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Sex-dependent behavioral functions of the Purkinje cell-specific Gαi/o binding protein, Pcp2(L7)

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Abstract

We previously reported motor and non-motor enhancements in a mouse mutant with an inactivated Purkinje cell-specific gene, *Pcp2(L7)*, that encodes a GoLoco domain-containing modulator of Gi/o protein-coupled receptors. Effects included elevated learning asymptote with repeated rotarod training, increased acquisition rate in tone-conditioned fear (TCF), and subtle male-specific changes in both acoustic startle habituation and pre-pulse inhibition. We have further analyzed this mutant strain for higher-order behavioral alterations this time with a focus on male-female differences, and here we report a sex-dependent anxiety-like phenotype: male mutants are less anxious, and female mutants more anxious, than same-sex wild-types. Similarly, the fear responses measured during the tone in TCF acquisition are decreased in male mutants and increased in female mutants relative to same-sex wild-types. Overall the dynamics of both acquisition and extinction of TCF is affected in mutants but memory was not affected. Mutants display normal sociability and do not differ from wild-types in the social novelty test; however, compositional analysis supports that both *L7* genotype and sex contribute to these behaviors. These results provide direct evidence of higher-order behavioral functions of the cerebellum due to the unambiguous cerebellar specificity of Pcp2(L7) expression, and the lack of any confounding motor defects in the mutant. We attempt to synthesize these new data with what is previously known both about Pcp2(L7) and about the effects of sex and sex hormones on anxiety and fear behaviors: specifically, L7 is a bidirectional and sex-dependent damper that regulates the amplitude and/or rate of sensorimotor responses, potentially acting as a mood stabilizer.

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Conflict of Interest Statement

The authors declare they have no conflicts of interest and are aware of no individuals or entity that would benefit from the studies described herein.

INTRODUCTION

Pcp2(L7) is a Purkinje cell-specific gene that encodes a modulator of Gi/o protein-coupled receptor (Gi/oPCR) signaling. Outside of the cerebellum the only other known site of expression is the retina, where L7 has been proposed to act as an activator of Gi/o function, possibly a GEF [Xu et al., 2008]. Biochemical studies mainly suggest the opposite, however: it acts as a GDI that decouples Gi/oPCR's [Webb et al., 2005]. An earlier study of ours using a Xenopus oocyte biochemical approach to reconstitute Gi/oPCR inhibition of the P/Q-type Ca^{2+} channel shows a possible resolution of this discrepancy: Pcp2(L7) acts bidirectionally, enhancing Gi/o inhibition of the channel at low L7 concentration but inhibiting it at high concentration [Kinoshita-Kawada et al., 2004].

At the transcriptional level the *Pcp2(L7)* gene is a direct target of the nuclear receptor RORα [Gold et al., 2003; Serinagaoglu et al., 2007], which is required for Purkinje cell growth and survival [Hamilton et al., 1996]. In a previous study of *Pcp2(L7)* mutant mice we observed no major anatomical defects and no loss of Purkinje cells, although the cerebellum was slightly smaller than normal, the Purkinje cell soma were smaller, and the dendrites were shorter [Iscru et al., 2009]. We also observed a robust alteration of the complex spike waveform, the so-called cerebellar "learning signal": both a reduced overall duration and a decreased number of somatic spikelets [Iscru et al., 2009]. Rather than motor defects, however, we reported motor and sensorimotor enhancements suggesting a damper function for L7. Effects included elevated learning asymptote with repeated rotarod training and increased acquisition rate in tone-conditioned fear (TCF) [Iscru et al., 2009]. In that study we also examined several anxiety behaviors, but no significant effects were observed. Nevertheless, while not significant, female mutants had reduced exploration time in an open-field relative to wild-types.

To explore this further, we began in the current study by analyzing additional cohorts of *Pcp2(L7)* mutant mice in spontaneous locomotor and TCF tests. Data from all cohorts were analyzed together using appropriate statistics in order to increase subject numbers and better assess curiosity/anxiety and fear memory acquisition, consolidation, and extinction, respectively. We also used the Porsolt forced swim test to assess motivation and depression, as it is possible that the elevated learning asymptote on the rotarod may reflect increased motivation rather than learning. Finally, we tested sociability and preference for social novelty, because imaging and lesion studies in humans have suggested the involvement of the cerebellum in social behaviors [Tavano et al., 2007; Riva and Georgia, 2000; Critchley et al., 2000], and because the cerebellum and the genetic switch RORα have been implicated in autism [Mostofsky et al., 2009; Sarachana et al., 2011]. We particularly focused on observation of sex differences in these behaviors as such differences, or behavioral effects of sex hormones, have long been described in open-field [Morgan and Pfaff, 2001] and TCF tests [Jasnow et al., 2006] as well as in eye-blink conditioning [Wood and Shors, 1998], a classical model of cerebellum-dependent associative learning. Such a focus is also predicated on the known dependence of Purkinje cell dendritic growth on the transient endogenous synthesis of sex hormones during early postnatal development [Sakamoto et al., 2001], on the observation that aromatase, a key enzyme in estrogen biosynthesis, is also a direct target of RORα [Odawara et al., 2009], and the possible differential influence of

androgen and estrogen on RORα expression [Sarachana et al., 2011], which implies a more long-lasting influence of sex on adult cerebellar function.

MATERIALS & METHODS

Mice

Generally we followed breeding standards for studies in mice as previously outlined by Crusio and colleagues [Crusio et al., 2009]. The L7KO line was originally obtained in May 2001 from Dr. James Morgan at St. Jude Childrens Research Hospital in a mixed B6/129 background [Vassileva et al., 1997]. All mice used in the current study were backcrossed more than 18 generations in C57BL/6NTac (Taconic) by successive crosses between heterozygotes and wild-types as we reported recently [Iscru et al., 2009]. The same breeding regimen of heterozygotes to commercially supplied wild-types (Taconic) is used for the purpose of line maintenance. However, all mutant animals in the current study were produced in-house from multiple homozygous parental crosses, and age-matched wild-types of both sexes were produced independently, also in-house, from multiple pairs of commercially obtained C57BL/6NTac stock breeders (Taconic). The homozygous mutant breeders did not exceed four generations of interbreeding in order to prevent genetic drift from the background strain; i.e., mutant breeders were constantly regenerated from heterozygote pairings. The pool of homozygous breeders was generated from multiple (heterozygous or homozygous) breeding pairs, and each tested animal cohort was generated from two or more breeding pairs. Each distinct cohort was generated from a non-overlapping set of breeding pairs as compared to other cohorts.

In some cases, as indicated in Results, data were analyzed together with old data reported previously (8 or more backcross generations; see Iscru et al., 2009), in which tested animals were generated by crosses between heterozygous parents. Data from heterozygous animals were removed from the old cohort datasets so that these old cohort data could be combined with the new cohort data in which no heterozygotes were produced. In all cases, however, animal cohort is included as a factor in the ANOVA analysis, and significant cohort differences, if observed, are reported. Whenever cohort data were combined we ensured that all cohorts and all animals that were ever tested were included, i.e., we did not exclude any cohorts or animals. All animals were 3–6 months old at the time of testing.

Behavioral tests

Mice were housed in standard polypropylene cages (27.8 × 7.5 × 13 cm) in a 14L:10D light cycle and given ad libitum access to food (Harlan Teklad 8640 rodent diet, Indianapolis, IN, USA) and filtered tap water. Mice were group housed (2–5 per cage), and were handled regularly by animal care staff during routine husbandry. Mice were also handled multiple times across several days prior to behavioral testing by researchers for preliminary assessment of general health and to acclimate to handling. Cohorts of mice, if tested in multiple behavioral tasks, were tested in increasing order of stressfulness as outlined below. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and are in compliance with guidelines established by the National Institutes of Health published in *Guide for the Care and Use of Laboratory Animals* (1996).

Activity in an open field arena—To assess spontaneous locomotor activity, early in the dark period mice were individually placed in a 40.5cm × 40.5cm × 36cm acrylic arena lined with fresh corncob bedding material housed in sound- and light-attenuating box (PAS-Openfield, San Diego Instruments, San Diego, CA, USA). A 16 × 16 × 2 IR grid tracked movement in 3 dimensions. Data collected over a 30 min period were analyzed for total number of beam breaks, percentage activity in the center of the arena (center = inner 6 × 6 beams on the grid), and number of rears. To prevent transmission of any olfactory cues, all chambers were cleaned between animals with 70% ethanol.

The open-field locomotor test was performed on three cohorts, two reported previously in Iscru et al., 2009 (cohort4505: 6 L7+/+ males, 3 L7−/− males and 3 L7+/+ females, 2 L7−/− females; cohort1105: 4 L7+/+ males, 3 L7−/− males, and 5 L7+/+ females, 10 L7−/− females) and one new one (cohort10208: 8 L7+/+ males, 10 L7−/− males, and 8 L7+/+ females, 8 L7−/− females).

Sociability and social preference testing—Social testing consisted of 3 phases, habituation, sociability, and preference for social novelty, as previously described in [Moy et al., 2004, 2008]. All phases of the testing were recorded to VHS tapes and videos were later scored, by an observer who was blind to the condition of the subjects, for time in each chamber, number of chamber entries, object sniffing time, and time spent sniffing a stimulus mouse using the automated Observer XT software (Noldus Information Technology, Leesburg, VA, USA).

Apparatus: The social testing apparatus consisted of a polycarbonate box 62cm L × 40cm W × 20cm H divided into 3 chambers of equal size with removable dividers with a 10cm W × 5cm H opening with a manual guillotine door between chambers, fabricated by The Ohio State University Department of Physics machine shop to specifications detailed in Nadler et al., 2004. Each side chamber contained a small wire cage (8.5cm diameter × 10cm H) with a 0.91 kg cylindrical weight on top to prevent fighting while allowing investigation between the subject mouse and the stimulus mouse. Between each test, the chambers and wire cages were cleaned with mild unscented detergent and rinsed with ddH2O.

Sociability test: During the early dark cycle, animals were brought into the testing room and allowed to habituate for a minimum of 15 min prior to testing. Under dim red lighting, mice were placed into the closed center chamber of the testing apparatus and were allowed to freely explore all 3 chambers for a 10 min habituation period. Mice were then closed in to the center chamber and a novel same-sex stimulus WT mouse, age and size matched, was placed under one of the wire cages. The doors were lifted and the mouse was again allowed to freely explore all 3 chambers for 10 min. The side placement of the stimulus mouse was alternated between subjects. During this phase of testing, the cage was classified as the "object", and the stimulus mouse as the "novel" stimulus.

Preference for social novelty: After 10 min of interaction with the first stimulus mouse, the subject was returned to the center chamber and another novel same-sex WT stimulus mouse was placed in the empty cage in the chamber opposite of the first stimulus mouse. The doors were lifted and the subject was allowed to freely explore all 3 chambers for 10 min. For this

phase of the test, the first stimulus mouse was now classified as “familiar” and the second mouse “novel”.

The above social behavior studies were performed on the same two cohorts as in forced swim (see below).

Olfaction—To verify that any potential differences in social testing were not due to deficits in olfaction, olfactory ability was assessed by testing latency to locate a hidden novel, carbohydrate rich food. To normalize for motivation all animals were tested after 24 hrs of food restriction. Early in the dark period mice were taken to a dimly lit room and allowed to acclimate for a minimum of 15 min prior to testing. Mice were placed in an acrylic arena (26cm W × 47.5cm D × 20cm H) filled 5 cm with fresh corncob bedding material with a piece of a cookie (Nabisco Nutter Butter, Atlanta, GA, USA) 1 cm^3 hidden 2 cm below the surface. Animals were allowed to freely explore the arena and the latency to locate the cookie was recorded by an observer blind to the genotype of the subjects. After each test, the arena was cleaned with 70% ethanol and refilled with fresh bedding.

Tone-conditioned fear—Tone-conditioned fear acquisition and retention were assessed as previously described [Iscru et al, 2009] using the Near-IR Fear Conditioning System (Med Associates Inc., St. Albans, VT, USA). Briefly, for acquisition of the tone-conditioned fear, mice were placed in the test chamber for 2 min free exploration with 68dB white noise. Mice were then exposed to a series of 8 conditional stimuli (80dB tone, CS) for 6 s with the last 2 s paired with a 0.6 mA foot shock (unconditioned stimulus, US). Mice remained in the chamber for an additional 60 s after the last CS/US pairing before being returned to their home cage. Freezing behavior was recorded by the software for the 2 min baseline, during the 4s of tone alone prior to the 2s shock during acquisition, during the 30 s interval between CS presentations, and for the 60 s after the final CS (sampling rate = 9.5 frames/sec). Testing chambers were cleaned after each subject with 70% ethanol. 3 h later, animals were tested for short-term fear retention as described above without receiving the US and freezing was measured during the 6 s tone and the 30 s interval between tones. Additionally, to avoid context-dependent freezing during the retention test, the chamber was modified via the addition of a smooth plastic floor, a semi-circular testing chamber, and the addition of vanilla extract odor to present the CS in a novel environment. Twenty-four hours after the initial acquisition session, animals were placed in the original unmodified chamber and freezing behavior was recorded for 2 min to assess contextual freezing. Approximately 5 h later (~26 h after the short-term retention test), mice were assessed for long-term fear retention exactly as described above for the short-term retention test.

TCF was performed on two cohorts, one reported previously in Iscru et al., 2009 (cohort07: 5 L7+/+ males, 5 L7−/− males, and 8 L7+/+ females, 5 L7−/− females) and one new one (cohort10208 as described above in open-field), and data pooled as described in Results.

Porsolt forced-swim task—To assess depressive-like responses, early in the dark period mice were taken to a dimly lit room and allowed to acclimate for a minimum of 15 min prior to testing. Mice were individually placed in an opaque cylinder (24cm diameter) filled 15 cm with clean 24°C water and behavior was recorded for 5 min. Videos were later analyzed by

an observer unaware of genotype and were scored for time spent swimming, time spent floating, and number of floating bouts (The Observer XT, Noldus Information Technology, Leesburg, VA, USA).

The Porsolt forced swim test was performed on two cohorts (cohort10208: as described above; and cohort31809: 8 L7+/+ males, 8 L7−/− males).

Statistical Methods

To identify potential outliers in our datasets [Rousseeuw and Leroy, 1987; Hoaglin et al 1986] we generated interquartile range plots for all groups to be analyzed. With the exception of two animals (male mutant 9.1 and male wild-type 4.2) in the social preference data, no measurements were located more than three interquartile ranges beyond the first or third quartile. I.e., other than male mutant 9.1 and male wild-type 4.2, there were no problematic outliers, and no other animals were removed from any other analysis. Exclusion of male mutant 9.1 and male wild-type 4.2 from the social preference data also seemed justified because they never entered the chamber with the familiar mouse or unfamiliar mouse, respectively, and consequently there was no measurable sniffing on the opposite side.

Data are displayed as means ± SEM. Data from analyses involving repeated measurements of the same subject were analyzed using linear mixed effects modeling (LME-ANOVA) [Van Dongen et al, 2004; Gur et al., 2007] with genotype, sex, cohort (when appropriate) and trial number as fixed effects and animal identity as a random variable. Count data were analyzed using a generalized linear model (GLM-ANOVA) with a logarithmic link and a quasipoisson model to account for overdispersion [McCullagh and Nelder, 1989; see also, Zeileis et al, 2008]. Standard (linear model) ANOVA (LM-ANOVA) was used to analyze data with a normal distribution. For these, the Tukey–Kramer procedure was used to perform post-hoc comparisons [Sokal and Rohlf, 1994]. All tests were implemented in R (R Development Core Team, 2007; http://cran.rproject.org/) using the nlme [Pinheiro and Bates, 2004] and pscl [Zeileis et al, 2008] packages.

In some cases as described above under “Mice” new cohort data were combined with old cohort data. This approach may ultimately improve the universality of findings by incorporating some degree of environmental heterogeneity into the behavioral analysis [Richter et al., 2009; Reed et al., 2010; Wahlsten et al., 2006]. In such cases cohort was included as a fixed factor in the data analysis. In most cases no significant cohort effect was observed, but when it was, we have reported it (for example, in the forced swim test). In such a case it does not negate other significant effects or the analytic procedure in that specific instance, but rather is an indication of how inter-batch variability, well established for these kinds of tests as referenced above, can be statistically controlled.

On data plots the main effects are indicated with single or multiple large asterisks. Unannotated large asterisks indicate a significant individual effect of genotype (open-field and forced swim tests), or large asterisks are annotated to indicate an alternative main effect including genotype interactions. Small asterisks refer to other important effects, for example, an individual effect of sex and are thus annotated.

Data from sociability and social preference testing were also analyzed using the log-ratio approach of Aitchinson [Aitchison 1986], to account for the fact that they have a multivariate, compositional structure. I.e., we measured three variates per animal that always added up to 600 sec (the total observational time). The data are represented as ternary diagrams, where the distance of individual data points, each representing an experimental animal, from the corners of the diagram indicate the time the animal spent on the choice represented by that corner. Means and 95% confidence intervals were calculated as described by Weltje [Weltje 2002]. Note that if confidence ellipses do not overlap, this also indicates that means are distinct on a 95% significance level (i.e., $p \leq 0.05$). Also, if confidence intervals do not span the midline, this indicates a side preference at the significance level of $p \leq 0.05$. Analyses and graphical presentation were implemented in R, using the package “compositions” [van den Boogaart and Tolosana-Delgado 2008].

RESULTS

Reduced spontaneous locomotor activity and rearing in female *Pcp2(L7)* mutants

We previously reported normal basal behavioral functions including locomotion, balance, grooming, and visual tracking in *Pcp2(L7)* mutant mice [Iscru et al., 2009]. In fact, in that study, rather than a motor defect we reported an improved learning asymptote on the rotarod, a common test of cerebellar control of balance. We wanted to explore in more detail whether this could be due to a general increase in activity or possibly improved motivation.

Locomotor activity in an open-field can indicate whether mutants are hyperactive, but also it can provide a measure of exploratory motivation as well as of anxiety. In a sideline within that earlier study we observed significantly more total locomotor activity in females than males suggesting that the behavior is sexually dimorphic [see Supplemental Table S1 in Iscru et al., 2009; for sex, $F(1,63) = 7.14$, $p = 0.0096$]. In addition, while we did not analyze for genotypic differences in the separate sexes, we noted a numerical decrease in total activity in mutant females relative to same-sex wild-types (Supplemental Figure S2A in Iscru et al., 2009). The purpose of the current study is to combine these old cohort data with more data obtained from additional cohorts to determine whether significant behavioral changes can be detected in the mutants by increasing the sample size. The legitimacy, even benefits, of this approach with appropriate statistics is supported by many recent studies [Wahlsten et al., 2006; Richter et al., 2009; Reed et al., 2010].

First, before considering the new cohort we extended the analysis of the old cohort data for each sex individually using GLM-ANOVA. Female mutants showed a numerically decreased total activity (5884 total beam breaks for WT’s vs 4791 for mutants) and also decreased vertical rearing (247 beam breaks for WT’s vs 197 for mutants), but in neither case was this significant ($p = 0.09$ or greater, with criterion set at $p < 0.05$ throughout this study). To follow this lead, we now examined an additional cohort of animals. When this new cohort was analyzed individually, the previously observed sex dimorphism for total activity could be readily reproduced (average of 6993 beam breaks for females, 4418 for males, inclusive of both genotypes; for sex, $F(1,30) = 14.96$, $p < 0.001$). Similarly, female mutants (but not males) showed a numerically decreased total activity (8181 total beam breaks for WT’s vs. 5806 for mutants) and vertical rearing events (289 vertical beam breaks for WT’s vs. 217 for

mutants), though, again, neither decrease was significant ($p = 0.12$ or greater). We should note here that we previously reported normal balance in the mutants using a number of tests, and they also had normal grip strength [Iscru et al., 2009]. Therefore decreased rearing is not due to impaired balance or weakness.

To take full advantage of multiple cohort testing the new data were combined with that from the previous study excluding the heterozygotes (see “Mice” in Methods) (Fig. 1A,B). The two datasets were not significantly different in activity in center, periphery, or total activity (for cohort, $p > 0.25$ in each case). In the combined data there is a significant contribution of sex in all measures except rearing (Table 1). Therefore, open-field exploration is generally sexually dimorphic, and females explore significantly more than males, but this is not the case for rearing (Fig. 1A,B). More importantly, in the pooled dataset inclusive of both sexes the mutants showed a significant decrease in both total activity (average of 5779±465 beam breaks for WT’s, 4926±175 for mutants; Table 1) and vertical rearing (average of 249±13.7 vertical beam breaks for WT’s, 214±14 for mutants; Table 1). These effects are largely driven by changes in the females (Fig. 1A,B), because when the sexes were analyzed separately no significant genotype contributions were observed in males in any activity measure including rearing, while female mutants showed a significant decrease relative to wild-types in both rearing and total exploratory activity (Table 1).

We conclude from these studies of open-field behavior that females normally explore more than males, but this sex difference is largely eliminated in *Pcp2(L7)* mutants due to reduced exploration in females.

Bidirectional and sexually dimorphic changes in anxiety-like behavior in *Pcp2(L7)* mutants

We also examined how the mutant animals distributed their time in the open-field. Activity in the center of the field relative to total activity is typically used as a measure of anxiety, as a less anxious animal will spend relatively more time in the center of the field [Sterneck et al., 1998; Lalonde et al., 2004]. We found a significant sex difference in this measure if only wild-types are considered (by t-test, $p = 0.0013$), and as shown in Fig. 1C wild-type males spend relatively less time in the center (more anxious) than wild-type females. Of note, mutant males are significantly less anxious than wild-type males in this measure, whereas mutant females show a trend towards more anxiety than wild-type females (Fig. 1C; Table 1). This different effect of the mutation in the two sexes is confirmed by ANOVA which indicates a significant genotype*sex interaction when either center activity or relative time in center is considered (Table 1). As described above for total activity and rearing the same effects were observed in both cohorts.

We conclude that the effect of the mutation in open-field anxiety is different and oppositely oriented in the two sexes. This is consistent with two other anxiety tests, light-dark and elevated plus maze, which we previously reported to show no significant effects of *L7* genotype [Iscru et al., 2009]. In retrospect, analysis of those prior data reveals the same trend: an increase of anxiety in mutant females and a decrease in mutant males (see Discussion).

Altered dynamics of memory acquisition and memory extinction in tone-conditioned fear

In our earlier study from 2009 we reported an enhanced initial rate of acquisition in tone conditioned fear (TCF), with no observable effect on consolidation of fear memory. Here we repeated this test on another cohort of *L7* mutants and wild-types. In brief, the animals were given eight tone-shock pairings, and acquisition of freezing was measured during the tone presentation phase of each tone-shock pair. In order to take full advantage of multiple cohort testing, data were pooled with those from the previous study, and analyzed over all 8 trials, over the first 3 trials, and over the last 3 trials using linear mixed effects model (LME) ANOVA [Van Dongen et al., 2004; Iscru et al., 2009], with genotype, sex, trial number, and dataset (cohort) as fixed factors, and animal identifier as the random variable (N = 28 mutants, 10 of which were from the old dataset, and 29 wild-types, 13 of which were from the old dataset; equal proportions of males and females within each genotype). The two datasets were not significantly different from one another in any of the three trial brackets (for cohort, p = 0.2 or greater). Over all 8 trials, as expected, there was a significant increase in freezing with repeated trials (Fig. 2A; Table 2A, left side for measures made during tone ("Tone"); see "Trial"). There was also a significant two-way interaction (Table 2A, trial*sex), indicating that sex significantly influences acquisition curve shape (defined as change in freezing over repeated trials), and a three-way interaction (Table 2A, genotype*sex*trial), indicating that curve shape is also significantly influenced by genotype. By examination of the acquisition plots this can be explained as a somewhat opposite effect of genotype in the two sexes, enhancing both acquisition rate and freezing amplitude in females but inhibiting both in males (Fig. 2A), similar to what we previously reported [see Fig. 3 in Iscru et al., 2009]. When the sexes are considered separately, no significant individual effect of genotype or interactions involving genotype are detectable in the males, but there is a genotype interaction in the females (Table 2A, genotype*trial). Therefore, the main effect of *L7* gene inactivation is clearly on curve shape or acquisition dynamics over trials as indicated by the significant genotype*trial interaction. The same general effects are observed when the two datasets are considered separately (not shown).

If only the initial acquisition rate is considered, i.e., the first 3 trials, inclusive of both sexes, then a trend indicating that genotype influences acquisition rate is observed (Table 2B, genotype*trial). However, when the sexes are considered separately over the first 3 trials there is a significant contribution of genotype in females (Table 2B, genotype*trial), but none in males. From the acquisition plots (Fig. 2B) this can be explained as an increase in acquisition rate over the first 3 trials that is more robust in females, as we reported before [Iscru et al., 2009]. Over the last 3 trials (i.e., acquisition asymptote) there is no significant individual effect of trial number, but there is a significant individual effect of sex (Table 2C, sex), the only trial bracket where this is true, and genotype also influences this trial bracket in a two-way interaction with sex (Table 2C, genotype*sex). The explanation for this, as revealed by the plots, is the same as discussed above for all 8 trials; i.e., there is an opposite effect of genotype in the two sexes. The same conclusion is arrived at if, instead, the animal average over the last three trials is calculated and analyzed using standard GLM-ANOVA (Fig. 2C; freezing asymptote measured during tone administration in acquisition session, "Asymp-Tone", for genotype*sex $F(1,52) = 4.49$, $p = 0.039$).

In the analysis of the two pooled datasets described above freezing was measured during the tone presentation in the acquisition trials. If freezing was measured instead during the 30 sec interval between tone trials, then there were no significant individual effects of genotype or interactions involving genotype (Fig. 2A-C, “Asymp-Interval”), indicating that the genotype contribution is specific to the tone presentation phase of acquisition and not due to changes in basal, shock-induced fear during training.

In our previous study we also examined short-term and long-term fear memory and reported no significant difference between mutants and wild-types [see Iscru et al., 2009]. Briefly, the test was performed as follows: 3 or 24 hrs after the acquisition session animals were presented with 8 trials of the tone (CS) alone and percent freezing was measured during the tone presentation or in the 30 sec interval between tone trials in a novel testing chamber to minimize contextual fear. However, to expand this analysis we pooled the old dataset with the new as described above. If the average of the first three trials is considered as a measure of initial memory and analyzed by GLM-ANOVA then there are no significant contributions of genotype in either short-term or long-term memory whether measured during the tone or during the inter-tone interval (Fig. 2D). Therefore memory consolidation and retention is normal in the mutants. There were also no differences in contextual fear (Fig. 2D).

However, in order to determine whether genotype contributed to changes over time in the memory sessions we analyzed the pooled data using LME-ANOVA with genotype, sex, trial number, and dataset (cohort) as fixed effects and animal identifier as the random variable. Over all 8 trials there was no significant individual effect of genotype or interactions involving genotype in short-term memory whether measured during the tone or inter-tone interval (not shown). In addition, there was a significant cohort effect in short-term memory, possibly suggesting a strong environmental contribution and behavioral instability so soon after acquisition (for cohort, $F(1,48) = 42.62$, $p < 0.0001$).

However, the situation was different in long-term memory. Fear (%-freezing) data measured during the tone were analyzed in three trial brackets as we did for the acquisition session: over all 8 trials, first 3 trials, and last 3 trials. In no trial bracket was there any significant difference between the two cohorts. In addition there was no individual effect of *Pcp2(L7)* genotype indicating that there was no overall defect in fear memory in the mutants, as we reported before [Iscru et al., 2009]. However, over all 8 trials a significant individual effect of trial number was detected, and a two-way interaction of trial number with genotype (Table 3A, left side for measures made during tone (“Tone”)). This genotype*trial interaction is largely driven by changes in males, since it is not significant in females considered individually, while it is in males (Table 3A). To illustrate the relative difference of the genotypes over time in the individual sexes we plotted the mean of each trial after subtracting the mean from the first trial such that trial 1 is arbitrarily set at 0 (Fig. 2E). The time courses suggest that this difference is explained by a gradual extinction of tone-dependent fear over time in wild-type males (presumably as they re-learn that no shock is forthcoming), but a relatively flat response over trials in *Pcp2(L7)* mutant males (Fig. 2E). Females are more complicated. While there is a significant increase in freezing from trial 1 to trial 2 when all sex and genotype combinations are considered together or when each sex is considered individually (Fig. 2E; for trial1vs2, $F(1,49) = 14.02$, $p = 0.0005$, inclusive of

both sexes), only wild-type females continue to increase over the first four trials (Fig. 2E; trial1vs4, wild-type females only, $F(1,15) = 17.95$, $p = 0.0007$). The transient increase in freezing at the beginning of the CS-alone memory trials is most likely an anticipatory response that is of longer duration in wild-type females. In fact, the change over trials is significantly different in mutant females versus wild-types when the first four trials are considered alone (see Fig. 2E; Table 3B, females only, genotype*trial). In addition, in the same trial bracket there is a significant three-way interaction supporting that genotype and sex both influence the change in fear with repeated trials, which is explained as an opposite effect of genotype in the two sexes over this trial bracket (Fig. 2E; Table 3B, genotype*sex*trial).

In the analysis of long-term fear memory described above, freezing was measured during the tone. If freezing is measured instead during the 30 sec interval between tones then no significant individual effect of genotype or interactions involving genotype are detected. Under these conditions all genotype and sex combinations show a progressive decrease in general fear that is of similar slope (Supplemental Data, Fig. S1). It should be noted that when measured during the tone there is no significant extinction over 8 trials, even in wild-type males, as measured by a comparison of trial 1 vs. trial 8 ($p = 0.4$ or greater, by t-test), even though overall there is a significant effect of trials (Fig. 2E; Table 3A). However, when freezing is measured during the inter-tone interval there is significant extinction (Fig. S1; for all animals, trial 1 average = 38.7% and trial 8 average = 27.0%, $p = 0.00081$ by t-test). The less robust extinction during the tone is most likely related to the greater amplitude of freezing when measured during the tone than during the inter-tone interval (Fig. 2D, right panel; 51.3% freezing during tone vs. 36.5% during interval, by t-test $p < 0.0001$), and the fact that no anticipation is observed during the inter-tone interval (Fig. S1). In future studies more trials will need to be added over a longer period of time to uncover the full range of tone-dependent extinction changes in the mutants.

All of these considerations in concert suggest that the contribution of Pcp2(L7) lies in the dynamic aspects of CS-US association and extinction re-learning during tone administration, and not in generalized or context-dependent fear or in memory storage. Pcp2(L7) normally contributes to the rate of both memory acquisition and extinction in TCF, reducing the rates in females while increasing them in males, resulting in opposite effects of the mutation in the sexes. In females the main role of L7 in extinction curve shape appears to be related to anticipation.

Depressive-like and Social Behavior Tests

Depressive-like behavior—We previously reported learning enhancement on the rotarod (Iscru et al., 2009). It is possible this is not a learning effect at all, but a change in motivation. To explore this further, we conducted a Porsolt forced swim test to measure the degree of behavioral despair. Two cohorts of males and one of females were tested and the time spent swimming was determined and analyzed by LM-ANOVA. Mutant males in cohort 1 have a significantly increased swim time relative to wild-types (200.8 sec for KO's vs 127.5 sec for WT's; Table 4A), but there is no significant genotype effect in cohort 2 males (neither shown graphically). When the two male cohort data are combined then a

significantly greater swim time is again observed in the mutant males relative to wild-types (Fig. 3; 209.7 sec for KO's vs 171.2 sec for WT's; Table 4A). There is no significant genotype effect for the single female cohort (Fig. 3). The observed increased swim time in mutant males is inconclusive, however, since the two male cohorts were significantly different from one another with a similar effect size as for genotype (total swim time of 164.2 sec for cohort 1 and 217.9 sec for cohort 2, inclusive of both genotypes; Table 4A). Therefore these data must necessarily be considered preliminary. Nevertheless, these results are reminiscent of our previously published rotarod study [Iscru et al., 2009]. In that study adolescent male and female mutants (4–5 wks old) both showed an increased learning asymptote with repeated training (both sexes combined, for genotype: $F(2,147) = 4.69$, $p = 0.011$). However, we also tested two older cohorts (>8 weeks of age, heterozygotes included) and found a significant individual effect of genotype in the males but not females [Iscru et al., 2009]. As they were not shown previously in graphic form the results from the older two cohorts alone are shown here in Fig. S2 (excluding the heterozygotes).

Social behavior—To our knowledge there are no available genetic models that have suggested any role of the cerebellum in the social domain. Here we examined sociability and preference for social novelty using the three chambered box test [Moy et al., 2008]. In the sociability test the amount of time the test mouse spends exploring an unfamiliar stimulus mouse placed in one chamber is compared to the time spent with an inanimate object in the other chamber. In the preference for social novelty test the amount of time spent exploring a familiar stimulus mouse (the same mouse from the sociability test) versus that spent with an unfamiliar (new) stimulus mouse is compared. In both tests the time spent on each side and time spent sniffing the two stimuli are measured. As sniff time is the more sensitive and direct measure of social interest [Moy et al., 2008], we mainly focused on this measure for this study. For both tests we scored for detectable preference in each group based on reaching statistical criterion for stimulus (i.e., $p < 0.05$ for animal 1 vs object or animal 1 vs animal 2) using a sample size previously shown to confidently reveal a preference in the C57Bl/6 strain [Moy et al., 2008]. Two distinct cohorts of male mice (tested in different sessions months apart) and one cohort of females were examined and data analyzed by LME-ANOVA (each cohort consisted of equal numbers of wild-types and mutants; see Methods). We selected LME-ANOVA rather than classical repeated measures ANOVA due to unbalanced numbers of males and females. If both sexes are considered together, both the wild-types (average WT sniff time is 171.8 sec. for the unfamiliar mouse and 52.3 sec. for the object) and *L7* mutants (average mutant sniff time is 156.4 sec. for the unfamiliar mouse vs. 48.9 sec. for the object) show a significant preference for the unfamiliar mouse compared to the object, and this is also true when the sexes are considered separately as shown in Fig. 4A (see also Table 4B). This is a sexually dimorphic behavior as males spend significantly more time sniffing the stimulus mouse than do females (inclusive of both genotypes, males have an average sniff time of 207 sec compared to 121 sec for females; Table 4B). We conclude from this analysis that mutants of both sexes have detectable sociability. There was no significant effect of genotype.

As for sociability there is no effect of genotype in the social novelty preference test. Nonetheless in contrast to their normal sociability, mutants do not show a significant

preference for an unfamiliar mouse compared to a familiar mouse either with sexes considered separately or together (Fig. 4B; Table 4C). However, a significant preference can be detected in wild-types (Fig. 4B; Table 4C). On average wild-type males spent 57.9 sec more time sniffing the stranger mouse than the familiar mouse, while for mutant males the difference was only 22.5 sec. Similarly wild-type females spent 52.8 sec more, while for mutant females the difference was only 31.5 sec.

To determine whether the loss of social novelty preference in the mutants may be due to a defect in olfaction we tested latency to find a buried cookie, a common olfactory test. *L7* mutants were able to find the cookie just as easily as wild-types (Fig. 4C), and unlike sniffing in a social context this olfactory behavior was not sex-dependent (for genotype, $F(1,28) = 0.2361$, $p = 0.6308$; for sex, $F(1,28) = 0.9166$, $p = 0.3466$).

In addition, it appears that sniffing in the social novelty preference test is a sex-dependent behavior as was shown above in the sociability test (Fig. 4B; Table 4C). However, interestingly, it seems that this effect is mainly driven by the mutants as the wild-types as a group do not reach statistical criterion for sex (Table 4C). This aspect is explored more vigorously in Fig. 4D,E (see below).

While ANOVA analysis of social behavior as just presented is commonly accepted in the animal behavior field, one problem with it is that it does not consider time spent not sniffing. Yet, by examination of Fig. 4B, one can see that a considerable effect of *L7* gene inactivation may be a reduction of overall time spent on sniffing, particularly in female mutants. In fact, as three time measures were actually made for each animal, this is clearly a multivariate dataset. Moreover, these data are really compositional in nature and represent relative as opposed to absolute measures. All reported times are part of a constant sum, or total (600 sec), and hence proportional. We therefore sought for a statistical approach that would consider both the multivariate and the compositional structure of these data better than classical parametric tests, which indeed are formally inappropriate for this kind of data [Aitchison 1986]. To this end we reexamined these data using the Aitchison log-ratio transformation method and present the data in triangle plots using the time not sniffing as a third parameter that now accounts for all time in the testing apparatus. Using non-overlap of the 95% confidence interval ellipses as a measure of significance [Weltje 2002] no significant effect of genotype was observed (not shown). There is, however, a significant sex difference with both genotypes considered together (Supplemental Fig. S3), as was observed using parametric analysis. However, when the data are analyzed by sex for the two genotypes independently a significant sex difference is only observed in the mutants and this is the case for both sociability (Fig. 4D) and preference for social novelty (Fig. 4E). From this analysis it would appear that the parametric analysis as performed in Fig. 4B overestimates the statistical significance of stranger preference in the wild-types, since the 95% confidence interval crosses the vertical midline in all cases (Fig. 4E). This is likely due to the fact that the two sniff times are highly correlated (i.e., along with time not sniffing they add up to a constant value) and this fact is not modelled in the standard parametric test whereas it is using compositional analysis. Also compositional analysis unmasks a subtle effect akin to a genotype-sex interaction. This effect may have two distinct components: a slight shift of the mutants away from a novel mouse toward an object or familiar mouse, and

a decrease in total interaction time especially in the females. The latter could be a further ramification of the reduced exploration and increased anxiety of female mutants as reflected in the open-field test. In toto these results suggest that further testing in the social arena is warranted and that compositional analysis, by not discarding important behavioral parameters, is better suited for this purpose.

DISCUSSION

Here we report that Pcp2(L7) contributes to anxiety and fear-related behaviors in a much more significant way than we previously appreciated [Iscru et al., 2009]. With respect to activity in the open-field, inactivation of *L7* significantly reduces female activity to levels typically observed in males. In addition female mutants spend less time in the center of the open-field, indicative of increased anxiety, while male mutants spend significantly more time in the center of the open-field, indicative of decreased anxiety. In accord with this result we add that in our previous study we also performed the light-dark anxiety test, and even though no significant genotype effects or interactions were reported (see Suppl. Table S1 in Iscru et al., 2009), in retrospect the average change is consistent with an opposite effect of the mutation in males and females. However, these modest changes are smaller than the standard deviation for each group. We also reported no significant genotype effects or interactions in the elevated plus maze in the previous study. In retrospect, though, the same trend can be observed: male KO's had a 9.7-fold increase in time in the open arms (decreased anxiety) and females a 36% decrease (increased anxiety) relative to same-sex WT's. In this case there may be a significant change in males and a trend towards a genotype-sex interaction, but too few animals were tested to rule out a spurious result (n = 3 female WT's, 2 KO's and 6 male WT's, 3 KO's). Should these impressions be verified by larger and balanced data sets this would extend our conclusions to other anxiety behaviors.

Similarly the effect of the mutation on fear amplitude in TCF is oppositely oriented in the sexes and in the same direction as for open-field anxiety. Here, however, the main effect is on the dynamics of TCF acquisition and extinction. There is no discernible effect on short-term or long-term memory per se. The most salient feature of this effect is that it is only observed when measured during the tone and not at all during the inter-trial interval. In addition, during the memory sessions we observed a robust anticipatory fear response that is only observed during the tone and may be perturbed in female mutants. This will need further exploration in the future, but anticipatory functions of the cerebellum at multiple time scales is well-described and of great interest [Tesche and Karhu, 2000; Mendoza et al., 2010]. With respect to the tone-dependence of the observed changes, we previously reported some very subtle sensorimotor changes in the *L7* mutants. For example in the acoustic startle reflex (ASR) test the male mutants showed a slight increase in pre-pulse inhibition relative to wildtypes at lower pre-pulse intensities suggesting improved sensorimotor gating. In addition, unlike wild-types, the male mutants showed habituation of the ASR over 60 trials [Iscru et al., 2009]. These measures could be correlates of decreased fear in TCF. In toto these experiments support that L7 contributes to adjusting the amplitude and/or rate, or force [Koziol et al., 2011], of sensory responses and their adaptations with experience. Granted, ASR and TCF are primarily controlled by amygdala (reviewed in Boele et al., 2010), but our data support a contribution of the cerebellum as elegantly revealed by

Sacchetti et al., 2002, 2004 for TCF. There are no known direct connections between the cerebellum and amygdala, but nevertheless such interactions could be secondarily mediated through reciprocal connections of the cerebellum with medial prefrontal cortex and/or hypothalamus, both of which connect to amygdala [reviewed in Strick et al., 2009; Haines et al., 1984].

The data we report here on depressive-like and social behaviors, while preliminary, nevertheless suggest that further analysis of the *L7* mutant is warranted. The increased swim time of mutant males in the forced swim test supports increased motivation as a possible root cause of the increased learning asymptote on the rotarod that we previously reported [Iscru et al., 2009]. In addition, the use of compositional analysis of the social arena data, by incorporating a third key parameter that is typically cast aside using classical parametric tests, has allowed us to reveal an anxiety component that would otherwise remain masked. Using this analysis it is quite clear that the most significant effect in the mutant is the accentuation of a male-female difference, especially in the preference for social novelty test, due mainly to decreased total sniff time in the mutant females. This result resonates well with the other tests showing increased anxiety in mutant females. As for a quantifiable change in preference for social novelty in the mutants, this cannot be excluded from the current data and, in fact, derives some support, but will require the testing of many more animals in the future.

The sex differences we report here cannot be due to some spurious effect due to the estrus state of the females. This was not specifically controlled in these studies. However, as we laid out in painstaking detail in our account of the open-field studies, both cohorts show a very robust reproducibility of the sex and anxiety effects. In addition, other anxiety tests as described above also point towards a sex difference, as do the changes in fear amplitude in the TCF tests. Lastly, as we describe next, these are all robust sex-dependent behaviors that are well-reported in the literature requiring no special normalizing hormonal conditions for sex differences to be detected.

Previous studies have demonstrated a strong contribution of sex in cerebellar-mediated tone-conditioned responses. For example, it is known that female rats have greater CR frequency asymptotes than males during acquisition trials in eyeblink conditioning (EBC), and the effect of stress on this behavior is opposite in the two sexes [Wood and Shors, 1998]. In the same study it was shown that the impairment of CR acquisition by stress in females was dependent on estradiol, as was the relatively greater CR acquisition in unstressed females relative to unstressed males. Also, estradiol enhances the amplitude of the TCF response in ovariectomized female mice [Jasnow et al., 2006]. TCF and EBC have similarities with respect to the cerebellar regions controlling the behaviors in mice, vermis and interposed nuclei in TCF [Sacchetti et al., 2002], simplex and adjacent HVI lobules and interposed nuclei in EBC [Van Der Giessen et al., 2008], and in addition the two behaviors are cooperatively controlled by amygdala and cerebellum [reviewed in Boele et al, 2010]. However, the modulation by sex hormones may extend to other cerebellar learning behaviors. Estradiol improved motor learning in ovariectomized females in a gain-reducing visuo-vestibular adaptation paradigm, and the animals had enhanced long-term potentiation (LTP) but not long-term depression (LTD) at the granule cell-Purkinje cell synapse

[Andreescu et al., 2007]. Similarly during TCF there is an enhancement of LTP at the same synapse supporting that this is an appropriate underlying physiological indicator of freezing behavior [Sacchetti et al., 2004]. Thus, females are intrinsically different from males in a variety of cerebellar learning paradigms and this appears to be linked to estrogen.

Similarly, it was previously shown that females normally explore more than males in an open-field test, and this difference can be eliminated by prenatal administration of testosterone [Talarovicova et al., 2009]; and it has been shown that estrogen enhances open-field anxiety and decreases exploration in ovariectomized females [Morgan and Pfaff, 2001]. Thus, based on the known anxiety- and fear-enhancing effects of estrogen in the open-field and TCF, respectively, one hypothesis is that L7 normally acts to counter the effects of estrogen in these behaviors, which can be tested in future studies.

What can be said about the mechanisms underlying non-motor functions of Pcp2(L7)? First, there is increasing evidence of cerebellum-related sex differences [reviewed in Nguon et al., 2005]. We have previously reported that the molecular layer is on average 7.3% thicker in males than females (inclusive of both WT's and *L7* mutants) [Iscru et al., 2009], suggesting a sex-specific influence on Purkinje cell dendritic arbor length. Developing Purkinje cells auto-synthesize progesterone and estrogen, which likely influences Purkinje cell dendritic growth [Sakamoto et al., 2001; Koibuchi et al., 2008]. The nuclear receptor, RORα, a key transcriptional switch required for Purkinje cell survival, has been shown to activate aromatase expression, the key enzyme in the final step of estrogen biosynthesis [Odawara et al., 2009]. RORα levels may be regulated transcriptionally by sex hormones [Sarachana et al., 2011] and in turn it is a key transcriptional modulator of a Ca^{2+}-related and signaling gene cluster that includes *Pcp2(L7)* [Gold et al., 2003; Serinagaoglu et al., 2007]. RORα is also the target locus of the classic cerebellar mouse mutation known as *staggerer* in which most Purkinje cells degenerate during early postnatal development [Hamilton et al., 1996]. However, there is no evidence of Purkinje cell death in *L7* mutants, although the dendrites were reported to be shorter and the soma diameter was reduced [Iscru et al., 2009]. With respect to the estrogen receptors ERα and ERβ, both are negligibly expressed in adult Purkinje cells [Merchentaler et al., 2004]. However, estrogen is known to modulate rapid changes in intracellular Ca^{2+} in neurons [Fricke et al., 2007], and a novel membrane-bound estrogen receptor, Gpr30, is expressed in Purkinje cells [Hazell et al., 2009] and can signal through Gi/o [Filardo et al., 2000].

In our initial electrophysiological analysis of Purkinje cells in *Pcp2(L7)* mutants we observed a robust and unique form of depression of the complex spike waveform relative to wild-types [Iscru et al., 2009]. The complex spike is the signature all-or-nothing spiking response of Purkinje cells (PCs) to climbing fiber (CF) stimulation, and is alternatively considered a "teaching", error, or timing signal depending upon the author [Simpson et al., 1996]. As a teaching signal it is often considered to be part of the cerebellar learning engram [Hansel et al., 1998; Koekkoek et al., 2003; however see Schonewille et al., 2011]. Our data certainly do not exclude compromised instructional capabilities of the complex spike in *L7* mutants, but do seem consistent with a role mainly affecting learning rate and/or response amplitude.

The sex-dependent bidirectional nature of the changes reported here is a key observation. In an earlier study using the Xenopus oocyte expression system we showed that Pcp2(L7) has a dose-dependent bidirectional effect on the P/Q-type voltage-dependent Ca^{2+} channel [Kinoshita-Kawada et al., 2004]. At low concentrations the protein enhanced inhibition of the channel by a Gi/oPCR, while at high concentrations the protein decoupled the channel inhibition thereby increasing channel activity. This is due to distinct effects of the Gβγ and Gα arms of the Gi/oPCR pathway. Based on our in vitro studies it seems reasonable to hypothesize that L7 modulates the dynamic range of Gi/oPCR influences on Purkinje cell physiology such that at high L7 the electrical state of the cell would be stabilized against changes in Gi/oPCR activation. Such a property could be ideal for a molecular damper. However, so far little if any difference is observed in global L7 protein levels in male and female mice (unpublished observations), which argues against L7 levels as an explanation of the sex effects reported here. Nevertheless, the opposite influence of sex in certain behaviors reported in the current study suggests a complex interplay between L7, sex steroids, and Gi/oPCR's that contributes to sex-dependent behaviors. Thus, as cerebellum-mediated motor learning appears to utilize both PF-PC LTD and LTP as putative plasticity mechanisms, depending on the particular behavior under consideration (e.g., LTD for EBC and VOR, and LTP for TCF [see Koekkoek et al., 2003; Sacchetti et al., 2004]), it may be that the bidirectional intrinsic plasticity of Purkinje cells is modulated regionally and is dependent upon the unique local hormonal and physiological milieu. Alternatively, we have previously shown that synthesis of the L7 protein is activity dependent at the post-transcriptional level [Wanner et al., 2000]. Therefore it is possible that L7 dose and/or post-translational changes may be modulated locally and post-synaptically by virtue of the fact that its mRNA is abundantly translocated into Purkinje cell dendrites [Bian et al., 1996].

The range of changes we have observed in the *Pcp2(L7)* mutants suggests they may be an excellent model for a cerebellar contribution to hormonally-driven anxiety and mood disorders. For example, a recent human study provides persuasive evidence that the cerebellum plays a role in premenstrual dysphoric behavior [Rapkin et al., 2011]. This provides at least a backdrop to consider a mood stabilizing function of the cerebellum. In addition, the sensorimotor enhancements such as increased learning asymptote on the rotarod as we reported previously and the decreased exploration/rearing reported here, may be endophenotypes for savant behaviors and restricted interests, respectively, potentially modeling developmental disorders. In fact recent advances in behavioral research in mice have begun to reveal the sex-dependent traits that may be considered to represent human autism [Moy et al., 2004; Moy et al., 2009], and the L7-KO model may shed further light on the contribution of the cerebellum to these behaviors [Courchesne 1997].

In conclusion, we believe Pcp2(L7) has the capacity to serve as an intrinsic damper of Gi/o effects on Purkinje cell physiology affecting higher brain function. The primary site of gene action related to these changes can be unambiguously assigned to Purkinje cells due to the known specificity of *Pcp2(L7)* gene expression. The effect of the mutation is clearly different in males and females. In females we hypothesize that it acts to limit the known fear and anxiety promoting actions of estrogen, while in males it may act to limit the effects of an unknown mechanism that normally reduces fear, anxiety and negative affect. This function is probably unique to mammals since the *Pcp2(L7)* gene is not found in birds, fish, frogs, or

insects (unpublished observations; see Simons et al., 2006). However, which Gi/o-linked receptor system or systems underlies the effects reported here is an open question. The novel Gpr30 estrogen receptor described above may be involved, or possibly some serotonin (5-HT) receptor subtypes and/or α2-adrenergic (norepinephrine; NE) receptors. In fact, it is well-established that estrogen can act as a neuromodulator through the 5-HT system [Joffe and Cohen, 1998 and Rubinow et al., 1998 for reviews], and abundant 5-HT and NE neuromodulatory input systems to the cerebellum have been described for decades with no clear understanding of their functional (i.e., behavioral) relevance [Bishop and Ho, 1985; Schweighofer et al., 2004 for review].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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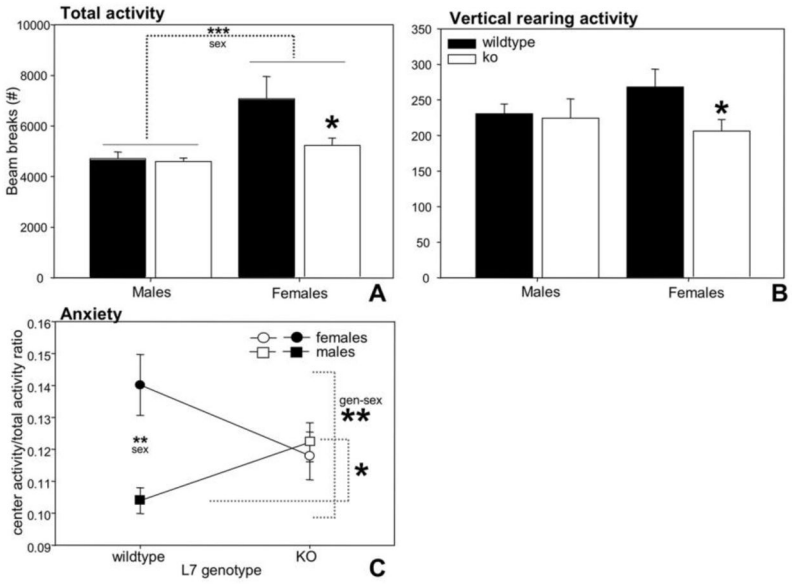

Figure 1.

Effects of *Pcp2(L7)* gene inactivation on activity and anxiety measures in an open-field test. **A**) There is a significant reduction in total activity in female mutants but not males. Also, activity in an open-field is sexually dimorphic. **B**) There is a significant decrease in vertical rearing behavior in female mutants but no effect in males. **C**) There is a sex-dependent bidirectional effect of *Pcp2(L7)* gene inactivation on anxiety. Compared to same-sex wild-types male mutants spend more relative time in the center of the field while female mutants spend less time there. In A-C: a single large unannotated asterisk indicates a significant genotype effect ($p < 0.05$), and, as annotated, two large asterisks indicate a significant genotype*sex interaction ("gen-sex": $p < 0.01$) and two or three small asterisks indicate a significant sex effect ("sex": **$p < 0.01$; ***$p < 0.001$). n = 18 L7+/+ males, 16 L7−/− males and 16 L7+/+ females, 20L7−/− females.

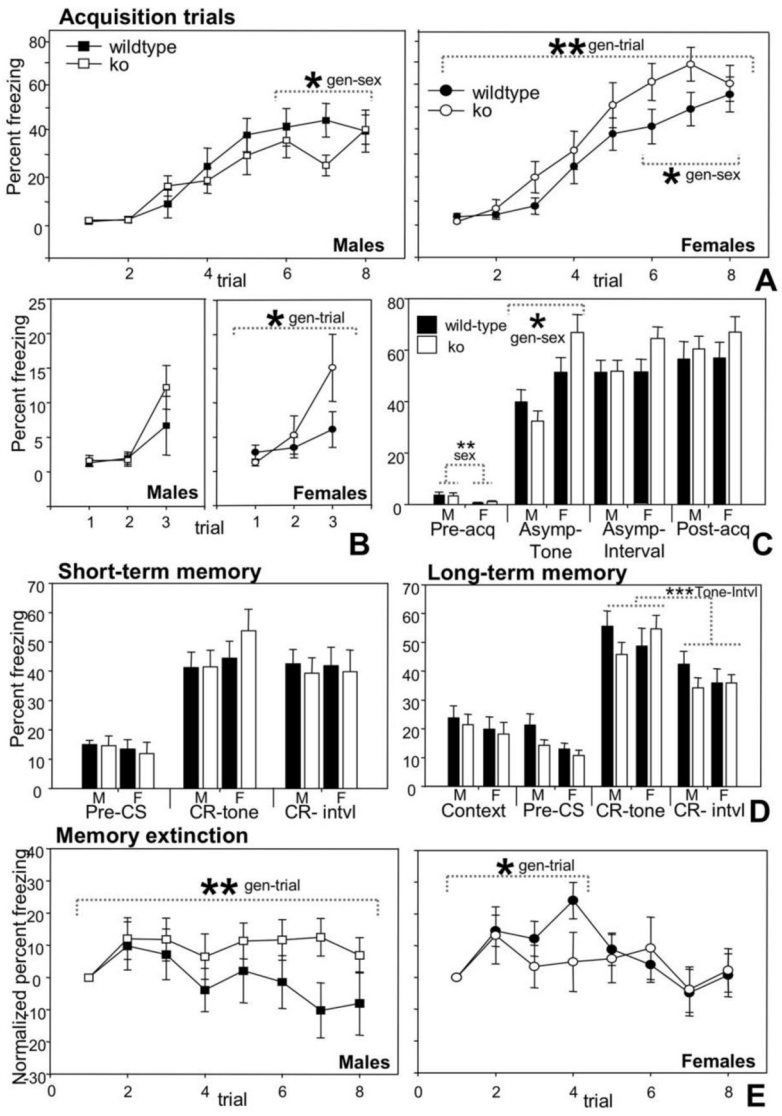

Figure 2.

Impact of *Pcp2(L7)* gene inactivation on dynamical aspects of TCF memory acquisition and extinction. **A**) Change in freezing response over repeated tone-shock pairings in the TCF acquisition session. Percent freezing was measured during the 4 s of the tone prior to shock. The main effect is a contribution of sex and genotype on curve shape as indicated by a significant 3-way interaction between genotype, sex, and trial number (see text), and by significant 2-way interactions as indicated in the figure (see also C). **B**) First three trials of acquisition session, showing a significant genotype-trial interaction indicating enhanced initial rate of association in female mutants relative to wild-types. **C**) Pre- and post-acquisition show no genotype contribution. Similarly there is no genotype contribution in freezing asymptote when freezing is measured during the inter-tone interval ("Asymp-Interval"), while in contrast there is a significant genotype-sex interaction indicating a sex-dependent bidirectional effect of genotype when freezing is measured during the tone application ("Asymp-Tone"). In both cases bars reflect animal averages of the last three

trials. **D**) Short- (left panel) or long-term (right panel) fear memory was determined using animal averages of the first 3 trials (out of 8 CS-alone trials), with %-freezing measured during either the tone presentation ("CR-tone") or the inter-trial interval ("CR-intvl"). While there is a trend in CR-tone for a sex-dependent bidirectional effect of genotype in long-term memory (right panel), similar to that observed in A, C, and E, it is not significant (no interactions involving genotype). As an aside, %-freezing is significantly greater when measured during the tone than during the inter-trial interval in the long-term memory test (right panel), but not in the short-term test. Lastly, there are no significant genotype contributions or interactions involving genotype in either contextual fear ("Context") or in Pre-CS. Slightly elevated pre-CS values are due to novelty-induced freezing since we used a shortened habituation period prior to onset of testing. **E**) Dynamics of long-term fear memory extinction are altered in *Pcp2(L7)* mutants. The CS alone was presented (6 second tone) eight times and freezing was measured during the tone presentation. For purposes of presentation trial 1 was arbitrarily set at 0 so changes relative to trial 1 can be compared; however, statistics were performed on un-normalized data. Symbol keys in A also apply to B & E. Symbol key in C also applies to D. Large asterisks indicate main effects as annotated (e.g., "gen-sex" = genotype-sex interaction, "gen-trial" = genotype-trial interaction), while small asterisks indicate results of posthoc t-tests for genotype in panel A or other effects not involving genotype as in panels C ("sex") and D ("Tone-Intvl" = significant difference between CR measured during tone vs interval between tones). * $p < 0.05$, ** $p < 0.01$, ***$p < 0.0001$; n = 13 L7+/+ males, 15 L7−/− males and 16 L7+/+ females, 13 L7−/− females.

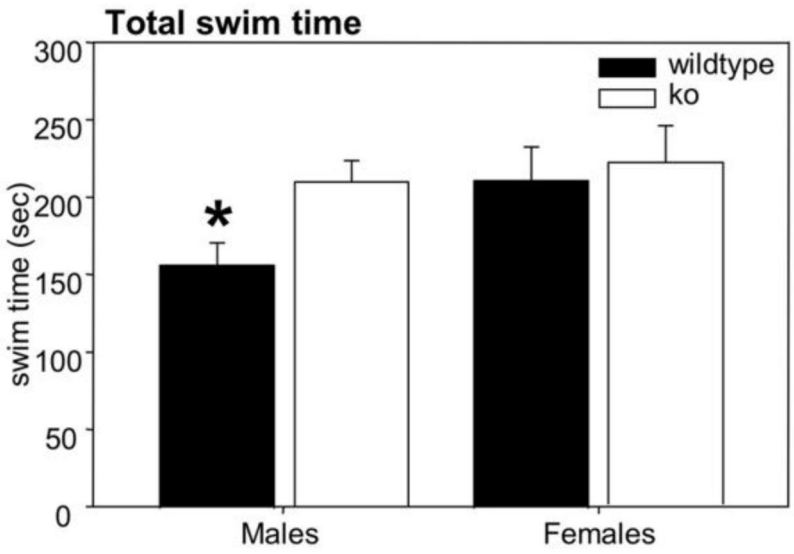

Figure 3.

Porsolt forced swim test: Increased swim time in adult male mutants. Mutant males show a significantly increased total swim time relative to wild-types, but there is no detectable effect in female mutants. Large asterisk indicates a significant genotype effect, * $p < 0.05$. This result is not considered conclusive because the effect size due to cohort is of similar magnitude as for genotype (see text). n = 16 L7+/+ males, 18 L7−/− males and 8 L7+/+ females, 8 L7−/− females.

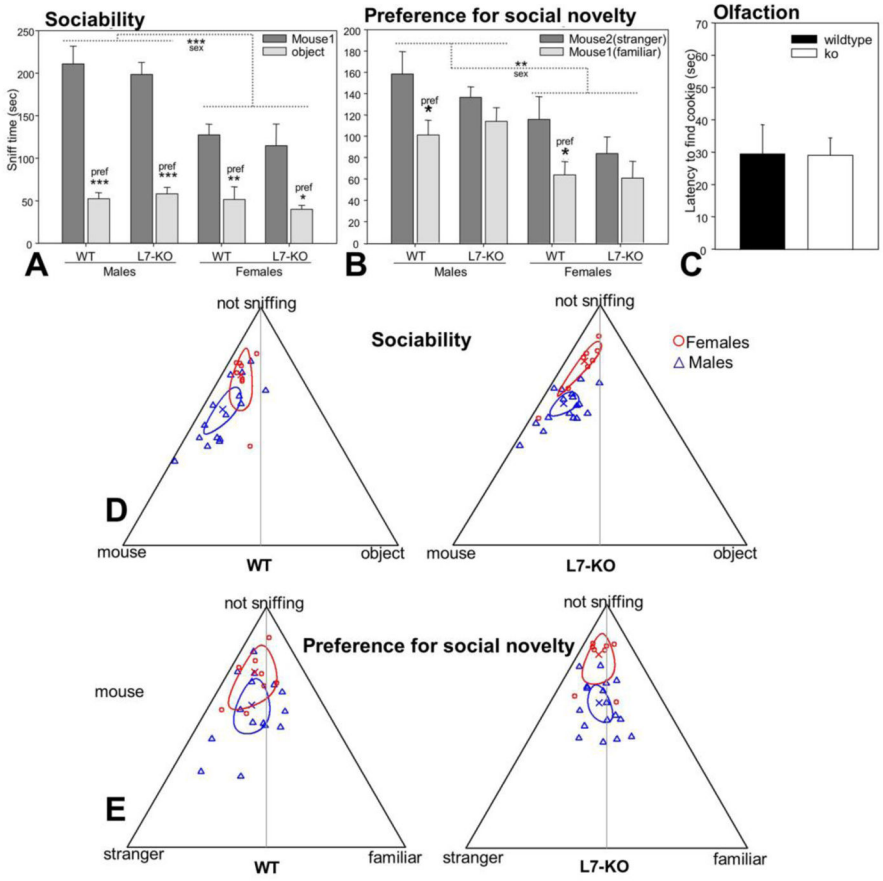

Figure 4.

Pcp2(L7) mutants have normal sociability, but do not reach statistical criterion for detection of a preference in social novelty. **A**) When presented with a choice between an object or an unfamiliar mouse both wild-types and mutants spend significantly more time sniffing the mouse. **B**) When presented with a choice between an unfamiliar or familiar mouse, wild-types spend significantly more time sniffing the unfamiliar mouse, while mutants do not. Sociability and preference for social novelty are both sexually dimorphic behaviors (A,B). **C**) There is no detectable difference in the ability of wild-type or mutant animals to find a cookie that is buried in the bedding material. Panels A & B: There are no significant individual effects of genotype or interactions involving genotype. Asterisks indicate significant effects of sex (“sex”) or stimulus (“pref”) as indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **D & E**) Alternative approach: compositional analysis of the sociability (D) and preference for social novelty (E) tests. Data are presented using triangle plots (Weltje, 2002). Symbol “X” indicates data mean, and ellipses indicate 95% confidence intervals. If two ellipses do not intersect this indicates a significant effect ($p \leq 0.05$). If ellipses do not cross the mid-line (dotted line) this indicates a significant preference. n = 16 L7+/+ males, 18 L7−/− males and 8 L7+/+ females, 8 L7−/− females.

Table 1

ANOVA Analysis of Open-Field Locomotor Behavior

	Total activity			Center activity			Rears			Relative time-in-center		
Both Sexes	**Df**	**F**	**P**	**Df**	**F**	**P**	**Df**	**F**	**P**	**Df**	**F**	**P**
Effects:												
Genotype	1	4.97	**0.030**	1	3.46	0.068	1	4.35	**0.041**	1	0.12	0.90
Sex	1	15.14	**<0.001**	1	12.06	**<0.001**	1	0.18	0.67	1	4.82	**0.028**
Genotype Sex*	1	3.10	0.084	1	5.16	**0.027**	1	1.17	0.28	1	8.23	**0.009**
Residual	58			58			58			58		
Females Only												
Effects:												
Genotype	1	5.39	**0.027**	1	5.21	**0.030**	1	5.20	**0.030**	1	2.58	0.12
Residual	30			30			30			30		
Males Only												
Effects:												
Genotype	1	0.0021	0.96	1	2.31	0.14	1	0.35	0.56	1	6.48	**0.017**
Residual	28			28			28			28		

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Table 2

ANOVA Analysis of Tone Conditioned Fear Acquisition

A. Acquisition-All trials

Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
Effects:					Effects:				
Genotype	1	49	0.13	0.72	*Genotype*	1	49	1.29	0.26
Sex	1	49	3.96	0.052	*Sex*	1	49	0.98	0.33
*Genotype*Sex*	1	49	0.39	0.54	*Genotype*Sex*	1	49	1.54	0.22
Trial	1	391	418.23	**<0.0001**	*Trial*	1	391	694.88	**<0.0001**
*Trial*Sex*	1	391	12.14	**<0.001**	*Trial*Sex*	1	391	4.81	**0.029**
*Genotype*Trial*	1	391	1.26	0.26	*Genotype*Trial*	1	391	1.31	0.25
*Genotype*Trial*Sex*	1	391	9.77	**0.0019**	*Genotype*Trial*Sex*	1	391	1.12	0.29
Males Only	**numDF**	**denDf**			**Males Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	24	0.00	0.96	*Genotype*	1	24	0.029	0.87
Trial	1	192	156.39	**<0.0001**	*Trial*	1	192	268.90	**<0.0001**
*Genotype*Trial*	1	192	1.42	0.23	*Genotype*Trial*	1	192	0.063	0.80
Females Only	**numDF**	**denDf**			**Females Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	25	0.84	0.37	*Genotype*	1	25	2.71	0.11
Trial	1	199	270.85	**<0.0001**	*Trial*	1	199	440.16	**<0.0001**
*Genotype*Trial*	1	199	8.58	**0.0038**	*Genotype*Trial*	1	199	2.78	0.097

B. Acquisition-1st Three Trials

Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
Effects:					Effects:				

B. Acquisition-1st Three Trials

Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
Genotype	1	49	0.45	0.51	*Genotype*	1	49	0.74	0.40
Sex	1	49	0.79	0.38	*Sex*	1	49	2.43	0.13
*Genotype*Sex*	1	49	1.07	0.31	*Genotype*Sex*	1	49	0.78	0.38
Trial	1	106	16.33	**<0.0001**	*Trial*	1	106	188.70	**<0.0001**
*Trial*Sex*	1	106	1.05	0.31	*Trial*Sex*	1	106	0.62	0.43
*Genotype*Trial*	1	106	3.77	0.055	*Genotype*Trial*	1	106	0.023	0.88
*Genotype*Trial*Sex*	1	106	2.02	0.16	*Genotype*Trial*Sex*	1	106	0.0088	0.93
Males Only	**numDF**	**denDf**			**Males Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	24	0.13	0.72	*Genotype*	1	24	0.037	0.85
Trial	1	52	2.78	0.10	*Trial*	1	52	81.80	**<0.0001**
*Genotype*Trial*	1	52	0.01	0.92	*Genotype*Trial*	1	52	0.0080	0.93
Females Only	**numDF**	**denDf**			**Females Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	25	0.63	0.43	*Genotype*	1	25	1.13	0.30
Trial	1	54	10.50	**0.0020**	*Trial*	1	54	122.90	**<0.0001**
*Genotype*Trial*	1	54	4.93	**0.031**	*Genotype*Trial*	1	54	0.0035	0.95

C. Acquisition-Last Three Trials

Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
Effects:					Effects:				
Genotype	1	49	0.10	0.75	*Genotype*	1	49	1.65	0.20
Sex	1	49	11.56	**0.0013**	*Sex*	1	49	1.91	0.17
*Genotype*Sex*	1	49	4.15	**0.047**	*Genotype*Sex*	1	49	1.71	0.20
Trial	1	106	1.10	0.30	*Trial*	1	106	0.092	0.76

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C. Acquisition-Last Three Trials

Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
*Trial*Sex*	1	106	0.36	0.55	*Trial*Sex*	1	106	0.0018	0.97
*Genotype*Trial*	1	106	0.30	0.59	*Genotype*Trial*	1	106	0.025	0.88
*Genotype*Trial*Sex*	1	106	1.32	0.25	*Genotype*Trial*Sex*	1	106	2.08	0.15
Males Only					**Males Only**				
Effects:	**numDF**	**denDf**			Effects:	**numDF**	**denDf**		
Genotype	1	24	1.38	0.25	*Genotype*	1	24	0.0052	0.94
Trial	1	52	0.07	0.79	*Trial*	1	52	0.030	0.86
*Genotype*Trial*	1	52	0.24	0.63	*Genotype*Trial*	1	52	1.24	0.27
Females Only					**Females Only**				
Effects:	**numDF**	**denDf**			Effects:	**numDF**	**denDf**		
Genotype	1	25	2.83	0.10	*Genotype*	1	25	3.35	0.079
Trial	1	54	1.55	0.22	*Trial*	1	54	0.065	0.80
*Genotype*Trial*	1	54	1.52	0.22	*Genotype*Trial*	1	54	0.79	0.38

* In A-C the measurements during tone presentation are indicated on the left (Tone), and during the interval between tones on the right (Interval)

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Table 3

ANOVA Analysis of Tone Conditioned Fear Memory Extinction

A. Long-term memory-All trials									
Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
Effects:					Effects:				
Genotype	1	49	0.17	0.68	*Genotype*	1	49	0.080	0.78
Sex	1	49	0.14	0.71	*Sex*	1	49	0.39	0.53
*Genotype*Sex*	1	49	0.88	0.35	*Genotype*Sex*	1	49	1.02	0.32
Trial	1	391	5.53	**0.019**	*Trial*	1	391	40.92	**<0.0001**
*Trial*Sex*	1	391	0.23	0.63	*Trial*Sex*	1	391	0.00	0.95
*Genotype*Trial*	1	391	6.15	**0.014**	*Genotype*Trial*	1	391	1.15	0.28
*Genotype*Trial*Sex*	1	391	1.41	0.24	*Genotype*Trial*Sex*	1	391	0.028	0.87
Males Only	**numDF**	**denDf**			**Males Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	24	0.10	0.75	*Genotype*	1	24	0.81	0.38
Trial	1	192	1.49	0.22	*Trial*	1	192	21.28	**<0.0001**
*Genotype*Trial*	1	192	7.27	**0.0076**	*Genotype*Trial*	1	192	0.79	0.37
Females Only	**numDF**	**denDf**			**Females Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	25	0.91	0.35	*Genotype*	1	25	0.22	0.64
Trial	1	199	4.23	**0.041**	*Trial*	1	199	19.87	**<0.0001**
*Genotype*Trial*	1	199	0.73	0.39	*Genotype*Trial*	1	199	0.40	0.53

B. Long-term memory-1st four trials

Tone					**Interval**				
Both Sexes	**numDF**	**denDf**	**F**	**P**	**Both Sexes**	**numDF**	**denDf**	**F**	**P**
Effects:					Effects:				
Genotype	1	53	0.33	0.57	*Genotype*	1	53	0.34	0.56
Sex	1	53	0.60	0.44	*Sex*	1	53	0.46	0.50
*Genotype*Sex*	1	167	1.01	0.32	*Genotype*Sex*	1	167	0.86	0.36
Trial	1	167	4.86	**0.029**	*Trial*	1	167	2.28	0.13
*Trial*Sex*	1	167	3.00	0.085	*Trial*Sex*	1	167	0.15	0.70
*Genotype*Trial*	1	167	0.93	0.34	*Genotype*Trial*	1	167	2.52	0.11
*Genotype*Trial*Sex*	1	167	5.75	**0.018**	*Genotype*Trial*Sex*	1	167	0.12	0.73
Males Only	**numDF**	**denDf**			**Males Only**	**numDF**	**denDf**		
Effects:	1				Effects:				
Genotype	1	26	1.27	0.27	*Genotype*	1	26	1.37	0.25
Trial	1	82	0.065	0.80	*Trial*	1	82	1.50	0.22
*Genotype*Trial*	1	82	1.29	0.26	*Genotype*Trial*	1	82	1.87	0.18
Females Only	**numDF**	**denDf**			**Females Only**	**numDF**	**denDf**		
Effects:					Effects:				
Genotype	1	27	0.11	0.74	*Genotype*	1	27	0.033	0.86
Trial	1	85	8.15	**0.0054**	*Trial*	1	85	0.84	0.36
*Genotype*Trial*	1	85	5.16	**0.026**	*Genotype*Trial*	1	85	0.84	0.36

* In A & B the measurements during tone presentation are indicated on the left (Tone), and during the interval between tones on the right (Interval)

Table 4

ANOVA Depressive-like and Social Behaviors

A. Porsolt forced swim			
Swim time			
Males: Cohort 1 Only	**DF**	**F**	**P**
Effects:			
Genotype	1	6.53	**0.021**
Residual	16		
Males: Cohort 1 & 2			
Effects:			
Genotype	1	4.88	**0.035**
Cohort	1	7.68	**0.0095**
Residual	30		
Females			
Effects:			
Genotype	1	0.08	0.78
Residual	14		

B. Sociability				
Sniff time				
Both Genotypes	**numDf**	**denDf**	**F**	**P**
Effects:				
Genotype	1	47	0.54	0.46
Sex	1	45	15.22	**<0.001**
Genotype Sex*	1	45	0.04	0.83
Stimulus	1	47	134.90	**< 0.0001**
*Stimulus * Sex*	1	47	9.65	**0.0032**
*Stimulus * Genotype*	1	47	0.49	0.83
Wild-types only				
Both sexes				
Stimulus	1	23	65.77	**<0.0001**
Sex	1	21	5.99	**0.023**
*Stimulus * Sex*	1	23	5.71	**0.026**
Males				
Stimulus	1	15	51.83	**<0.0001**
Females				
Stimulus	1	8	15.75	**0.0041**
Mutants only				
Both sexes				
Stimulus	1	24	69.38	**<0.0001**
Sex	1	24	9.59	**0.0049**
*Stimulus * Sex*	1	24	3.94	0.059

B. Sociability				
Sniff time				
Both Genotypes	**numDf**	**denDf**	**F**	**P**
Males				
Stimulus	1	17	65.97	**<0.0001**
Females				
Stimulus	1	7	8.37	**0.023**

C. Preference for Social Novelty				
Sniff time				
Both Genotypes	**numDf**	**denDf**	**F**	**P**
Effects:				
Genotype	1	43	0.60	0.44
Sex	1	43	17.16	**<0.001**
Genotype Sex*	1	43	0.55	0.46
Stimulus	1	45	13.04	**<0.001**
*Stimulus * Sex*	1	45	0.01	0.93
*Stimulus * Genotype*	1	45	1.91	0.17
Wild-types only				
Both sexes				
Stimulus	1	22	9.14	**0.0062**
Sex	1	20	4.19	0.054
*Stimulus * Sex*	1	22	0.02	0.90
Males				
Stimulus	1	14	5.33	**0.037**
Females				
Stimulus	1	8	4.55	0.066
Mutants only				
Both sexes				
Stimulus	1	23	3.93	0.059
Sex	1	23	17.49	**<0.001**
*Stimulus * Sex*	1	23	0.11	0.75
Males				
Stimulus	1	16	1.98	0.18
Females				
Stimulus	1	7	2.34	0.17