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Measures of anxiety, sensorimotor function, and memory in male and female mGluR4^{-/-} mice

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Abstract

Metabotropic glutamate receptors (mGluRs) are coupled to second messenger pathways via G proteins and modulate synaptic transmission. Of the eight different types of mGluRs (mGluR1-mGluR8), mGluR4, mGluR6, mGluR7, and mGluR8 are members of group III. Group III receptors are generally located presynaptically, where they regulate neurotransmitter release. Because of their role in modulating neurotransmission, mGluRs are attractive targets for therapies aimed at treating anxiety disorders. Previously we showed that the mGluR4-selective allosteric agonist VU 0155041 reduces anxiety-like behavior in wild-type male mice. Here, we explore the role of mGluR4 in adult (6-month-old) and middle-aged (12-month-old) male and female mice lacking this receptor. Compared to age- and sex-matched wild-type mice, middle-aged mGluR4^{-/-} male mice showed increased measures of anxiety in the open field and elevated zero maze and impaired sensorimotor function on the rotarod. These changes were not seen in adult 6-month old male mice. In contrast to the male mice, mGluR4^{-/-} female mice showed reduced measures of anxiety in the open field and elevated zero maze and enhanced rotarod performance. During the hidden platform training sessions of the water maze, mGluR4^{-/-} mice swam farther away from the platform than wild-type mice at 6, but not at 12, months of age. mGluR4^{-/-} mice also showed enhanced amygdala-dependent cued fear conditioning. No genotype differences were seen in hippocampus-dependent contextual fear conditioning. These data indicate that effects of mGluR4 on sensorimotor function and measures of anxiety, but not cued fear conditioning, are critically modulated by sex and age.

Keywords

mGluR4; anxiety; Group-III mGluR; learning; memory

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1. Introduction

The fast actions of the excitatory neurotransmitter glutamate are mediated by glutamate-gated ion channels (ionotropic Glu receptors). Metabotropic glutamate receptors (mGluRs) are coupled to second messenger pathways via G proteins and modulate glutamatergic and GABAergic neurotransmission [1, 2]. Of the eight different types of mGluRs (mGluR1-mGluR8), mGluR4, mGluR6, mGluR7, and mGluR8 are members of group III. Except for mGluR6, which is expressed in retinal bipolar cell dendrites, group III receptors are generally located presynaptically, where they regulate neurotransmitter release [3]. Because of their role in modulating neurotransmission, mGluRs are attractive targets for therapies aimed at treating anxiety disorders [4]. Previously, higher measures of anxiety were shown in male mice lacking mGluR8 (mGluR8^{-/-}) [5-8]. The anxiety phenotype showed some sex-dependency. While 12-month-old mGluR8^{-/-} male mice showed increased measures of anxiety in the elevated plus maze, 12-month-old mGluR8^{-/-} female mice showed decreased measures of anxiety in this maze. Sex-dependent hippocampus-dependent cognitive impairments were also seen at this age. Both mGluR8^{-/-} female and male mice showed impairments in novel location recognition [7]. However, mGluR8^{-/-} female, but not male, mice showed impairments in spatial memory retention in the water maze [7]. In contrast to measures of anxiety and hippocampus-dependent cognition, no impairments in mGluR8^{-/-} female or male mice were seen in sensorimotor function, as assessed on the rotarod [7].

Acute pharmacological stimulation with the mGluR8 agonist (S)-3,4-dicarboxyphenylglycine (DCPG) or the positive allosteric modulator (PAM) AZ12216052 reduces measures of anxiety in wild-type male mice [9]. While the mGluR4-selective allosteric agonists VU 0155041 [8] also reduce measures of anxiety in wild-type male mice, relatively little is known about the potential sex differences in behavioral phenotypes in mGluR4^{-/-} mice. Young (2-4 month-old) mGluR4^{-/-} mice showed impaired rotarod performance [10], consistent with the high expression of mGluR4 at the striatopallidal synapse within the indirect pathway [11, 12], but it is not clear from that study whether one sex or both sexes were used. In addition, while the mice were analyzed in the open field, it doesn't appear that measures of anxiety in the open field were examined [10]. Therefore, in this study the behavioral and cognitive phenotypes of 6- and 12-month-old mGluR4^{-/-} female and male mice were assessed.

2. Materials and methods

2.1. Animals

mGluR4-null mice were obtained from Jackson Laboratory (stock #003619) and bred with wild-type C57BL/6 mice. Heterozygote mice were crossed to generate related mGluR4^{-/-} and mGluR4^{+/+} (WT) mice. mGluR4^{-/-} crosses and WT crosses were then made to generate the mice and the parents of the mice used for behavioral experiments. Experimentally naïve 6- and 12-month-old mGluR4^{-/-} and WT female and male were used. Animals were group housed in standard shoebox cages until one day prior to the start of behavioral testing and subsequently singly housed. The mice were maintained on a 12:12 light/dark schedule with laboratory chow (PicoLab Rodent diet 20, # 5,053; PMI Nutrition International, St. Louis MO, USA) and water provided *ad libitum*. The animals were tested in the following order of testing: open field (day 1), elevated zero maze (day 2), rotarod (days 3-5), Morris water maze (days 8-12), and fear conditioning (days 15 and 16). The elevated zero maze and acoustic startle tests were performed in the morning, between 9 and 11 a.m. Experimenters were blinded to the genotype and sex of the animals. All procedures were conducted in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee at Oregon Health & Sciences University.

2.2. Behavioral analysis

2.2.1. Open Field—Exploratory behavior and measures of anxiety were first assessed in the open field test, as described previously [13]. Briefly, mice were placed individually into the center of a square Plexiglas enclosure (40 × 40 cm) and allowed to explore for 10 minutes. The arena had a smooth, white plastic floor and the center of the arena was 100 ± 10 lux. Movement was tracked via infrared photocells (16 × 16) using Kinder Scientific Motor-Monitor software (Kinder Scientific, Poway, CA). Outcome measures were general locomotor activity analyzed as total distance moved (cm) and time spent in the center of the open field. The time spent in the conceptually defined zones was assessed using a zone map, which defined the periphery and the center of the open field.

2.2.2. Elevated Zero Maze—Measures of anxiety were also assessed in the elevated zero maze. The custom built elevated zero maze (Kinder Scientific, Poway, CA) consisted of two enclosed areas and two open areas. Each of the four sections was 6 cm wide. The circumference of the maze was 340 cm and elevated 64 cm above the ground. Mice were placed into an open section and allowed to explore for 10 minutes. Photobeams underneath the maze were used to track mouse movements using Motor Monitor software (version 10183-19). The outcome measures were total distance moved (cm) and time spent in the open sections (s) of the maze.

2.2.3. Rotarod—Sensorimotor performance was assessed on a Rotarod. Mice were placed on an elevated rotating rod (diameter: 3 cm, elevated: 45 cm, Rotamex-5, Columbus Instruments, Columbus, OH), initially rotating at 5.0 RPM. The rod accelerated 1.0 RPM every 3 seconds. A line of photobeams beneath the rod recorded the latency to fall (s). Each mouse receives three trials per day, with no delay between trials, on three consecutive days.

2.2.4. Morris Water Maze—Hippocampus-dependent spatial learning and memory were assessed in the Morris water maze [14]. The maze consisted of a circular pool (diameter 140 cm), filled with water (18 ± 1°C). The center of the arena averaged 100 lux. Mice are first trained to locate a visible escape platform (Plexiglas circle, 6 cm radius) submerged 0.5 cm below the surface of the water containing a cue (a colored cylinder, 2.5 cm radius, 8 cm height) over two days and subsequently to locate a hidden platform over three days. For both the visible and hidden platform training days, there were two daily sessions, morning and afternoon, which were separated by an inter-session interval of 2 hours. Each session consisted of three trials, with 5-10-minute inter-trial intervals. To control for a procedural learning bias, during the visible sessions the platform was rotated among four conceptually defined quadrants. The platform remained in the same location during the hidden trials. Mice were placed into the water facing the edge of the pool in one of nine randomized locations (consistent for each mouse). A trial ended when the mouse located the platform and remained on it for 3 seconds. Mice that failed to locate the platform within the 60 s trial, were led to the platform by placing a finger in front of their swim path.

The visible training trials were used to determine potential genotype differences in task learning, swim speeds, motivation, and vision. Following the visible platform training, the mice were trained to locate a hidden platform using extra-maze spatial cues. During the hidden trials, the water was made opaque by adding white chalk, and the platform was submerged 1.5 cm below the water surface. Ninety minutes after the end of the last hidden platform training trial on each day of hidden platform training, the platform was removed and spatial memory retention assessed during a probe trial. During all trials, the experimenter remained behind a curtain.

The swimming patterns of the mice were recorded and analyzed using Ethovision 2.3 video tracking software (Ethovision XT, Noldus, Netherlands) set at 6 samples/second. The average cumulative distance to the platform was calculated as performance measure during the training and probe trials. Thigmotaxis, or time spent near the periphery of the maze, was calculated to assess potential anxiety differences in the water maze. Also, swim speeds during the visible and hidden platform training trials were analyzed.

2.2.5. Fear Conditioning—Finally, the mice were tested for fear conditioning using Med Associates NIR Video and automated analysis (Med Associates, St. Albans, Vermont) utilizing Med Associates Video Freeze automated scoring system. This system is described in detail and validated against traditional hand scoring methods [15]. Pavlovian fear conditioning is a versatile and well-understood method of assessing associative learning [16]. In this task, mice learn to associate a conditioned stimulus (CS, e.g. a tone) with an unconditioned stimulus (US, e.g. foot shock). CS-US pairings are preceded by a short habituation period, from which a baseline measure of locomotor activity and other behavior can be scored. Contextual fear conditioning is thought of as hippocampus and amygdala dependent, while fear conditioning to a cue is considered to be amygdala-dependent but hippocampus-independent [17]. Post-exposure freezing, defined as absence of all movement with the exception of respiration, is a widely used indicator of a conditioned fear response [18]. On day 1, the mice were placed inside a white LED lit (100 lux) fear conditioning chamber (Context A). There was a 150 second baseline followed by two CS-US pairings. A 2.8 kHz, 80 dB tone (CS) was presented at 150 and 270 seconds. Both CS presentations co-terminated with a 2-sec 0.35 mA footshock (US). On day 2, hippocampus dependent associative learning was assessed during re-exposure to Context A for 300 seconds. Three hours later, mice were exposed to Context B. Context B consisted of a smooth white floor, a black plastic triangular insert for the walls, scented with vanilla. The mice were allowed to habituate for 180 seconds (Pre-CS period), and then exposed to the tone for 180 seconds (Post-CS period). Associative learning was measured as the percent time spent freezing in response to the contextual environment or tone. Motion during shock (arbitrary units) was measured to explore potential genotype differences in response to the aversive stimulus.

2.6. Statistical Analysis

Data are reported as means $\pm$ the standard error of the mean (SEM) and were analyzed using SPSS 16.0 software (IBM, Armonk, NY). Three-way ANOVAs were conducted using age, sex, and genotype as between-subjects variables. Additional one-way and two-way ANOVAs were conducted to analyze significant interaction effects. Repeated-measures ANOVAs were used to analyze the water maze learning curves and response to the US during fear conditioning. Data were log-transformed if they did not meet normality and/or equal variances assumptions required by the ANOVA. Mice were removed from the analysis if the outcome measure was greater than 2.5 standard deviations from the mean of the group. As a result, two mice were removed from the open field analysis. Results were considered significant at an α level of 0.05.

3. Results

3.1. Animal health and body weights

There were no obvious genotype differences in physical health, drinking or feeding. Body weights were taken just before the animals were euthanized (Table 1). In the female mice, there was an effect of age on body weight ($F(1, 22) = 9.899, p = 0.005$) with older mice weighing more than younger mice. In male mice, there was a trend towards an effect of age ($F(1, 23) = 3.469, p = 0.075$). There was no effect of genotype on body weight in female or male mice.

3.2. mGluR4^{-/-} mice show age- and sex-dependent effects on measures of anxiety

Percent time spent in the center of the open field was measured to assess levels of anxiety (Fig. 1A). There was an effect of age ($F(7, 76) = 10.149, p = 0.002$) and there were age $\times$ genotype ($F(7, 69) = 8.498, p = 0.005$) and a sex $\times$ genotype ($F(7, 76) = 5.709, p = 0.020$) interactions. Therefore, male and female data were analyzed separately. In the male mice, there was an effect of age ($F(1, 36) = 7.127, p = 0.011$), and a significant interaction between age and genotype ($F(1, 36) = 4.711, p = 0.037$). Next, age was analyzed separately. In 6-month-old male mice ($F(1, 18) = 1.080, p = 0.312$) there was no genotype difference, but there was a significant genotype effect in 12-month-old male mice ($F(1, 18) = 5.217, p = 0.035$); mGluR4^{-/-} mice ($7.0 \pm 1.4\%$) spent less time in the center of the open field than wild-type mice ($13.5 \pm 3.1\%$).

In the female mice, there was an effect of genotype ($F(1, 33) = 8.331, p = 0.007$), and a trend towards an age $\times$ genotype interaction ($p = 0.059$) for time spent in the center of the open field (Fig. 1A). Irrespective of age and in contrast to male mice, mGluR4^{-/-} female mice ($18.1 \pm 2.0\%$) spent more time in the center of the open field than wild-type mice ($11.2 \pm 1.5\%$).

When activity in the open field was analyzed, there also was an effect of age ($F(7, 76) = 17.0, p < 0.0001$), and age $\times$ genotype ($F(7, 69) = 4.558, p = 0.036$) and a sex $\times$ genotype ($F(7, 76) = 4.083, p = 0.047$) interactions (Fig. 1B). Six-month-old mice (6178 ± 419 cm) moved more than 12-month-old mice (4435 ± 265 cm). In the male mice, there was a main effect of age ($F(1, 36) = 6.548, p = 0.015$), with 6-month-old mice (5489 ± 194) moving more than 12-month-old mice (4697 ± 192) (Fig. 1B). Additionally, there was an effect of genotype ($F(1, 36) = 7.538, p = 0.009$) but no age by genotype interaction ($p = 0.541$). Wild-type male mice (5619 ± 247 cm) moved more than mGluR4^{-/-} male mice (4743 ± 151 cm). In contrast to wild-type female mice, there was no effect of age on distance moved in mGluR4^{-/-} female mice ($p = 0.482$).

Next, measures of anxiety were assessed in the elevated zero maze (Fig. 1C). The data were log transformed as the groups had significantly unequal variances. There also was an effect of age ($F(7, 76) = 20.223, p < 0.0001$), and age $\times$ genotype ($F(7, 69) = 46.763, p = 0.011$) and a sex $\times$ genotype ($F(7, 76) = 4.898, p = 0.030$) interactions. In the male mice, there was a main effect of age ($F(1, 35) = 4.528, p = 0.040$), and an interaction between age and genotype ($F(1, 35) = 5.095, p = 0.030$), but no main effect of genotype ($p = 0.451$). While there was no genotype difference in 6-month-old male mice ($p = 0.316$), there was an effect of genotype in 12-month-old male mice ($F(1, 17) = 5.022, p = 0.039$). Twelve-month-old mGluR4^{-/-} male mice ($0.89 \pm 0.06\%$) spent less time in the open areas of the zero maze than wild type mice ($1.19 \pm 0.12\%$).

In female mice, there was a main effect of age ($F(1, 32) = 17.878, p < 0.001$) and a main effect of genotype ($F(1, 32) = 5.594, p = 0.024$), but no interaction between age and genotype ($p = 0.165$) (Fig. 1C). Regardless of age, mGluR4^{-/-} female mice spent more time in the open areas of the elevated zero maze than wild-type female mice. Also, 12-month-old female mice spent more time in the open areas of the elevated zero maze than 6-month-old female mice.

When activity in the elevated zero maze was analyzed in the male mice, there were main effects of age ($F(7, 67) = 7.671, p = 0.007$) and sex ($F(7, 67) = 4.742, p = 0.033$), and an interaction between age and genotype ($F(7, 67) = 5.737, p = 0.019$) (Fig. 1D). When activity in the elevated zero maze was analyzed in the male mice, there was a main effect of age ($F(1, 35) = 4.528, p = 0.040$) and an interaction between age and genotype ($F(1, 35) = 5.422, p = 0.026$) (Fig. 1D). Splitting the analysis by age revealed that there was an effect of

genotype in 6-month-old male mice ($F(1, 18) = 6.162, p = 0.023$), with mGluR4^{-/-} mice (1197.7 ± 58.34 cm) moving less than wild-type mice (1520 ± 116 cm). In contrast, there was no effect of genotype in 12-month-old male mice ($p = 0.341$).

For distance moved in the female mice, there was a main effect of age ($F(1, 32) = 4.326, p = 0.046$), but no main effect of genotype or interaction between age and genotype. Six-month-old female mice (1157 ± 97 cm) moved less than 12-month-old female mice (1406 ± 68 cm).

3.3. mGluR4^{-/-} mice have altered motor function on the rotarod test

The primary outcome measure for rotarod performance was average fall latency. There was a main effect of sex ($F(7, 71) = 36.98, p < 0.0001$) and a trend towards an effect of age ($F(7, 71) = 3.900, p = 0.052$). In addition, there were interactions between age and genotype ($F(7, 71) = 8.027, p = 0.006$), sex and genotype ($F(7, 71) = 16.262, p < 0.0001$), and a trend towards an age $\times$ sex $\times$ genotype interaction ($F(7, 71) = 3.510, p = 0.065$). In the male mice, there was a main effect of age ($F(1, 37) = 4.718, p = 0.036$), and an interaction between age and genotype ($F(1, 37) = 11.979, p = 0.001$) (Fig. 2). While there was no effect of genotype in 6-month-old male mice ($p = 0.348$), there was an effect of genotype in the 12-month-old male mice ($F(1, 19) = 14.337, p = 0.001$). Twelve-month-old mGluR4^{-/-} mice (23.0 ± 1.4 sec) were not able to stay on the rod as long as age-matched wild-type male mice (35.2 ± 3.8 sec).

In the female mice, there was a main effect of genotype ($F(1, 34) = 12.089, p = 0.001$), but no interaction between genotype and age (Fig. 2). Irrespective of age, female mGluR4^{-/-} mice (44.1 ± 1.7 sec) showed enhanced sensorimotor function compared to wild-type female mice (35.8 ± 1.7 sec).

3.4. Six-month-old, but not 12-month-old, mGluR4^{-/-} male mice show impaired learning of the hidden platform

For average swim speeds during the visible platform training sessions, there was an effect of sex ($F(7, 64) = 46.45, p < 0.0001$) and an age $\times$ genotype interaction ($F(7, 71) = 4.333, p = 0.041$) (Table 1). While at 6 months of age, wild-type mice swam faster than mGluR4^{-/-} mice, and 12 months of age mGluR4^{-/-} mice swam faster than wild-type mice (Table 1). Therefore, the mean swim speeds during the visible sessions were used as a covariate in the analysis of the visible and hidden water maze learning curves. Anxiety does not seem to be a factor on water maze performance as thigmotaxis levels were not significantly different across genotype and age in both sexes (Table 1).

The cumulative distance to the target platform decreased during the visible training sessions (within subjects effect of session, $p < 0.001$), indicating that the mice learned the task (Fig. 3). During the hidden platform training sessions, there was a genotype $\times$ age interaction ($F(1, 63) = 8.477, p = 0.005$). At 6 months of age, mGluR4^{-/-} mice swam farther away from the platform than wild-type mice ($F(1, 34) = 7.282, p = 0.011$). At 12 months of age, there was a trend towards mGluR4^{-/-} mice swimming closer to the platform than wild-type mice ($F(1, 30) = 3.628, p = 0.066$). When the two genotypes were analyzed separately, there were effects of sex ($F(1, 33) = 7.400, p = 0.010$) and age ($F(1, 33) = 9.488, p = 0.004$) in mGluR4^{-/-} but not in wild-type mice. Thus, while the performance of mGluR4^{-/-} mice during the hidden sessions changed between 6 and 12 months of age, that of wild-type mice did not.

When spatial memory retention was assessed in the probe trials, there was an effect of probe trial ($F(2, 128) = 10.130, p < 0.001$), indicating that the mice improved their performance with additional training, but there were no other significant effects or interactions.

3.5. mGluR4^{-/-} mice exhibit enhanced cued fear conditioning

In the training session, prior to CS and US administration (i.e. baseline), mice had 120 seconds to explore the chamber. For average baseline movement, there were interactions for age $\times$ genotype ($F(7, 71) = 7.761, p = 0.007$) and age $\times$ sex $\times$ genotype ($F(7, 71) = 4.639, p = 0.035$) and a trend towards an effect of genotype ($F(7, 71) = 36.609, p = 0.062$). In male mice, there were no effects of age, genotype, or age $\times$ genotype interaction for average baseline movement (Fig. 4A). However, in the female mice there was an age by genotype interaction ($F(1, 34) = 16.860, p < 0.001$). When the younger and older female mice were analyzed separately, there was a main effect of genotype ($F(1, 17) = 10.843, p < 0.004$) in 6-month-old female mice (Fig. 4A); mGluR4^{-/-} female mice were less active than wild-type female mice. In 12-month-old female mice there also was a main effect of genotype ($F(1, 17) = 6.035, p = 0.025$), but the effect was opposite to that seen at 6 months of age. Twelve-month-old mGluR4^{-/-} female mice were more active than the age-matched wild-type female mice.

Sensitivity to the shocks was assessed using the measure of average activity (arbitrary units) during the 2 second delivery of the shocks (Fig. 4B). A repeated measures ANOVA was used to account for potential shock habituation differences between the groups. There was a within-subjects effect of shock ($F(1, 70) = 24.29, p < 0.0001$), but no other effects or interactions. There were between effects of sex ($F(1, 70) = 5.988, p = 0.017$) and genotype ($F(1, 70) = 10.189, p = 0.002$) and an age $\times$ sex interaction ($F(7, 71) = 7.502, p = 0.008$) and a trend towards an effect of age ($F(1, 70) = 3.956, p = 0.051$).

Twenty-four hours after the CS-US delivery in context A, mice were re-exposed to context A to assess hippocampus-dependent contextual fear conditioning (Fig. 5A). There were no interaction effects and there was a trend towards an effect of sex ($F(7, 68) = 3.497, p = 0.066$).

Three hours after the contextual test, mice were put into a new environment, context B, to assess amygdala-dependent, but hippocampus-independent, cued fear conditioning (Fig. 5B). Percent time freezing during the pre-CS period and post-CS was recorded. Freezing during the pre-CS period was essentially zero (data not shown). There was a main effect of genotype on post-CS freezing ($F(7, 71) = 6.516, p = 0.013$). mGluR4^{-/-} mice ($31.7 \pm 3.4\%$) showed enhanced cued fear conditioning compared to wild-type controls ($17.5 \pm 2.2\%$).

4. Discussion

This study shows sex-dependent alterations in measures of anxiety and rotarod performance in middle-aged mGluR4^{-/-} mice. While mGluR4^{-/-} male mice showed increased measures of anxiety in the open field and elevated zero maze and impaired sensorimotor function on the rotarod, mGluR4^{-/-} female mice showed reduced measures of anxiety in the open field and elevated zero maze and enhanced rotarod performance. During the hidden platform training sessions of the water maze, mGluR4^{-/-} mice swam farther away from the platform than wild-type mice at 6, but not at 12, months of age. mGluR4^{-/-} mice also showed enhanced amygdala-dependent cued fear conditioning, consistent with an amygdala phenotype. No genotype differences were seen in hippocampus-dependent contextual fear conditioning. Thus, effects of mGluR4 on measures of anxiety and sensorimotor function are critically modulated by sex.

The accelerating rotarod performance test requires motor coordination and balance [19]. The mGluR4 receptor densely populates the molecular layer of the cerebellum and is moderately present in the globus pallidus and substantia nigra [11], brain areas that are important for motor control. Consistent with earlier studies [10], mGluR4^{-/-} male mice showed impaired

rotarod performance. However, in this study with mice on a mixed C57Bl6/J background this was seen in middle-aged, but not young, mice, while in the earlier studies this was seen in 2-month-old mice on the CD-1 background [10]. The difference in the genetic background of the mice and differences in the amount of rotarod training trials might have contributed to these divergent findings. The rotarod impairment in mGluR4^{-/-} male mice might be due to enhanced GABA release from the striatum to the external segment of the globus pallidus (GPe) within the indirect pathway. In Parkinson's disease, this release is increased due to degeneration of dopamine neurons in the substantia nigra pars compacta. Activation of mGluR4 reduces GABA release and likely normalize the function of the basal ganglia [20]. In contrast to mGluR4^{-/-} male mice, mGluR4^{-/-} female mice showed enhanced rotarod performance. The sex difference is interesting based on the increased susceptibility of males compared to females to develop Parkinson's disease [21, 22]. These data support that mGluR4 is involved in the pathogenesis of Parkinson's disease [20, 23] and might contribute to the sex difference in susceptibility to develop this condition.

Previously, we reported that the intraperitoneal administration of the mGluR4 positive allosteric modulator (PAM) VU 0155041 reduced measures of anxiety on the elevated zero maze [9]. Based on the rotarod data in the mGluR4^{-/-} mice, it will be important to determine whether acute mGluR4 stimulation also affects rotarod performance in wild-type mice in a sex-dependent fashion. Stimulation of mGluR4 might also be protective in other neurodegenerative conditions [24]. For example, the mGluR4 positive allosteric modulator (-)-PHCCC showed neuroprotection against β amyloid peptide and NMDA toxicity in mixed cultures of murine cultured neurons [25].

Both mGluR4^{-/-} and mGluR8^{-/-} [6] male mice show anxiety phenotypes, but there are clear differences between the anxiety phenotypes of the two mutant models. While mGluR8^{-/-} male mice show increased measures of anxiety already at 6 months of age, mGluR4^{-/-} male mice do not show increased measures of anxiety at this age but only at 12 months of age. At 6 months of age, there is a trend towards reduced measures of anxiety in the open field and elevated zero maze in mGluR4^{-/-} male mice. Age-related changes in middle-aged mice might be important for revealing the mGluR4^{-/-} anxiety phenotype in male mice. For example, brain levels of apolipoprotein E, a protein important in cholesterol transport and metabolism, are increased in middle-aged animals [26] and mice lacking apoE show increased measures of anxiety [27]. ApoE might modulate the role of mGluR4 in the regulation of anxiety. ApoE might also affect the ability of mGluR8 to regulate anxiety. While acute pharmacological stimulation with the mGluR8 agonist DCPG reduces measures of anxiety in wild-type than is needed in *ApoE*^{-/-} male mice [9], a lower dose of DCPG is effective in wild-type than *ApoE*^{-/-} mice [8]. As apoE levels in humans [28] and human apoE targeted replacement mice [29-31] are modulated in an apoE isoform-dependent fashion, higher doses of mGluR4 or mGluR8 agonists or allosteric modulators might be required to reduce anxiety levels in patients with lower apoE levels.

In contrast to mGluR4^{-/-} mice, there are no genotype differences in rotarod performance in mGluR8^{-/-} mice. Anatomical specificity in receptor expression might cause the distinct effects on rotarod performance. There are very high relative densities of [³H]L-AP4 binding, which binds both mGluR4 and mGluR8, in the molecular layer of the cerebellar cortex, the nucleus basalis, the outer layer of the superior colliculus, and the substantia nigra, while very low levels of binding is seen in mGluR4^{-/-} mice [32]. In addition, there are moderate levels of binding in wild-type mice in the molecular layer of the hippocampal dentate gyrus and in the thalamus which are not seen in mGluR4^{-/-} mice [32]. Comparable binding levels were seen in most of the cerebral cortex, caudate putamen, and globus pallidus of mGluR4^{-/-} and wild-type mice, likely reflecting mGluR8 binding. Using immunocytochemistry, there was strong mGluR4 immunoreactivity in the cerebellar cortex, basal ganglia, the sensory

relay nuclei of the thalamus, and the dentate molecular layer and CA1-3 regions of the hippocampus [12].

Potential interactions of mGluR4 with membrane estrogen receptors involving rapid estradiol signaling might contribute to the sex-dependent effects in measures of anxiety and rotorod performance. Similar to its effects on group I receptors [33], estradiol might stimulate group II and group III receptors. The group II/III mGluR antagonist CPPG eliminated the effect of estradiol on L-type calcium channel-mediated cAMP response element binding protein (CREB) phosphorylation in hippocampal neurons [33]. The effects of estradiol to attenuate L-type calcium channel-mediated CREB phosphorylation were only seen in culture from female rat pups. Similar sex-dependent effects of estradiol on mGluRs might occur in other brain regions. Although female mice become acyclic around 12 months of age [13], sex-dependent effects of estradiol earlier in life might also still contribute to the sex-dependent effects on measures of anxiety and rotarod performance seen in middle-aged mGluR4^{-/-} mice.

The altered measures of anxiety in mGluR4^{-/-} mice might involve alterations in the amygdala, a brain area critical in the regulation of anxiety. Consistent with involvement of the amygdala, amygdala-dependent cued fear conditioning was enhanced in mGluR4^{-/-} mice. Interestingly, the mGluR7 allosteric agonist AMN082 blocked acquisition and facilitated extinction of fear learning in the amygdala-dependent fear-potentiated startle paradigm, while downregulation of mGluR7 blocked extinction of amygdala-dependent conditioned taste aversion [34]. Similar to mGluR7, mGluR4 might also play a role in acquisition and/or extinction of cued fear conditioning.

In summary, while both female and male mGluR4^{-/-} mice show enhanced cued fear conditioning, female and male mGluR4^{-/-} mice show sex-dependent effects on measures of anxiety and sensorimotor function. These data indicate that there is no simple relationship between measures of anxiety and cued fear conditioning and underline the importance to include female mice in behavioral and cognitive analyses. Future efforts are warranted to investigate the mechanisms underlying these sex-dependent effects.

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Research Highlights

1. Middle-aged mGluR4^{-/-} male mice show increased measures of anxiety.
2. Middle-aged mGluR4^{-/-} male mice show impaired sensorimotor function.
3. mGluR4^{-/-} female mice show reduced measures of anxiety.
4. mGluR4^{-/-} female mice show enhanced rotarod performance.
5. mGluR4^{-/-} male mice show enhanced cued fear conditioning.

Fig. 1.

Measures of anxiety and activity of male and female, adult (6-month-old) and middle-aged (12-month-old), mGluR4^{-/-} mice in the open field test and elevated zero maze. Percent time in the center or open was used as a marker for anxiety behavior. Total distance moved was measured to assess general locomotor activity. (A) Percent time in the center of the open field. Middle-aged male mGluR4^{-/-} mice exhibited more anxiety-like behavior than wild-type controls. In females, mGluR4^{-/-} mice showed reduced anxiety-like behavior. (B) Total distance moved (cm) in the open field. In the males, in general mGluR4^{-/-} mice moved less than wild-type mice, also adult mice moved more than middle-aged mice. Only in female wild-type mice, adult mice moved more than middle-aged mice. (C) Percent time in the open areas of the elevated zero maze. In females, mGluR4^{-/-} mice exhibited lower anxiety-like behavior than wild-type mice. In addition, adult mice were more anxious than middle-aged mice. Middle-aged male mGluR4^{-/-} mice exhibited more anxiety-like behavior than wild-type mice. (D) Total distance moved (cm) in the elevated zero maze. Adult male mGluR4^{-/-} mice moved less than wild-type mice. But female adult mice, regardless of genotype, moved less than middle-aged mice. * $p < 0.05$ versus age- and sex-matched wild-type mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, indicating a main effect of age; 'a' versus 'b', indicating a main effect of genotype, $p < 0.05$. Error bars represent SEM values.

Fig. 2.

Sensorimotor function of male and female, adult (6-month-old) and middle aged (12-month-old), mGluR4^{-/-} mice on the rotarod. Mice were placed on the accelerating rotarod for 3 trials per day on 3 consecutive days. Performance on the rotarod was assessed by averaging the fall latencies for each mouse across all nine trials. Middle-aged male mGluR4^{-/-} mice had impaired rotarod performance compared to wild-type controls. In female mice, mGluR4^{-/-} showed enhanced rotarod performance compared to wild-type controls. *** $p < 0.001$ versus age- and sex-matched wild-type mice; 'a' versus 'b', indicates main effect of genotype, $p < 0.05$. Error bars represent SEM values.

Fig. 3. Spatial learning of male and female, adult (6-month-old) and middle aged (12-month-old), in the Morris water maze. Mice were placed in a pool of water and trained to locate a visible platform during four sessions followed by hidden platform training during six subsequent sessions. There were no genotype differences in performance during the visible platform sessions in adult or middle-aged mice. However, during the hidden platform sessions, adult $mGluR4^{-/-}$ mice performed worse than wild-type mice (A, C). In contrast, in middle-aged mice, there was a trend towards $mGluR4^{-/-}$ mice performing better than wild-type controls during the hidden (B, D) platform training trials. Error bars represent SEM values.

Fig. 4.

Day 1 (training) fear conditioning of adult (6-month-old) and middle aged (12-month-old), mGluR4^{-/-} mice. Mice received two tone (CS)-shock (US) pairings on day 1. (A) Average baseline motion was recorded to assess general locomotor activity. There were no age or genotype effects in male mice. In the females, adult mGluR4^{-/-} mice moved less than wild type controls, but middle-aged mGluR4^{-/-} mice moved more than wild-type controls. * $p < 0.05$, ** $p < 0.01$ versus sex- and age-matched wild-type mice. Error bars represent SEM values.

Fig. 5.

Day 2 fear conditioning (testing) of adult (6-month-old) and middle aged (12-month-old), mGluR4^{-/-} mice. Contextual freezing (A) and cued freezing during the tone (B). There were no effects of age or genotype on freezing levels in the contextual test. However, in the cued test, mGluR4^{-/-} mice froze more to the cue than wild-type controls. Error bars represent the SEM.

Table 1
Water maze performance and body weights of male and female mGluR^{-/-} and wild-type mice.

	Male				Female			
	6 month		12 Month		6 Month		12 Month	
	WT	mGluR4 ^{-/-}	WT	mGluR4 ^{-/-}	WT	mGluR4 ^{-/-}	WT	mGluR4 ^{-/-}
Cumulative Distance To Target (cm):								
P1	14670 ± 979	14138 ± 937	14660 ± 947	14991 ± 1045	15299 ± 1349	14194 ± 606	15260 ± 1038	14358 ± 875
P2	13690 ± 441	12191 ± 941	12265 ± 2381	13402 ± 892	12229 ± 772	13087 ± 772	12840 ± 982	13189 ± 806
P3	13494 ± 1075	11498 ± 1876	12352 ± 1258	12610 ± 1053	12253 ± 472	13558 ± 990	13618 ± 1197	11483 ± 691
Thigmotaxis (% time)	8.51 ± 1.30	8.61 ± 0.76	8.15 ± 1.10	9.53 ± 0.94	9.26 ± 1.86	7.91 ± 1.34	9.74 ± 1.78	11.07 ± 1.15
Visible session swim speeds (cm/s)	24.44 ± 0.61	22.89 ± 0.62	23.51 ± 0.89	24.32 ± 0.68	27.09 ± 1.14	26.7 ± 0.68	27.07 ± 0.59	28.68 ± 0.66
Hidden session swim speeds (cm/s)	21.41 ± 0.50	17.98 ± 0.75	19.50 ± 1.19	20.88 ± 1.03	24.17 ± 1.22	23.13 ± 1.22	21.88 ± 0.72	25.36 ± 1.02
Body Weights (g)	32.53 ± 0.03	34.53 ± 0.96	35.97 ± 1.14	37.01 ± 1.01	28.33 ± 0.83	26.24 ± 1.06	31.69 ± 0.82	31.34 ± 1.65

Data are presented as means ± SEM