) = 0.1202, p = 0.7315  
Time*Sex*Genotype F (4, 108) = 0.3982, p = 0.8096 |
| normal, learning | 3 Way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (3.432, 92.68) = 1.055, p = 0.3778  
Sex F (1, 27) = 3.444, p = 0.0744  
Genotype F (1, 27) = 1.476, p = 0.2346  
Time*Genotype F (4, 108) = 0.3233, p = 0.5075  
Time*Genotype F (4, 108) = 1.401, p = 0.2386  
Sex*Genotype F (1, 27) = 0.9628, p = 0.3352  
Time*Sex*Genotype F (4, 108) = 1.632, p = 0.1715 |
| normal, reversal | 3 Way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (3.908, 105.5) = 2.622, p = 0.0401  
Sex F (1, 27) = 3.849, p = 0.0662  
Genotype F (1, 27) = 3.603, p = 0.0684  
Time*Sex F (5, 135) = 0.5075, p = 0.7702  
Time*Genotype F (5, 135) = 1.994, p = 0.0835  
Sex*Genotype F (1, 27) = 0.3262, p = 0.5726  
Time*Sex*Genotype F (5, 135) = 1.334, p = 0.2534 |
| normal, learning | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Sex F (1, 27) = 2.637, p = 0.1160  
Genotype F (1, 27) = 3.093, p = 0.0900  
Sex*Genotype F (1, 27) = 0.08495, p = 0.7729 |
| normal, reversal | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Sex F (1, 27) = 3.849, p = 0.0602  
Genotype F (1, 27) = 3.603, p = 0.0684  
Sex*Genotype F (1, 27) = 0.3262, p = 0.5726 |
| normal, learning | 3 Way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (2.835, 75.84) = 3.066, p = 0.0356  
Sex F (1, 27) = 2.197, p = 0.1499  
Genotype F (1, 27) = 9.849, *p = 0.0041  
Time*Sex F (4, 107) = 2.615, p = 0.0392  
Time*Genotype F (4, 107) = 1.243, p = 0.2972  
Sex*Genotype F (1, 27) = 1.633, p = 0.2122  
Time*Sex*Genotype F (4, 107) = 2.190, p = 0.0750 |
| normal, reversal | 3 Way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9 | Time F (4.046, 109.2) = 1.749, p = 0.1437  
Sex F (1, 27) = 0.1645, p = 0.6882  
Genotype F (1, 27) = 6.623, *p = 0.0159  
Time*Sex F (5, 135) = 0.2910, p = 0.9173  
Time*Genotype F (5, 135) = 1.716, p = 0.0135  
Sex*Genotype F (1, 27) = 0.1365, p = 0.7207  
Time*Sex*Genotype F (5, 135) = 0.9189, p = 0.4708 |
| Figure 4f, bottom | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9  
Tukey multiple comparisons  
Males:WT vs. Males:KO, *p = 0.0228  
Males:KO vs. Females:WT, *p = 0.0241 | Sex F (1, 27) = 2.059, p = 0.1627  
Genotype F (1, 27) = 9.560, *p = 0.0046  
Sex*Genotype F (1, 27) = 1.757, p = 0.1961 |
| --- | --- | --- |
| Figure 4g, top | Mixed-effects model | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9  
Time F (3.444, 92.04) = 12.09, ****p<0.0001  
Sex F (1, 27) = 0.6334, p = 0.4330  
Genotype F (1, 27) = 5.254, *p = 0.0299  
Time*Sex F (4, 107) = 2.009, p = 0.0984  
Time*Genotype F (4, 107) = 0.7231, p = 0.5780  
Sex*Genotype F (1, 27) = 1.080, p = 0.3080  
Time*Sex*Genotype F (4, 107) = 0.03886, p = 0.9971 |
| Figure 4g, bottom | 3 Way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9  
Time F (4.092, 110.5) = 5.794, ***p = 0.0003  
Sex F (1, 27) = 1.683, p = 0.2055  
Genotype F (1, 27) = 2.900, p = 0.1001  
Time*Sex F (5, 135) = 0.2336, p = 0.9472  
Time*Genotype F (5, 135) = 0.3898, p = 0.8483  
Sex*Genotype F (1, 27) = 4.395e-006, p = 0.9983  
Time*Sex*Genotype F (5, 135) = 1.038, p = 0.3982 |
| Figure 4g, bottom | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9  
Sex F (1, 27) = 0.5944, p = 0.4474  
Genotype F (1, 27) = 5.149, *p = 0.0315  
Sex*Genotype F (1, 27) = 1.029, p = 0.3194 |
| Figure 4g, bottom | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9  
Sex F (1, 27) = 1.308, p = 0.2628  
Genotype F (1, 27) = 3.053, *p = 0.0919  
Sex*Genotype F (1, 27) = 2.694, p = 0.1123 |
| Figure 4g, bottom | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 7  
N for female KO = 9  
Sex F (1, 27) = 1.683, p = 0.2055  
Genotype F (1, 27) = 2.900, p = 0.1001  
Sex*Genotype F (1, 27) = 4.395e-006, p = 0.9983 |
<table>
<thead>
<tr>
<th>Figure</th>
<th>Time</th>
<th>ANOVA</th>
<th>N for male WT</th>
<th>N for male KO</th>
<th>N for female WT</th>
<th>N for female KO</th>
<th>Result</th>
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<td>3-way</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>Time F (3.014, 111.5) = 154.4, ****P&lt;0.0001</td>
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<td>Sex F (1, 37) = 0.4984, P=0.4846</td>
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<td>Genotype F (1, 37) = 0.3381, P=0.5644</td>
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<td>Time x Sex F (5, 185) = 1.301, P=0.2654</td>
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<td>Time x Genotype F (5, 185) = 0.3270, P=0.8962</td>
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<td>Sex x Genotype F (1, 37) = 0.9528, P=0.3354</td>
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<td>Time x Sex x Genotype F (5, 185) = 0.7126, P=0.6147</td>
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<td>5b</td>
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<td>Interaction F (1, 37) = 0.1562, P=0.6950</td>
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<td>Sex F (1, 37) = 0.02669, P=0.8711</td>
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<td>Genotype F (1, 37) = 1.640, P=0.2083</td>
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<td>5c</td>
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<td>Interaction F (1, 37) = 3.856, P=0.0571</td>
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<td>Sex F (1, 37) = 0.2707, P=0.6059</td>
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<td>Genotype F (1, 37) = 0.0007571, P=0.9782</td>
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</tbody>
</table>
| Figure 5d | normal, freezing time | 3-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 9  
N for female KO = 9  
Time F (3.188, 89.26) = 139.2, ***P<0.0001  
Sex F (1, 28) = 0.1210, P=0.7306  
Genotype F (1, 28) = 1.115, P=0.3000  
Time x Genotype F (5, 140) = 6.870, **P=0.0001  
Time x Sex F (5, 140) = 0.6086, P=0.6934  
Sex x Genotype F (1, 28) = 3.724, P=0.0638  
Time x Sex x Genotype F (5, 140) = 1.333, P=0.2539 |
| Figure 5e | normal, episodes | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 9  
N for female KO = 9  
Interaction F (1, 28) = 0.1741, P=0.6797  
Sex F (1, 28) = 0.4272, P=0.5187  
Genotype F (1, 28) = 0.01431, P=0.9056 |
| Figure 5f | normal, bout | 2-way ANOVA | N for male WT = 8  
N for male KO = 7  
N for female WT = 9  
N for female KO = 9  
Interaction F (1, 28) = 2.456, P=0.1283  
Sex F (1, 28) = 1.207, P=0.2812  
Genotype F (1, 28) = 1.199, P=0.2829 |
| Figure 6 | Correlation data | 2-way ANOVA | N for male WT = 26  
N for male KO = 31  
N for female WT = 16  
N for female KO = 24  
Interaction F (1, 93) = 1.285, P=0.2599  
Sex F (1, 93) = 0.2465, P=0.6207  
Genotype F (1, 93) = 0.4801, P=0.4901 |
| Figure 7b | normal, amplitude | 2-way ANOVA | N for male WT = 26  
N for male KO = 32  
N for female WT = 16  
N for female KO = 24  
Interaction F (1, 94) = 1.207, P=0.2748  
Sex F (1, 94) = 0.03845, P=0.8450  
Genotype F (1, 94) = 1.047, P=0.3087 |
| Figure 7c | normal, rise time | 2-way ANOVA | N for male WT = 37  
N for male KO = 43  
N for female WT = 16  
N for female KO = 24  
Interaction F (1, 94) = 0.3068, P=0.5714  
Sex F (1, 94) = 13.45, ***P=0.0004  
Genotype F (1, 94) = 0.04758, P=0.8278  
Tukey post-hoc:  
Male WT vs Females WT, *p = 0.0181  
Male WT vs. Female KO, *p = 0.0262 |
| Figure 7d   | normal, decay time | 2-way ANOVA | n for male WT = 37  
n for male KO = 43  
n for female WT = 16  
n for female KO = 24 | Interaction F (1, 94) = 3.839, P=0.0530  
Sex F (1, 94) = 15.77, "***"P=0.0001  
Genotype F (1, 94) = 0.4680, P=0.4956  
Tukey post-hoc:  
Male WT vs Females WT, "*"p = 0.0011  
Male KO vs. Female WT, "*p = 0.0109 |
| Figure 7e   | normal, RMP      | 2-way ANOVA | n for male WT = 37  
n for male KO = 43  
n for female WT = 16  
n for female KO = 24 | Interaction F (1, 94) = 0.4267, P=0.5152  
Sex F (1, 94) = 2.363, P=0.1276  
Genotype F (1, 94) = 8.877, "***"P=0.0037 |
| Figure 8a   | normal, area     | 2-way ANOVA | N for male WT = 6  
N for male KO = 6  
N for female WT = 6  
N for female KO = 6 | Interaction F (1, 20) = 6.251, "*"P=0.0212  
Sex F (1, 20) = 2.087, P=0.1641  
Genotype F (1, 20) = 0.9083, P=0.3519 |
| Figure 8c   | normal           | Hierarchical bootstrapping | N for male WT = 134  
N for male KO = 127  
N for female WT = 175  
N for female KO = 293 |  
| Figure 8d   | normal           | Hierarchical bootstrapping | N for male WT = 30  
N for male KO = 30  
N for female WT = 30  
N for female KO = 30 |  
| Figure 8e   | normal, area     | 2-way ANOVA | N for male WT = 3  
N for male KO = 3  
N for female WT = 3  
N for female KO = 3 | Interaction F (1, 8) = 1.305, P=0.2863  
Sex F (1, 8) = 0.6448, P=0.4452  
Genotype F (1, 8) = 4.515, P=0.0663 |
| Figure 8f   | normal, cell density | 2-way ANOVA | N for male WT = 3  
N for male KO = 3  
N for female WT = 3  
N for female KO = 3 | Interaction F (1, 8) = 17.33, "***"P=0.0032  
Sex F (1, 8) = 29.89, "***"P=0.0006  
Genotype F (1, 8) = 26.11, "***"P=0.0009  
Tukey Post-hoc:  
Male WT vs. Female KO, "*"p = 0.0003  
Male KO vs. Female KO, "*"p = 0.0006  
Female WT vs Female KO, "*"p = 0.0008 |
| Figure 8h   | normal           | Hierarchical bootstrapping | N for male WT = 30  
N for male KO = 30  
N for female WT = 30  
N for female KO = 30 |  
| Figure 8i   | normal           | Hierarchical bootstrapping | N for male WT = 30  
N for male KO = 30  
N for female WT = 30  
N for female KO = 30 |  

**Note:** The table above summarizes the results from various statistical analyses conducted on different experimental conditions. The figures represent different aspects of the experimental data, and the tables provide the statistical significance of the observed differences. The specific details of the analyses, including the F-values and p-values, are indicated in the table.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Data structure</th>
<th>Type of test</th>
<th>Number of samples</th>
<th>Statistical report</th>
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<td><strong>Rnf216 KO</strong></td>
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<tr>
<td>Figure 1a</td>
<td>normal</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 8</td>
<td>t(14)= 3.583, **p = 0.0030</td>
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<td>normal</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 8</td>
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<tr>
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<td>normal</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 8</td>
<td>t(14)= 3.676, **p = 0.0025</td>
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<td>normal</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 8</td>
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<tr>
<td>Figure 1c</td>
<td>normal, peak force</td>
<td>2-way ANOVA</td>
<td>N for WT = 49</td>
<td>Interaction F (1, 96) = 2.261, P=0.1359</td>
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<td>normal, distance</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 49</td>
<td>Time F (1, 96) = 210.8, ****P&lt;0.0001</td>
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<tr>
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<td>normal, rearing</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 20</td>
<td>t (40) = 1.684, p = 0.0999</td>
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<td>normal, latency</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 23</td>
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<tr>
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<td>normal, day 1</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 20</td>
<td>t (40) = 0.7746, p = 0.4431</td>
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<td>normal, speed</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 23</td>
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<td>Figure 1f</td>
<td>normal, rearing</td>
<td>2-way ANOVA</td>
<td>N for WT = 20</td>
<td>Interaction F (4, 160) = 2.653, *P=0.0351</td>
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<td></td>
<td>normal, latency</td>
<td>2-way ANOVA</td>
<td>N for KO = 22</td>
<td>Time F (2.83, 111.3) = 36.46, ****P&lt;0.0001</td>
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<td>N for WT = 20</td>
<td>Genotype F (1, 40) = 0.5417, P=0.4660</td>
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<td>2-way ANOVA</td>
<td>N for KO = 22</td>
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<td>Figure 1g</td>
<td>normal, rearing</td>
<td>2-way ANOVA</td>
<td>N for WT = 17</td>
<td>Interaction F (4, 124) = 0.4443,P=0.7763</td>
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<td>N for KO = 16</td>
<td>Time F (2.689, 83.36) = 11.99, ****P&lt;0.0001</td>
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<td>2-way ANOVA</td>
<td>N for WT = 17</td>
<td>Genotype F (1, 31) = 0.00760, P=0.9299</td>
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<td>Figure 3b</td>
<td>normal, speed</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 20</td>
<td>t(39)= 0.1161, p = 0.9081</td>
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<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 21</td>
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<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 20</td>
<td>t(39)= 1.280, p = 0.2081</td>
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<td>Figure 3c</td>
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<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 20</td>
<td>t(39)= 0.6991, p = 0.4948</td>
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<td>Unpaired t-test, two-tailed</td>
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<td>Figure 3d</td>
<td>normal, training</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 20</td>
<td>t(39)= 1.042, p = 0.3037</td>
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<td>Unpaired t-test, two-tailed</td>
<td>N for KO = 21</td>
<td>t(39)= 0.8512, p = 0.3999</td>
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| Figure 3e, top | normal, reversal | Mixed-effects model | N for WT = 20  
N for KO = 21 | Time F (3.667, 142.1) = 2.898, *P=0.0279  
Genotype F (1, 39) = 2.111, P=0.1543  
Time\*Genotype (1, 155) = 1.603, P = 0.1763 |
| | normal | 2-way ANOVA | N for WT = 20  
N for KO = 21 | Interaction F (4, 156) = 1.829, P = 0.1259  
Time F (3.797, 148.1) = 1.151, P=0.3344  
Genotype F (1, 39) = 3.230, P=0.0800 |
| | normal | 2-way ANOVA | N for WT = 20  
N for KO = 21 | Interaction F (5, 195) = 0.8804, P = 0.4953  
Time F (4.101, 159.9) = 3.495, **P=0.0086  
Genotype F (1, 39) = 0.03942, P=0.8436 |
| Figure 3e, bottom | normal, training | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)=1.476, p = 0.1479 |
| | normal, learning | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)=1.797, p = 0.0800 |
| | normal, reversal | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)= 0.1985, p = 0.8436 |
| Figure 3f, top | normal, training | Mixed-effects model | N for WT = 20  
N for KO = 21 | Time F (3.573, 138.4) = 6.025, ***P=0.0003  
Genotype F (1, 39) = 0.6367, P=0.4298  
Time\*Genotype (1, 155) = 1.403, P = 0.2356 |
| | normal, learning | 2-way ANOVA | N for WT = 20  
N for KO = 21 | Interaction F (4, 156) = 1.215, P = 0.3088  
Time F (3.509, 136.9) = 1.495, P=0.2127  
Genotype F (1, 39) = 2.607, P=0.1145 |
| | normal, reversal | 2-way ANOVA | N for WT = 20  
N for KO = 21 | Interaction F (5, 195) = 1.161, P = 0.3298  
Time F (4.685, 182.7) = 0.7862, P=0.5534  
Genotype F (1, 39) = 0.8415, P=0.3646 |
| Figure 3f, bottom | normal, training | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)=0.7864, p = 0.4364 |
| | normal, learning | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)=1.614, p = 0.1145 |
| | normal, reversal | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)= 0.9173, p = 0.3646 |
| Figure 3g, top | normal, training | Mixed-effects model | N for WT = 20  
N for KO = 21 | Time F (3.397, 131.6) = 16.09, ****P <0.0001  
Genotype F (1, 39) = 0.2071, P= 0.6516  
Time\*Genotype (1, 155) = 3.204, P = 0.0147 |
| | normal, learning | 2-way ANOVA | N for WT = 20  
N for KO = 21 | Interaction F (4, 156) = 1.282, P = 0.2795  
Time F (3.297, 128.6) = 1.045, P=0.3790  
Genotype F (1, 39) = 0.1037, P=0.7491 |
| | normal, reversal | 2-way ANOVA | N for WT = 20  
N for KO = 21 | Interaction F (5, 195) = 1.114, P = 0.3541  
Time F (4.463, 174.0) = 4.640, ***P=0.0009  
Genotype F (1, 39) = 2.218, P=0.1444 |
| Figure 3g, bottom | normal, training | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)=0.4062, p = 0.6226 |
| | normal, learning | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)=0.3221, p = 0.7491 |
| | normal, reversal | Unpaired t-test, two-tailed | N for WT = 20  
N for KO = 21 | t(39)= 1.489, p = 0.1444 |
| Figure 4b | normal, training | Unpaired t-test, two-tailed | N for WT = 15  
N for KO = 16 | t(29)=0.9360, p = 0.3570 |
| | normal, learning | Unpaired t-test, two-tailed | N for WT = 15  
N for KO = 16 | t(29)=0.8138, p = 0.4224 |
| | normal, reversal | Unpaired t-test, two-tailed | N for WT = 15  
N for KO = 16 | t(29)= 1.970, p = 0.0584 |
| Figure 4c | normal, training | Unpaired t-test, two-tailed | N for WT = 15  
N for KO = 16 | t(29)=0.7390, p = 0.4658 |
| | normal, learning | Unpaired t-test, two-tailed | N for WT = 15  
N for KO = 16 | t(29)=0.3862, p = 0.7021 |
| | normal, reversal | Unpaired t-test, two-tailed | N for WT = 15  
N for KO = 16 | t(29)=0.5783, p = 0.5675 |
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<th>Figure</th>
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<th>Statistical Test</th>
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<th>N for KO</th>
<th>t-value</th>
<th>p-value</th>
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<td>t(29) = 0.9695</td>
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<td>N for WT = 15</td>
<td>N for KO = 16</td>
<td>Time F (3.401, 97.78) = 3.522, <em>p= 0.0141 Genotype F (1, 29) = 0.03604, P= 0.8508 Time</em>Genotype (4, 115) = 0.6327, P = 0.6402</td>
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<td>2-way ANOVA</td>
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<td>N for KO = 16</td>
<td>Interaction F (4, 116) = 1.508, P = 0.2044 Time F (3.612, 104.7) = 0.9876, P= 0.4122 Genotype F (1, 29) = 1.064, P= 0.3108</td>
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<td>2-way ANOVA</td>
<td>N for WT = 15</td>
<td>N for KO = 16</td>
<td>Interaction F (5, 145) = 2.027, P = 0.0782 Time F (3.924, 113.8) = 2.439, P= 0.0521 Genotype F (1, 29) = 2.807, P= 0.1046</td>
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<td>t(29) = 1.032, p = 0.3108</td>
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<td>t(29) = 1.675, p = 0.1046</td>
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<td>Mixed-effects model</td>
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<td>Time F (2.770, 79.62) = 2.583, P= 0.0637 Genotype F (1, 29) = 8.306, **p= 0.0074 Time*Genotype (4, 115) = 1.121, P = 0.3502</td>
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<td>Interaction F (4, 116) = 0.9341, P = 0.4468 Time F (3.436, 99.63) = 1.508, P= 0.9463 Genotype F (1, 29) = 8.537, **p= 0.0067 Sidak Post-hoc: Week 7, *p = 0.0309 Week 10, **p = 0.0030</td>
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<td>Interaction F (5, 145) = 1.849, P = 0.1070 Time F (4.011, 116.3) = 1.960, P= 0.0150 Genotype F (1, 29) = 6.954, **p= 0.0133 Sidak Post-hoc: Week 14, **p = 0.0050</td>
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<td>Unpaired t-test, two-tailed</td>
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<td>t(29) = 2.838, **p= 0.0082</td>
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<td>N for KO = 16</td>
<td>t(29) = 2.984, **p= 0.0057</td>
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<td>N for WT = 15</td>
<td>N for KO = 16</td>
<td>t(29) = 2.648, *p= 0.0130</td>
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<td>Mixed-effects model</td>
<td>N for WT = 15</td>
<td>N for KO = 16</td>
<td>Time F (3.475, 99.90) = 11.99, ****p &lt; 0.0001 Genotype F (1, 29) = 5.205, <em>p= 0.0300 Time</em>Genotype (4, 115) = 0.5664, P = 0.6875</td>
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<td>N for KO = 16</td>
<td>Interaction F (4, 116) = 1.286, P = 0.2796 Time F (3.458, 100.3) = 0.7795, P= 0.5244 Genotype F (1, 29) = 3.102, P= 0.0887</td>
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<td>N for KO = 16</td>
<td>Interaction F (5, 145) = 0.3698, P = 0.8687 Time F (4.166, 120.8) = 6.101, ***p= 0.0001 Genotype F (1, 29) = 3.410, P= 0.0750</td>
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<td>N for KO = 16</td>
<td>t(29) = 2.252, *p = 0.0320</td>
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<td>N for KO = 16</td>
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<td>t(29) = 1.847, p= 0.0750</td>
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**Figure 5a**  

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<th>Interaction</th>
<th>Time</th>
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<td>N for KO = 21</td>
<td>Interaction F (5, 195) = 0.3179, P=0.9018</td>
<td>Time F (3.053, 119.1) = 154.4, ****P&lt;0.0001</td>
<td>Genotype F (1, 39) = 0.3919, P=0.5349</td>
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<td>N for KO = 22</td>
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<td>t(39) = 0.03080, p = 0.9756</td>
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**Figure 5b**  

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<td>Genotype F (1, 39) = 1.422, P=0.2403</td>
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<td>t(39) = 1.171, p = 0.2487</td>
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**Figure 5c**  

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<td>Interaction F (5, 195) = 0.5639, P=0.7276</td>
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**Figure 5d**  

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<th>Time</th>
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<td>Interaction F (5, 150) = 0.3752, P=0.8651</td>
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**Figure 5e**  

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**Figure 5f**  

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**Figure 6**  

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<th>2-way ANOVA</th>
<th>N for WT</th>
<th>N for KO</th>
<th>Interaction</th>
<th>Time</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal, amplitude</td>
<td>Unpaired t-test, two-tailed</td>
<td>n for WT = 53</td>
<td>n for KO = 67</td>
<td>t (95) = 0.9494, p = 0.3448</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal, frequency</td>
<td>Unpaired t-test, two-tailed</td>
<td>n for WT = 53</td>
<td>n for KO = 67</td>
<td>t (96) = 0.8840, p = 0.4008</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 7a**  

<table>
<thead>
<tr>
<th>Group</th>
<th>2-way ANOVA</th>
<th>N for WT</th>
<th>N for KO</th>
<th>Interaction</th>
<th>Time</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal, rise time</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 42</td>
<td>N for KO = 56</td>
<td>t (96) = 0.5442, p = 0.5876</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal, decay time</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 42</td>
<td>N for KO = 56</td>
<td>t (96) = 0.1154, p = 0.9084</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal, RMP</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 42</td>
<td>N for KO = 56</td>
<td>t (96) = 3.076, **p = 0.0027</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 8a**  

<table>
<thead>
<tr>
<th>Group</th>
<th>2-way ANOVA</th>
<th>N for WT</th>
<th>N for KO</th>
<th>Interaction</th>
<th>Time</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal, area</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 12</td>
<td>N for KO = 12</td>
<td>t (22) = 0.8397, p = 0.4101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal, cell density</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 12</td>
<td>N for KO = 12</td>
<td>t (22) = 2.111, *p = 0.0464</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 8e**  

<table>
<thead>
<tr>
<th>Group</th>
<th>2-way ANOVA</th>
<th>N for WT</th>
<th>N for KO</th>
<th>Interaction</th>
<th>Time</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal, area</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 6</td>
<td>N for KO = 6</td>
<td>t (10) = 2.130, p = 0.0590</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal, cell density</td>
<td>Unpaired t-test, two-tailed</td>
<td>N for WT = 6</td>
<td>N for KO = 6</td>
<td>t (10) = 2.175, p = 0.0547</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2: Cerebellar Weights

<table>
<thead>
<tr>
<th>Cerebellar Weights</th>
<th>16 weeks</th>
<th>n</th>
<th>p value</th>
<th>&gt;52 weeks</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rnf216</em> global knockout</td>
<td>WT = 20.0 ± 0.48 KO = 21.2 ± 0.71</td>
<td>WT= 18 KO = 11</td>
<td>0.16</td>
<td>WT = 21.0 ± 0.82 KO = 20.5 ± 0.82</td>
<td>WT= 18 KO = 25</td>
<td>0.70</td>
</tr>
</tbody>
</table>
A. 3 Weeks  9 Weeks  41 Weeks

♂
WT
- 82.86% 0
- 17.14% 1

KO
- 88.46% 0
- 11.54% 1

♀
WT
- 97.22% 0
- 2.78% 1

KO
- 93.75% 0
- 6.25% 1

B. 3 Weeks  9 Weeks  41 Weeks

♀
WT
- 50.00% 0
- 50.00% 1

KO
- 45.83% 1
- 4.17% 2

♂
WT
- 42.86% 0
- 57.14% 1

KO
- 50.00% 0
- 50.00% 1

♀
WT
- 34.62% 0
- 34.62% 1
- 34.62% 0

KO
- 53.85% 1
- 11.54% 2

- 45.83% 1
- 4.17% 2

- 50.00% 0
- 50.00% 1
A. Male
WT
KO
Female

B. Amplitude (pA)
Frequency (Hz)

C. Rise time (ms)

D. Decay time (ms)

E. RMP (mV)