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GAL$_3$ receptor knockout mice exhibit an alcohol-preferring phenotype

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ABSTRACT

Galanin is a neuropeptide which mediates its effects via three G-protein coupled receptors (GAL_{1-3}). Administration of a GAL_3 antagonist reduces alcohol self-administration in animal models while allelic variation in the *GAL_3 gene* has been associated with an increased risk of alcohol use disorders in diverse human populations. Based on the association of GAL_3 with alcoholism, we sought to characterise drug-seeking behavior in *GAL_3*-deficient mice for the first time. In the two-bottle free choice paradigm, *GAL_3*-KO mice consistently showed a significantly increased preference for ethanol over water when compared to wildtype (WT) littermates. Furthermore, male *GAL_3*-KO mice displayed significantly increased responding for ethanol under operant conditions. These differences in alcohol seeking behavior in *GAL_3*-KO mice did not result from altered ethanol metabolism. In contrast to ethanol, *GAL_3*-KO mice exhibited similar preference for saccharin and sucrose over water; and a similar preference for a high fat diet over a low fat diet as WT littermates. No differences in cognitive and locomotor behaviors were observed in *GAL_3*-KO mice to account for increased alcohol seeking behavior. Overall, these findings suggest genetic ablation of *GAL_3* in mice increases alcohol consumption.

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INTRODUCTION

Galanin (GAL) is a 29 amino acid neuropeptide (30 in humans) which has a widespread presence in the peripheral and central nervous systems, including the kidney, stomach, lung, spinal cord and brain (Branchek *et al.* 2000; Lang *et al.* 2015). Due to this diverse distribution, GAL has been implicated in an array of physiological functions and behaviors including gastrointestinal motility (Anselmi *et al.* 2005), neuroendocrine function (Melander *et al.* 1987), feeding (Fang *et al.* 2012), and anxiety and depression (Fang *et al.* 2012; Swanson *et al.* 2005).

There are currently three known G-protein coupled GAL receptor subtypes, GAL receptor-1 (GAL_1), GAL receptor-2 (GAL_2), and GAL receptor-3 (GAL_3) (Burgevin *et al.* 1995; Wang *et al*. 1997a; Wang *et al.* 1997b). These receptors each have a varied distribution in the body and preferentially bind to different fragments of the GAL peptide (Lang *et al.* 2015). As such, these receptors have been individually implicated in different physiological actions of GAL (Branchek *et al.* 1998; Webling *et al*. 2012).

The GAL peptide and receptors are found in regions of the brain with important implications in affective disorders, learning and memory processes, as well as the formation and maintenance of drug dependence, specifically the ventral tegmental area (VTA), amygdala (AMG), hippocampus (HIP), nucleus accumbens (NAc), and locus coeruleus (LC; Barreda-Gómez *et al.* 2005; Lu *et al*. 2005; Waters & Krause 1999). GAL receptors act at these regions to modulate neurotransmitter release, for example, preventing noradrenaline

release in the LC, inhibiting serotonin (5-HT) function in the dorsal raphe nucleus, and selectively stimulating dopaminergic activity in the VTA (Ericson & Ahlenius 1999; Hökfelt *et al.* 1998; Pieribone *et al.* 1995). Thus, the GAL system has been investigated in regards to affective disorders.

Central administration of the active N terminal fragment GAL(1-15) was found to induce anxiogenic and depressent-like behaviours in rats, as indicated by the open field, forced swim and tail suspension tests (Millon *et al.* 2014). Further, a study by Swanson and colleagues revealed that rats treated with 30 mg/kg of the GAL_3 selective antagonist, SNAP 37889, via intraperitoneal injection for 14 days, displayed anxiolytic- and antidepressant-like behavior in the social interaction and forced swim tests respectively (Swanson *et al.* 2005). It has been proposed that anxiety and depression have a shared neurobiology with addiction due to the brain regions involved as well as the high co-morbidity of mood disorders with substance dependence (Koob 2008). Low blood alcohol concentrations generally contribute to a decrease in anxiety and have subsequently earned alcoholic beverages a reputation as a 'social lubricant', which may explain the high correlation between anxiety and alcohol consumption (Koob 2014; Zhao *et al.* 2013). Recent investigations have further described a role of GAL in alcohol use disorder.

After an initial study revealed that haplotypes of GAL were associated with alcohol use disorder (Belfer *et al.* 2006), the *GAL_3* gene was implicated in alcohol addiction among two ethnically and geographically diverse human populations (Belfer *et al.* 2007). Of the GAL receptors, only a single nucleotide polymorphism (SNP) of *GAL_3* conferred susceptibility to

alcohol use disorders by an increased odds ratio of 2.4 (Belfer *et al.* 2007). Combination of this SNP with GAL risk haplotypes increases the odds ratio of developing alcohol use disorder by 2.4, while the SNP of *GAL$_3$* in conjunction with GAL risk diplotypes increased this odds ratio to 4.6 (Belfer *et al.* 2007).

Work in our laboratory has since shown that rats treated with the GAL_3 selective antagonist, SNAP 37889 (30 mg/kg, i.p.), significantly reduced lever pressing under operant conditions indicating a reduced motivation to acquire alcohol (Ash *et al.* 2011; Ash *et al.* 2014). A similar study in mice yielded concurrent results (Scheller *et al.* 2017) which, taken together, support a role of GAL_3 in alcohol dependence.

Given the recent availability of *GAL$_3$*-KO mice (Brunner *et al.* 2014), the aim of the current study was to investigate alcohol-seeking behavior in *GAL$_3$*-deficient mice. Previous characterization of *GAL$_3$*-KO mice revealed they exhibit an anxiogenic phenotype with normal development, growth and reproduction (Brunner *et al.* 2014). We sought to further comprehensively characterize *GAL$_3$*-KO mice using a battery of behavioral tests for cognition and psychosis-like behavior given the overlapping neurochemical circuitry between addiction and psychosis.

MATERIALS AND METHODS

Animals

GAL3-KO mice were originally obtained from the Paracelsus Medical University in Salzburg, Austria (Brunner *et al.* 2014) and a breeding colony was established at the La Trobe Animal

Research and Teaching Facility, Melbourne, Australia. All mice were genotyped by Transnetyx (Cordova, TN, USA). Male and female *GAL$_3$*-KO mice and WT littermates aged 10-13 weeks were used for all experiments. Mice were familiarized to the experimenter by regular handling and to the laboratory conditions (relative humidity 40-50%, temperature 20 ± 1°C) for 1 week prior to any behavioral testing. All mice had *ad libitum* access to food and water throughout the study. Mice participating in behavioral characterization tests (n=192) were housed (maximum of 5 per cage) in individually ventilated cages (IVC, Techniplast, Buguggiate, Italy) under normal lighting conditions (12-hour light/dark cycle with lights on 7.00 – 19.00). Mice were divided into 4 cohorts of 48 (n=12/sex/genotype in each cohort) for testing in different behavioral paradigms with at least a week break between tests. Cohort 1 underwent Y maze, social interaction, prepulse inhibition and two-bottle free choice testing for ethanol; cohort 2 was assessed using elevated plus-maze, fear conditioning and locomotor activity protocols; cohort 3 completed two-bottle free choice testing for saccharin and sucrose; and cohort 4 were assessed for preference of a high fat (HFD) versus low fat diet (LFD).

The operant self-administration cohort consisted of an additional 25 mice (7 male *GAL$_3$*-KO, 5 male WT, 6 female *GAL$_3$*-KO, 7 female WT) which were housed under reverse light cycle conditions (12-hour light/dark cycle with lights on 19.00 – 7.00).

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Treatments

Apomorphine, a dopamine receptor agonist (3 mg/kg), and MK-801, a glutamate receptor antagonist (0.2 mg/kg), were obtained from Sigma Aldrich (St Louis, MO, USA). Methamphetamine (1 mg/kg and 3 mg/kg) was sourced from the National Measurement Institute (Sydney, Australia). All injections were delivered i.p. using Terumo 26 gauge needles and 1 mL syringes.

Alcohol Self-Administration

Two-Bottle Free Choice

After completing a battery of behavioral tests, mice were transferred to open top cages and singly housed one week prior to administration of any experimental solutions. During this habituation period two identical bottles (Techniplast, Italy) filled with tap water were placed on the cage. After acclimatization, the first cohort had one water bottle replaced with a bottle containing a 5% v/v ethanol solution diluted from 100% ethanol (AR grade, Univar, Redmond, WA, USA) in tap water. Both bottles were weighed daily to the nearest 0.1 g, Monday to Friday, at approximately 2 pm. Daily positioning of bottles were put back randomly to avoid place-preference. Data were collected for 10 days, after which the 5% ethanol solution was replaced with a 10% ethanol solution. This continued until data were collected for a further 10 days for each of the test concentrations of ethanol (5%, 10%, 15% and 20%). A second cohort of mice were tested for saccharin and sucrose preference. After

the initial week of habituation, one of the bottles on the cage was replaced with one containing 0.1% saccharin for 10 days, after which the saccharin was replaced with a second water bottle for 10 days. The second water bottle was then replaced with a bottle containing 5% sucrose and both bottles were weighed daily for 10 days.

Diet Preference

Mice were singly housed and given ad libitum access to pre-weighed high fat diet (HFD; SF04-001, Specialty Feeds, Glen Forrest, Western Australia) and low fat diet (LFD; SF13-081, Specialty Feeds, Glen Forrest, Western Australia) food pellets for 14 days. Each diet was placed at opposite ends of the feeder and the side was alternated daily. Remaining food was weighed at approximately 3pm each day, diets were replenished, and mice weighed daily.

Operant Responding

Operant chambers (Med Associates Inc., Fairfax, VT, USA), were used to test motivation to obtain alcohol as previously described (Walker *et al.* 2015). Briefly, mice underwent 90-min operant sessions 5 times per week. A drop of vanilla essence was positioned beneath the floor of the chamber underneath the active lever and a light was used to indicate reward delivery, providing an olfactory cue and a visual cue, respectively. The first 3 days involved a single lever which the mice had to press once for each reward delivery of 10% w/v sucrose (5 µL over 1.7-sec). The following 5 days required the mice to distinguish between the active lever, which dispensed rewards, and an inactive lever, which resulted in no reward delivery.

Mice that correctly distinguished between the active and inactive lever for at least 60% of lever presses were allowed to continue in the study. Of the 87 mice screened, 25 mice (n=7 male *GAL$_3$*-KO, n=5 male WT, n=6 female *GAL$_3$*-KO, n=7 female WT) reached criteria and proceeded through the full protocol while the remaining mice were excluded from further analysis. A sucrose fade protocol gradually incorporated ethanol into the reward solution starting with 4 days of 5% sucrose, 5% ethanol; 3 days with 2% sucrose, 7.5% ethanol; 3 days with 2% sucrose, 10% ethanol; and finally, 4 days with 10% ethanol and no sucrose. Mice then began lever pressing at a fixed ratio of 3 (FR3) for 20 sessions after which they had a single session of progressive ratio. During this session, the number of lever presses required to obtain reward increased incrementally with each reward delivery.

Alcohol Metabolism

A separate cohort of mice (n=6/sex/genotype) were injected with 20% ethanol (volume equivalent to 1% of body weight) 5 hours after light onset. Blood samples were taken via tail bleed 1, 2 and 3 hours post-injection and stored in heparinized capillaries. Samples were centrifuged (3000 rpm for 15-min at 4°C) and plasma was collected and frozen until further analysis. Blood ethanol concentration was measured by an Analox Instruments (Stourbridge, UK) GL5 analyzer against ethanol standards.

Behavioural Testing

Y-Maze

Y-maze testing was conducted using a grey plexiglass Y-maze which consisted of three arms measuring 10.5 x 31.5 x 15.5 cm (width x length x height) with each arm set at a 120-degree angle from the next, as previously described (Jaehne *et al.* 2017). Briefly, the Y-maze was setup in a quiet, isolated room under normal lighting conditions. For the acquisition phase, mice were placed at the distal end of the start arm, and allowed to explore the start and an open (familiar) arm for 10-min, with the remaining arm blocked by a plexiglass barrier. After a 1-hour inter-trial interval (ITI), mice were returned to the maze for a second trial of 5-min in which they were free to explore all three maze arms. Ethovision XT software (Noldus Information Technology, Wageningen, The Netherlands) analyzed the movements of each mouse for time spent in each arm.

Social Interaction

A custom-made acrylic social interaction chamber was used, measuring 43 x 64 x 22.5 cm (width x length x height) and separated into three equal compartments by acrylic walls, with entryways allowing mice access into each compartment. Two 'stranger' cages, measuring 10 x 9 cm (height x diameter), were placed in the left and right compartments and each was weighed down by an 8 cm high ceramic cup. Social interaction testing was adapted from a protocol previously described (Jaehne *et al.* 2017). Briefly, the test mouse was placed in the center compartment and allowed to explore all three chambers for 5-min. The mouse was then returned to the center compartment while a stranger mouse was then placed in the stranger cage in either the left or right compartment. The test mouse was then allowed to

explore all three compartments for a further 5-min. The test mouse was again returned to the center compartment while a second, novel, stranger mouse was moved into the empty stranger cage. The test mouse was free to explore all compartments for another 5-min. Ethovision XT software (Noldus Information Technology, Wageningen, The Netherlands) analyzed the time spent in each compartment, as well as time spent in the immediate vicinity (within a 2.5 cm radius) of each stranger cage.

Prepulse Inhibition of Acoustic Startle

Prepulse inhibition (PPI), a measure of sensorimotor gating which is disrupted in psychotic illness, was completed as previously described (Manning & van den Buuse, 2013) to assess genotype differences during sensorimotor gating. Startle response was measured using SR-LAB startle chambers (San Diego Instruments, San Diego, CA, USA). The sound-attenuating isolation chamber consisted of a 12.7 x 3.81 cm (length x diameter) acrylic cylinder sitting on a platform connected to a piezoelectric transducer to measure whole body startle in response to acoustic noise bursts. Mice underwent a pre-test to obtain baseline data and habituate mice to the enclosures prior to drug trials. Three days later, mice were randomly assigned to receive an injection of saline, apomorphine (3 mg/kg) or MK-801 (0.2 mg/kg). MK-801 was administered 20-min prior to testing while apomorphine was administered immediately before mice were placed in the chambers. Half of the saline-injected mice were randomly assigned for administration 20-min prior to testing while the remaining mice received the saline injection immediately prior to allow for any variation in results based on

timing of injection. There were at least 3 days between all testing to allow for wash-out of any remaining drug. Each PPI session consisted of 104 randomized trials running an average length of 35-min. Each session included 8 no-stimulus trials, 32 pulse-alone trials, and 64 prepulse-pulse trials. The prepulse-pulse trials involved 8 trials at each prepulse intensity of 2, 4, 8 or 16 decibels (dB) above the 70 dB background noise followed 30 or 100-msec afterwards by a 115 dB startle pulse. ITI ranged from 12-28-sec to prevent a habituated response to startle.

Fear Conditioning

Fear memory was measured over 3 consecutive days using fear conditioning chambers from Med Associates Inc. (Fairfax, VT, USA), as previously described (Jaehne *et al*. 2017). Briefly, mice were randomly assigned to one of two contexts, which differed by lux, scent, bedding, and structure of chamber. During the first 6-min session, mice were placed in the chamber and presented with three pairings of the conditioned stimulus (tone, 30-sec duration, 7500 Hz, 70 dB) and unconditioned stimulus (foot-shock, 1-sec duration, 0.7 mA). There was a 30-sec ITI between each presentation of the conditioned and unconditioned stimulus combination. The next day, mice were returned to the same context in which they were conditioned. No stimuli were presented and the amount of time freezing was measured, with freezing interpreted as a complete lack of movement for at least 1-sec, excluding respiration. Activity was recorded and quantified using Video Freeze software (Med Associates Inc.). During the final session, mice were placed in the alternate context and

were presented three times with the conditioned tone stimulus. Freezing behavior was measured.

Methamphetamine-Induced Locomotor Hyperactivity

Psychotomimetic drug-induced locomotor hyperactivity, a measure of psychosis-like behavior and subcortical dopaminergic hyperactivity, was assessed over three sessions using 27 x 27 x 40 cm (width x length x height) locomotor photocell arenas (Med Associates Inc., Fairfax, VT, USA). Protocol was adapted from that previously described (Jaehne *et al.* 2017). During each 2-hr session, mice were placed in the arena and baseline activity was recorded for 30-min. Mice were then removed from the arena and injected with saline (5 mL/kg), low dose methamphetamine (1 mg/kg), and high dose methamphetamine (3 mg/kg) in consecutive sessions. Mice were immediately returned to the arena to explore for a further 90-min. Photocells recorded and analyzed horizontal movement and expressed data as distance moved (in cm) per 5-min interval. A minimum 4-day gap was allowed between sessions to ensure the wash-out of any residual drug.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 24 (Armonk, New York, NY, USA). Results were assessed for differences between sexes or genotypes via analysis of variance (ANOVA), with repeated measures where applicable. If a statistically significant main effect of sex was not observed, male and female data sets were combined. Graphs

were generated using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, California, USA). Data are expressed as the mean ± standard error of the mean (SEM) and a value of $p<0.05$ was considered to be of statistical significance.

RESULTS

GAL$_3$-KO mice display a selective increase in preference for ethanol

GAL$_3$-KO mice were analyzed for alcohol preference using a continuous-access, two-bottle free choice paradigm. Analysis of ethanol intake revealed a main effect of sex [$F_{(1,43)} = 32.40$, $p<0.0001$), as well as a sex x genotype interaction [$F_{(1,43)} = 5.53$, $p=0.023$], therefore, data were assessed separately for male and female mice.

Male *GAL$_3$*-KO mice consumed a comparable amount of ethanol to WT littermates at all concentrations tested (Fig. 1a), however they consumed significantly less water when given 10% or 20% ethanol (see Fig. S1). Conversely, ethanol intake of female *GAL$_3$*-KO mice revealed a main effect of genotype [$F_{(1, 43)} = 11.45$, $p=0.02$; Fig. 1b], indicating a significantly increased intake of ethanol compared to WT littermates for all concentrations assessed, with no interaction between concentration and genotype. Both male [$F_{(1,18)} = 40.79$, $p<0.001$] and female [$F_{(1,18)} = 95.16$, $p<0.001$] *GAL$_3$*-KO mice displayed a significantly increased preference for ethanol when compared to WT littermates at all concentrations tested (Fig. 1c and d).

A separate cohort of mice underwent further two-bottle free choice testing with saccharin and sucrose. Analysis revealed no statistical interactions with sex, therefore,

further analyses were run with male and female data combined. Average intake of both saccharin and sucrose solutions were not statistically different across genotypes (Fig. 2a). Similarly, analysis of preference revealed no genotype difference for either solution (Fig. 2b).

Mice were also assessed for HFD versus LFD preference. Analysis revealed no main effect of sex, thus male and female data were assessed collectively. All mice, regardless of genotype, displayed a significant preference for a HFD over LFD (Fig. 2c) with no significant genotype difference in body weight (Fig. 2d).

GAL_3-KO mice show an increased self-administration of ethanol

Operant responding was used to investigate differences between GAL_3-KO mice and WT littermates in motivation to obtain alcohol. After an initial period of training, mice maintained stable responding on a fixed ratio-3 (FR3) schedule, where 3 lever presses delivered one reward. Analysis revealed a main effect of sex during this period [$F_{(1,21)} = 5.89$, p=0.024], therefore, male and female data was analyzed separately. Male GAL_3-KO mice pressed significantly more on the active lever than WT littermates during stable responding [$F_{(1,345)} = 6.57$, p=0.01, Fig. 3a], while no significant differences were observed between female GAL_3-KO and WT mice (Fig. 3b). A single session of progressive ratio found no genotype difference in either male (Fig. 3c) or female (Fig. 3d) mice in motivational breakpoint for ethanol.

Alcohol metabolism is not impacted by *GAL$_3$* absence

Blood ethanol concentrations were analyzed to account for any difference in alcohol metabolism in determining alcohol preference in an additional cohort of *GAL$_3$*-KO mice. Analysis revealed no main effect of sex, thus male and female data were assessed collectively. A comparable rate of alcohol breakdown was observed after receiving an acute dose of 20% ethanol for both *GAL$_3$*-KO and WT mice (Fig. 4).

***GAL$_3$* ablation does not affect spatial memory, sociability, emotional memory or locomotor activity**

Statistical analysis of behavioural data revealed no main effect of sex, therefore, male and female data were combined for y-maze, social interaction, fear conditioning and locomotor activity analyses.

The Y-maze test was used to determine any genotype differences between *GAL$_3$*-KO mice and their WT littermates in short-term spatial memory. Mice were assessed for time spent in the novel versus familiar arm. Time spent in the home arm was excluded from analysis as all mice began trials in the same arm. Time spent in the individual test arms of the Y-maze revealed a main effect of arms [$F_{(1, 44)}$ = 26.34, p<0.001], indicating a significant preference for the novel arm over the familiar arm, with no interaction between time in arms and genotype (Fig. 5a).

A social interaction test was conducted to determine if sociability and social novelty preference differed between *GAL$_3$*-KO mice and WT littermates. During the initial trial of

sociability, a main effect of compartment was observed with both *GAL$_3$*-KO and WT mice showing a significant preference for interaction with the stranger mouse over the empty stranger cage [$F_{(1,44)}$ = 157.65, p<0.001, Fig. 5b]. Further, during the social novelty preference trial, all mice spent significantly more time interacting with the novel stranger mouse (WT 43.43 ± 2.65, *GAL$_3$*-KO 44.34 ± 3.31 s) when compared to the familiar stranger mouse (WT 27.08 ± 2.69, *GAL$_3$*-KO 30.43 ± 2.86 s), regardless of genotype [main effect of social zone, $F_{(1, 46)}$ = 26.74, p<0.001, data not shown].

Differences in fear memory were investigated using a fear conditioning protocol. No significant alterations were found in the percentage of time freezing during context or tone memory trials between WT and *GAL$_3$*-KO mice (Fig. 5c).

Mice were studied for any genotype differences in methamphetamine-induced locomotor activity. A main effect of treatment was observed, indicating a significant methamphetamine dose-dependent increase in average distance travelled by both *GAL$_3$*-KO and WT mice [$F_{(2,88)}$=13.25, p<0.001, Fig. 5d]. However, there was no significant interaction of treatment and genotype indicating *GAL$_3$*-KO had no effect on the response to methamphetamine.

Sensorimotor gating is normal in *GAL$_3$* KO mice

PPI was used to examine any genotype differences in sensorimotor gating between *GAL$_3$*-KO mice and WT controls, as well as the response to apomorphine, a dopamine receptor agonist, and MK-801, a glutamate receptor antagonist. Analysis revealed a significant main

effect of sex at the 30 ms inter-stimulus interval [$F_{(1,43)}$ = 4.17, p=0.047] reflecting that females had lower PPI than males, however, no significant interactions of sex with either genotype, treatment or prepulse level were observed. Thus, data for male and female mice were combined for further analysis. A main effect of treatment was detected for saline versus apomorphine [$F_{(1,44)}$ = 25.52, p<0.001 for 30 ms ISI; $F_{(1,43)}$ = 10.57, p=0.002 for 100 ms ISI] and saline versus MK-801 [$F_{(1,44)}$ = 27.54, p<0.001 for 30 ms ISI; $F_{(1,43)}$ = 15.82, p<0.001 for 100 ms ISI]. However, no significant genotype differences were observed after treatment with saline, apomorphine and MK-801 either at inter-stimulus intervals of 30 ms or 100 ms (Fig. 6a and b).

DISCUSSION

Alcohol Self-Administration

The main findings of the current study were that *GAL*$_3$-KO mice displayed an alcohol-preferring phenotype. *GAL*$_3$-KO mice given free access to ethanol during a two-bottle free choice paradigm showed a significant preference for ethanol over water when compared to WT littermates. Operant self-administration results were concurrent with these findings as *GAL*$_3$-KO male mice displayed significantly increased responding for ethanol than WT littermates.

Further investigation found no genotype difference in preference for sucrose, saccharin or a high fat diet. This implies that the increased preference observed in *GAL*$_3$-KO mice is specific for ethanol. Blood samples collected 1, 2 and 3 hours after acute ethanol exposure

demonstrated no difference in alcohol metabolism between *GAL$_3$*-KO and WT mice, indicating that this difference in consumption is also not the result of enhanced breakdown of alcohol in *GAL$_3$*-KO animals.

A genetic association study revealed variation in the *GAL$_3$* gene specifically appears to influence alcohol dependence in two ethnically and geographically diverse populations (Belfer *et al.* 2007). Since this discovery, several studies have investigated the effect of pharmacologically blocking *GAL$_3$* on voluntary self-administration of ethanol. We have previously demonstrated that, in rats, treatment with SNAP 37889 decreased operant responding for ethanol compared to vehicle treatment (Ash *et al.* 2011). Further, SNAP 37889 treatment reduced breakpoint under progressive ratio as well as significantly decreasing relapse response to cue-induced reinstatement, indicative of a decreased motivation to obtain ethanol (Ash *et al.* 2014). Similar results were also observed when SNAP 37889 was administered to mice during a scheduled high alcohol consumption paradigm (Scheller *et al.* 2017).

The findings of the present study using a genetic knockout model were in contrast to those found when pharmacologically blocking *GAL$_3$* with SNAP 37889. One potential explanation for this disparity may be a compensatory increase in GAL peptide abundance in response to the absence of *GAL$_3$* in these KO mice (GAL is well documented to stimulate the consumption of alcohol in animal models, discussed further below), a phenomenon not uncommon in KO lines (Carter & Shieh 2010). However, previous studies on GAL peptide expression in *GAL$_3$*-KO and WT mice revealed no difference in GAL expression in six brain

regions assessed, including the hypothalamus, hippocampus and AMG (Brunner *et al.* 2014). These findings therefore do not support this idea. In addition, no differences were found in GAL_1 and GAL_2 receptor expression in *GAL*$_3$-KO and WT mice, nor were there any differences found in the related 5-HT system (Brunner *et al*. 2014), which has also been implicated in alcohol seeking and dependence (Hoplight *et al.* 2006; Wang *et al.* 2017). In addition, the effects of *GAL*$_3$ ablation from conception on alcohol dependence may differ from acute *GAL*$_3$ antagonism using a pharmacological agent in adult rodents.

Interestingly, a recent study showed that central GAL(1–15) administration decreased voluntary intake of ethanol in rats, an effect thought to be mediated by the GAL_2 receptor since this effect was blocked by the specific GAL_2 antagonist, M871 (Millon *et al.* 2017). While the results exclude compensatory mechanisms of the GAL and 5-HT systems (such as GAL_2) in mediating the increase seen in alcohol-seeking in the current study, they do not eliminate the possibility that other perturbations may have occurred in other neurochemical systems that could contribute to this alcohol-preferring phenotype, such as dopaminergic and glutamatergic systems, both of which are known to modulate alcohol consumption (Ding *et al.* 2013; Trantham-Davidson & Chandler 2015). While preliminary findings in the locomotor activity and PPI tests indicate no alterations in the dopaminergic and glutamatergic systems respectively, further investigation is required to confirm these pathways are not impacted by *GAL*$_3$ ablation. Given the novel finding that *GAL*$_3$-KO mice show an alcohol-preferring phenotype, further research is required to dissect the neurochemical basis of this phenotype.

A number of interesting sex differences in the alcohol self-administration paradigms were observed in the current study. Female *GAL$_3$*-KO mice showed significantly increased intake and preference for ethanol during the two-bottle free choice test at all concentrations assessed, unlike males who only revealed preference for ethanol. Similarly, statistical analysis revealed that female mice of both genotypes operant responded significantly more for ethanol than male mice. It is possible that sex hormones may have played a role in the increased intake of ethanol observed in the female *GAL$_3$*-KO mice. Estrogen, for example, has a well-documented stimulatory effect on GAL expression (Kaplan *et al.* 1988; Vrontakis *et al.* 1989). One such study found that ovariectomised female rats and male rats treated with a therapeutic dose of estrogen (17ß-estradiol) exhibited an up to 4000-fold increase in GAL expression in the anterior pituitary (Kaplan *et al.* 1988). Several reports have since supported this finding, with each displaying a significantly increased expression of the GAL peptide compared to control mice (Horvath *et al.* 1995; Shen *et al.* 1998). This theory requires further investigation; however, it still does not take into account the increased preference for ethanol observed in male *GAL$_3$*-KO mice during the two-bottle free choice paradigm.

Intake of ethanol increases expression of the GAL peptide in rats (Leibowitz *et al.* 2003), and this increase in GAL augmented ethanol consumption. The paraventricular nucleus (PVN) and dorsomedial nucleus were revealed as being highly receptive to the stimulatory effects of ethanol on GAL when compared to other hypothalamic nuclei (Leibowitz *et al.* 2003). The PVN in particular has been implicated in the relationship between GAL and fat

intake (Barson & Leibowitz 2016). Alcohol is the only drug of abuse that has a caloric content and the consumption of alcohol results in an increase in circulating lipids as seen in high fat diets (Chang *et al.* 2007). This suggests the proposed positive feedback loop between GAL and ethanol share similar underlying mechanisms (Leibowitz 2007). This is of particular interest as increased circulating triglyceride levels have previously been described in *GAL$_3$*-KO mice (Brunner *et al.* 2014). Taken together, the increased triglyceride levels in *GAL$_3$*-KO mice may feed the cycle between GAL expression and ethanol consumption, negating the expected reduction of ethanol intake in the *GAL$_3$*-KO mice.

Behavioral Phenotyping

An initial investigation utilizing the *GAL$_3$*-KO mice revealed these mice display an anxiety-like phenotype, as determined by elevated plus maze, open field and light/dark box tests. Alcohol use disorders are highly co-morbid with anxiety, with data showing an increased prevalence of alcohol abuse among individuals with an anxiety disorder (Boschloo *et al*. 2011). Rodent studies have similarly indicated an increased consumption of ethanol in rats that display anxiety-like behaviour (Chappell *et al.* 2013). The observed anxiety-like phenotype previously observed in the GAL$_3$-KO mouse strain (Brunner *et al.* 2014) may therefore account for the increased ethanol intake observed in these mice in the present study. We employed a battery of behavioral tests in order to further characterize the phenotype of *GAL$_3$*-KO mice. Short-term spatial memory, fear memory and social novelty preference memory were investigated in order to detect any deficits in cognition caused by

deletion of *GAL$_3$*. No differences were observed between genotypes in any of the paradigms, indicating preserved cognitive function.

All three GAL receptor subtypes are found in brain regions important to cognitive function including the HIP, basal forebrain and AMG (Rustay *et al.* 2005). Several studies have investigated GAL receptor KO mice in order to observe any correlation of specific GAL receptors with learning and memory deficits. An investigation using GAL$_1$-KO mice found no significant changes in the Morris water maze task and fear conditioning protocols (Wrenn *et al.* 2004). Another study by Gottsch and colleagues assessed GAL$_2$-KO mice using a fear conditioning paradigm and also failed to find an effect of GAL$_2$ ablation on memory function (Gottsch *et al.* 2005). The present study revealed that deletion of *GAL$_3$* does not cause a deficit in learning, either in spatial memory, fear memory or social preference memory, consistent with GAL$_1$ and GAL$_2$ KO mice.

In conclusion, the present study demonstrates both male and female *GAL$_3$*-KO mice displayed increased preference and self-administration of ethanol. The increased alcohol-preferring phenotype of *GAL$_3$*-KO mice was not accounted for by changes in ethanol metabolism, cognitive or locomotor behaviors assessed, reinforcing that these animals may be a useful model of alcohol abuse disorders.

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Author contributions

ED conceived the project and designed the research; SGG, KJS, EJJ, BJT and ED performed the research; SGG, ED, EJJ and MvdB analyzed data. SMB and BK provided new transgenic animals. SGG and ED wrote the manuscript. All authors contributed to critical reading and editing of the manuscript. The authors declare no conflict of interest.

References

Anselmi L, Stella SL, Lakhter A, Hirano A, Tonini M, Catia Sternini M (2005) Galanin receptors in the rat gastrointestinal tract. *Neuropeptides* 39: 349-352. doi:10.1016/j.npep.2004.12.023

Ash BL, Quach T, Williams SJ, Lawrence AJ, Djouma E (2014) Galanin-3 receptor antagonism by SNAP 37889 reduces motivation to self-administer alcohol and attenuates cue-induced reinstatement of alcohol-seeking in iP rats. *J Pharmacol Sci* 125: 211-216.

Ash BL, Zanatta SD, Williams SJ, Lawrence AJ, Djouma E (2011) The galanin-3 receptor antagonist, SNAP 37889, reduces operant responding for ethanol in alcohol-preferring rats. *Regul Pept* 166: 59-67. doi:10.1016/j.regpep.2010.08.009

Barreda-Gómez G, Giralt MT, Rodríguez-Puertas R (2005) G protein-coupled galanin receptor distribution in the rat central nervous system. *Neuropeptides* 39: 153-156. doi:10.1016/j.npep.2004.12.014

Barson JR and Leibowitz SF (2016) Hypothalamic neuropeptide signaling in alcohol addiction. *Prog Neuropsychopharmacol Biol Psychiatry* 65: 321-329. doi:10.1016/j.pnpbp.2015.02.006

Belfer I, Hipp H, Bollettino A, McKnight C, Evans C, Virkkunen M, Albaugh A, Max MB, Goldman D, Enoch MA (2007) Alcoholism is associated with GALR3 but not two other galanin receptor genes. *Genes Brain Behav* 6: 473-481.

Belfer I, Hipp H, McKnight C, Evans C, Buzas B, Bollettino A, Albaugh B, Virkkunen M, Yuan Q, Max MB, Goldman D, Enoch MA (2006) Association of galanin haplotypes with alcoholism and anxiety in two ethnically distinct populations. *Mol Psychiatry* 11: 301-311.

Boschloo L, Vogelzangs N, Smit JH, van den Brink W, Veltman DJ, Beekman ATF, Penninx WJH (2011). Comorbidity and risk indicators for alcohol use disorders among persons with anxiety and/or depressive disorders: Findings from the Netherlands Study of Depression and Anxiety (NESDA). *J Affect Disord* 131: 233-242.

Branchek TA, Smith KE, Walker MW (1998) Molecular biology and pharmacology of galanin receptors. *Ann N Y Acad Sci* 863: 94-107.

Branchek TA, Smith KE, Gerald C, Walker, M. W. (2000). Galanin receptor subtypes. *Trends Pharmacol Sci,* 21: 109-117. doi:10.1016/S0165-6147(00)01446-2

Brunner SM, Farzi A, Locker F, Holub BS, Drexel M, Reichmann F, Lang AA, Mayr JA, Vilches JJ, Navarro X, Lang R, Sperk G, Holzer P, Kofler B. (2014) GAL3 receptor KO mice exhibit an anxiety-like phenotype. *Proc Natl Acad Sci USA* 111: 7138-7143. doi:10.1073/pnas.1318066111

Burgevin M, 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 Neurosci* 6: 33-41. doi:10.1007/BF02736757

Carter M and Shieh J (2010) *Guide to Research Techniques in Neuroscience*. Elsevier: Oxford.

Chang G, Karatayev O, Ahsan R, Avena NM, Lee C, Lewis MJ, Hoebel BG, Leibowitz SF (2007) Effect of ethanol on hypothalamic opioid peptides, enkephalin, and dynorphin: relationship with circulating triglycerides. *Alcohol Clin Exp Res* 31: 249-259. doi:10.1111/j.1530-0277.2006.00312.x

Chappell AM, Carter E, McCool BA, Weiner JL (2013). Adolescent rearing conditions influence the relationship between initial anxiety-like behaviour and ethanol drinking in male Long Evans rats. *Alcohol Clin Exp Res* 37: E394-E403.

Ding ZM, Rodd ZA, Engleman EA, Bailey JA, Lahiri DK, McBride WJ (2013) Alcohol drinking and deprivation alter basal extracellular glutamate concentrations and clearance in

the mesolimbic system of alcohol-preferring (P) rats. *Addict Biol* 18: 297-306. doi:10.1111/adb.12018

Ericson E and Ahlenius S (1999) Suggestive evidence for inhibitory effects of galanin on mesolimbic dopaminergic neurotransmission. *Brain Research* 822: 200-209. doi:10.1016/S0006-8993(99)01144-0

Fang P, Yu M, Guo L, Bo P, Zhang Z, Shi M (2012) Galanin and its receptors: A novel strategy for appetite control and obesity therapy. *Peptides* 36: 331-339. doi:http://dx.doi.org/10.1016/j.peptides.2012.05.016

Gottsch ML, Zeng H, Hohmann JG, Weinshenker D, Clifton DK, Steiner RA, (2005) Phenotypic Analysis of Mice Deficient in the Type 2 Galanin Receptor (GALR2). *Mol Cell Biol* 25: 4804-4811.

Hökfelt T, Xu ZQ, Shi TJ, Holmberg K, Zhang X (1998) Galanin in ascending systems. Focus on coexistence with 5-hydroxytryptamine and noradrenaline. *Ann N Y Acad Sci* 863: 252-263.

Hoplight BJ, Sandygren NA, Neumaier JF (2006) Increased expression of 5-HT 1B receptors in rat nucleus accumbens via virally mediated gene transfer increases voluntary alcohol consumption. *Alcohol* 38: 73-79. doi:10.1016/j.alcohol.2006.04.003

Horvath TL, Leranth C, Kalra SP, Naftolin F (1995) Galanin neurons exhibit estrogen receptor immunoreactivity in the female rat mediobasal hypothalamus. *Brain Research* 675: 321-324. doi:10.1016/0006-8993(94)01374-Q

Jaehne EJ, Ameti D, Paiva T, van den Buuse M (2017) Investigating the role of serotonin in methamphetamine psychosis: Unaltered behavioral effects of chronic methamphetamine in 5-HT 1A knockout mice. *Front Psychiatry 8*. doi:10.3389/fpsyt.2017.00061

Kaplan LM, Gabriel SM, Koenig JI, Sunday ME, Spindel ER, Martin JB, Chin WW (1988) Galanin is an Estrogen-Inducible, Secretory Product of the Rat Anterior Pituitary. *Proc. Natl. Acad. Sci. U.S.A.* 85: 7408-7412.

Koob GF (2008) A Role for Brain Stress Systems in Addiction. *Neuron* 59: 11-34. doi:10.1016/j.neuron.2008.06.012

Koob GF (2014) *Drugs, Addiction, and the Brain*. Elsevier Science: Saint Louis.

Lang R, Gundlach AL, Holmes FE, Hobson SA, Wynick D, Hökfelt T, Kofler B (2015) Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity. *Pharmacol Rev* 67: 118-175. doi:10.1124/pr.112.006536

Leibowitz SF (2007) Overconsumption of dietary fat and alcohol: mechanisms involving lipids and hypothalamic peptides. *Physiol Behav* 91: 513-521. doi:10.1016/j.physbeh.2007.03.018

Leibowitz SF, Avena NM, Chang GQ, Karatayev O, Chau DT, Hoebel BG (2003) Ethanol intake increases galanin mRNA in the hypothalamus and withdrawal decreases it. *Physiol Behav* 79: 103-111. doi:10.1016/S0031-9384(03)00110-0

Lu X, Mazarati A, Sanna P, Shinmei S, Bartfai T (2005) Distribution and differential regulation of galanin receptor subtypes in rat brain: effects of seizure activity. *Neuropeptides* 39: 147-152. doi:10.1016/j.npep.2004.12.011

Manning EE and van den Buuse M (2013) BDNF deficiency and young-adult methamphetamine induce sex-specific effects on prepulse inhibition regulation. *Front Cell Neurosci 7*. doi:10.3389/fncel.2013.00092

Melander T, Fuxe K, Harfstrand A, Eneroth P, Hokfelt T (1987) Effects of intraventricular injections of galanin on neuroendocrine functions in the male rat. Possible involvement of hypothalamic catecholamine neuronal systems. *Acta Physiol Scand* 131: 25-32. doi:10.1111/j.1748-1716.1987.tb08201.x

Millón C, Flores-Burgess A, Narváez M, Borroto-Escuela DO, Santín L, Parrado C, Narváez JA, Fuxe K, Díaz-Cabiale Z (2014) A role for galanin N-terminal fragment (1-15) in anxiety- and depression-related behaviors in rats. *Int J Neuropsychopharmacol* 18: 1-13.

Millón C, Flores-Burgess A, Castilla-Ortega E, Gago B, Garcia-Fernandez M, Serrano A, Rodriguez de Fonseca F, Narvaez JA, Fuxe K, Santin L, Diaz-Cabiale Z (2017) Central administration of galanin N-terminal fragment 1-15 decreases the voluntary alcohol intake in rats. *Addict Biol* DOI: 10.1111/adb.12582

Pieribone VA, Xu ZQ, 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. doi:10.1016/0306-4522(94)00450-J

Rustay NR, Wrenn CC, Kinney JW, Holmes A, Bailey KR, Sullivan TL, Harris AP, Long KC, Saavedra MC, Starosta G, Innerfield CE, Yang RJ, Dreiling JL, Crawley JN (2005) Galanin impairs performance on learning and memory tasks: Findings from galanin transgenic and GAL-R1 knockout mice. *Neuropeptides* 39: 239-243. doi:10.1016/j.npep.2004.12.026

Scheller KJ, Williams SJ, Lawrence AJ, Djouma E (2017) The galanin-3 receptor antagonist, SNAP 37889, suppresses alcohol drinking and morphine self-administration in mice. *Neuropharmacology* 118: 1-12. doi:10.1016/j.neuropharm.2017.03.004

Shen ES, Meade EH, Pérez MC, Deecher DC, Negro-Vilar A, López FJ (1998) Expression of functional estrogen receptors and galanin messenger ribonucleic acid in immortalized luteinizing hormone-releasing hormone neurons: estrogenic control of galanin gene expression. *Endocrinology* 139: 939-948.

Swanson CJ, Blackburn TP, Zhang X, Zheng K, Xu ZQD, Hökfelt T, . . . Branchek TA (2005) Anxiolytic- and antidepressant-like profiles of the galanin-3 receptor (Gal3) antagonists SNAP 37889 and SNAP 398299. *Proc Natl Acad Sci USA* 102: 17489-17494.

Trantham-Davidson H and Chandler LJ (2015) Alcohol-induced alterations in dopamine modulation of prefrontal activity. *Alcohol* 49: 773-779. doi:10.1016/j.alcohol.2015.09.001

Vrontakis ME, Yamamoto T, Schroedter IC, Nagy JI, Friesen HG (1989) Estrogen induction of galanin synthesis in the rat anterior pituitary gland demonstrated by in situ hybridization and immunohistochemistry. *Neurosci Lett* 100: 59-64. doi:10.1016/0304-3940(89)90660-5

Walker AW, Smith CM, Gundlach AL, Lawrence AJ (2015) Relaxin-3 receptor (Rxfp3) gene deletion reduces operant sucrose- but not alcohol-responding in mice. *Genes Brain Behav* 14: 625-634. doi:10.1111/gbb.12239

Wang FL, Chassin L, Bates JE, Dick D, Lansford JE, Pettit GS, Dodge KA (2017) Serotonin functioning and adolescents' alcohol use: A genetically informed study examining mechanisms of risk. *Dev Psychopathol* 1-21. doi:10.1017/S095457941700058X

Wang S, Hashemi T, He C, Strader C, Bayne M (1997a) Molecular cloning and pharmacological characterization of a new galanin receptor subtype. *Mol Pharmacol* 52: 337-343.

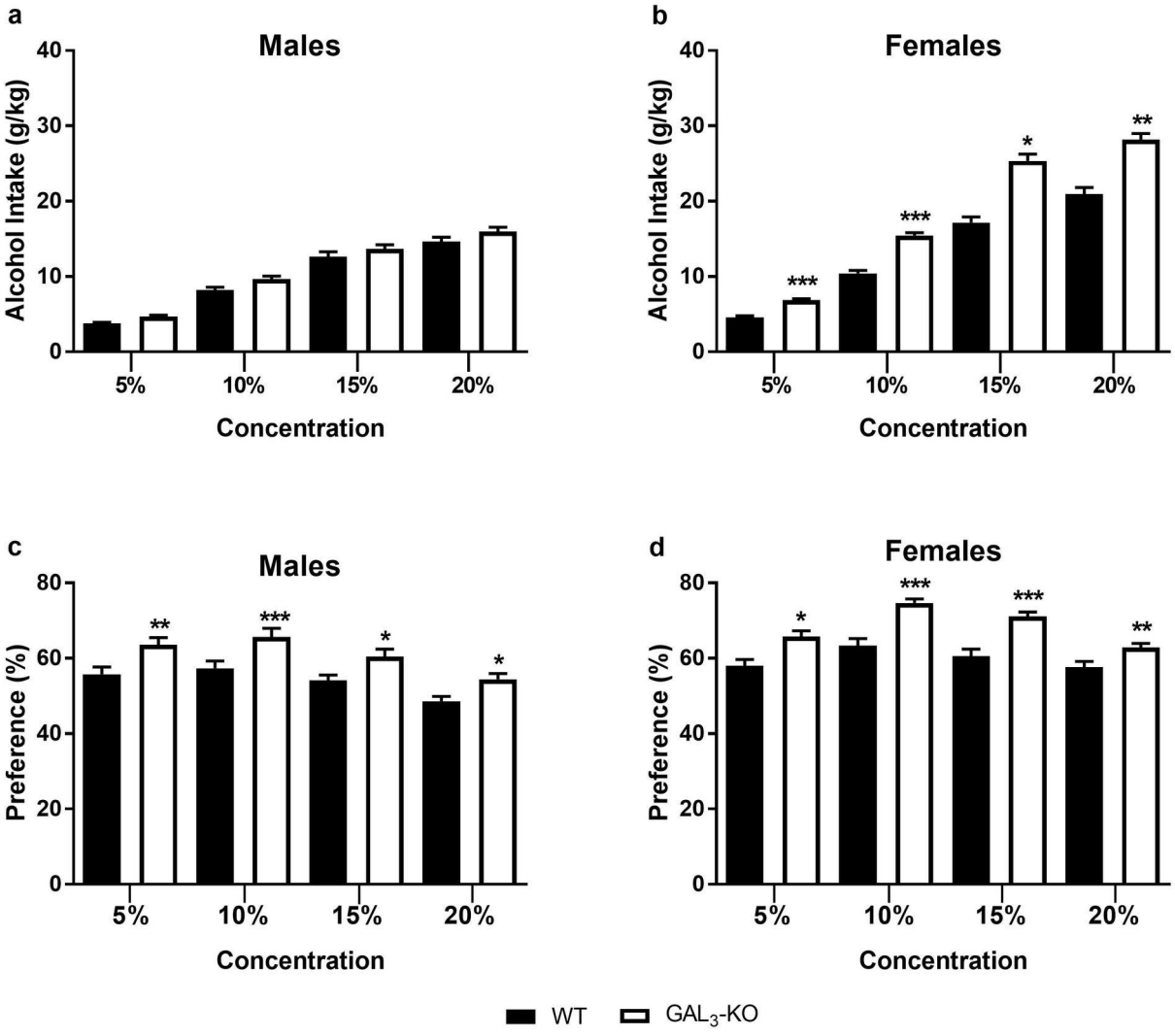
Wang S, He C, Hashemi T, Bayne M (1997b) Cloning and expressional characterization of a novel galanin receptor. Identification of different pharmacophores within galanin for the three galanin receptor subtypes. *J Biol Chem* 272: 31949-31952.

Waters SM and Krause JE (1999) Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. *Neuroscience* 95: 265-271. doi:10.1016/S0306-4522(99)00407-8

Webling KEB, Runesson J, Bartfai T, Langel Ü (2012) Galanin Receptors and Ligands. *Front Endocrinol 3*. doi:10.3389/fendo.2012.00146

Wrenn CC, Kinney JW, Marriott LK, Holmes A, Harris AP, Saavedra MC, Starosta G, Innerfield CE, Jacoby AS, Shine J, Iismaa TP, Wenk GL, Crawley JN (2004) Learning and memory performance in mice lacking the GAL-R1 subtype of galanin receptor. *Eur J Neurosci* 19: 1384-1396.

Zhao X, Seese RR, Yun K, Peng T, Wang Z (2013) The role of galanin system in modulating depression, anxiety, and addiction-like behaviors after chronic restraint stress. *Neuroscience* 246: 82-93. doi:10.1016/j.neuroscience.2013.04.046



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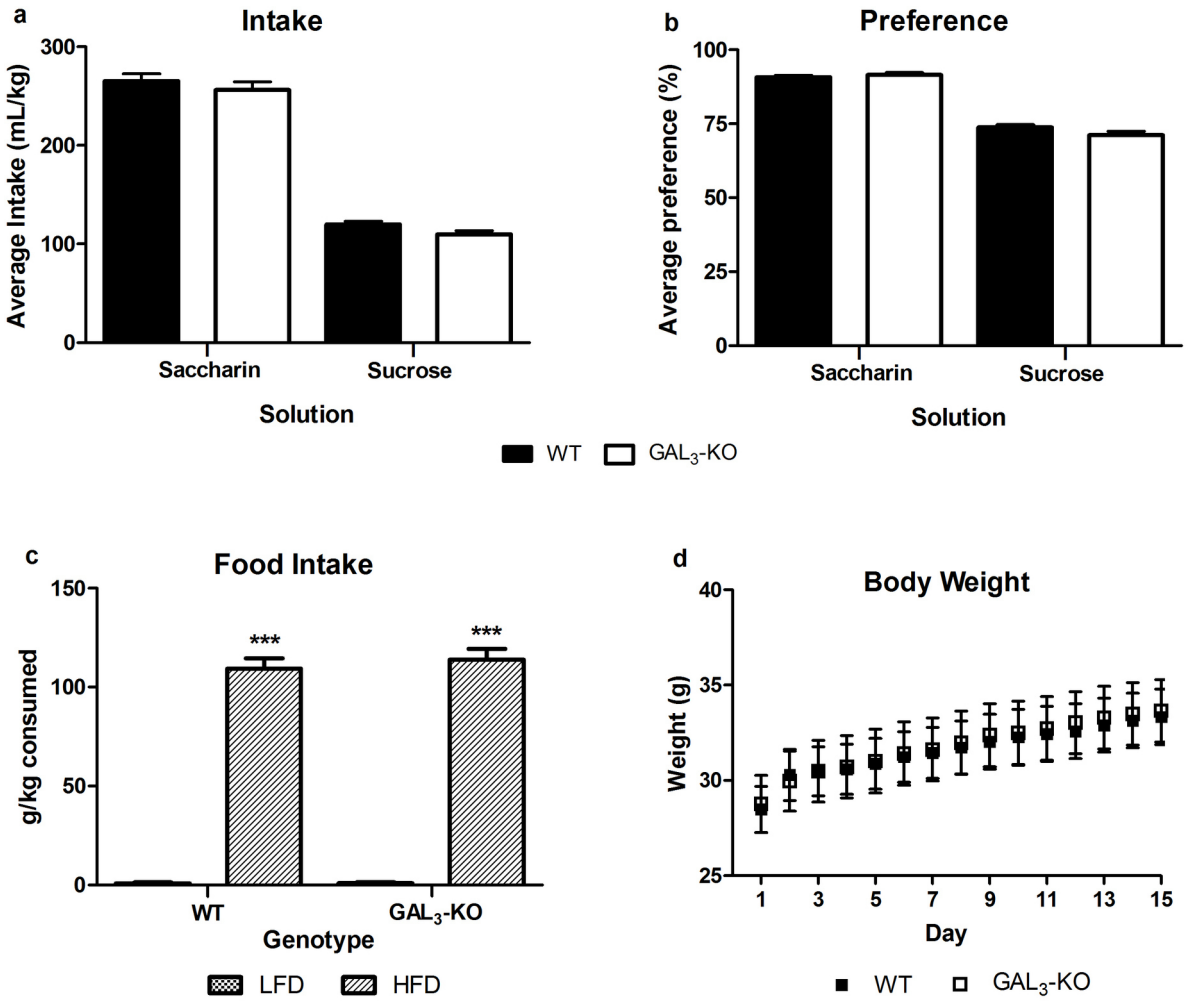

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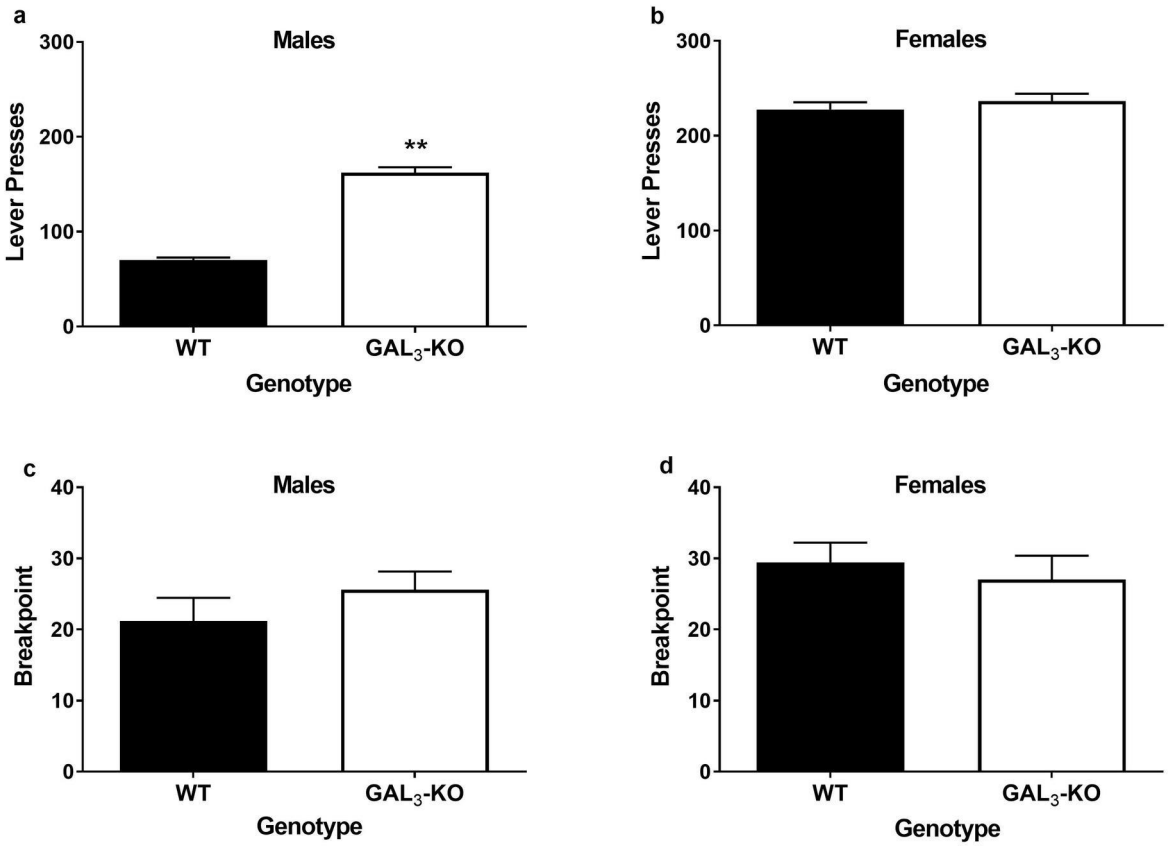

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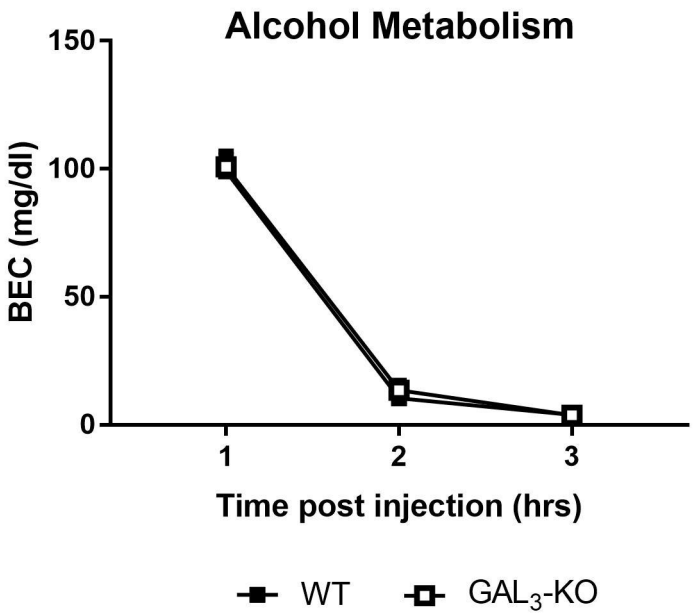

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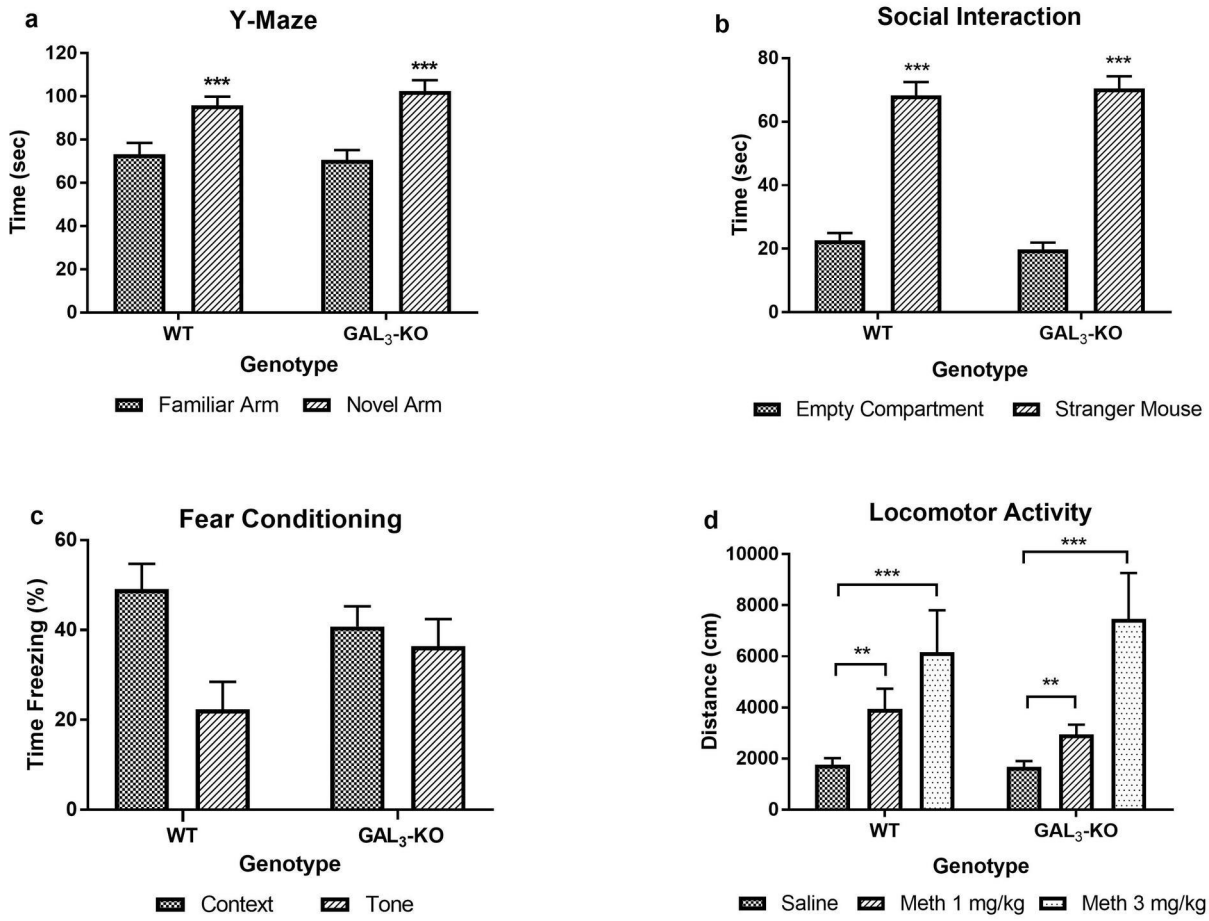

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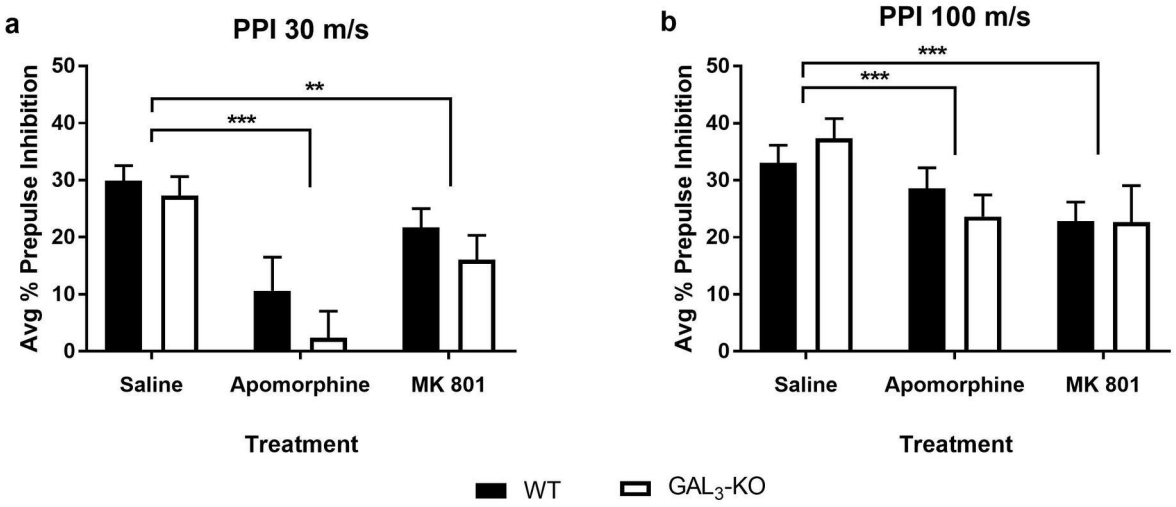

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