

# Learning and memory performance in mice lacking the GAL-R1 subtype of galanin receptor

Craige C. Wrenn,<sup>1</sup> Jefferson W. Kinney,<sup>1</sup> Lisa K. Marriott,<sup>2</sup> Andrew Holmes,<sup>1</sup> Ashley P. Harris,<sup>1</sup> Maria C. Saavedra,<sup>1</sup> Grzegorz Starosta,<sup>1</sup> Caitlin E. Innerfield,<sup>1</sup> Arie S. Jacoby,<sup>3</sup> John Shine,<sup>3</sup> Tiina P. Iismaa,<sup>3</sup> Gary L. Wenk<sup>2</sup> and Jacqueline N. Crawley<sup>1</sup>

<sup>1</sup>Laboratory of Behavioural Neuroscience, National Institute of Mental Health, Bethesda, MD 20892–1375, USA

<sup>2</sup>Division of Neural Systems, Memory and Ageing, University of Arizona, Tucson, AZ, USA

<sup>3</sup>The Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia

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## Abstract

The neuropeptide galanin induces performance deficits in a wide range of cognitive tasks in rodents. Three G-protein-coupled galanin receptor subtypes, designated GAL-R1, GAL-R2 and GAL-R3, have been cloned. The present study examined the role of GAL-R1 in cognition by testing mice with a null mutation in *Galr1* on several different types of learning and memory tasks. Assessments of general health, neurological reflexes, sensory abilities and motor functions were conducted as control measures. Mutant mice were unimpaired in social transmission of food preference and the Morris water maze. In tests of fear conditioning, mutant mice were unimpaired in a delay version of cued fear conditioning. However, mice homozygous for the null mutation were impaired in a trace version of cued fear conditioning. Mutant mice were unimpaired in contextual fear conditioning, whether training was by the delay or trace protocol. General health, neurological reflexes, sensory abilities and motor functions did not differ across genotypes, indicating that the trace fear conditioning deficit was not an artifact of procedural disabilities. The findings of normal performance on several cognitive tasks and a selective deficit in trace cued fear conditioning in homozygous GAL-R1 mutant mice are discussed in terms of hypothesized roles of the GAL-R1 subtype. The generally normal phenotype of GAL-R1 null mutants supports the use of this line for identification of the receptor subtypes that mediate the cognitive deficits produced by exogenous galanin.

## Introduction

Galanin is a 29 amino acid neuropeptide that is widely distributed in the central nervous system (Tatemoto *et al.*, 1983; Hökfelt *et al.*, 1998). Galanin modulates many physiological functions, including gastrointestinal motility (Guerrini *et al.*, 2000), pituitary hormone release (Hohmann *et al.*, 1998), neurotrophic actions (Wynick *et al.*, 1999; Holmes *et al.*, 2000; Mahoney *et al.*, 2003), nociception (Liu & Hökfelt, 2000, 2002), anxiety-like behaviour (Holmes *et al.*, 2002b), feeding (Kyrkouli *et al.*, 1986; Crawley *et al.*, 1990; Kyrkouli *et al.*, 1990; Corwin *et al.*, 1993; Crawley, 1999) and cognition (Robinson & Crawley, 1993; Steiner *et al.*, 2001; Wrenn & Crawley, 2001).

The effects of galanin on cognition are of particular interest because of the overexpression of galanin observed in the basal forebrain of Alzheimer's disease (AD) patients (Chan-Palay, 1988; Beal *et al.*, 1990; Bowser *et al.*, 1997). A number of studies in the rat have shown that central injection of galanin produces performance deficits in working memory, fear conditioning and spatial tasks (Mastroianni *et al.*, 1988; Malin *et al.*, 1992; Robinson & Crawley, 1993; McDonald & Crawley, 1996; Ögren *et al.*, 1996; Stefani & Gold, 1998). This consistent body of literature has led to the hypothesis that endogenous galanin overexpression contributes to the cognitive dysfunction that is

characteristic of AD (Hökfelt *et al.*, 1987; Counts *et al.*, 2001; Wrenn & Crawley, 2001). Further support of this hypothesis was contributed by our laboratory's discovery that transgenic mice that overexpress galanin are impaired in cognitive tasks, including the Morris water maze probe trial, social transmission of food preference and trace cued fear conditioning (Steiner *et al.*, 2001; Kinney *et al.*, 2002; Wrenn *et al.*, 2003).

The many effects of galanin, including those on cognition, are mediated by three cloned receptor subtypes designated GAL-R1, GAL-R2 and GAL-R3 (Habert-Ortoli *et al.*, 1994; Burgevin *et al.*, 1995; Fathi *et al.*, 1997; Howard *et al.*, 1997; Wang *et al.*, 1997a, b, c; Bloomquist *et al.*, 1998; Pang *et al.*, 1998; Smith *et al.*, 1998). Studies of mRNA expression have shown that each of the galanin receptors is expressed in regions of the brain critically involved in cognitive function such as the cortex, hippocampus, amygdala and cholinergic basal forebrain (Fathi *et al.*, 1997, 1998; O'Donnell *et al.*, 1999; Waters & Krause, 2000; Mennicken *et al.*, 2002).

The determination of which galanin receptor subtypes mediate which effects of galanin has been hampered by the lack of readily available, subtype-specific galaninergic drugs. An alternative approach to the investigation of receptor subtype function is the characterization of mutant mice having a null mutation of the receptor of interest. In the current study we have adopted the approach of generating a mouse line having a null mutation of the GAL-R1 gene (GAL-R1 KO mice) with the purpose of elucidating the cognitive functions of this galanin receptor subtype. Previous studies of the

Correspondence: Dr C. C. Wrenn, as above.  
E-mail: wrennc@intra.nimh.nih.gov

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GAL-R1 KO mice have shown that the mice are viable, and that they breed, nurse and grow normally (Jacoby *et al.*, 2002). Observed differences in GAL-R1 KO mice compared to wild-type littermate controls (+/+) included reduced levels of circulating insulin-like growth factor (Jacoby *et al.*, 2002), increased tonic-clonic spontaneous seizures (Jacoby *et al.*, 2002) and increased anxiety-like behaviour in the elevated plus-maze (Holmes *et al.*, 2003). Given the role of galanin in learning and memory, the purpose of the present study was to evaluate the cognitive abilities of GAL-R1 KO mice using different types of learning and memory tasks in conjunction with the appropriate control tasks and measurements.

## Methods

### Mice

Mice with a null mutation of the gene coding for the GAL-R1 subtype of galanin receptor were generated at the Garvan Institute, Sydney Australia as previously described (Jacoby *et al.*, 2002). Briefly, the targeting vector was comprised of a [*SpeI* + *KpnI*] 780 bp fragment of *Galr1* that contained the majority of exon 1 and some 5' sequence, a PGK-neo<sup>r</sup> selection cassette inserted in reverse orientation from *Galr1* transcription and a [*KpnI* + *SpeI*] 3.7 kb fragment that extended from the 3' end of exon 1 into intron 1 of *Galr1*. This construct was injected into W9.5 embryonic stem cells. Embryonic stem cells positive for the null mutation were injected into C57BL/6J blastocysts. In order to avoid complications of a genetically heterogeneous stock, heterozygous mutant mice were backcrossed into the C57BL/6J line for five generations at the Garvan Institute. The line was then rederived at the Jackson Laboratory (Bar Harbor, ME) and backcrossed into C57BL/6J for two generations. Genotype was determined by polymerase chain reaction analysis at the Jackson Laboratory, and identification of the mice was by subcutaneously implanted microchip and scanner. Identified mice were transported postweaning to NIMH, Bethesda, MD. Wild-type (+/+), heterozygous (+/-) and homozygous (-/-) littermates were group housed by sex in the NIH vivarium under controlled temperature, humidity, and a 12 h light : 12 h dark cycle, with lights on between 6 : 00 am and 6 : 00 pm. Food and water were available *ad lib.*, except where otherwise indicated. In experiments that included both sexes, the number of male and female subjects of each genotype was approximately equal.

The present experiments were conducted with three separate shipments of mice, designated Cohorts 1, 2 and 3. All behavioural experiments were performed between 9 : 00 am and 5 : 00 pm. Behavioural analyses of Cohorts 1 and 2 were conducted using a multi-tiered approach as previously described (Crawley & Paylor, 1997; Crawley *et al.*, 1997). Behavioural analyses included assessments of general health, neurological reflexes, motor functions, sensory abilities, anxiety-like behaviour and cognitive performance. The general health, neurological, motor and sensory results from Cohort 1 and the anxiety-like behaviour of Cohorts 1 and 2 are described in a separate report (Holmes *et al.*, 2003).

The present report describes the general health, neurological, motor and sensory function of Cohort 2 and the cognitive performance of Cohorts 1 and 2. Cohort 3 was used only for an additional cognitive experiment, delay cued and contextual fear conditioning. A separate cohort was used for this experiment to avoid complications of conducting both delay and trace fear conditioning in the same animals. Methods for the behavioural tests are given in the order in which they were performed. Experimenters were uninformed of genotype during all experiments. All procedures were approved by the National Institute of Mental Health Animal Care and Use Committee, and followed the NIH Guidelines 'Using Animals in Intramural Research'.

### Behavioural tests

#### General health and neurological reflexes

Measures of general health and neurological reflexes were assessed in Cohort 2 using methods as previously described (Crawley & Paylor, 1997; Crawley *et al.*, 1997). Briefly, general health was determined by assessment of fur condition, whisker condition, body and limb tone and observation of home cage behaviour. Neurological reflexes assessed were trunk curl, forepaw reaching, eye-blink, pinna twitch, vibrissae response, toe pinch response and the righting reflex. Behavioural reactivity was assessed by attempted escape during petting, struggling and/or vocalization during handling and biting of a wooden dowel. Empty cage behaviour was assessed by placing each subject in a clean, empty cage for a period of 3 min, and noting the occurrence of freezing, wild running, stereotypies, exploration and grooming.

#### Body weight

The body weights of Cohort 1 and Cohort 2 were recorded longitudinally. Cohort 1 was weighed every 2 weeks from the ages of 9–15 weeks to the ages of 17–23 weeks. Cohort 2 was weighed every 8 weeks from the ages of 12–16 weeks to the ages of 36–40 weeks. In Cohort 1, the number of subjects per group was: male +/+,  $n = 19$ ; female +/+,  $n = 11$ ; male +/-,  $n = 25$ ; female +/-,  $n = 24$ ; male -/-,  $n = 15$ ; female -/-,  $n = 17$ . In Cohort 2, the number of subjects per group was: male +/+,  $n = 19$ ; female +/+,  $n = 20$ ; male +/-,  $n = 19$ ; female +/-,  $n = 21$ ; male -/-,  $n = 17$ ; female -/-,  $n = 15$ .

#### Wire hang

The wire hang test of limb strength was performed in Cohort 2 by placing the mouse on a wire cage lid and then inverting the lid for a maximum of 60 s. The latency of the mouse to lose its grip and fall from the cage lid to a padded surface served as the measure of limb strength. The total number of subjects per group was:  $n = 46$  (+/+);  $n = 66$  (+/-);  $n = 38$  (-/-).

#### Rotarod

The rotarod test of motor coordination and motor learning was performed in Cohort 2 once a day for three consecutive days by placing each mouse on a rotating drum (Ugo Basile, Stoelting, Wood Dale, IL) that accelerated from 4 to 40 r.p.m. over a 5-min period. The latency to fall from the drum was the measure of motor coordination. Improvement across trials was the measure of motor learning. The total number of subjects per group was:  $n = 20$  (+/+);  $n = 24$  (+/-);  $n = 21$  (-/-).

#### Open field activity

The spontaneous motor activity of Cohort 2 was assessed in an automated open field as previously described (Holmes *et al.*, 2001). The open field was a square arena (40 × 40 × 35 cm) with clear Plexiglas walls and floor, evenly illuminated by white light. Each subject was placed in the centre of the open field and allowed to explore for 30 min. Activity was measured by a computer-assisted Digiscan optical animal activity system (RXYZCM, Omnitech Electronics, Accuscan, Columbus, OH). Eight photocell beams were located on each of two sides of the arena, at right angles to one another, forming a grid of 64 equally sized squares. A third set of eight photocell beams was located above the square grid to detect vertical movements. The numbers of horizontal and vertical beam breaks were taken as measures of horizontal and vertical activity, respectively. The total number of subjects per group was:  $n = 25$  (+/+);  $n = 33$  (+/-);  $n = 23$  (-/-).

### Tail flick

Nociception was assessed in Cohort 2 by measuring the latency to flick the tail away from a heat stimulus. The subject was held in place by hand on the tail flick apparatus (Model number 0570–001 L Columbus Instruments, Columbus, OH) with the tail overlying an aperture that contained a heat source. Heat was applied to the tail until the subject flicked the tail away or 10 s elapsed, whichever came first. The latency to flick the tail was timed using a stopwatch. A heat intensity setting of 10 (arbitrary units) was used. The total number of subjects per group was:  $n = 25$  (+/+);  $n = 30$  (+/-);  $n = 26$  (-/-).

### Hot plate

For the hot plate test, each subject from Cohort 2 was placed on a hot plate (ITTC Inc., Woodland Hills, CA) heated to 55 °C. The latency to either shake or lick the hind paw was timed with a stopwatch. To prevent tissue damage there was a maximum cutoff latency of 30 s. The total number of subjects per group was:  $n = 25$  (+/+);  $n = 30$  (+/-);  $n = 25$  (-/-).

### Acoustic startle and prepulse inhibition of acoustic startle

Acoustic startle and prepulse inhibition of acoustic startle of Cohort 2 were measured in separate experiments using the SR-Laboratory System (San Diego Instruments, San Diego, CA) as described previously (Paylor *et al.*, 1998; Holmes *et al.*, 2001). Test sessions began by placing each mouse in the Plexiglas holding cylinder for a 5 min acclimation period. For acoustic startle, mice were presented with four 120 dB bursts to measure startle reactivity and gross hearing ability. For prepulse inhibition of acoustic startle responses, mice were presented with each of seven trial types across six discrete blocks for a total of 42 trials, over a 10.5 min test session. One trial measured the response to no stimulus (i.e. baseline movement). One trial presented the startle stimulus alone (i.e. acoustic amplitude), which was a 40 ms 120 dB sound burst. The other 5 trials were acoustic prepulse + acoustic startle stimulus trials. Prepulse tones were 20 ms at 74, 78, 82, 86 and 90 dB, presented 100 ms before the startle stimulus. For prepulse inhibition experiments, the order in which trial types were presented was randomized within each trial-block. The interval between trials was 10–20 s. Startle amplitude was measured every 1 ms, over a 65 ms period beginning at the onset of the startle stimulus. The maximum startle amplitude over this sampling period was taken as the dependent variable. A background level of 70 dB white noise was maintained over the duration of the test sessions. The total number of subjects per group was:  $n = 20$  (+/+);  $n = 24$  (+/-);  $n = 21$  (-/-).

### Social transmission of food preference

Olfactory learning and memory was assessed in Cohorts 1 and 2 using the social transmission of food preference (STFP) task, as previously described (Steiner *et al.*, 2001; Holmes *et al.*, 2002a; Wrenn *et al.*, 2003). STFP experiments were conducted in three phases: habituation to powdered food, interaction between demonstrator and observer mice, and test of the food preference of the observer mice. The mice were habituated to eat powdered rodent chow (AIN-93 M, Dyets, Inc, Bethlehem, PA) from 4 oz. (~ 125 g) glass food jar assemblies (Dyets, Inc., Bethlehem, PA) that minimized digging or spilling of the food from the jar (see Wrenn *et al.* (2003) for full description). Habituation was performed in the home cage after removing the pellet chow for 18 h. Two weighed jars of powdered chow were placed in each cage for 1 h or until a minimum of 0.2 g of food per mouse was eaten.

For the interaction phase, a demonstrator mouse was chosen from each cage. The genotype of the demonstrator mice, with a few exceptions, was +/--. Demonstrators were housed singly in a room separate

from the colony room for 18 h with free access to water but not food. At this time, observer mice were placed in cages in which the interaction with the demonstrator would occur, and these cages were placed in a different test room. At the end of 18 h, each demonstrator was presented with a jar of 1% cinnamon (Safeway Inc., Pleasanton, CA) or 2% Dutch processed cocoa (Hershey Foods Corp., Hershey, PA). These concentrations of powdered flavoring mixed with powdered chow were previously found to be equipalatable in tests of innate flavor preference (Wrenn *et al.*, 2003). Half the demonstrators received cocoa-flavored food, and the other half received cinnamon-flavored food. The demonstrators were allowed to eat the flavored food for 1 h. The jars were weighed before and after presentation to the demonstrators. The criterion for inclusion in the experiment was consumption of  $\geq 0.2$  g. After eating the flavored food, each demonstrator was placed into the interaction cage containing its cage mate observer mice and allowed to interact with the observer mice for 30 min. During the interaction phase, the experimenter constantly watched the demonstrator, and recorded the number of times that each observer sniffed the muzzle of the demonstrator. A sniff was defined as close (<2 cm) orientation of the observer's nose towards the front or side of the demonstrator's muzzle. After the interaction period, the demonstrator mouse was removed from the interaction cage and returned to its individual cage.

In the final phase of the STFP experiments, the food preference of the observer mice was tested 24 h after the end of the interaction with the demonstrator. Eighteen hours before the preference test, observer mice were caged individually with free access to water, but not food, in the same room where the interaction was performed. The 1 h preference test consisted of presenting each observer with a pair of weighed food jars in the individual cage. One jar contained the flavor of food eaten by the demonstrator (cued); the other contained the novel flavor of the pair (novel). Thus, half the observers were tested with the cinnamon-flavored cued food eaten by their demonstrator vs. the novel cocoa-flavored food; the other half of the observers were tested with the cocoa-flavored cued food eaten by their demonstrator vs. the novel cinnamon-flavored food. The location of the jars in the test cages was balanced by placing the cued flavor jar in the front of the cage in half the cages and in the back of the cage in the other half. The novel flavor jar was placed in the opposite end of the cage. After 1 h, both food jars were removed and weighed to quantify the food preference of the observer mice. During the preference testing of the observers, the experimenter scored each observer mouse at 30-s intervals and recorded the number of eating bouts of each food. The total number of subjects, combining cohorts, was:  $n = 55$  (+/+);  $n = 37$  (+/-);  $n = 50$  (-/-).

### Buried food olfactory test

Olfaction was assessed in the +/+, +/- and -/- mice from Cohort 2 by measuring the latency to find a buried piece of food (Alberts & Galef, 1971; Harding & Wright, 1979; Stowers *et al.*, 2002). Mice were first habituated to eat butter-flavored cookies (Bud's Best Cookies, Inc., Hoover, AL) by placing pieces of the cookie in the home cages overnight. The next day, mice were food deprived for 22.5–24 h. The test was conducted in clean rat cages (47.5 × 25.8 × 21.0 cm) divided by tape into nine rectangles of equal area. One of the rectangles (1 × 1 × 0.5 cm) was randomly chosen as the area in which the piece of cookie would be buried. The food was buried by placing it in the centre of the rectangle and then covering the entire bottom of the rat cage with litter (wood shavings) at an even depth of 2.5 cm. One mouse was then placed into the cage and the latency to find the cookie and commence eating was timed using a stopwatch. A maximum time limit of 15 min was used. The total number of subjects per group was:  $n = 20$  (+/+);  $n = 21$  (+/-);  $n = 15$  (-/-).

### Habituation-dishabituation olfactory test

Mice from Cohort 2 were tested in an habituation-dishabituation olfactory test (Schellinck *et al.*, 1992; Guan *et al.*, 1993; Luo *et al.*, 2002). The odourant stimuli were tap water, cinnamon, and cocoa. Stimuli were presented by dipping a cotton-tipped applicator into the stimulus and then placing the applicator through the wire grid lid of the cage. A plastic weigh boat (4.5 × 4.5 cm), through which the applicator had been pushed, served to stabilize the applicator at a level 4.4 cm from the bottom of the cage. Each stimulus was presented for 3 min and then replaced by a new applicator, three times in succession for a total of nine presentations. The water presentations (3 ×) were always first and were followed by the cinnamon (3 ×) and cocoa (3 ×) presentations in counterbalanced order. An experimenter using a stopwatch recorded the cumulative time that the mouse spent sniffing the cotton-tipped applicator. Sniffing was defined as tilting the head upward with the nose orientated towards and within 2 cm of the applicator, rearing with the nose orientated towards and within 2 cm of the applicator, or physical contact of the muzzle to the applicator if the mouth was closed. Occasional open mouth contacts were considered to be chewing and not included in the cumulative sniff time.

### Trace and delay cued and contextual fear conditioning

For trace cued and contextual fear conditioning of Cohorts 1 and 2, methods were as previously described (Holmes *et al.*, 2002a; Kinney *et al.*, 2002) and similar to those of the established literature for this task (Crestani *et al.*, 1999; Huerta *et al.*, 2000). The chamber used for fear conditioning and scoring of freezing to context was a 26 × 23 × 17 cm clear Plexiglas box with a metal grid floor for foot shock delivery (Freeze Monitor, San Diego Instruments, San Diego, CA). The chamber for scoring of novel context and auditory cued fear was a triangular box, 50 × 35.5 × 25 cm, constructed of white plastic. McCormick vanilla extract (~0.5 mL) was painted onto one of the walls of the novel chamber. A Dell Optiplex computer interfaced to a shock stimulator and speaker delivered the auditory stimulus (conditioned stimulus, CS) and shock (unconditioned stimulus, US). auditory stimulus generated by the speaker was white noise at 80 dB. The shock was a 0.5-mA AC current for 1 s. All procedures were carried out using San Diego Instruments software.

On the training day, each subject was placed into the conditioning chamber and presented with four CS (white noise)–US (footshock) pairings. Each pairing was preceded and followed by a 2 min exploration period. The CS–US pairings were comprised of 30 s of white noise (CS), a 2.5-s trace interval, and a 1-s footshock. The experimenter observed the mice every 10 s during the 2 min before the first CS–US pairing and the 2 min after the last CS–US pairing for the presence or absence of freezing.

Twenty-four hours after training, subjects were individually removed from their home cages and taken to a different room for the cued fear test. Each subject was placed in the triangular box (novel context) for 6 min. The first 3 min consisted of baseline exploration in the absence of the CS (precue period). During the next 3 min, the CS tone previously used in training was presented (cue period). After the termination of the CS, the subject was allowed to explore the novel context for and an additional 90 s (postcue period). Presence or absence of freezing behaviour was scored every 10 s.

Forty-eight hours after training, subjects were individually removed from their home cages and taken to the original training room for the contextual fear test. Each subject was placed into the training context under conditions identical to the training day, with the exception that the CS and US were not presented. Freezing behaviour was scored at 10 s intervals over a 5 min session.

For assessment of standard delay CCF of Cohort 3, the procedures utilized were identical to the trace CCF procedure, with the exception of US onset and number of CS and US pairings (Chen *et al.*, 1996; Wehner *et al.*, 1997; Impey *et al.*, 1998). In this version of the task, the training session consisted of two CS–US pairings, each of which was preceded and followed by 2 min exploration periods. The CS–US pairings consisted of a 30 s presentation of the auditory stimulus and a one second footshock which began during the last second of CS presentation and coterminated with the CS.

The total number of subjects per group in the trace conditioning experiment, combining cohorts, was:  $n = 53$  (+/+);  $n = 64$  (+/-);  $n = 52$  (-/-). The total number of subjects per group in the delay conditioning experiment (Cohort 3) was:  $n = 28$  (+/+);  $n = 29$  (+/-);  $n = 22$  (-/-).

### Morris water maze

Spatial learning and memory was assessed in male mice from Cohorts 1 and 2 in the Morris water task using standard methods and equipment as previously described (Holmes *et al.*, 2001; Steiner *et al.*, 2001). Mice were tested on three components in the following order: visible platform training, hidden platform training and probe trial testing. A circular pool, 1.4 m in diameter (Nalge, Rochester, NY) was filled with water and rendered opaque by the addition of nontoxic white paint. Videotracking was conducted with a videocamera focused on the full diameter of the pool. Navigation parameters were analysed by using NIH Image software, originally developed by Wayne Rasband, National Institute of Mental Health (NIMH), and modified by Tsuyoshi Miyakawa, NIMH. Training on the visible and hidden platform tasks consisted of placing the mouse, facing the wall of the pool, in a new quadrant on each successive trial. Mice were allowed a maximum of 60 s to reach the platform, and then removed from the platform after 15 s. Four trials per day were administered for 3 days of training on the visible platform task and for 8 days of training on the hidden platform task. Three hours after the completion of training on day 8 of the hidden platform task, the platform was removed and a 60-s probe trial was administered. Parameters analysed for the visible and hidden platform trials included latency to reach the platform, swim speed, distance swum and thigmotaxis (time spent in the outer 8 cm of the pool). Time spent in each quadrant of the pool was analysed for the probe trial. The total number of subjects per group, combining cohorts, was:  $n = 26$  (+/+);  $n = 24$  (+/-);  $n = 24$  (-/-).

### Biochemical assays

Approximately 20 weeks after the end of behavioural testing, Cohort 2 was killed by cervical dislocation and the brain was quickly removed and rinsed in ice cold saline (0.9%). Both hippocampi, the entire right cortical hemisphere and the left frontal cortex were dissected over ice, placed in Eppendorf tubes, and frozen in dry ice. Samples were stored at -80 °C. The right hippocampus and the right cortex were designated for galanin radioimmunoassay (RIA). The left hippocampus and left frontal cortex were designated for assay of choline acetyltransferase (ChAT) activity. The total number of subjects per group for the hippocampal galanin RIA was:  $n = 31$  (+/+);  $n = 31$  (+/-);  $n = 25$  (-/-). The total number of subjects per group for the cortical galanin RIA was:  $n = 24$  (+/+);  $n = 31$  (+/-);  $n = 24$  (-/-). The total number of subjects per group for the hippocampal ChAT assay was:  $n = 31$  (+/+);  $n = 32$  (+/-);  $n = 25$  (-/-). The total number of subjects per group for the cortical ChAT assay was:  $n = 31$  (+/+);  $n = 32$  (+/-);  $n = 25$  (-/-). Samples were shipped on dry ice by overnight courier from the NIH campus in Bethesda, MD to the campus of the University of Arizona Medical Center in Tucson, AZ.

Frozen samples designated for galanin RIA were sonicated in ice-cold (4 °C) 0.1 M HCl and centrifuged (10 min at 8160g) in an Eppendorf microcentrifuge. Duplicate aliquots of the supernatant were frozen (-70 °C), lyophilized, and assayed for galanin using a commercial <sup>125</sup>I-RIA kit (Peninsula Laboratories, Belmont, CA). Protein content was assayed (Lowry *et al.*, 1951) to allow calculation of galanin concentrations as pg/mg protein.

ChAT activity was assayed in triplicate by measuring the formation of [<sup>14</sup>C]-acetylcholine from [<sup>14</sup>C]-acetyl-coenzyme-A (New England Nuclear, Boston, MA) as previously described (Fonnum, 1969). Briefly, frozen samples designated for assay of ChAT activity were homogenized in 0.4% Triton X-100:10 mM Na<sub>2</sub>EDTA. The homogenate and substrate were incubated for 15 min at 37 °C. Protein content was assayed (Lowry *et al.*, 1951) to allow calculation of ChAT activity as nmoles/h/mg protein.

### Statistical analysis

Data were analysed by analysis of variance (ANOVA) using StatView (SAS Institute Inc., Cary, NC). Initial between-subjects analyses included cohort, sex and genotype as factors in the ANOVA. When no genotype–sex interaction and no genotype–cohort interaction was detected, data were subsequently combined across cohorts and sex to increase the statistical power of the ANOVA for detecting a main effect of genotype. Repeated measures ANOVA was used to analyse the within-subjects data where appropriate (body weight, latency to fall from the rotarod, prepulse inhibition of acoustic startle, cued vs. novel amount eaten in STFP, cued vs. novel eating bouts in STFP, latency to find platform in water maze acquisition trials, percentage quadrant time in water maze probe trials, and platform crossings in water maze probe trials). The Tukey test was used for post hoc analyses where appropriate. Threshold of significance in all tests was  $P < 0.05$ .

## Results

### General health and neurologic function

The assessment of the general health and neurologic function in GAL-R1+/+, GAL-R1+/- and GAL-R1-/- mice is shown in Table 1. The genotypes were indistinguishable on all measures, as reported previously (Holmes *et al.*, 2003).

TABLE 1. Assessment of general health and neurologic function in the three groups of mice

	GAL-R1+/+ mice (n = 46)	GAL-R1+/- mice (n = 66)	GAL-R1-/- mice (n = 38)
General health			
Fur condition (3 point scale)	1.9	1.9	2.0
Bald patches (%)	15	21	8
Missing whiskers (%)	7	17	5
Piloerection (%)	0	0	0
Body tone (3 point scale)	2.0	2.0	2.0
Limb tone (3 point scale)	2.0	2.0	2.0
Motoric abilities			
Positional passivity (%)	0	0	0
Trunk curl (%)	100	100	100
Reflexes			
Forepaw reaching (%)	100	100	100
Righting reflex (%)	100	100	100
Corneal (%)	100	100	100
Pinna (%)	100	100	100
Vibrissae (%)	98	92	97
Toe pinch (%)	100	100	100
Reactivity			
Petting escape (%)	100	100	100
Struggle/vocalization (%)	70	68	68
Dowel biting (3 point scale)	2.1	2.0	1.8
Empty cage behaviour			
Transfer freezing (%)	0	2	3
Wild running (%)	0	0	0
Stereotypies (%)	0	0	0
Exploration (3 point scale)	2.3	2.2	2.3
Grooming (3 point scale)	1.3	1.5	1.4

### Body weight

Figure 1 shows longitudinal body weights from Cohorts 1 and 2. In Cohort 1 (Fig. 1A), There was no significant main effect of genotype on body weight. There were significant main effects of sex ( $F_{1,105} = 392.27$ ,  $P < 0.001$ ) and age ( $F_{4,420} = 395.70$ ,  $P < 0.001$ ). There was a significant age–sex interaction ( $F_{4,420} = 26.20$ ,  $P < 0.001$ ). Post hoc analysis determined that this interaction was due to a slightly faster weight gain in males.

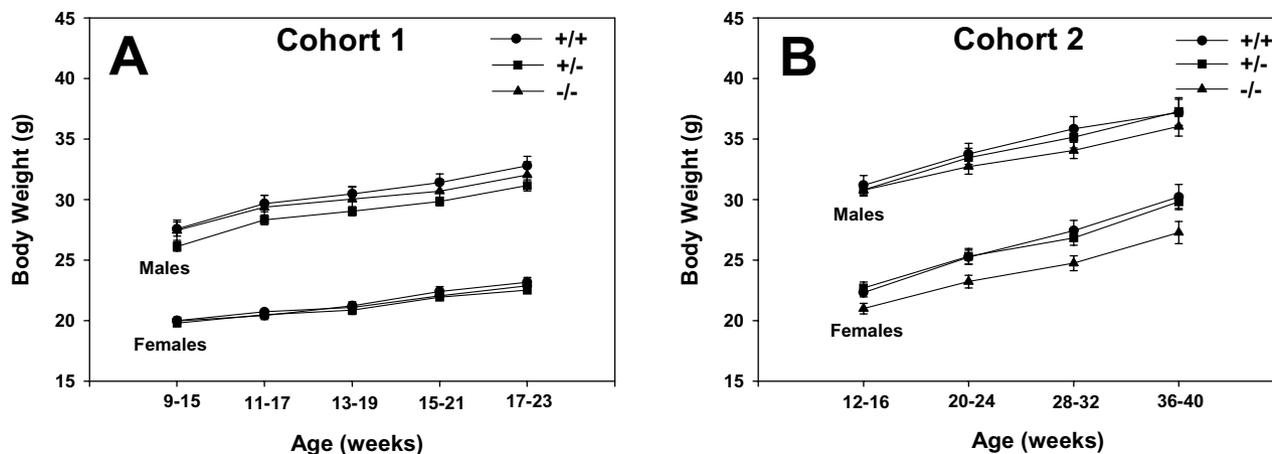


FIG. 1. Longitudinal body weight of (A) Cohort 1 and (B) Cohort 2 of GAL-R1 null mutants (-/-), heterozygotes (+/-) and wild-type littermate controls (+/+). There was no effect of genotype on body weight. Data are presented as mean  $\pm$  SEM. The number of mice per group in Cohort 1 was: male+/, n = 19; female+/, n = 11; male+/-, n = 25; female+/-, n = 24; male-/-, n = 15; female-/-, n = 17. In Cohort 2: male+/, n = 19; female+/, n = 20; male+/-, n = 19; female+/-, n = 21; male-/-, n = 17; female-/-, n = 15.

TABLE 2. Motor function, sensory function and control measures for cognitive tests in the three groups of mice

	GAL-R1+/+ mice (n = 46)	GAL-R1+/- mice (n = 66)	GAL-R1-/- mice (n = 38)
<b>Motor function</b>			
Wire hang latency (s)	56.6 ± 1.6	57.2 ± 1.2	57.7 ± 1.1
Rotarod latency (s)			
Day 1	92.9 ± 12.1	110.1 ± 15.0	104.2 ± 16.1
Day 2	134.4 ± 12.7	149.6 ± 16.7	167.8 ± 17.1
Day 3	164.1 ± 16.4	188.7 ± 16.4	183.6 ± 16.7
Open field – horizontal activity	6255.8 ± 360.1	6734.2 ± 245.9	7740.3 ± 775.7
Open field – vertical activity	263.6 ± 33.0	321.9 ± 28.0	394.9 ± 44.2*
<b>Sensory function</b>			
Tail flick latency (s)	2.59 ± 0.59	2.60 ± 0.47	2.56 ± 0.58
Hot plate latency (s)	10.50 ± 2.40	11.44 ± 3.04	11.46 ± 3.38
Acoustic startle amplitude (120dB)	506.9 ± 182.5	517.8 ± 173.3	585.6 ± 282.7
Prepulse inhibition of acoustic startle	Normal	Normal	Normal
Buried food latency (s)	381.3 ± 37.9	403.4 ± 75.3	388.8 ± 86.6
Olfactory habituation-dishabituation	Normal	Normal	Normal
<b>Control measures for cognitive tests</b>			
STFP – total eaten (g)	0.75 ± 0.04	0.80 ± 0.04	0.84 ± 0.04
STFP – sniffs of demonstrator muzzle	19.47 ± 0.85	20.89 ± 1.05	21.32 ± 1.14
Water maze swim speed (hidden platform, Day 8) (cm/s)	24.28 ± 0.58	21.81 ± 1.06	23.61 ± 0.66
Water maze thigmotaxis (hidden platform, Day 8) (% time within 8 cm of wall)	2.27 ± 0.67	2.78 ± 0.56	4.52 ± 2.07
Water maze visible platform latency (s)			
Day 1	37.04 ± 3.50	38.62 ± 4.15	34.34 ± 3.11
Day 2	19.59 ± 7.12	22.69 ± 3.75	17.91 ± 3.29
Day 3	11.07 ± 1.79	16.93 ± 2.93	12.67 ± 2.06
Day 4	7.58 ± 1.29	8.74 ± 2.07	8.57 ± 1.44

Data are means ± SEM. \* $P < 0.05$ .

In Cohort 2 (Fig. 1B) there was no significant main effect of genotype and significant main effects of sex ( $F_{1,111} = 227.92$ ,  $P < 0.001$ ) and age ( $F_{3,333} = 431.26$ ,  $P < 0.001$ ) on body weight. There was a significant age–sex interaction ( $F_{3,333} = 3.23$ ,  $P = 0.02$ ). Pair-wise comparisons found that body weight was significantly different between all ages within both sexes and between both sexes at all ages.

### Motor function

Results of motor function assessment in GAL-R1+/+, GAL-R1+/- and GAL-R1-/- mice are shown in Table 2. There was not a statistically significant effect of genotype on performance in the wire hang test (a test of limb strength), the rotarod (test of co-ordination and motor learning), or on horizontal activity in the open field. There was a significant main effect of genotype on vertical activity in the open field ( $F_{2,78} = 3.225$ ,  $P = 0.045$ ). Pair-wise comparisons showed that the main effect of genotype was due to significantly higher vertical activity in the -/- mice compared to the +/+ mice ( $P < 0.05$ ).

### Sensory function

Results of sensory function assessment in GAL-R1+/+, GAL-R1+/- and GAL-R1-/- mice are shown in Table 2. There was no statistically significant effect of genotype on the latency to respond to heat stimulus in the tail flick test, on the latency to respond to heat stimulus in the hot plate test, on acoustic startle, on the latency to find buried food, on odour habituation/dishabituation, or on prepulse inhibition of acoustic startle.

### Learning and memory: social transmission of food preference

Results from the STFP choice phase are shown in Fig. 2. There was a significant main effect of food type ( $F_{1,139} = 18.29$ ,  $P < 0.0001$ ) but no main effect of genotype on the amount of food eaten (Fig. 2A). There was not a significant interaction between food type and genotype. These data suggest that all genotypes preferred the cued food to the novel food.

Analysis of eating bout data from the STFP choice phase yielded similar results (Fig. 2B). There was a significant main effect of food type ( $F_{1,139} = 24.21$ ,  $P < 0.001$ ) and genotype ( $F_{2,139} = 3.37$ ,  $P = 0.04$ ). Food type and genotype did not interact. Despite the main effect of genotype on the number of eating bouts, post hoc analysis found no significant pair-wise differences between the genotypes for the number of eating bouts (all  $P > 0.06$ ).

There were no differences between genotypes on controls measures of STFP performance or olfaction (Table 2). There were no differences across genotype on sniffs of the demonstrator's muzzle during the interaction phase (measure of social interaction) or on the total amount eaten during the test phase (measure of ingestive behaviour) of STFP. In the buried food test, there was no genotype effect on the latency to find buried food. GAL-R1 null mutant mice performed the same as controls in the olfactory habituation and dishabituation test.

### Learning and memory: conditioned fear

Figure 3 shows the results from trace cued and contextual fear conditioning experiment. In the training phase (Fig. 3A), mice generally froze significantly more during the 2 min after the cue–shock pairings than in the 2 min before the cue–shock pairings ( $F_{1,168} = 1,097$ ,  $P < 0.0001$ ). There was no main effect of genotype on freezing during training and the effect of the cue–shock pairings did not interact with genotype.

In the novel context test (Fig. 3B), two-way repeated measures ANOVA using cue (levels: precue, cue and postcue) and genotype as factors, determined that there was a significant effect of both cue ( $F_{2,168} = 106.06$ ,  $P < 0.001$ ) and genotype ( $F_{2,168} = 4.13$ ,  $P = 0.02$ ) on freezing. There was a significant interaction between cue and genotype ( $F_{4,168} = 3.10$ ,  $P = 0.02$ ). Post hoc analysis determined that mice of each genotype significantly increased freezing in response to the cue (all  $P < 0.001$ ), but that the -/- mice froze significantly less during cue presentation than both the +/+ mice ( $P < 0.001$ ) and the +/- mice ( $P = 0.04$ ). The genotypes did not differ in their levels of freezing

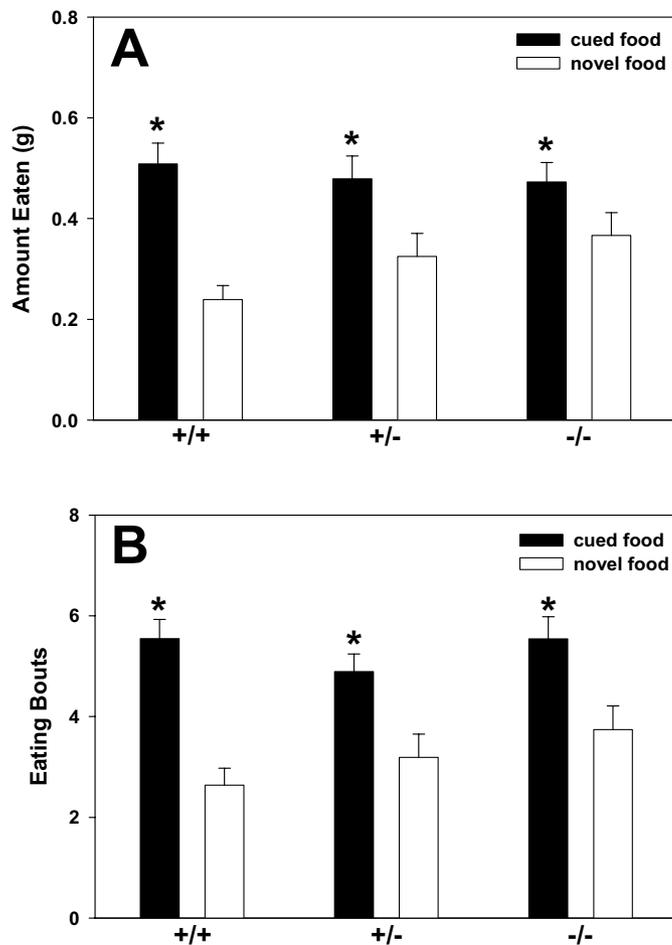


FIG. 2. STFP in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ). (A) All genotypes ate more of the cued food than of the novel food ( $*P < 0.0001$ ). Data are presented as mean  $\pm$  SEM. (B) All genotypes performed more eating bouts of the cued food than the novel food. The number of mice per group was:  $n = 55$  ( $+/+$ );  $n = 37$  ( $+/-$ );  $n = 50$  ( $-/-$ ).

during the precue and postcue portions of the session. These data indicate that trace cued conditioning is impaired in the  $-/-$  mutant mice.

In the same context test (Fig. 3C), there was no effect of genotype on freezing when the mice were placed in the same environment as that used during training ( $F_{2,97} = 0.99$ ,  $P = 0.38$ ).

A separate cohort of mice (Cohort 3) was tested in a delay version of the conditioned fear task. In the training phase (Fig. 4A), the mice generally froze significantly more during the 2 min after the cue–shock pairings than during the 2 min before the cue–shock pairings ( $F_{1,76} = 138.02$ ,  $P < 0.0001$ ). There was no main effect of genotype on freezing during training ( $F_{2,76} = 0.07$ ,  $P = 0.93$ ) and the effect of the cue–shock pairings did not interact with genotype ( $F_{2,76} = 0.12$ ,  $P = 0.89$ ).

In the novel context test (Fig. 4B), there was a significant main effect of cue presentation on freezing ( $F_{1,76} = 51.86$ ,  $P < 0.0001$ ). There was no main effect of genotype ( $F_{2,76} = 0.05$ ,  $P = 0.95$ ) and the effect of cue presentation did not interact with genotype ( $F_{2,76} = 0.96$ ,  $P = 0.39$ ). These data indicate that standard delay cued fear conditioning is unaffected in GAL-R1 KO mice.

In the same context test (Fig. 4C), there was no effect of genotype on freezing when the mice were placed in the same context as that

## Trace Fear Conditioning

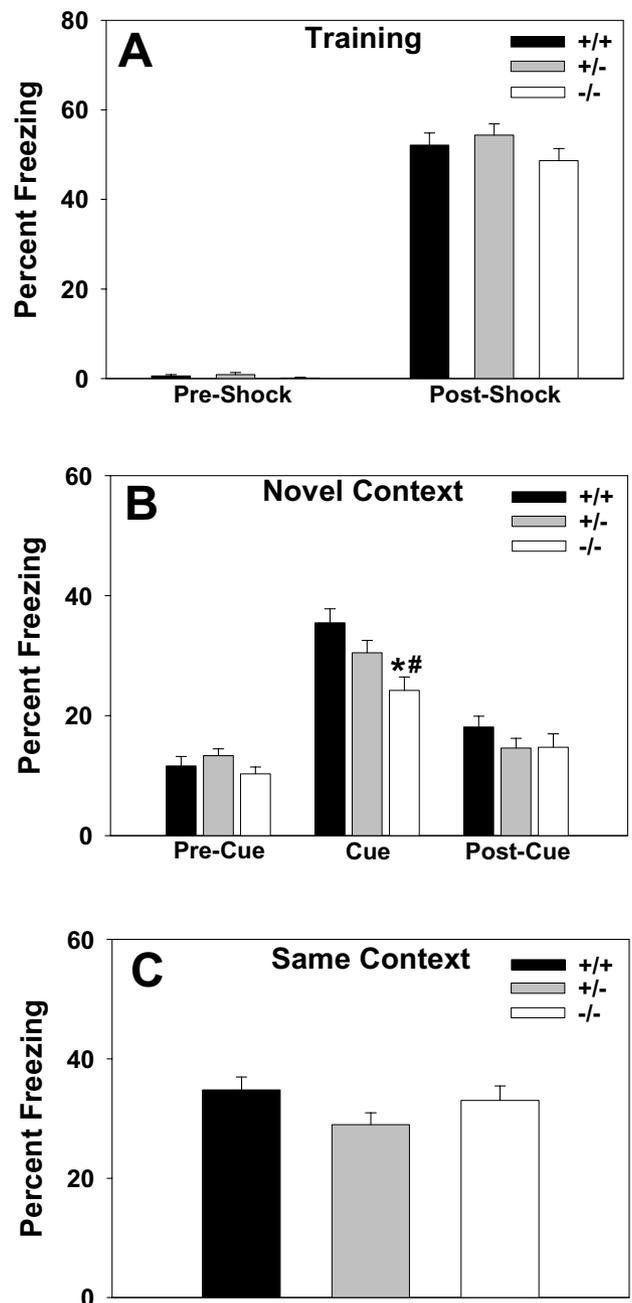


FIG. 3. Trace cued and contextual fear conditioning in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ). (A) Proportion of observations in which freezing was detected during training prior to and after the CS–US pairings. Freezing was similar across genotypes during training. (B) Proportion of observations in which freezing was detected in the novel context testing before (Pre-Cue), during (Cue) and after (Post-Cue) the presentation of the CS (auditory cue) identical to that presented during training. GAL-R1  $-/-$  mice froze significantly less than both  $+/+$  ( $*P < 0.001$ ) and  $+/-$  mice ( $\#P = 0.04$ ) during cue presentation. (C) Proportion of observations in which freezing was detected during testing of contextual conditioning. Freezing was similar across genotypes during testing of contextual conditioning. Data are presented as mean  $\pm$  SEM. The number of mice per group was:  $n = 53$  ( $+/+$ );  $n = 64$  ( $+/-$ );  $n = 52$  ( $-/-$ ).

## Delay Fear Conditioning

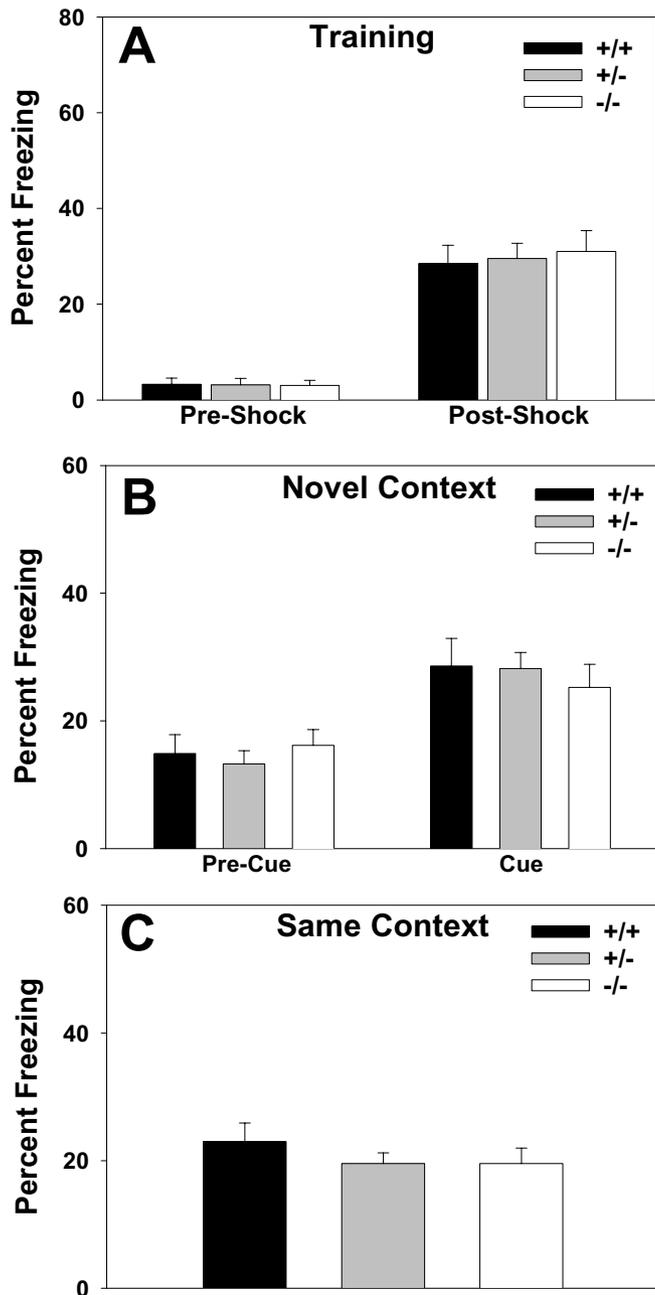


Fig. 4. Standard delay cued and contextual fear conditioning in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ) from Cohort 3. (A) Proportion of observations in which freezing was detected during standard delay CCF training prior to and after the CS-US pairings. (B) Proportion of observations in which freezing was detected during novel context testing before (Pre-Cue) and during (Cue) CS presentation. (C) Proportion of observations in which freezing was detected during testing of contextual conditioning. Freezing was similar across genotypes at all phases of the experiment. Data are presented as mean  $\pm$  SEM. The number of mice per group was:  $n = 28$  ( $+/+$ );  $n = 29$  ( $+/-$ );  $n = 22$  ( $-/-$ ).

used during training ( $F_{2,76} = 0.71$ ,  $P = 0.49$ ). These data indicate that contextual conditioning was unaffected in GAL-R1 KO mice.

### Learning and memory: water maze

Water maze acquisition data is shown in Fig. 5A and B. There was a main effect of training day on the latency to find the hidden platform ( $F_{7,497} = 8.78$ ,  $P < 0.001$ , Fig. 5A) in which latency decreased with training, as expected. There was no main effect of genotype on latency and no interaction between training day and genotype. Similarly, when the acquisition data were analysed in terms of distance swum (Fig. 5B), there was main effect of day ( $F_{7,497} = 14.82$ ,  $P < 0.0001$ ) but no main effect of genotype or interaction between day and genotype. These data indicate that the  $+/+$ ,  $+/-$  and  $-/-$  mice reduced their latencies and distance swum similarly during training.

The water maze probe trial results are shown in Fig. 5C and D. A  $3 \times 4$  mixed ANOVA (factors, genotype  $\times$  quadrant) for percentage quadrant time determined that there was a significant main effect of quadrant location ( $F_{3,71} = 21.86$ ,  $P < 0.0001$ , Fig. 5C). There was not a significant quadrant-genotype interaction indicating that the effect of quadrant location did not depend on genotype. Post hoc analysis determined that the mice spent significantly more time in the target quadrant than each of the three other quadrants (all  $P < 0.001$ ).

Analysis of platform crossings (Fig. 5D) during the probe trial gave a similar picture. A  $3 \times 4$  mixed ANOVA determined that there was a significant main effect of platform location ( $F_{3,71} = 14.41$ ,  $P < 0.0001$ ). There was not a significant platform location-genotype interaction indicating that the effect of platform location did not depend on genotype. Post hoc analysis determined that the mice crossed the former platform location significantly more than the equivalent locations in the three other quadrants (all  $P < 0.001$ ).

Several control measures (Table 2) were taken in the water maze experiments to rule out nonmnemonic explanations of any observed differences in performance. These included swim speed, thigmotaxis and latency to find a visible platform. There was no significant effect of genotype on any of these measures (all  $P > 0.05$ ).

### Galanin RIA

Hippocampal and cortical galanin peptide levels in  $+/+$ ,  $+/-$  and  $-/-$  mice are shown in Table 3. Galanin peptide levels were not significantly different between genotypes in either brain region.

### ChAT activity

Hippocampal and cortical ChAT activity in  $+/+$ ,  $+/-$  and  $-/-$  mice are shown in Table 3. ChAT activity was not significantly different between genotypes in either brain region.

### Discussion

The principal finding of the present study is that GAL-R1 KO mice are unimpaired on a number of tests of learning and memory with the exception of the trace version of cued fear conditioning. These results indicate that the GAL-R1 subtype of galanin receptor is not essential for the performance of many standard learning and memory tasks in mice. Normal scores on measures of general health and neurological function rule out a gross abnormality as a cause of the deficit in trace conditioning. The impairment in the GAL-R1 KO mice in this hippocampal-dependent (Solomon *et al.*, 1986; Moyer *et al.*, 1990; Sutherland & McDonald, 1990; McEchron *et al.*, 1998; Weiss *et al.*, 1999; Ryou *et al.*, 2001) task and the lack of impairment in other hippocampal-dependent tasks suggests a small but specific role for GAL-R1 in hippocampal function.

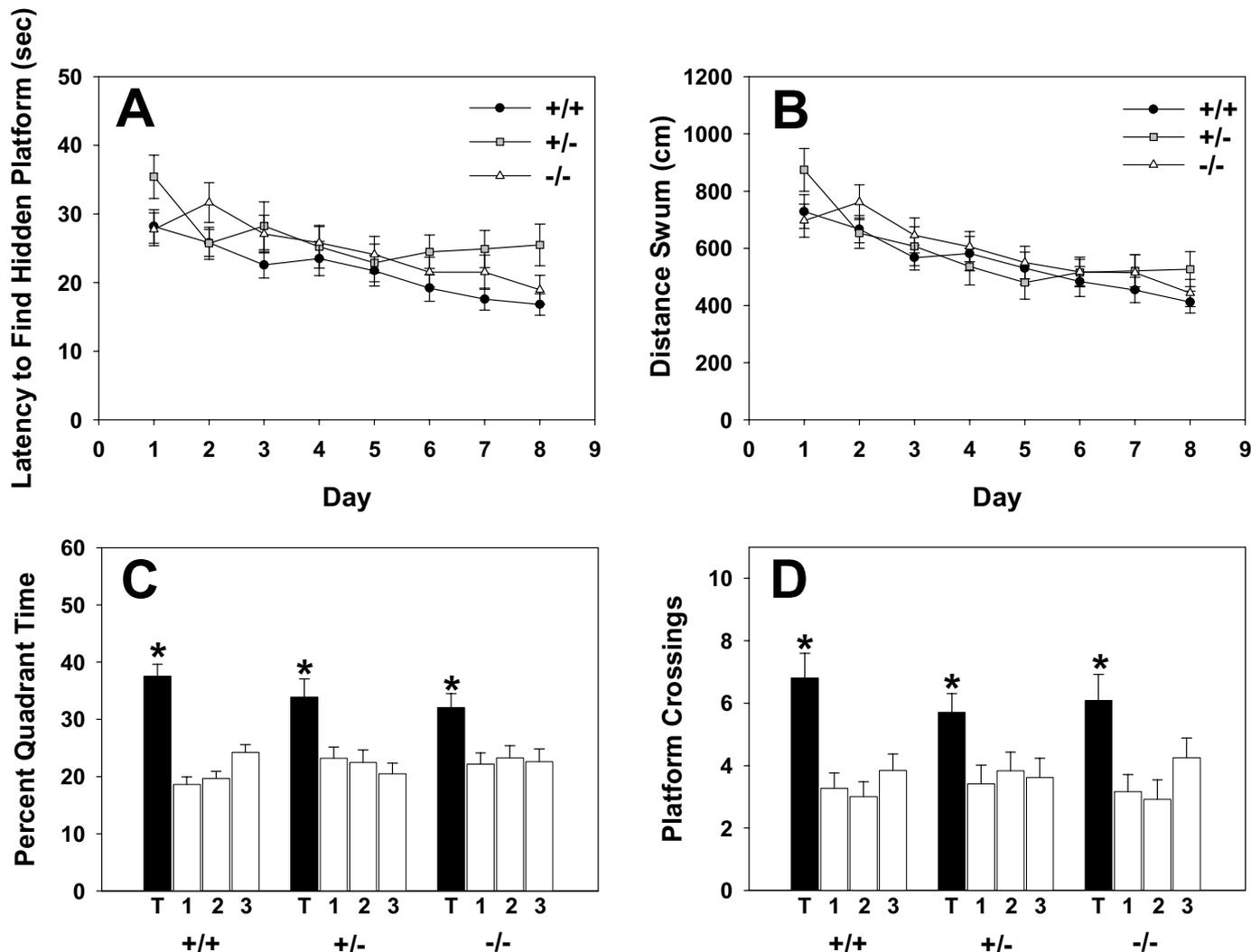


FIG. 5. (A) Morris water maze hidden platform latency in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ). There was no effect of genotype on latency to find the hidden platform in the acquisition trials. (B) Morris water maze hidden platform distance swum in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ). There was no effect of genotype on distance swum in the hidden platform trials. (C) Percentage quadrant time during the probe trial of the Morris water maze in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ). All genotypes spent significantly more time in the target quadrant compared to all three other quadrants ( $*P < 0.001$ ). (D) Platform crossings during the probe trial of the Morris water maze in GAL-R1 null mutants ( $-/-$ ), heterozygotes ( $+/-$ ) and wild-type littermate controls ( $+/+$ ). All genotypes crossed the target platform location significantly more than the equivalent location in the 3 other quadrants. Data are presented as mean  $\pm$  SEM. The number of mice per group was:  $n = 26$  ( $+/+$ );  $n = 24$  ( $+/-$ );  $n = 24$  ( $-/-$ ).

While GAL-R1 KO mice showed impairment in trace fear conditioning, these mice were unimpaired in delay fear conditioning. This dissociation should be interpreted with caution as the training protocols differed in the number of CS–US pairings (2 in delay, 4 in trace). Notably, freezing of the  $+/+$  mice, but not the  $-/-$  mice, was slightly higher during cue presentation in the novel context after trace con-

ditioning compared to after delay conditioning. Thus, freezing to the cue in the delay experiment may be low enough to preclude the detection of a genotype difference. Further, it should be considered that enhanced vertical activity in the GAL-R1 KO mice may interact with the greater training of the trace conditioning protocol to interfere with freezing behaviour. This explanation seems unlikely as the

TABLE 3. Galanin peptide levels and choline acetyltransferase activity in hippocampus and cortex of the three groups of mice

	GAL-R1 $+/+$ mice ( $n = 46$ )	GAL-R1 $+/-$ mice ( $n = 66$ )	GAL-R1 $-/-$ mice ( $n = 38$ )
Galanin – hippocampus (pg/mg protein)	121.6 $\pm$ 15.2	103.6 $\pm$ 11.9	122.6 $\pm$ 14.6
Galanin – cortex (pg/mg protein)	14.9 $\pm$ 1.2	16.0 $\pm$ 1.6	17.9 $\pm$ 2.0
ChAT – hippocampus (nmole/h/mg protein)	61.6 $\pm$ 1.2	61.0 $\pm$ 1.4	65.2 $\pm$ 1.7
ChAT – cortex (nmole/h/mg protein)	123.9 $\pm$ 3.3	129.2 $\pm$ 4.1	124.9 $\pm$ 3.6

enhanced vertical activity was not seen in a previous cohort (Holmes *et al.*, 2003), but the impairment in trace conditioning is seen in both cohorts tested so far.

It is interesting to speculate on the possible mechanism(s) underlying a highly specific deficit in trace conditioning in the GAL-R1 KOs. Of note is our previously reported finding that the GAL-R1 mice have increased anxiety-like behaviour in the elevated plus maze (Holmes *et al.*, 2003), but not in other, putatively less stressful, tests of anxiety-like behaviour. Thus, it appears that the behavioural effects of the loss of GAL-R1 are not global in scope but are detectable only under certain conditions. The limited nature of the behavioural changes in GAL-R1 KO mice may be related to the physiological observation that the release of galanin requires relatively high rates of neuronal firing (Consolo *et al.*, 1994) consistent with the frequency coding hypothesis of neuropeptide release (Hökfelt *et al.*, 1987). Perhaps the behavioural effects of the loss of GAL-R1 are most readily manifest in the elevated plus maze and trace conditioning because it is only in those tasks that neuronal activity is high enough to bring about the release of galanin and the subsequent activation of GAL-R1.

The selective effect of the GAL-R1 null mutation on trace conditioning is not the first observation of a dissociation between trace fear conditioning and other hippocampal dependent tasks. For example, inhibition of neurogenesis using the antimetabolic agent, methylazoxymethanol acetate, reduced trace fear conditioning but had no effect on contextual fear conditioning or spatial navigation in the water maze (Shors *et al.*, 2002). Shors *et al.* (2002) suggest that these data indicate a special dependence of trace conditioning on newly generated granule cells, the survival of which is enhanced by training in hippocampus-dependent tasks (Gould *et al.*, 1999). It is therefore tempting to speculate that the impairment in trace conditioning in GAL-R1 KO mice may reflect an important role for the GAL-R1 receptor in the survival of these neurons. Precedent for this hypothesis is provided by the neurotrophic effects of galanin in other systems (Holmes *et al.*, 2000; O'Meara *et al.*, 2000; Mahoney *et al.*, 2003). Studies of hippocampal neurogenesis in galanin knockout, galanin receptor knockout and galanin transgenic mice could shed light on this issue.

The impairment in trace conditioning in GAL-R1 KO mice may reflect a role for GAL-R1 in the electrophysiologic function of pyramidal neurons of the CA1 subfield of the hippocampus. These neurons play a critical role in the neural mechanisms of trace conditioning. Mutations of NMDA receptors in CA1 led to impairment in trace fear conditioning (Huerta *et al.*, 2000). Further, a significant percentage of CA1 neurons show maximal firing at latencies similar to trace interval duration (McEchron *et al.*, 2003). These data suggest that the trace interval is encoded by the activity of CA1 pyramidal cells. Interestingly, GAL-R1 is expressed by CA1 pyramidal cells in the ventral hippocampus of the rat (O'Donnell *et al.*, 1999; Burazin *et al.*, 2000). Thus, GAL-R1 may participate in the regulation of activity of these neurons and in turn play a critical role in the acquisition of trace conditioning.

The present finding of a trace conditioning impairment in GAL-R1 KO mice is similar to that which has been observed in galanin-overexpressing mice (GAL-tg) (Kinney *et al.*, 2002). It is unexpected to see the same impairment in a transgenic line that overexpresses a neuropeptide transmitter and a knockout line that lacks the receptor for that transmitter. One potential explanation is that GAL-R1 may regulate galanin peptide levels as an inhibitory autoreceptor (Pieribone *et al.*, 1995; Burazin *et al.*, 2001; Ma *et al.*, 2001; Hohmann *et al.*, 2003; Larm *et al.*, 2003). Thus, the absence of GAL-R1 may result in increased galanin peptide levels in the brain. To test this hypothesis, we performed RIA for galanin from dissected samples of hippocampus and cortex. We found no differences in galanin peptide levels between

+/, +/- and -/- mice in either of these regions. These data provide no evidence that GAL-R1 regulates galanin peptide levels; however, RIA of dissected brain regions may be unlikely to detect a dynamic change in the regulation of synaptic release of galanin. An *in vivo* technique, such as microdialysis for galanin in a behaving animal, will be necessary to test this hypothesis further.

Another possible explanation for the impairment in trace conditioning in GAL-R1 KO mice rests on the hypothesized neurotrophic role for galanin. Neurotrophic effects of galanin have been reported (Holmes *et al.*, 2000; Mahoney *et al.*, 2003) including involvement in the survival of the cholinergic basal forebrain (CBF) (O'Meara *et al.*, 2000). Interestingly, pharmacological studies have shown that cholinergic function is critical for trace variants of classical conditioning (Kaneko & Thompson, 1997; Disterhoft *et al.*, 1999; Seager *et al.*, 1999). It seems possible that if the trophic effects of galanin on the CBF are mediated by GAL-R1, then a null mutation of *Galr1* might result in neurodegeneration or abnormal development of the CBF. Defects in CBF anatomy could lead to impaired trace conditioning. To test this hypothesis, we assessed cholinergic function in the GAL-R1 KO mice by assaying ChAT activity in the hippocampus. We found no differences in ChAT activity between +/+, +/- and -/- mice. These results suggest that cholinergic function is unaffected by the loss of GAL-R1. Further studies using immunohistochemistry in conjunction with morphometrics and stereology are needed to confirm normal CBF anatomy in the GAL-R1 KO mice.

An interesting negative finding from our assessment of the general health of the GAL-R1 KO mice was the lack of a genotype effect on body weight. Galanin is known to stimulate feeding in satiated rats when injected into the lateral ventricles (Crawley *et al.*, 1990), paraventricular nucleus of the hypothalamus (Crawley *et al.*, 1990), the central nucleus of the amygdala (Corwin *et al.*, 1993) and nucleus tractus solitarius/area postrema (Koegler & Ritter, 1998). The galanin receptor subtype that mediates this effect is unknown. The present data indicate that GAL-R1 is not necessary for the regulation of lifetime body weight. Further, in the STFP experiment, GAL-R1 KO mice ate the same amount of food as +/+ controls after an 18 h fast. It should be noted that the present data do not preclude a role for GAL-R1 in feeding behaviour or body weight regulation as compensatory changes may mitigate the effects of the null mutation. Possible compensatory changes include changes in expression of GAL-R2 and GAL-R3. Although rigorous quantification of GAL-R2 and GAL-R3 expression in GAL-R1 KO mice has not yet been done, initial results indicate no major differences in GAL-R2 and GAL-R3 expression between GAL-R1 KO mice and +/+ controls (Jacoby *et al.*, 2002). Investigation of changes in GAL-R2 expression may be especially interesting as there are reports of GAL-R1 and GAL-R2 being oppositely regulated (Zhang *et al.*, 1998; Burazin *et al.*, 2001; Hohmann *et al.*, 2003).

In the initial report describing the GAL-R1 KO mice, Jacoby *et al.* (2002) showed that -/- GAL-R1 KO mice have decreased plasma levels of insulin-like growth factor, an increased incidence of spontaneous tonic-clonic seizures, and increased seizure-related deaths. The seizure phenotype has also been reported by Fetissov *et al.* (2003), and inhibitory actions of galanin on seizures have been documented in other reports (Mazarati *et al.*, 2000; Haberman *et al.*, 2003). The consequences of these phenotypes and their relationship, if any, to the behavioural findings reported herein is unclear. Interestingly, seizures in GAL-R1 KO mice have rarely been observed in our laboratory. Further, susceptibility to pentylenetetrazole-induced seizures was not greater in GAL-R1 KO mice (unpublished observations). However, it remains possible that the GAL-R1 KO mice used in the present experiments are experiencing subthreshold seizure activity, undetect-

able by the behavioural methods employed. The effect of laboratory environment and/or the number of backcrosses into C57BL/6J offer possible, but unexplored, explanations for the discrepancy.

The nociceptive threshold of GAL-R1 KO mice has been tested in a battery of pain tests by Blakeman *et al.* (2003). Using different modalities of stimulation, these studies found that  $-/-$  mice had decreased hot plate latency, an enhancement in cold response, and increased duration of pain-like behaviours after sciatic nerve injury. The  $-/-$  mice were unaffected in tail flick, radiant heat stimulation of the paw and mechanical sensitivity (Blakeman *et al.*, 2003). In a separate study, GAL-R1 KO mice were shown to not differ from  $+/+$  controls in the excitability of the flexor reflex or in sensitization of the reflex by repetitive C-fibre stimulation (Grass *et al.*, 2003). Together, these data suggest that the role of GAL-R1 in baseline nociception is relatively minor. Alternatively, there may be significant plasticity in the galanin system such that other subtypes can compensate for the loss of GAL-R1. The differing results in the hot plate test in the present study and the study discussed above may be explained by methodological issues such as the lower temperature of hot plate used in the previous study (54 °C vs. 55 °C). Higher hot plate latencies in the  $+/+$  control group in the study by Blakeman *et al.* (2003) compared to those of the present study suggest an interaction between the null mutation and this test parameter.

The relatively minor differences in behavioural phenotype between GAL-R1 KO mice and  $+/+$  littermates provide an opportunity to discover which of the myriad behavioural functions of exogenously administered galanin are mediated by GAL-R1. Studies using GAL-R1 KO mice to test the actions of centrally microinjected galanin and subtype-selective galanin agonists will greatly increase our understanding of the functions of the galanin receptor subtypes. Investigations of mice with mutations in *Galr2* or *Galr3*, combined with pharmacological studies using subtype selective compounds, will provide additional insights into the behavioural and physiological actions of galanin at each of its receptors. This understanding will inform the development of galaninergic drugs as therapeutics for a diverse range of clinical problems including Alzheimer's disease, mood disorders, eating disorders and nerve regeneration.

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## Abbreviations

ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; CBF, cholinergic basal forebrain; CCF, cued and contextual fear; ChAT, choline acetyltransferase; CS, conditioned stimulus; GAL-R1 KO mice, GAL-R1 knockout mice; GAL-tg mice, galanin-overexpressing transgenic mice; IR, immunoreactivity; MAPK, mitogen activated protein kinase; RIA, radioimmunoassay; SEM, standard error of the mean; STFP, social transmission of food preference.

## References

Alberts, J.R. & Galef, B.G. (1971) Acute anosmia in the rat: a behavioral test of a peripherally-induced olfactory deficit. *Physiol. Behav.*, **6**, 619–621.  
 Beal, M.F., MacGarvey, U. & Swartz, K.J. (1990) Galanin immunoreactivity is increased in the nucleus basalis of Meynert in Alzheimer's disease. *Ann. Neurol.*, **28**, 157–161.  
 Blakeman, K.H., Hao, J.X., Xu, X.J., Jacoby, A.S., Shine, J., Crawley, J.N., Iismaa, T. & Wiesenfeld-Hallin, Z. (2003) Hyperalgesia and increased

neuropathic pain-like response in mice lacking galanin receptor 1 receptors. *Neuroscience*, **117**, 221–227.  
 Bloomquist, B.T., Beauchamp, M.R., Zhelmin, L., Brown, S.E., Gore-Willse, A.R., Gregor, P. & Cornfield, L.J. (1998) Cloning and expression of the human galanin receptor GalR2. *Biochem. Biophys. Res. Commun.*, **243**, 474–479.  
 Bowser, R., Kordower, J. & Mufson, E. (1997) A confocal microscopic analysis of galaninergic hyperinnervation of cholinergic basal forebrain neurons in Alzheimer's disease. *Brain Pathol.*, **2**, 723–730.  
 Burazin, T.C.D., Larm, J.A. & Gundlach, A.L. (2001) Regulation by osmotic stimuli of galanin-R1 receptor expression in magnocellular neurones of the paraventricular and supraoptic nuclei of the rat. *J. Neuroendocrinol.*, **13**, 358–370.  
 Burazin, T.C.D., Larm, J.A., Ryan, M.C. & Gundlach, A.L. (2000) Galanin-R1 and -R2 receptor mRNA expression during the development of rat brain suggests differential subtype involvement in synaptic transmission and plasticity. *Eur. J. Neurosci.*, **12**, 2901–2917.  
 Burgevin, M.C., Loquet, I., Quarteronet, D. & Habert-Ortoli, E. (1995) Cloning, pharmacological characterization, and anatomical distribution of a rat cDNA-encoding for a galanin receptor. *J. Mol. Neuroscience*, **6**, 33–41.  
 Chan-Palay, V. (1988) Galanin hyperinnervates surviving neurons of the human basal nucleus of Meynert in dementias of Alzheimer's and Parkinson's disease: a hypothesis for the role of galanin in accentuating cholinergic dysfunction in dementia. *J. Comp. Neurol.*, **273**, 543–557.  
 Chen, C., Kim, J.J., Thompson, R.F. & Tonegawa, S. (1996) Hippocampal lesions impair contextual fear conditioning in two strains of mice. *Behav. Neurosci.*, **110**, 1177–1180.  
 Consolo, S., Baldi, G., Russi, G., Civenni, G., Bartfai, T. & Vezzani, A. (1994) Impulse flow dependency of galanin release in-vivo in the rat ventral hippocampus. *Proc. Natl Acad. Sci. USA*, **91**, 8047–8051.  
 Corwin, R.L., Robinson, J.K. & Crawley, J.N. (1993) Galanin antagonists block galanin-induced feeding in the hypothalamus and amygdala of the rat. *Eur. J. Neurosci.*, **5**, 1528–1533.  
 Counts, S.E., Perez, S.E., Kahl, U., Bartfai, T., Bowser, R.P., Decher, D.C., Mash, D.C., Crawley, J.N. & Mufson, E.J. (2001) Galanin: neurobiologic mechanisms and therapeutic potential for Alzheimer's disease. *CNS Drug Rev.*, **7**, 445–470.  
 Crawley, J.N. (1999) The role of galanin in feeding behavior. *Neuropeptides*, **33**, 369–375.  
 Crawley, J.N., Austin, M.C., Fiske, S.M., Martin, B., Consolo, S., Berthold, M., Langel, Ü., Fisone, G. & Bartfai, T. (1990) Activity of centrally administered galanin fragments on stimulation of feeding behavior and on galanin receptor binding in the rat hypothalamus. *J. Neurosci.*, **10**, 3695–3700.  
 Crawley, J.N., Belknap, J.K., Collins, A., Crabbe, J.C., Frankel, W., Henderson, N., Hitzemann, R.J., Maxson, S.C., Miner, L.L., Silva, A.J., Wehner, J.M., Wynshaw-Boris, A. & Paylor, R. (1997) Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology*, **132**, 107–124.  
 Crawley, J.N. & Paylor, R. (1997) A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. *Hormones Behav.*, **31**, 197–211.  
 Crestani, F., Lorez, M., Baer, K., Essrich, C., Benke, D., Laurent, J.P., Belzung, C., Fritschy, J.M., Luscher, B. & Mohler, H. (1999) Decreased GABA (A) -receptor clustering results in enhanced anxiety and a bias for threat cues. *Nature Neurosci.*, **2**, 833–839.  
 Disterhoft, J.F., Kronforst-Collins, M., Oh, M.M., Power, J.M., Preston, A.R. & Weiss, C. (1999) Cholinergic facilitation of trace eyeblink conditioning in aging rabbits. *Life Sci.*, **64**, 541–548.  
 Fathi, Z., Battaglino, P.M., Iben, L.G., Li, H., Baker, E., Zhang, D.L., McGovern, R., Mahle, C.D., Sutherland, G.R., Iismaa, T.P., Dickinson, K.E.J. & Zimanyi, I.A. (1998) Molecular characterization, pharmacological properties and chromosomal localization of the human GALR2 galanin receptor. *Mol. Brain Res.*, **58**, 156–169.  
 Fathi, Z., Cunningham, A.M., Iben, L.G., Battaglino, P.B., Ward, S.A., Nichol, K.A., Pine, K.A., Wang, J.C., Goldstein, M.E., Iismaa, T.P. & Zimanyi, I.A. (1997) Cloning, pharmacological characterization and distribution of a novel galanin receptor. *Mol. Brain Res.*, **51**, 49–59.  
 Fetissov, S.O., Jacoby, A.S., Brumovsky, P.R., Shine, J., Iismaa, T.P. & Hökfelt, T. (2003) Altered hippocampal expression of neuropeptides in seizure-prone GALR1 knockout mice. *Epilepsia*, **44**, 1022–1033.  
 Fonnum, F. (1969) Radiochemical microassays for the determination of choline acetyltransferase and acetylcholinesterase activities. *Biochem. J.*, **115**, 465–472.  
 Gould, E., Beylin, A., Tanapat, P., Reeves, A. & Shors, T.J. (1999) Learning enhances adult neurogenesis in the hippocampal formation. *Nature Neurosci.*, **2**, 260–265.

- Grass, S., Jacoby, A.S., Iismaa, T.P., Crawley, J.N., Xu, X.J. & Wiesenfeld-Hallin, Z. (2003) Flexor reflex excitability in mice lacking galanin receptor galanin-R1. *Neurosci. Lett.*, **345**, 153–156.
- Guan, X., Blank, J.L. & Dluzen, D.E. (1993) Role of olfactory bulb norepinephrine in the identification and recognition of chemical cues. *Physiol. Behav.*, **53**, 437–441.
- Guerrini, S., Raybould, H.E., Reeve, J.R. & Sternini, C. (2000) Different galanin receptor subtypes mediate galanin effects on gastric motility in anesthetized rats. *Gastroenterology*, **118**, A877–A877.
- Haberman, R.P., Samulski, R.J. & McCown, T.J. (2003) Attenuation of seizures and neuronal death by adeno-associated virus vector galanin expression and secretion. *Nature Med.*, **9**, 1076–1080.
- Habert-Ortoli, E., Amiranoff, B., Loquet, I., Laburthe, M. & Mayaux, J.F. (1994) Molecular cloning of a functional human galanin receptor. *Proc. Natl Acad. Sci. USA*, **91**, 9780–9783.
- Harding, J.W. & Wright, J.W. (1979) Reversible effects of olfactory nerve section on behavior and biochemistry in mice. *Brain Res. Bull.*, **4**, 17–22.
- Hohmann, J.G., Clifton, D.K. & Steiner, R.A. (1998) Galanin. Analysis of its coexpression in gonadotropin-releasing hormone and growth hormone-releasing hormone neurons. In Hökfelt, T., Bartfai, T. & Crawley, J.N. (Eds), *Galanin: Basic Research Discoveries and Therapeutic Implications*. The New York Academy of Sciences, New York, pp. 221–235.
- Hohmann, J.G., Jureus, A., Teklemichael, D.N., Matsumoto, A.M., Clifton, D.K. & Steiner, R.A. (2003) Distribution and regulation of galanin receptor 1 messenger RNA in the forebrain of wild type and galanin-transgenic mice. *Neuroscience*, **117**, 105–117.
- Hökfelt, T., Bartfai, T. & Crawley, J.N. (1998) *Galanin: Basic Research Discoveries and Therapeutic Implications*. The New York Academy of Sciences, New York.
- Hökfelt, T., Millhorn, D., Seroogy, K., Tsuruo, Y., Ceccatelli, S., Lindh, B., Meister, B., Melander, T., Schalling, M., Bartfai, T. & et al. (1987) Coexistence of peptides with classical neurotransmitters. *Experientia*, **43**, 768–780.
- Holmes, A., Hollon, T.R., Gleason, T.C., Liu, Z., Dreiling, J., Sibley, D.R. & Crawley, J.N. (2001) Behavioral characterization of dopamine D-5 receptor null mutant mice. *Behav. Neuroscience*, **115**, 1129–1144.
- Holmes, A., Kinney, J.W., Wrenn, C.C., Li, Q., Yang, R.J., Ma, L., Vishwanath, J., Saavedra, M.C., Innerfield, C.E., Jacoby, A.S., Shine, J., Iismaa, T.P. & Crawley, J.N. (2003) Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. *Neuropsychopharmacology*, **28**, 1031–1044.
- Holmes, F.E., Mahoney, S., King, V.R., Bacon, A., Kerr, N.C.H., Pachnis, V., Curtis, R., Priestley, J.V. & Wynick, D. (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc. Natl Acad. Sci. USA*, **97**, 11563–11568.
- Holmes, A., Wrenn, C.C., Harris, A.P., Thayer, K.E. & Crawley, J.N. (2002a) Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. *Genes Brain Behav.*, **1**, 55–69.
- Holmes, A., Yang, R.J. & Crawley, J.N. (2002b) Evaluation of an anxiety-related phenotype in galanin overexpressing transgenic mice. *J. Mol. Neurosci.*, **18**, 151–165.
- Howard, A.D., Tan, C., Shiao, L.L., Palyha, O.C., McKee, K.K., Weinberg, D.H., Feighner, S.D., Cascieri, M.A., Smith, R.G., VanDerPloeg, L.H.T. & Sullivan, K.A. (1997) Molecular cloning and characterization of a new receptor for galanin. *FEBS Lett.*, **405**, 285–290.
- Huerta, P.T., Sun, L.D., Wilson, M.A. & Tonegawa, S. (2000) Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons. *Neuron*, **25**, 473–480.
- Impey, S., Smith, D.M., Obrietan, K., Donahue, R., Wade, C. & Storm, D.R. (1998) Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nature Neurosci.*, **1**, 595–601.
- Jacoby, A.S., Hort, Y.J., Constantinescu, G., Shine, J. & Iismaa, T.P. (2002) Critical role for GALR1 galanin receptor in galanin regulation of neuroendocrine function and seizure activity. *Mol. Brain Res.*, **107**, 195–200.
- Kaneko, T. & Thompson, R.F. (1997) Disruption of trace conditioning of the nictitating membrane response in rabbits by central cholinergic blockade. *Psychopharmacology*, **131**, 161–166.
- Kinney, J.W., Starosta, G., Holmes, A., Wrenn, C.C., Yang, R.J., Harris, A.P., Long, K.C. & Crawley, J.N. (2002) Deficits in trace cued fear conditioning in galanin-treated rats and galanin-overexpressing transgenic mice. *Learn. Memory*, **9**, 178–190.
- Koegler, F.H. & Ritter, S. (1998) Galanin injection into the nucleus of the solitary tract stimulates feeding in rats with lesions of the paraventricular nucleus of the hypothalamus. *Physiol. Behav.*, **63**, 521–527.
- Kyrkouli, S.E., Stanley, B.G. & Leibowitz, S.F. (1986) Galanin – stimulation of feeding induced by medial hypothalamic injection of this novel peptide. *Eur. J. Pharmacol.*, **122**, 159–160.
- Kyrkouli, S.E., Stanley, B.G., Seirafi, R.D. & Leibowitz, S.F. (1990) Stimulation of feeding by galanin: anatomical localization and behavioral specificity of this peptide's effects in the brain. *Peptides*, **11**, 995–1001.
- Larm, J.A., Shen, P.-J. & Gundlach, A.L. (2003) Differential galanin receptor-1 and galanin expression by 5-HT neurons in dorsal raphe nucleus of rat and mouse: evidence for species-dependent modulation of serotonin transmission. *Eur. J. Neurosci.*, **17**, 481–493.
- Liu, H.X. & Hökfelt, T. (2000) Effect of intrathecal galanin and its putative antagonist M35 on pain behavior in a neuropathic pain model. *Brain Res.*, **886**, 67–72.
- Liu, H.X. & Hökfelt, T. (2002) The participation of galanin in pain processing at the spinal level. *Trends Pharmacol. Sci.*, **23**, 468–474.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- Luo, A.H., Cannon, E.H., Wekesa, K.S., Lyman, R.F., Vandenbergh, J.G. & Anholt, R.R.H. (2002) Impaired olfactory behavior in mice deficient in the  $\alpha$  subunit of G<sub>o</sub>. *Brain Res.*, **941**, 62–71.
- Ma, X., Tong, Y.-G., Schmidt, R., Brown, W., Payza, K., Hodzic, L., Pou, C., Godbout, C., Hökfelt, T. & Xu, Z.-Q.D. (2001) Effects of galanin receptor agonists on locus coeruleus neurons. *Brain Res.*, **919**, 169–174.
- Mahoney, S.A., Hosking, R., Farrant, S., Holmes, F.E., Jacoby, A.S., Shine, J., Iismaa, T.P., Scott, M.K., Schmidt, R. & Wynick, D. (2003) The second galanin receptor GalR2 plays a key role in neurite outgrowth from adult sensory neurons. *J. Neurosci.*, **23**, 416–421.
- Malin, D., Novy, B., Lett-Brown, A., Plotner, R., May, B., Radulescu, S., Crothers, M., Osgood, L. & Lake, J. (1992) Galanin attenuates one-trial reward learning. *Life Sci.*, **50**, 939–944.
- Mastropaolo, J., Nadi, N.S., Ostrowski, N.L. & Crawley, J.N. (1988) Galanin antagonizes acetylcholine on a memory task in basal forebrain-lesioned rats. *Proc. Natl Acad. Sci. USA*, **85**, 9841–9845.
- Mazarati, A.M., Hohmann, J.G., Bacon, A., Liu, H., Sankar, R., Steiner, R.A., Wynick, D. & Wasterlain, C.G. (2000) Modulation of hippocampal excitability and seizures by galanin. *J. Neurosci.*, **20**, 6276–6281.
- McDonald, M.P. & Crawley, J.N. (1996) Galanin receptor antagonist M40 blocks galanin-induced choice accuracy deficits on a delayed-nonmatching-to-position task. *Behav. Neurosci.*, **110**, 1025–1032.
- McEchron, M.D., Bouwmeester, H., Tseng, W., Weiss, C. & Disterhoft, J.F. (1998) Hippocampectomy disrupts auditory trace fear conditioning and contextual fear conditioning in the rat. *Hippocampus*, **8**, 638–646.
- McEchron, M.D., Tseng, W. & Disterhoft, J.F. (2003) Single neurons in CA1 hippocampus encode trace interval duration during trace heart rate (fear) conditioning in rabbit. *J. Neurosci.*, **23**, 1535–1547.
- Mennicken, F., Hoffert, C., Pelletier, M., Ahmad, S. & O'Donnell, D. (2002) Restricted distribution of galanin receptor 3 (GalR3) mRNA in the adult rat central nervous system. *J. Chem. Neuroanatomy*, **24**, 257–268.
- Moyer, J.R., Deyo, R.A. & Disterhoft, J.F. (1990) Hippocampectomy disrupts trace eye-blink conditioning in rabbits. *Behav. Neurosci.*, **104**, 243–252.
- O'Donnell, D., Ahmad, S., Wahlestedt, C. & Walker, P. (1999) Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: distinct distribution from GALR1. *J. Comp. Neurol.*, **409**, 469–481.
- O'Meara, G., Coumis, U., Ma, S.Y., Kehr, J., Mahoney, S., Bacon, A., Allen, S.J., Holmes, F., Kahl, U., Wang, F.H., Kearns, I.R., Ove-Ögren, S., Dawbarn, D., Mufson, E.J., Davies, C., Dawson, G. & Wynick, D. (2000) Galanin regulates the postnatal survival of a subset of basal forebrain cholinergic neurons. *Proc. Natl Acad. Sci. USA*, **97**, 11569–11574.
- Ögren, S.O., Kehr, J. & Schott, P.A. (1996) Effects of ventral hippocampal galanin on spatial learning and on in vivo acetylcholine release in the rat. *Neuroscience*, **75**, 1127–1140.
- Pang, L., Hashemi, T., Lee, H.J.J., Maguire, M., Graziano, M.P., Bayne, M., Hawes, B., Wong, G. & Wang, S.K. (1998) The mouse GalR2 galanin receptor: genomic organization, cDNA cloning, and functional characterization. *J. Neurochem.*, **71**, 2252–2259.
- Paylor, R., Nguyen, M., Crawley, J.N., Patrick, J., Beaudet, A. & Orr-Urtreger, A. (1998) alpha 7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice. *Learn. Memory*, **5**, 302–316.
- Pieribone, V.A., Xu, Z.Q., Zhang, X., Grillner, S., Bartfai, T. & Hökfelt, T. (1995) Galanin induces a hyperpolarization of norepinephrine-containing locus coeruleus neurons in the brainstem slice. *Neuroscience*, **64**, 861–874.
- Robinson, J.K. & Crawley, J.N. (1993) Intraventricular galanin impairs delayed nonmatching-to-sample performance in rats. *Behav. Neurosci.*, **107**, 458–467.

- Ryou, J.W., Cho, S.Y. & Kim, H.T. (2001) Lesions of the entorhinal cortex impair acquisition of hippocampal-dependent trace conditioning. *Neurobiol. Learn. Memory*, **75**, 121–127.
- Schellinck, H.M., West, A.M. & Brown, R.E. (1992) Rats can discriminate between the urine odors of genetically identical mice maintained on different diets. *Physiol. Behav.*, **51**, 1079–1082.
- Seager, M.A., Asaka, Y. & Berry, S.D. (1999) Scopolamine disruption of behavioral and hippocampal responses in appetitive trace classical conditioning. *Behav. Brain Res.*, **100**, 143–151.
- Shors, T.J., Townsend, D.A., Zhao, M.R., Kozorovitskiy, Y. & Gould, E. (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus*, **12**, 578–584.
- Smith, K.E., Walker, M.W., Artymyshyn, R., Bard, J., Borowsky, B., Tamm, J.A., Yao, W.J., Vaysse, P.J.J., Branchek, T.A., Gerald, C. & Jones, K.A. (1998) Cloned human and rat galanin GALR3 receptors – pharmacology and activation of G-protein inwardly rectifying K<sup>+</sup> channels. *J. Biol. Chem.*, **273**, 23321–23326.
- Solomon, P.R., Vanderschaaf, E.R., Thompson, R.F. & Weisz, D.J. (1986) Hippocampus and trace conditioning of the rabbits classically conditioned nictitating-membrane response. *Behav. Neurosci.*, **100**, 729–744.
- Stefani, M. & Gold, P. (1998) Intra-septal injections of glucose and glibenclamide attenuate galanin-induced spontaneous alternation performance deficits in the rat. *Brain Res.*, **813**, 50–56.
- Steiner, R.A., Hohmann, J.G., Holmes, A., Wrenn, C.C., Cadd, G., Jureus, A., Clifton, D.K., Luo, M., Gutshall, M., Ma, S.Y., Mufson, E.J. & Crawley, J.N. (2001) Galanin transgenic mice display cognitive and neurochemical deficits characteristic of Alzheimer's disease. *Proc. Natl Acad. Sci. USA*, **98**, 4184–4189.
- Stowers, L., Holy, T.E., Meister, M., Dulac, C. & Koentges, G. (2002) Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science*, **295**, 1493–1500.
- Sutherland, R.J. & McDonald, R.J. (1990) Hippocampus, amygdala, and memory deficits in rats. *Behav. Brain Res.*, **37**, 57–79.
- Tatemoto, K., Rokaeus, A., Jornvall, H., McDonald, T.J. & Mutt, V. (1983) Galanin – a novel biologically active peptide from porcine intestine. *FEBS Lett.*, **164**, 124–128.
- Wang, S., Hashemi, T., He, C.G., Strader, C. & Bayne, M. (1997a) Molecular cloning and pharmacological characterization of a new galanin receptor subtype. *Mol. Pharmacol.*, **52**, 337–343.
- Wang, S.K., He, C.G., Hashemi, T. & Bayne, M. (1997b) Cloning and expression characterization of a novel galanin receptor – identification of different pharmacophores within galanin for the three galanin receptor subtypes. *J. Biol. Chem.*, **272**, 31949–31952.
- Wang, S.K., He, C.G., Maguire, M.T., Clemmons, A.L., Burrier, R.E., Guzzi, M.F., Strader, C.D., Parker, E.M. & Bayne, M.L. (1997c) Genomic organization and functional characterization of the mouse GalR1 galanin receptor. *FEBS Lett.*, **411**, 225–230.
- Waters, S.M. & Krause, J.E. (2000) Distribution of galanin-1,-2 and-3 receptor messenger RNAs in central and peripheral rat tissues. *Neuroscience*, **95**, 265–271.
- Wehner, J.M., Radcliffe, R.A., Rosmann, S.T., Christensen, S.C., Rasmussen, D.L., Fulker, D.W. & Wiles, M. (1997) Quantitative trait locus analysis of contextual fear conditioning in mice. *Nature Genet.*, **17**, 331–334.
- Weiss, C., Bouwmeester, H., Power, J.M. & Disterhoft, J.F. (1999) Hippocampal lesions prevent trace eyeblink conditioning in the freely moving rat. *Behav. Brain Res.*, **99**, 123–132.
- Wrenn, C.C. & Crawley, J.N. (2001) Pharmacological evidence supporting a role for galanin in cognition and affect. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, **25**, 283–299.
- Wrenn, C.C., Harris, A.P., Saavedra, M.C. & Crawley, J.N. (2003) Social transmission of food preference in mice: methodology and application to galanin-overexpressing transgenic mice. *Behav. Neurosci.*, **117**, 21–31.
- Wynick, D., Priestley, J.V., McMahon, S.B., Mufson, E., Davies, C. & Dawson, G. (1999) Galanin is a growth factor to the central and peripheral nervous system. *Brain Res.*, **848**, A15–A16.
- Zhang, X., Xu, Z.Q., Shi, T.J., Landry, M., Holmberg, K., Ju, G., Tong, Y.G., Bao, L., Cheng, X.P., Wiesenfeld-Hallin, Z., Lozano, A., Dostrovsky, J. & Hökfelt, T. (1998) Regulation of expression of galanin and galanin receptors in dorsal root ganglia and spinal cord after axotomy and inflammation. In Hökfelt, T., Bartfai, T. & Crawley, J.N. (Eds), *Galanin: Basic Research Discoveries and Therapeutic Implications*. The New York Academy of Sciences, New York, pp. 402–413.