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Galanin receptor subtype 2 (GalR2) null mutant mice display an anxiogenic-like phenotype specific to the elevated plus-maze

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Abstract

The neuropeptide galanin has been implicated in anxiety-related behaviors, cognition, analgesia, and feeding in rodents. Neuromodulatory actions of galanin are mediated by three G-protein coupled receptors, GalR1, GalR2, and GalR3. The present study investigates the role of the GalR2 receptor by evaluating behavioral phenotypes of mice with a targeted mutation in the GalR2 gene. A three-tiered behavioral phenotyping approach first examined control measures of general health, body weight, neurological reflexes, sensory abilities and motor function. Mice were then assessed on several tests for cognitive and anxiety-like behaviors. GalR2 null mutants and heterozygotes were not significantly different from wildtype littermates on two cognitive tests previously shown to be sensitive to galanin manipulation: acquisition of the Morris water maze spatial task, and trace cued and contextual fear conditioning, an emotional learning and memory task. Two independent cohorts of GalR2 null mutant mice demonstrated an anxiogenic-like phenotype in the elevated plus-maze. No genotype differences were detected on several other measures of anxiety-like behavior. The discovery of an anxiogenic phenotype specific to the elevated plus-maze, similar to findings in GalR1 null mutants, highlights the potential therapeutic efficacy of targeting GalR1 and GalR2 receptors in treating anxiety disorders.

Keywords: Galanin; GalR2 null mutant; Anxiogenic-like; Mice; Learning; Memory; Nociception; Neuropeptide

Converging evidence from many laboratories implicates galanin and galanin receptors in anxiety-like and depression-related behaviors, via modulation of neuroendocrine and noradrenergic systems (Barrera et al., 2005; Echevarria et al., 2005; Holmes et al., 2002, 2003; Khoshbouei et al., 2002a,b). Rats administered galanin intracerebroventricularly (ICV) showed a significant increase in punished responding in the Vogel punished drinking test (Bing et al., 1993). Conversely, intra-amygdala administration of galanin produced a dose dependent decrease in punished drinking without affecting unpunished drinking or behavior in a second conflict-based test, the elevated plus-maze (Möller et al., 1999). Restraint stress in rats induced anxiogenic-like behavior in a social recognition task and in the elevated plus-maze (EPM; Khoshbouei et al., 2002a); while galanin pretreatment bilaterally into central amygdala produces anxiolytic-like EPM behavior (Khoshbouei et al., 2002b). Injection of the non-selective galanin receptor antagonist M40 bilaterally into the bed nucleus of the stria terminalis or the lateral septum attenuated these anxiolytic actions of galanin (Echevarria et al., 2005; Khoshbouei et al., 2002a). In rats, chronic fluoxetine pretreatment showed antidepressant-like effects in the forced swim test, which were abolished by central administration of M40 (Lu et al., 2005). Similarly, Galmic, a GalR1 specific agonist, produced antidepressant-like behavior in rats in the forced swim test (Bartfai et al., 2004). In contrast, administration of a new GalR3 selective antagonist to rats, guinea pigs and mice produced anxiolytic- and antidepressant-like effects in a wide range of anxiety-like and depression-related behavioral tests (Barr et al., 2006; Swanson et al., 2005).

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Studies with transgenic and knockout mice offer additional evidence that galanin may play an important role in modulating anxiety-like and depression-related behaviors. Mice overexpressing galanin (Gal-OE) under the platelet derived growth factor B (PDGF-B) promotor displayed increased immobility in the Porsolt forced swim test as young adults (Kuteeva et al., 2005), and exacerbated forced swim immobility in aged Gal-OE mice (Pirondi et al., 2005), consistent with increased depression-related behaviors. In addition, as compared to wildtype littermates, these mice showed greater stress-induced increases in the release of norepinephrine and serotonin, two neurotransmitters implicated in affective states (Yoshitake et al., 2004). Mice overexpressing galanin (Gal-tg) under the control of a dopamine β-hydroxylase (DBH) promoter displayed anxiolytic-like behavior when pretreated with yohimbine, a noradrenergic alpha 2 adrenoreceptor antagonist (Holmes et al., 2002). Mice lacking the GalR1 receptor demonstrated increased anxiety-like behavior specific to the elevated plus-maze (Holmes et al., 2003). Interestingly, ICV administration of galanin to normal C57BL/6J mice had no effect on anxiety-related behaviors (Karlsson et al., 2005).

In addition to a role in anxiety-like and depression-related behaviors, galanin has also been implicated in the cognitive and memory impairments associated with Alzheimer's disease (Counts et al., 2001, 2003; Kinney et al., 2002; Malin et al., 1992; McDonald et al., 1998a,b; Mufson et al., 1998, 2000, 2005; Steiner et al., 2001; Wrenn et al., 2003). During mid- to late-stage Alzheimer's disease, galanin immunoreactive fibers hyperinnervate the remaining cholinergic neurons of the nucleus basalis of Meynert, galanin peptide levels increase in the cortex and hippocampus, and galanin receptor binding increases in the basal forebrain (Chan-Palay, 1988; Counts et al., 2001, 2003; Mufson et al., 1998, 2000). It has been theorized that the inhibitory effect of galanin on cholinergic transmission contributes to the cognitive impairments characteristic of Alzheimer's disease (Chan-Palay, 1988; Counts et al., 2003, 2006; McDonald et al., 1998a; Mufson et al., 1998, 2000, 2005; Rustay et al., 2005). Studies in rodents have examined the role of galanin in cognition and memory processing (for reviews see McDonald et al., 1998b; Rustay et al., 2005). Rats administered exogenous galanin display cognitive deficits in spatial maze learning (Gleason et al., 1999; Kinney et al., 2003; Malin et al., 1992; Ögren et al., 1996, 1999; Schött et al., 1998, 2000; Sundstrom et al., 1988), operant delayed non-matching to position (McDonald and Crawley, 1996; McDonald et al., 1997; Robinson and Crawley, 1993, 1994), T-maze alternation (Givens et al., 1992; Mastropaolo et al., 1988) and passive avoidance learning (Ukai et al., 1995). Gal-tg mice showed impairments in spatial memory in the Morris water maze (Steiner et al., 2001), olfactory memory in social transmission of food preference (Wrenn et al., 2003), and emotional memory in trace cued fear conditioning (Kinney et al., 2002). GalR1 null mutant mice show impaired performance in trace cued and contextual fear conditioning, yet normal learning and memory on the delay version of this test, normal performance on spatial learning and memory, and normal olfactory memory, suggesting that some cognitive impairments resulting from excess galanin may involve actions at the subtype 1 receptor (Wrenn et al., 2004). Other important biological actions of galanin and galanin receptors, supported by convergent evidence from many labs, include effects on ingestive behaviors (Crawley et al., 1993; Kyrkouli et al., 1986, 1990; Leibowitz, 2005; O'Donnell et al., 1999; Tan et al., 2005) and nociception (Holmes et al., 2005; Kerr et al., 2001; Malkmus et al., 2005; Wiesenfeld-Hallin et al., 1993, 2005).

The physiological actions of galanin on neurotransmitter activity are mediated by three G protein-coupled receptors identified as GalR1, GalR2 and GalR3 (Bloomquist et al., 1998; Fathi et al., 1997, 1998; Habert-Ortoli et al., 1994; Howard et al., 1997; Kolakowski et al., 1998; Pang et al., 1998; Smith et al., 1997; Wang et al., 1998). All three galanin receptor subtypes are expressed at differing levels in the amygdala (Mennicken et al., 2002; O'Donnell et al., 1999) a brain structure involved in fear and anxiety (Walker et al., 2003). In addition, varying expression levels of GalR1, GalR2, and GalR3 are found in limbic structures implicated in learning and memory including the medial septum, bed nucleus of the stria terminalis, and the diagonal band of Broca, in hypothalamic nuclei mediating feeding, and in peripheral neurons mediating nociception (Mennicken et al., 2002; O'Donnell et al., 1999). The three galanin receptor subtypes activate distinct G protein-coupled pathways. GalR1 and GalR3 inhibit adenylyl cyclase I activity through the Gi pathway while GalR2 can activate three separate pathways, Go, Gq and Gi, primarily increasing phospholipase C activity and inositol phosphate production (Wang et al., 1998).

While the anatomical distribution and the distinct G protein-coupling of galanin receptor subtypes 1 and 2 have been elucidated, it remains unclear which galanin receptor or combination of galanin receptor subtypes may be mediating the behavioral effects of galanin on anxiety-related behaviors, learning and memory, feeding, and analgesia. The present experiments sought to elucidate the role of the GalR2 receptor in these processes, by comprehensive behavioral phenotyping of mice with a null mutation of the galanin subtype 2 receptor (GalR2 KO mice), previously generated by retroviral mutagenesis (Krasnow et al., 2004). The first functional study of these GalR2 mice reported normal reproduction and survival rates, normal susceptibility to seizures, and normal scores on several behavioral tasks (Gottsch et al., 2005). The present experiments extend the initial findings in GalR2 knockout mice with a larger set of mouse behavioral measures, using our established behavioral phenotyping strategy. This three-tiered phenotyping approach employs multiple complementary behavioral tests within each behavioral domain of interest (i.e. cognition and affect) In addition, potentially confounding variables are assessed, the experimental history of animals is controlled, and age-matched, same sex littermates are employed as the appropriate comparison groups (Bailey et al., 2006; Crawley, 1999, 2000; Crawley and Paylor, 1997).

The present experiments were designed to evaluate GalR2 null mutants, heterozygotes, and wildtype littermates on general health, body weight, neurological reflexes, sensory abilities including pain sensitivity, and motor functions. All genotypes

were evaluated on open field exploration, hotplate and tail flick testing, rotorod performance, and four specific tests of anxiety-like behavior. Elevated plus-maze testing was conducted prior to other behavioral measures to ensure that all mice were naïve prior to the test. Performance on the plus-maze has been shown to be affected by repeated testing, order of testing and differences in experimental test history (Holmes and Rodgers, 1998; McIlwain et al., 2001; Võikar et al., 2004). Cognitive ability was assessed in trace cued and contextual fear conditioning and the Morris water maze, tests that have proven sensitive to galanin manipulation (Kinney et al., 2002; Steiner et al., 2001; Wrenn et al., 2004).

1. Methods

1.1. Subjects

Mice with a null mutation of the gene coding for the galanin subtype-2 receptor (GalR2) were obtained from Nura Inc., Seattle, WA. The mice were generated by retrovial mutagenesis as previously described (Krasnow et al., 2004). Briefly, 129S1Sv/ImJ embryonic stem cells with the GalR2 null mutation were injected into C57BL/6J blastulas and then transferred into d2.5 pseudopregnant CD-1 female mice. The resulting chimeric mice were bred with 129S1Sv/ImJ mice, producing the GalR2 null mutation on a homogeneous inbred background. Genotyping of rodent tail DNA was conducted using standard PCR methods with GalR2 specific primers (5′-TCACTGCTCTGCAAG-GCCGTTCA-3′ and 5′-AGATTGGCCAGCTGCGACT-GACTGT-3′) as described previously (Gottsch et al., 2005; Krasnow et al., 2004). Heterozygous mice were mated to produce +/+, +/− and −/− mice. Two cohorts of mice (Cohort 1, 101 mice, ~8 weeks of age; Cohort 2, 68 mice, 4 months) were shipped to NIMH in Bethesda, MD for behavioral testing.

Upon arrival, mice were group-housed (4 per cage) with same sex littermates, resulting in mixed genotype cages. All mice were maintained in an NIH vivarium under humidity and temperature controlled conditions on a 12/12 light cycle (lights on at 6:00 am). During behavioral testing food and water were available ad libitum. All experiments included both male and female mice of approximately equal numbers in each of the three genotypes. Behavioral testing was conducted between the hours of 8:00 am and 6:00 pm. All procedures were approved by the NIMH Animal Care and Use Committee.

Behavioral analysis of general health, home cage behavior, neurological reflexes, sensory abilities, motor function and body weight were conducted on Cohort 1. In addition, Cohort 1 completed tests of anxiety-like behaviors, motor coordination and exploratory activity, and cognitive abilities. Mice in Cohort 2 were tested on body weight, elevated plus-maze, and rotorod. A third cohort of mice was bred in-house from heterozygotes in Cohort 2. This third cohort was tested on elevated zero-maze, elevated plus-maze, and stress-induced hyperthermia.

1.2. Behavioral tests

Tests were conducted in the following order: observations of home cage behavior, elevated plus-maze, general health measures including body weight measurements every 4 weeks, neurological reflexes, sensory and motor function, light ↔ dark exploration test, rotorod, open field exploration, trace cued and contextual fear conditioning, and spatial navigation in the Morris water maze. Two additional tests of anxiety-like behavior, elevated zero-maze and stress-induced hyperthermia, were conducted at the end of all other behavioral testing in the Cohort 3 GalR2 mice bred at NIH. There was a minimum of one week between behavioral tests except for tests included in Table 1 (i.e. measures of general health, neurological reflexes and sensory and motor function which were conducted on the same day). Rotorod testing, also included in Table 1, followed the one-week constraint described above. Identification of each animal was determined after testing to ensure that the experimenter remained blind to the genotype of the test subject. Before beginning a test session and between experimental subjects, each piece of testing equipment was wiped down with a solution of 70% ethanol and wiped dry.

1.3. Elevated plus-maze

Testing in the elevated plus-maze followed previously described procedures (Holmes et al., 2002). The elevated (40 cm) plus-maze consists of two open arms (30 cm × 5 cm) and two closed arms (30 × 5 × 15 cm) extending from a central

Table 1
General health and neurological screening of GalR2 +/+, +/−, −/− mice

Genotypes	+/+ N=22	+/− N=23	−/− N=19
General health			
Fur condition (3 pt scale)	2.0	2.0	2.0
Bald patches (%)	23	26	16
Missing whiskers (%)	68	43	21 (*)
Piloerection (%)	0	0	0
Body tone (3 pt scale)	2.0	2.0	2.0
Limb tone (3 pt scale)	2.0	2.0	2.0
Empty cage behavior			
Transfer freezing (%)	0	4	0
Wild running (%)	0	0	0
Stereotypies (%)	0	0	0
Exploration (3 pt scale)	1.6	1.7	1.7
Grooming (3 pt scale)	1.1	1.2	1.4
Motoric abilities			
Positional passivity (%)	23	8	21
Trunk curl (%)	100	100	100
Rotorod (latency sec)	129.7	127.8	115.7
Reflexes			
Forepaw reach (%)			
Righting reflex (%)	100	100	100
Corneal (%)	100	100	100
Pinna (%)	100	95	95
Vibrissae (%)	100	100	100
Toe pinch (%)	82	79	83
Reactivity			
Petting escape (%)	59	57	74
Struggle/vocalization (%)	50	61	68
Dowel biting (3 pt scale)	1.3	1.2	1.1

There were no significant differences on any measure of general health or neurological, sensory or motor reflex except missing vibrissae (*). Gal −/− had significantly fewer mice with missing vibrissae than wildtype mice [χ^2=9.107, p=.0105].

(5×5 cm) area. A raised lip (0.25 cm) around the open arms minimized the likelihood that a mouse would fall from the maze. Mice (Cohort 1 N=22 +/+, 23 +/−, 19 −/−; Cohort 2 N=14 +/+, 12 +/−, 17 −/−) were placed in the central area facing an open arm and allowed to traverse the maze freely for 5 min. Room lighting was ~20 lux. Arm entries (all 4 paws in the arm) and time spent in the arms were scored by a trained observer using (Hindsight, version 1.4) ethological recording and analysis software.

1.4. Light ↔ dark exploration test

The light ↔ dark exploration test was conducted as previously described (Crawley and Goodwin, 1980; Holmes et al., 2001). The test apparatus consists of a standard polypropylene cage (48×20×20 cm^3) divided into two unequal compartments by a black partition with a small opening at the base. The larger compartment is transparent, open from above and illuminated by a 75 W incandescent light. The smaller compartment is covered and the walls are painted black. Mice (N=22 +/+, 23 +/− and 17 −/−) were placed centrally in the open compartment facing away from the partition. Photocells located in the opening of the partition signaled transitions into the dark compartment and activated a timer recording time spent in the dark compartment.

1.5. Elevated zero-maze

The elevated zero-maze was conducted as described previously (Heisler et al., 1998). The maze consisted of an elevated (63 cm) circular runway (5.5 cm wide) approximately 43 cm in diameter, divided into 4 quadrants. Two opposing open quadrants with raised inner and outer lips (2 mm) minimized the chance of a mouse slipping off the runway. The opposing closed quadrants have opaque walls 15 cm high. Room lighting was ~30 lx. Mice (15 +/+, 14 +/−, 17 −/−) were placed in the middle of one of the closed quadrants and were allowed to explore the maze freely for 5 min. Quadrant entries and total time in the open quadrants were scored by a trained observer using Hindsight (version 1.4) ethological recording and analysis software.

1.6. Stress-induced hyperthermia test

Stress-induced hyperthermia testing was conducted, with slight modification, as previously described (Bouwknecht and Paylor, 2002). Group housed GalR2 littermates were singly housed for a minimum of 6 h prior to testing in an isolated staging area nearby to the test room. Singly housed mice (N=10 +/+, 10 +/−, and 13 −/−) were brought into the testing room, gently restrained by the tail while a baseline body temperature was obtained using a rectal probe (Thermalert TH-5 system Physitemp, Clifton, NJ, USA) inserted approximately 2 cm. After testing, mice were moved to the hall outside the testing room. After each use, the probe was cleaned with 70% ethanol, thoroughly dried and coated with a lubricant to minimize discomfort. Ten minutes after the baseline temperature reading was obtained, mice were returned to the testing room and a second temperature was taken. The change in temperature between the two time points reflects an unconditioned physiological response to the stress of prior handling and testing.

1.7. Open field test

Exploratory locomotor activity was examined in an automated open field test (Accuscan, Columbus, OH). Open field chambers consisted of clear Plexiglas sides and floor approximately 40×40×30.5 cm^3. Mice (N=38 +/+, 41 +/−, and 36 −/−) were placed in the center of the open field and allowed to explore the chamber for 30 min. Lower photocells, recording horizontal activity, were aligned 8 to a side, dividing the chamber into 64 equal squares. Vertical activity was assessed by an additional 8 aligned photocells placed slightly above the horizontal photocells. Rearing and exploratory activity, recorded as photocell beam breaks, were collected using the Versamax activity monitor and analyzer software system.

1.8. General health, neurological reflexes, sensory and motor ability

The general health of mice in Cohort 1 was evaluated using measures described previously (Crawley, 1999; Crawley and Paylor, 1997). Briefly, home cage observations involved scoring the activity of all mice in a home cage for approximately 15 min at three different daily time points (9:00 am, 3:00 pm, and 8:00 pm). The experimenter specifically noted incidence of excessive fighting, grooming, stereotypies, isolated mice, lack of huddling and quality of nest building. Empty cage behavior was scored in a separate session by placing the mouse into a clean, empty cage and noting incidents of transfer freezing, wild running, stereotypies, and grooming and exploration levels. General health assessment included assessing fur and whisker condition as well as limb and body tone. Limb strength was evaluated by placing mice on a wire cage lid that was then inverted over a standard mouse cage lined with a layer of bedding, for a maximum of 60 s. The latency to fall from the wire cage lid to the bedding below was used as the measure of limb strength. Neurological reflex tests included forepaw reaching, righting reflex, trunk curl, whisker twitch, pinna twitch, eyeblink response, and toe pinch. The reactivity level of the mice was assessed with tests measuring responsiveness to petting, intensity of a dowel biting response and level of vocalization during handling. Body weight of GalR2 mice was assessed longitudinally every 4 weeks from 16–32 weeks in Cohort 1, and 20–32 weeks in Cohort 2.

Responsiveness to painful stimuli was measured using the tail flick and hotplate tests on mice from Cohorts 1 and 2. For the tail flick test, mice were gently restrained with the tail placed in the groove of the tail flick test apparatus (Columbus Instruments, Columbus, OH). An intense lightbeam was focused on the tail and the latency to move the tail from the lightbeam was recorded. To prevent any tissue damage, a maximum latency cutoff of 10 s was used. For the hotplate test, the mouse was placed on the hotplate surface at a constant

temperature of 55 °C (IITC Life Science Inc., Woodland Hills, CA). Latency to first response was recorded. To prevent tissue damage, mice were removed from the test apparatus at a maximum cut-off latency of 30 s. The numbers of mice tested per genotype on the hotplate test were males +/+=7, +/−=10, −/−=9; females +/+=15 +/−=12, −/−=9; and on the tail-flick were males +/+=16, +/−=27, −/−=19; females +/+=21, +/−=23, −/−=18.

Motor coordination was evaluated in mice from Cohorts 1 and 2 using an accelerating rotorod (Ugo Basile, Stoelting, Wood Dale, IL) test. Mice were placed on the rotating drum for a 5 min test, during which the rotating drum gradually accelerated from 4 to 40 rpm. Mice from Cohorts 1 (~16 weeks) and 2 (~27 weeks) were tested in three consecutive trials during one test session on separate days. Average latency to fall from the drum was used as the measure of motor coordination. The numbers of mice per genotype were (Cohort 1) males +/+=7, +/−=10, −/−=9; females +/+=15, +/−=13, −/−=9; and (Cohort 2) males +/+=9, +/−=17, −/−=10; females +/+=6, +/−=13, −/−=9.

The body weight of mice in Cohorts 1 and 2 was recorded ~every 4 weeks from 16 – 32 and 20 – 32 weeks, respectively. The number of mice in Cohort 1 were: male +/+=7, +/−=10, −/−=9, female +/+=14, +/−=12, −/−=9; the total number of mice in Cohort 2 were: male +/+=9, +/−=6, −/−=9, females +/+=6, +/−=6, −/−=8.

1.9. Trace cued and contextual fear conditioning

Trace cued and contextual fear conditioning was conducted as previously described (Kinney et al., 2002; Wrenn et al., 2004), with slight modifications. Mice (N=10 +/+, 10 +/−, and 12 −/−) were trained and scored for freezing behavior to the same environmental context in a clear Plexiglas chamber (26×26×18) with a metal rod floor for foot shock delivery (Freeze Monitor, San Diego Instruments, San Diego, CA). A Dell Optiplex computer connected to the shock generator delivered the unconditioned foot shock stimulus (0.5 mA, AC current, 1 s duration). The conditioned auditory stimulus (80 dB) was provided by a white noise generator (Research Services Branch, NIH/NIMH) using a toggle switch manual control consisting of 30 s presentations, which preceded the foot shock by 2.5 s in this trace fear conditioning procedure. Timing of auditory cue presentation and foot shock delivery were coordinated through San Diego Instruments software.

The novel context chamber used for scoring cued fear conditioning consisted of a white plastic triangular shaped chamber (36×36×51 cm) with 26 cm high walls and a solid floor. A novel odor (diluted McCormick vanilla extract) was spread with a cotton-tipped applicator on one sidewall insert prior to the start of each test subject. Freezing was manually scored as complete absence of movement except respiration, at 10 s intervals, during the scoring periods on each day of training and testing. On the training day, mice were brought individually to the testing room, placed into the conditioning chamber, and presented with four pairings of auditory white noise (CS) and foot shock (US). CS–US pairings were preceded and followed by 2 min exploration periods. Freezing was scored every 10 s during the initial 2 min exploration period prior to CS–US pairings and during the final 2 min after (4) CS–US pairings by a highly trained observer.

Twenty-four hours after training, mice were brought individually to the original test room and returned to the training chamber (same context), with the test room environment identical to the training day, for the contextual test. Mice were placed in the chamber and allowed to explore for 5 min in the absence of the auditory cue and foot shock. Freezing behavior was scored every 10 s over the 5 min test period.

Forty-eight hours post-training, mice were placed in clean cages, brought to a different test room, and placed in the triangular chamber (novel context) for cued context testing. The session consisted of a 3 min exploration period followed by 3 min of 80 dB white noise. At the termination of the auditory stimulus a trained observer scored freezing behavior every 10 s for an additional 90 s.

1.10. Morris water maze spatial navigation

Spatial learning and memory were assessed in the Morris water maze using established procedures and equipment (Holmes et al., 2001; Wrenn et al., 2004). Order of training was: visible platform trials, hidden platform trials and a final probe trial with the platform removed.

Testing was conducted in a circular pool (120 cm diameter) filled 45 cm deep with tap water rendered opaque with the addition of non-toxic white (Crayola) paint. Trials were videotaped and scored with Actimetrics video tracking software (Actimetrics, Inc. Wilmette, IL). Visible and hidden platform training consisted of four trials per session, with the mouse starting facing the pool edge, in a new quadrant on each trial. During visible training, the platform was moved to a new quadrant location on each trial. During hidden platform training, the platform remained in the same quadrant for all trials across all sessions. Trials lasted for 60 s. If a mouse did not successfully locate the platform by the completion of the trial, it was guided to the platform by the experimenter. Mice remained on the platform for 15 s before being placed under a warming light for the 30–45 s intertrial interval.

Three days of visible platform training preceded seven days of hidden platform training. Mice were tested on the probe trial 3 h after completing hidden platform testing on day seven. Parameters recorded included latency to reach the platform, swim speed, and total distance traveled. Probe trial selective quadrant search was assessed by time spent in each quadrant and the number of crossings over the trained quadrant platform location compared to the analogous locations in the non-trained quadrants. Total number of mice tested in the Morris water maze were 22 +/+, 20 +/− and 18 −/−.

2. Statistical analyses

Most behavioral results were analyzed using a between-subjects design Analysis of Variance (ANOVA). Significant overall ANOVAs were further analyzed using appropriate post hoc tests (StatView, SAS Institute Inc., Cary, NC). Comparisons of within-subject effects (i.e. longitudinal measures of body weight, water maze probe trial quadrant preference) were analyzed using a

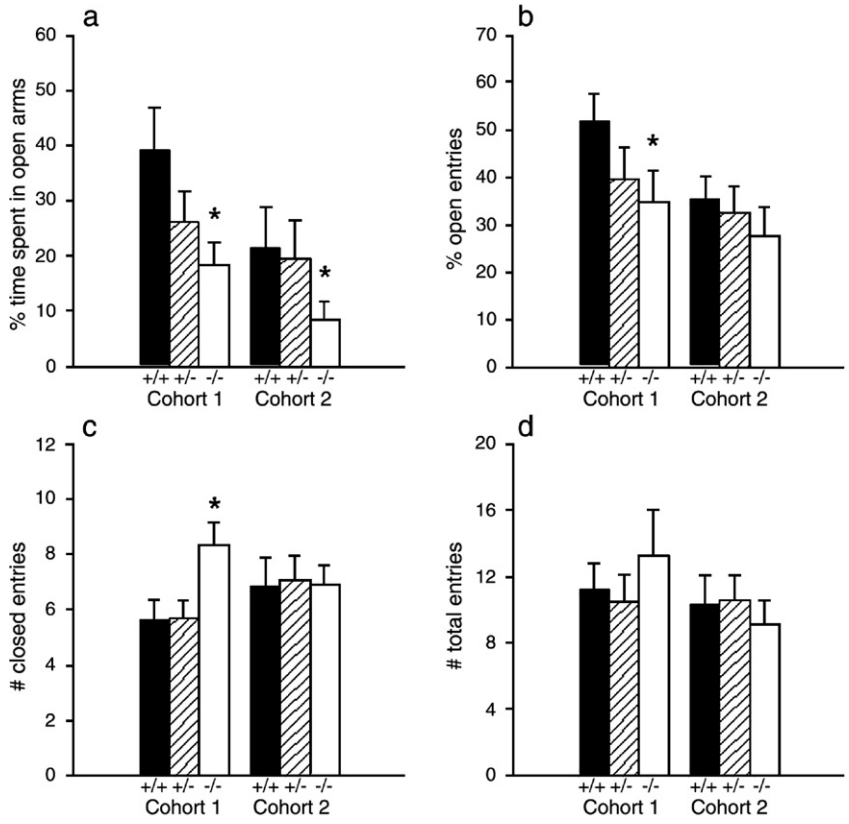

Fig. 1. Anxiogenic-like phenotype of GalR2 on the elevated plus-maze. Two independent cohorts of GalR2 −/− displayed an anxiogenic-like phenotype compared to their +/+ littermates in the elevated plus-maze. GalR2 −/− mice spent significantly * less time in the open arms (a) and made fewer entries into the open arms (b) than +/+ mice. The −/− mice in experiment 1 made significantly * more entries into the closed arms (c), while total arm entries was similar across genotypes (d) suggesting that less exploration of open arms did not reflect lower overall exploratory behavior. The genotypes did not differ on total arm entries (c). For the figures in graphs a–d Cohort 1 N=22 +/+, 23 +/−, 19 −/−; Cohort 2 N=14 +/+, 12 +/−, 17 −/−. *p<.05 as compared to +/+.

repeated measures ANOVA design. Stress-induced hyperthermia test scores were analyzed for each group using paired T-tests.

3. Results

3.1. Elevated plus-maze

As shown in Fig. 1a, GalR2 −/− mice of both Cohort 1 and Cohort 2 spent significantly less time in the open arms [Cohort 1: $F(2,61)=4.575$, $p=.0141$. Fisher's PLSD post hoc analysis $p<.05$ +/+ vs. −/−; Cohort 2: $F(2,40)=3.254$, $p=.0490$. Fisher's PLSD post hoc analysis +/+ vs. −/− $p<.05$]. Cohort 1 −/− mice made fewer open arm entries than +/+ mice [$F(2,61)=3.660$, $p=.0315$, post hoc analysis $p<.05$ +/+ vs. +/− and −/−]. A trend toward fewer open arm entries was seen in Cohort 2 but this did not reach statistical significance (Fig. 1b). Cohort 1 GalR2 −/− mice made significantly more closed arm entries [$F(2,61)=4.661$, $p=.0131$, post hoc analysis $p<.05$ −/− vs. +/+ and +/−], indicating that the reduced open arm entries were not caused by low general exploration (Fig. 1c). In addition, females in Cohort 1 made significantly fewer total arm entries than males [$F(1,58)=4.657$, $p=.0353$ (not shown)]. There was no significant effect of genotype on total entries [$F(2,58)=1.502$, $p=.2313$ and $F(2,40)=.501$, $p=.6096$] in either Cohort (Fig. 1d).

3.2. Light ↔ dark exploration test

As shown in Fig. 2a and b, there was no significant genotype effect on the number of transitions between the light and dark chambers, [$F(2,56)=1.225$, $p=.3014$], or the amount of time spent in the dark chamber [$F(2,56)=1.318$, $p=.2759$] in the light ↔ dark exploration test.

3.3. Elevated zero-maze

Mice that never moved from the original closed quadrant (5 +/+, 3 +/−, 6 −/−) were excluded from the final analysis. There were no genotype differences on time spent in the open quadrants (mean+standard error of the mean) +/+=16.55±3.80; +/−=14.81±3.80; −/−=10.87±2.79 [$F(2,29)=0.697$, $p=.5064$], or on the total number of transitions between open and closed quadrants +/+=19.80±4.50; +/−=16.18±3.92; −/−=13.46±3.34 [$F(2,29)=0.647$, $p=.5308$].

3.4. Stress-induced hyperthermia

GalR2 +/+, +/−, and −/− mice were tested for differences in physiological responding as measured by a change in body temperature resulting from handling and temperature measurement. All genotypes demonstrated a significant increase in body

temperature (Fig. 2f) between the basal temperature reading and a subsequent reading 10 min later (paired T test all p values $< .05$). However, there were no significant differences between the genotypes in the amount of change [$F(2,30) = .728$, $p = .4911$]. GalR2 −/− mice demonstrated a similar physiological response to +/+ littermates on this measure of mild stress.

3.5. Open field activity

GalR2 +/+, +/− and −/− mice were assessed for exploratory activity and anxiety-like behavior in the open field test. No genotype differences were detected on vertical [$F(2,110) = .881$, $p = .42$ (not shown)] or horizontal activity [$F(2,109) = 2.704$, $p = .0714$, Fig. 2d]. GalR2 −/− displayed an anxiogenic-like phenotype on center time exploration that did not reach statistical significance [$F(2,112) = 1.827$, $p = .17$, Fig. 2e]. There was a significant effect of sex on total distance [$F(2,109) = 11.642$, $p = .0009$, Fig. 2c,d] and horizontal activity $F(2,109) = 8.562$, $p = .0042$ (females were less active overall). For total distance (Fig. 2c), sex and genotype interacted, +/+ and −/− males traveled significantly greater distances than females while heterozygous males and females had similar total distance scores. The single genotype × sex interaction, on total distance only, and not related to gene-dose, suggests that open field activity is generally not affected by the mutation.

3.6. General health and neurological reflexes

GalR2 −/−, GalR2 +/− and GalR2 +/+ were examined on measures of general health, reflexes and sensory function, as

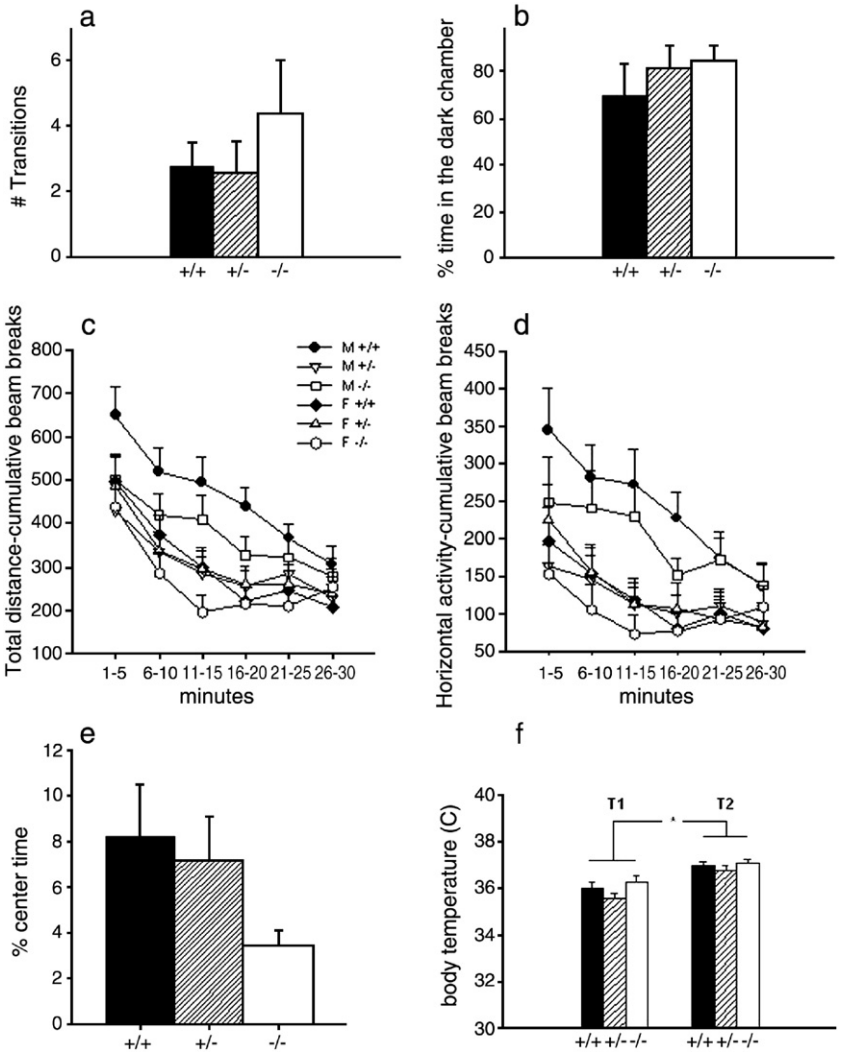

Fig. 2. Light ↔ dark exploration, open field exploration and stress-induced hyperthermia. The genotypes did not differ significantly on the number of transitions between the light and dark compartments (a) or in the percentage of time spent in the dark compartment (b) demonstrating similar responding across the genotypes in the light ↔ dark exploration test. For Fig. 2a,b N=+/+=22, +/−=23 and −/−=17. There was no significant effect of genotype on horizontal activity (c) or total distance traveled (d) in the open field exploration test. There was a significant effect of sex on both of these measures indicating that females traveled significantly shorter distances than males. GalR2 −/− mice showed decreased center exploration time (e) consistent with an anxiogenic-like phenotype that did not reach statistical significance ($p > .05$) N=+/+=38, +/−=41, −/−=36. There was no significant effect of genotype on the increase in body temperature in the second rectal probe measurement as compared to the baseline measurement (f). Handling and prior temperature probe measurement significantly increased the body temperature of all genotypes (*$p < .05$). N=+/+=10, +/−=10, −/−=13.

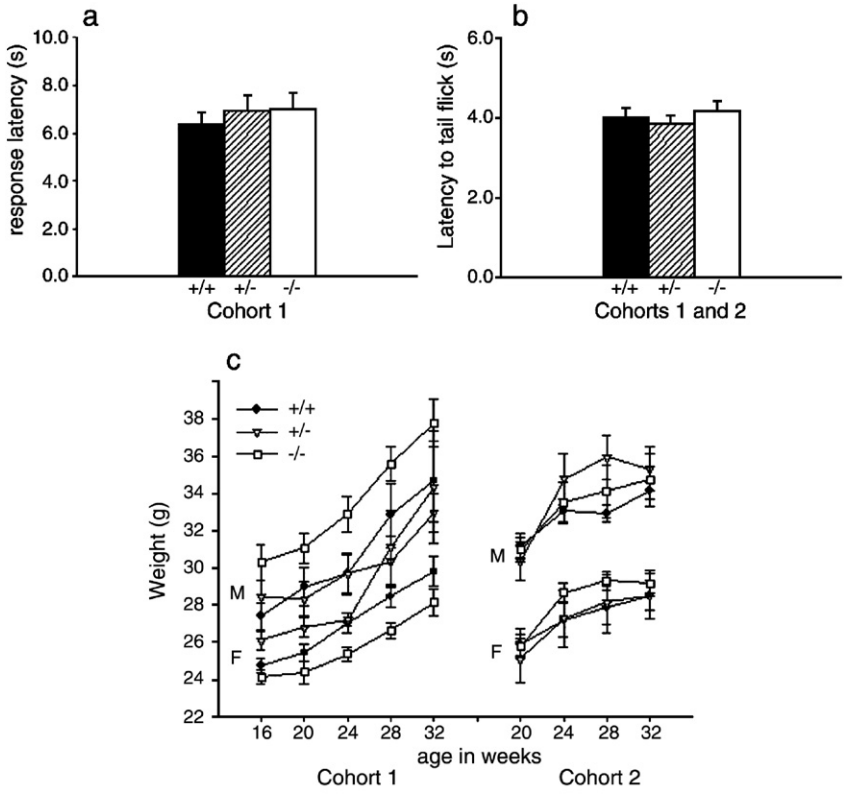

Fig. 3. Hotplate, tail flick, and body weight. There was no significant genotype difference in latency to respond on the hotplate test (a) Cohort 1 N=+/+=22, +/−=23, −/−=18. GalR2 −/− mice in Cohort 1 and Cohort 2 N=+/+=15, +/−=27, −/−=19 showed similar tail flick response latencies compared to +/+ controls (b). All genotypes increased in weight across time (c). GalR2 −/− males (Cohort 1) were significantly heavier than +/− mice at 28 and 32 weeks of age.GalR2 +/− females (Cohort 1) were significantly heavier than −/− at all ages and significantly heavier than +/+ littermates at 16 and 32 weeks. No significant differences in body weights were detected in Cohort 2 mice.

shown in Table 1. The genotypes did not differ significantly on any measure with the exception of missing vibrissae. Gal −/− had significantly fewer missing vibrissae than wildtype mice [χ^2=9.107, p=.0105].

3.7. Body weight

As shown in Fig. 3c, all mice gained weight across time. In Cohort 1 males there was a significant effect of genotype [F(2,22)=3.681, p=.0418] and age [F(4,88)=66.517, p<.0001] and a significant interaction [F(8,88)=2.468, p=.0184]. One way ANOVAs at each time point showed that the GalR2 −/− male mice of Cohort 1 weighed significantly more than +/− littermates at 28 and 32 weeks of age. There was a significant interaction of age and genotype in Cohort 1 female mice. One way ANOVAs at each time point showed that GalR2 +/− females were significantly heavier than GalR2 −/− at all measurement points and significantly heavier than +/+ mice at 16 and 32 weeks. In addition, +/+ females were significantly heavier than −/− mice at 24 weeks. In Cohort 2 males, there was a significant effect of age [F(3,36)=37.511, p<.0001] that interacted with genotype [F(6,36)=2.472, p=.0418]. One way ANOVAs showed no significant differences at any time point between the genotypes. There were no genotype differences and no interaction with age in Cohort 2 female mice.

3.8. Hotplate and tail flick tests

As shown in Fig. 3a and b, there was no significant effect of genotype on latency to respond to a painful thermal stimulus during testing on the hotplate [F(2,56)=.161, p=.6901] or tail flick tests [F(2,121)=.466, p=.6283].

3.9. Rotorod

As shown in Table 1, there was no significant effect of genotype on latency to fall from the rotorod in Cohort 1 [F(2,57)=.460, p=.6338], this finding was replicated in Cohort 2 [F(2, 58)=1.939, p=.1530 (not shown)].

3.10. Trace cued contextual fear conditioning

As shown in Fig. 4a–c, there was no significant effect of genotype and no interaction of genotype and training (baseline versus post training) on freezing behavior (All F values were less than 1.0). All genotypes showed significantly more freezing during the final 2 min of the training session than in the initial 2 min prior to 4 tone (CS)+foot shock (US) pairings [F(1,29)= 311.82, p<.0001]. When returned to the same environmental context 24 h later, there were no significant genotype differences on freezing behavior to the training environment [F(2,29)=0.681, p=.5141]. In a novel context, 48 h post-

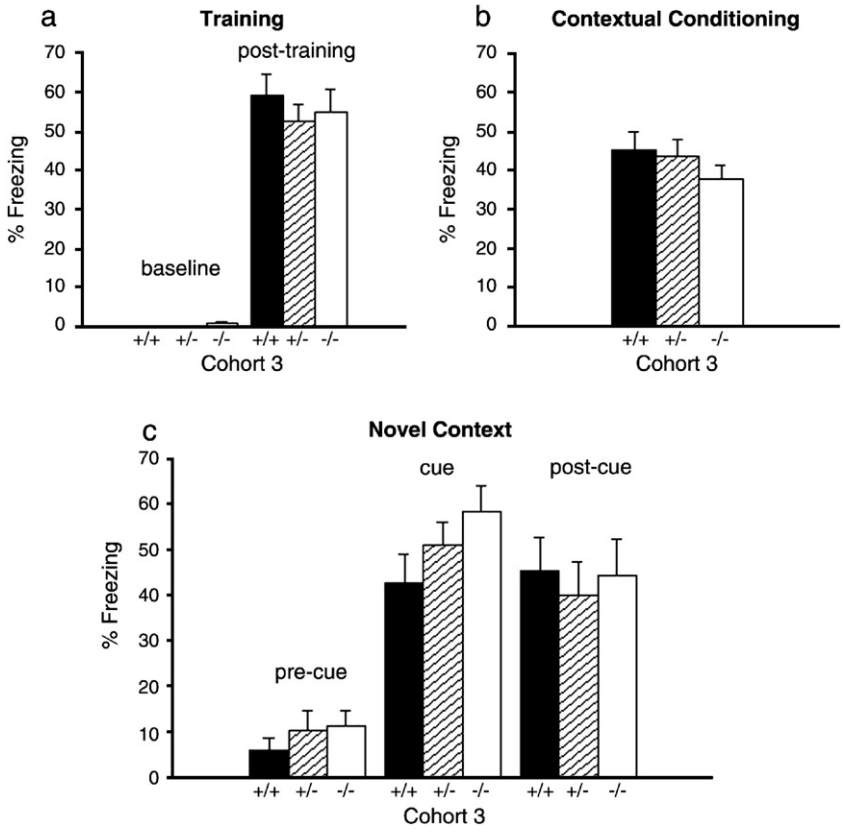

Fig. 4. Trace cued contextual fear conditioning. GalR2 −/ − mice showed similar levels of freezing as wildtype littermates in all phases of the trace cued fear conditioning test: Post training levels (a); contextual test 24 hours later (b) and during the novel context auditory cued test 48 h post training (c). N=+/+=10, +/−=10, −/−=12.

training there was a significant increase in freezing during the cue presentation for all genotypes compared with pre-cue freezing levels [$F(1,29)=216.535$, $p<.0001$]. The main effect of genotype and the interaction effect were not significant (all comparison p values>.05).

3.11. Morris water maze

There was no significant effect of genotype on spatial acquisition of a platform location in the Morris water maze (all comparison p values>0.05). As shown in Fig. 5a, GalR2 −/− mice performed as well as their +/+ and +/− littermates on visible and hidden trial acquisition. During probe trial testing (Fig. 5b, c), all genotypes spent significantly more time (all comparison p values<0.01) and made significantly more platform crosses (all comparison p values<0.0001) in the trained quadrant than all other quadrants.

4. Discussion

Comprehensive behavioral phenotyping of GalR2 null mutants, heterozygotes, and wildtype littermates in the present experiments detected no significant genotype differences on a wide range of behavioral tasks. On measures of general health, all mice demonstrated normal home cage activity, nest building, huddling, and grooming. There was an effect of genotype on the presence of vibrissae, GalR2 −/− had significantly more vibrissae than their wildtype littermates. This difference may indicate a higher level of social barbering in +/+ mice than −/− mice. There were no genotype differences in body or limb tone, or in corneal, pinnae and righting reflexes. Hotplate and tail flick results indicated no significant differences between genotypes on nociception. Sensory abilities were similar across genotypes on forepaw reaching, startle reactivity, and toe pinch response. Motor functions were in the normal range on trunk curl, rotorod, and open field activity. These results are consistent with previous findings in the GalR2 null mutant mice reporting normal performance on tests examining home cage behavior, feeding and body weight, motor control and balance, open field exploration and sensory and pain processing (Gottsch et al., 2005). In addition to these measures, Gottsch and colleagues (2005) found no difference between GalR2 null mutant mice and wildtype controls on seizure susceptibility, ethanol sensitivity, or prepulse inhibition of the acoustic startle response. Our present data and previous findings (Gottsch et al., 2005) in GalR2 knockout mice do not support a major modulatory role for the GalR2 receptor in baseline responsiveness to a brief painful thermal stimulus.

Significantly higher body weights were detected in GalR2 −/− males at older ages, and in GalR2 +/− females at younger ages, as compared to age and sex matched +/+ littermates, in Cohort 1. However, body weights were not significantly

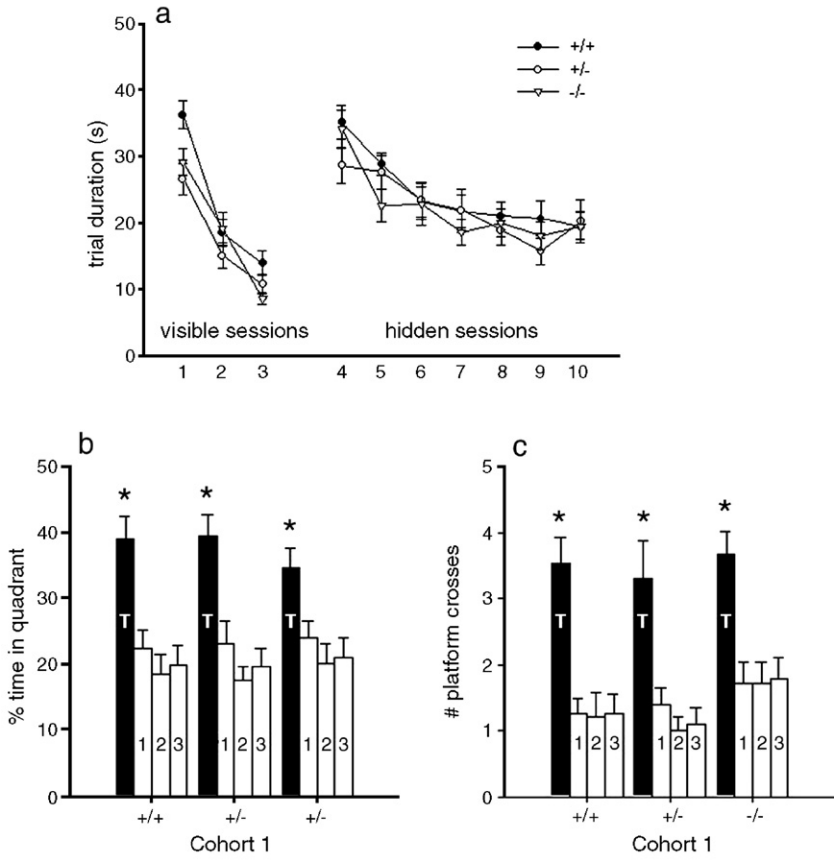

Fig. 5. Morris water maze. Latency to locate the visible and hidden platforms during Morris water maze acquisition was similar for all genotypes (a). All genotypes demonstrated spatial learning during the probe trial by spending significantly more time in the trained quadrant (b). All genotypes made significantly more platform crossings in the trained quadrant (c) than in the other three quadrants. N=+/+=22, +/−=20, −/−=18.

different across genotypes in Cohort 2. Absence of replication of body weight differences in two independent GalR2 cohorts, and the lack of consistent genotype effects in both sexes in Cohort 1, limit the interpretation of the small effects seen.

If the GalR2 receptor subtype is necessary for the detrimental effects of galanin on cognitive function, we predicted that GalR2 null mutants would display enhanced performance levels on these learning and memory tasks. Task designs in the present experiments incorporated sufficient difficulty to allow detection of an increase in performance for both the Morris water maze probe trial and trace fear conditioning. Results from the current study indicate normal freezing scores in GalR2 null mutants, heterozygotes, and wildtype controls on trace cued contextual fear conditioning, and normal acquisition and probe trial performance in the water maze. These findings are consistent with a previous report of normal fear conditioning in GalR2 knockouts (Gottsch et al., 2005), indicating that the deleterious effects of excess galanin on cognitive performance may act through the GalR1 or GalR3 receptor subtypes. However, two alternative explanations for the negative findings are useful to consider. 1) The loss of the GalR2 receptor throughout development may trigger compensatory changes in systems that minimize the functional significance of the deficient receptor, e.g. other galanin receptor subtypes, cholinergic receptors, glutamatergic receptors, and the synthesis or release of galanin and other neurotransmitters. It has yet to be determined in GalR2 null mutant mice if GalR1 or GalR3 receptor levels or galanin peptide levels are altered. Jacoby et al. (2002) demonstrated normal GalR2 receptor levels in GalR1 null mutant mice, suggesting that functional loss of one receptor may not trigger compensatory changes in other receptor levels. In contrast, mice chronically overexpressing galanin (Gal-tg) display increased GalR1 mRNA levels in the CA1 region of the hippocampus while levels of GalR2 and GalR3 mRNA were unchanged (He et al., 2005). One way to further test for compensatory interactions between GalR1 and GalR2 is to generate double knockouts deficient in both of these receptor subtypes. 2) Release of endogenous galanin during task performance may fail to reach levels necessary to sufficiently activate GalR2 receptors, resulting in similar performance of wildtype and GalR2 null mutant mice. It may be necessary to challenge GalR2 null mutant mice with pharmacological doses of exogenously administered galanin, before completely ruling out a role for the GalR2 subtype in mediating the inhibitory actions of galanin on cognitive functions.

The main positive finding in the present study is an anxiety-like phenotype of the GalR2 null mutants on the elevated plus-maze. An anxiolytic action of exogenously administered galanin has been reported in some, but not all, anxiogenic and stress-related paradigms in rats and mice (Bartfai et al., 2004; Bing et al.,

1993; Echevarria et al., 2005; Karlsson et al., 2005; Khoshbouei et al., 2002a,b; Lu et al., 2005; Möller et al., 1999; Ukai et al., 1995). Our comprehensive analysis of anxiety-like behaviors using multiple tasks, in several independent groups of GalR2 null mutant, heterozygote, and wildtype littermates, both males and females, revealed an anxiogenic-like phenotype compared with wildtype controls on the elevated plus-maze, but not on the elevated zero-maze, light ↔ dark exploration, stress-induced hyperthermia, or open field center time. In two independent GalR2 cohorts, the null mutants displayed lower percentages of time spent on the open arms of the elevated plus-maze, while total arm entries showed no genotype differences. Lack of genotype differences on stress-induced hyperthermia was similarly reported by Gottsch and coworkers (2005) in GalR2 null mutant mice.

It is interesting to speculate on the unique properties of the elevated plus-maze in detecting phenotypic differences in galanin receptor knockout mice. Similar specificity for the elevated plus-maze was discovered in GalR1 mutant mice (Holmes et al., 2003). GalR1 null mutants spent less time in the open arms and made fewer entries in the open arms than controls, but did not differ on total entries or closed arm entries (Holmes et al., 2003). Plasma levels of corticosterone and ACTH were measured in GalR1 mice after performance of the elevated plus-maze, light ↔ dark, and emergence tests. The highest levels of these stress hormones were seen after the elevated plus-maze task. In addition, a factor analysis of these anxiety-related tasks in GalR1 mice indicated a unique factor on the elevated plus-maze task as compared to open field, emergence, and light ↔ dark exploration (Holmes et al., 2003). Our similar findings of an anxiety-like phenotype in GalR2 deficient mice on the elevated plus-maze may indicate that the unique approach/avoidance demands of this test activate a quantitatively or qualitatively different stress-induced galanin response compared to other conflict paradigms.

A secondary issue that can confound the interpretation of a behavioral phenotype is the contribution of the background strain on which the mutation was bred. The GalR2 mutation was bred on a 129S1/SvImJ background. Behavioral studies with this background suggest relatively low levels of locomotor activity in open field exploration (Bolivar et al., 2000) and slower swim speeds in the Morris water maze (Clapcote and Roder, 2004). In the present experiments, lower levels of ambulatory activity were consistently observed for all genotypes in open field exploration, light ↔ dark test, elevated zero-maze, and swim speed in the Morris water maze as compared to C57BL/6J. Failure to detect an anxiety-like phenotype in GalR2 −/− in the elevated zero-maze may be an artifact of the maze design combined with reduced exploratory behavior displayed by this GalR2 mutation generated on a 129S1/SvImJ background. However, the GalR1 mutation was bred on a B6 background, and GalR1 null mutants displayed a similar deficit on elevated plus-maze performance, failing to support the 129S1/SvImj background strain as the critical factor in the GalR2 phenotype. Lastly, as described above, putative compensatory mechanisms and insufficient release of endogenous galanin could explain the lack of genotype differences on anxiety-related tasks other than the elevated plus-maze.

Recent development of the small molecule GalR3 specific antagonists SNAP 37889 and SNAP 398299 contribute persuasive evidence of a modulatory role for galanin in anxiety-like and depression-related behavior (Barr et al., 2006; Swanson et al., 2005). Acute treatment in rats, mice, and guinea pigs produced anxiolytic-like activity in multiple tests designed to measure anxiety-like behavior. Chronic administration of these compounds produced antidepressant-like effects, as seen in increased social interaction time in the social interaction test and increased swim time and decreased immobility in the forced swim paradigm (Swanson et al., 2005). Since blocking the GalR3 receptor produced anxiolytic-like and antidepressant-related actions in these tasks, the interpretation would be that endogenous galanin increases anxiety-related and depression-related behaviors when acting at the GalR3 subtype. The neural circuitry mediating the putative opposite effects of galanin at the GalR3 versus the GalR1 and GalR2 receptors remains to be determined.

In summary, the present experiments indicate a highly selective anxiogenic-like phenotype of GalR2 null mutant mice on the elevated plus-maze. The present findings replicate and extend the scope of the GalR2 behavioral phenotypes (Gottsch et al., 2005) by showing normal pain sensitivity, relatively normal body weights across ages, normal learning and memory in trace cued and contextual fear conditioning and normal spatial learning and memory in the Morris water maze. The present findings, taken together with the recent GalR3 antagonist report (Swanson et al., 2005), suggest that targeting galanin receptors may provide a unique therapeutic approach to treating anxiety disorders.

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