Published in final edited form as:

Eur J Neurosci. 2014 March ; 39(6): 999–1008.

Epigenetic and pharmacological regulation of 5HT3 receptors controls compulsive ethanol seeking

Jacqueline M Barker, PhD^{1,2}, Huiping Zhang, PhD^{1,3}, J Joshua Villafane¹, Tiffany L Wang¹, Mary M Torregrossa, PhD¹, and Jane R Taylor, PhD¹

¹Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06519

²Interdepartmental Neuroscience Program, Yale University School of Medicine, New Haven, Connecticut 06519

³VA Connecticut Healthcare System, West Haven, Connecticut 06516

Abstract

Factors underlying individual vulnerability to develop alcoholism are largely unknown. In humans, risk for alcoholism is associated with elevated cue reactivity. Recent evidence suggests that in animal models, reactivity to reward-paired cues is predictive of addictive behaviors. To model cue reactivity in mice, we used a Pavlovian approach (PA) paradigm in which mice were trained to associate a cue with delivery of a food reinforcer. We then investigated the relationship between PA status with habitual and compulsive-like ethanol seeking. After training mice to respond for 10% ethanol, habitual behavior was investigated using both an outcome devaluation paradigm, in which ethanol was devalued via association with lithium chloride induced malaise, and a contingency degradation paradigm in which the relationship between action and outcome was disrupted. Compulsive-like behavior was investigated in a modified conditioned place preference paradigm in which footshock was paired with the reward-paired chamber. We saw that high PA was predictive of habitual and compulsive-like ethanol seeking. Additionally, innate risk status was related to epigenetic changes in the gene encoding the requisite subunit of the 5HT3 receptor, *Htr3a*, as well as 5HT3A protein expression in the amygdala. We then used pharmacological tools to demonstrate that risk status determines the ability of a 5HT3 antagonist to reduce compulsive ethanol seeking. These data indicate that risk status can be identified prior to any alcohol exposure by assessment of cue reactivity, and further, that this endophenotype may be predictive of response to pharmacological treatment for components of alcoholism.

Keywords

5HT3; individual differences; alcohol; addiction; habit; compulsive reward-seeking

Corresponding author: Jane R Taylor, Jane.taylor@yale.edu, 34 Park Street, Connecticut Mental Health Center, Ribicoff Labs, New Haven, CT 06519.

Conflict of Interest: Research was funded by the NIH; the authors report no conflict of interest.

Introduction

Prediction of genetic risk for disease is needed for personalized medicine. Few studies have investigated the potential shared genetic/epigenetic factors that predict efficacious pharmacological approaches for psychiatric illnesses, which are often treatment insensitive and highly variable, and by their nature are associated with behavioral phenotypes rather than somatic symptoms (Robison & Nestler, 2011; McMahon & Insel, 2012). Alcoholism is a common and debilitating neurobiological illness in terms of lifetime prevalence and economic burden. Despite high evidence of heritability and genetic risk, there are few effective therapeutic agents. While genotype-based personalized psychiatry is still in its infancy, phenotype-based approaches have not been exploited.

The transition to alcoholism is associated with loss of control over drinking. Initially, alcohol consumption is mediated by its rewarding properties, but some individuals develop inflexible stimulus-mediated habits, that are insensitive to changes in action-outcome relationships or outcome value (Dickinson, et al, 2002), and compulsive alcohol-seeking behaviors that occur despite adverse consequences. Importantly, not all casual drinkers will transition to these potentially harmful drug-seeking strategies (Becker, 2008). By identifying individuals at risk for habitual and compulsive alcohol-seeking behavior prior to alcohol exposure, we have the potential to develop successful, individualized pharmacotherapeutics.

In humans, elevated risk for alcoholism is related to enhanced cue reactivity (Kareken et al., 2010). Rodent models have demonstrated a relationship between incentive motivation, drug reward, and differences in dopamine signaling (Flagel, et al., 2009; Saunders & Robinson, 2013). We have shown that the ability of reward-paired cues to invigorate behavior predicts resistance to extinction increased relapse-related behavior for ethanol (Barker, et al., 2012). Here, we assessed whether innate differences in the ability of a food-paired cue to elicit automatic behavior (Pavlovian approach (PA); Balleine & Dickinson, 1998; Berridge & Robinson, 2003) predicted the subsequent formation of habitual and compulsive-like ethanol seeking.

Much work has suggested that genetic and epigenetic differences in serotonergic systems are related to alcohol use disorders (e.g., Enoch, 2012; Zhang et al., 2013). While ethanol exposure itself causes some related epigenetic changes (Barker, et al., 2013), others report that preexisting differences in serotonergic signaling contribute to alcoholism risk (e.g., Heilig, et al., 2011; Yaakob, et al., 2011). The 5HT₃ receptor has been the subject of targeted treatment for alcoholism with limited success. Ethanol potentiates the effects of serotonin at 5HT₃ (Lovinger, 1999), and amygdalar 5HT₃ signaling may play a role in ethanol consumption (Lynch, et al., 2011). Notably, it has been argued that individual characteristics of alcohol users may predict the efficacy of 5HT₃ antagonists at reducing alcohol consumption and craving (Johnson, 2000). These data suggest that abnormal transcription of the serotonin receptor 3A gene (*HTR3A*), encoding the requisite subunit of 5HT₃, could influence vulnerability to alcoholism. To determine whether preexisting differences in epigenetic regulation of 5HT₃ are mechanistically related to the propensity to develop habitual and compulsive-like ethanol seeking, we assessed promoter region methylation of *htr3a* in limbic-corticostriatal circuitry that mediates addictive behavior as

well as the effects of 5HT3 manipulation on inflexible ethanol seeking in high and low PA animals.

Materials and methods

Subjects

Male CD1 mice were supplied from Charles River and delivered to the Yale University/Connecticut Mental Health Center mouse vivarium between 56–70 days of age. This outbred strain of mice was chosen because they have been previously shown to self-administer ethanol and because the individual variability in this strain is expected to be higher than in inbred mouse populations. These mice were allowed to acclimate for one week with *ad libitum* access to food and water. All experiments were approved by the Yale University IACUC and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Instrumental Conditioning Chambers

Instrumental chambers were identical to those described by Barker et al., 2010. Briefly, twelve mouse instrumental chambers (15 cm deep X 17 cm wide X 12 cm high) housed within a sound-attenuating box, were used for these experiments (Med-Associates; Georgia, VT). The side walls of each chamber were made of stainless steel panels and the front door, ceiling and back wall were made of clear Plexiglas. Each chamber was equipped with a 28V house light located at the top of the middle panel on the left side wall, three adjacent nosepoke apertures located at the bottom of the left side wall, and a magazine located at the bottom of the middle panel on the right side wall. Retractable levers were extended during relevant sessions on either side of the magazine. Liquid reinforcers were presented in the magazine via a motorized dipper that held 50 μ L of liquid. Grain pellet reinforcers were delivered to the same magazine. Nosepoke apertures and magazine were equipped with a light and photobeam sensor. A fan provided background noise and ventilation.

Behavioral Screening of cue reactivity

Pavlovian Training—Prior to starting acquisition training, mice were placed on a food restriction schedule in which they were maintained at 90–92% of their free feeding weight throughout the duration of all experiments. Water was available *ad libitum*. After five days of food restriction, mice received a single 15 min session of magazine training in which grain pellets were delivered on a fixed interval 60 sec schedule. Mice then received 4 30 min Pavlovian training sessions. During these sessions, a cue (tone) was played on a fixed interval schedule (120 sec off, 60 sec on). A single grain pellet was delivered during each Tone On interval on a random interval schedule so that delivery could not be predicted. No reinforcers were delivered during the Tone Off intervals. Importantly, no levers or nosepokes were available during these sessions and no operant response was required to initiate reinforcer delivery. Magazine entries were measured to assess Pavlovian approach (PA) to the magazine during Tone On versus Tone Off intervals.

Instrumental Training—After Pavlovian training, mice learned to perform a response (lever press) for access to the same reinforcer (grain pellets) in the same operant chambers

as during the Pavlovian training component. Mice were first trained on a fixed ratio 1 (FR1) schedule (1 pellet delivered for each active lever press). Sessions were terminated at 30 min or after the mouse received 30 pellets. Responses on the inactive lever had no consequence. Mice advanced to a random interval 30 sec (RI30) schedule after 3 consecutive days of acquiring all 30 pellets. During 4 sessions of RI training, reinforcement could be earned every 30 sec on average, although the actual duration of each interval was randomly determined; reinforcement availability was not predictable. The first active lever press following completion of the interval resulted in delivery of a grain reinforcer. The duration of the next random interval was then generated automatically. These sessions were terminated after 30 min or acquisition of 30 grain pellets.

Pavlovian- to- Instrumental Transfer Test—The PIT test took place in the same operant chambers as training. During the 6 min session, both the active and inactive levers were extended into the chambers and the mouse was able to respond for the duration of the test. The cue (tone) was played on a fixed interval schedule that was distinct from that used during the Pavlovian training component (90 sec off, 30 sec on). Responding during the Tone On and Tone Off intervals was recorded. Tests were conducted in extinction; no reinforcers were delivered.

Ethanol Seeking Habit Experiments

Instrumental Training—After completion of PA and PIT experiments, CD1 mice were trained to respond for a 10% v/v ethanol reinforcer on a moderate instrumental schedule used to detect differences in a population. That is, we have chosen a schedule that does not bias toward goal-directed or habitual response strategies in order to assess which individuals show rapid ethanol habit formation. Mice were trained in boxes identical to those in which PIT training occurred, but levers were retracted and nose pokes were available. One nose poke was assigned as the active nose poke, where a response resulted in reinforcer delivery, and the others designated as inactive nose pokes. Prior to any ethanol instrumental training, mice were placed in a novel cage with a water bottle containing 10% ethanol in tap water for 30 minutes. Intake was measured to assure that all animals consumed ethanol. After one day of exposure, instrumental acquisition began. The training schedule consisted of 1 day magazine training, 3 days fixed ratio 1 training, and 2 days random interval 30 sec training. During each daily training session, the houselight and fan were on. Magazine training was 30 minutes in length. All other training sessions were 45 min in duration. This schedule is based on that reported by Barker and colleagues (2010) to promote “moderate training” that only produces habitual responding in sensitive populations, but we have shortened the length of the training based on the finding that chromosomal males show accelerated habit formation.

Habit Testing—Two measures of habitual behavior were used in these experiments. We employed a contingency degradation paradigm in which the acquired action-outcome relationship was degraded by providing noncontingent access to ethanol reinforcers. In addition, we used an outcome devaluation procedure in which the ethanol reinforcer was paired with lithium chloride-induced malaise.

Contingency Degradation Test—One half of mice were returned to the same chambers as during training one day after the last RI 30 instrumental session. During these sessions, conditions were identical except that the 10% ethanol reinforcer was delivered on a noncontingent schedule determined by each individual animal's reinforcement rate on the day prior. Reinforcer delivery was spaced equivalently across the 30 min session. Responses on the active and inactive nose pokes were recorded, but did not result in reinforcer delivery. Responding in the degradation session was compared to responding in a nondegraded session. We expect that animals performing in a goal-directed manner will reduce responding (learn the change in action-outcome contingency and update behavior), while habitual animals will continue to respond despite the degradation of the action-outcome relationship.

Conditioned Taste Aversion (CTA)—One day after the last RI 30 instrumental acquisition session, mice were placed into a novel cage with *ad libitum* access to 10% ethanol in a water bottle. The mice were left undisturbed for a 45 min period during which the animals freely consumed ethanol. Immediately after removal from the novel cage, mice were injected with either 0.9% saline ("valued" mice) or 0.15M lithium chloride ("devalued" mice; 40ml/kg i.p.). Mice were then returned to their homecage. Approximately 3.5 hours after injection, mice were allowed 1.5 hour of homecage food access. Approximately 3 hours following homecage feeding, "valued" mice received an injection of 0.15M lithium chloride (40ml/kg i.p.) and "devalued" mice received an equal volume injection of 0.9% saline in order to normalize exposure to LiCl without pairing illness with ethanol or food chow in valued mice. This procedure was performed once daily across three consecutive days. Consumption was measured by weighing the water bottles immediately before and after the 45 min session.

Instrumental Testing—One day after the last CTA session, testing was conducted in the same instrumental conditioning chamber used for instrumental acquisition. The test session lasted 5 minutes and was performed in extinction (no reinforcers were available), but was otherwise identical to conditions during acquisition. Performance in the 5 min test was calculated as a percentage of baseline responding with the number of active nose pokes during the final training day serving as a baseline. Immediately following the instrumental test, animals received a final 45 min "post-test" consumption test to assess group differences in *ad libitum* ethanol consumption.

Individual differences in compulsive-like ethanol seeking

An additional cohort of male CD1 mice that was trained on the PA/PIT paradigm was allowed *ad libitum* access to food and water and returned to free feeding weights prior to training in a compulsive ethanol-seeking paradigm. Conditioning chambers were standard three chamber boxes with retractable doors (Med Associates; Georgia, VT). Chambers had distinct walls (vertical black and white stripes or diagonal marble and black stripes) and floors (wire mesh or grid). The conditioning chambers were separated by a neutral, gray chamber. Photocell beam breaks were used to calculate time spent in each chamber, latency to enter the chamber and number of entries by Med-PC IV software. During habituation, mice were placed in the neutral chamber with both doors retracted such that mice could

freely explore all chambers. During conditioning, mice were treated with 2g/kg ethanol in saline immediately prior to placement in the paired chamber with the door closed on days 1, 3, and 5. The mouse was confined in this conditioning chamber for 20 min. On days 2, 4, and 6, mice received saline injections prior to placement in the opposite conditioning chamber for 20 min.

On day 7, mice were placed in the neutral chamber with both doors retracted and allowed to freely explore all chambers for 5 min. This time point was chosen because we were able to examine entry into both chambers and latency to enter, but minimal extinction was expected to occur. Latency to enter the chambers was measured.

On the following day, mice were confined to the ethanol-paired chamber. One min after placement, mice received a 0.8mA shock 2 sec in duration. Mice remained in the chamber for 60 sec after the shock and then were returned to their home cage. To test compulsive ethanol seeking, mice were returned to the gray chamber and allowed to freely enter both chambers and latency to enter the chamber was assessed.

Compulsive –like food seeking was assessed in this paradigm with the following changes: pairing sessions were 30 min in duration. On pairing days (1, 3, 5) mice received access to a dish with 30 sucrose pellets in the paired chamber. On unpaired days (2, 4, 6), an empty dish was placed in the chamber. Pretest and posttest sessions, as well as the shock session, were identical.

Pharmacological manipulations of compulsive-like ethanol seeking

To investigate the effects of 5HT3 manipulations, the 5HT3 agonist mCPBG and the antagonist, ondansetron, were used. Drugs were only injected on day 7 (pre shock test) and on day 9 (post shock test). 5HT3 manipulations *did not* occur during the acquisition of the conditioned place preference, during exposure to ethanol, or during footshock. To assess whether 5HT3 agonism would promote compulsive ethanol seeking, training occurred as described above except that mice received an i.p. injection of 5mg/kg mCPBG in saline 5 min prior to placement in the chamber. The ability of 5HT3 antagonism to reduce compulsive ethanol seeking was assessed in a distinct training paradigm where differences between high and low PA mice were minimized. In this paradigm, training sessions (day 2–6) were extended to 30 min. Test sessions were identical to those described above. Separate groups of mice received i.p. injections of .01, .05, and .1 mg/kg ondansetron 20 min prior to test sessions.

Drinking in the Dark

A cohort of male CD1 mice that was trained on the PA/PIT paradigm was allowed ad libitum access to water and food and was returned to free feeding weights prior to the initiation of drinking in the dark experiments. Mice were assigned to control or ethanol conditions based on their PA and PIT statuses. All mice were weighed at 1830h, 30 min prior to the end of the light cycle. Three hours after the start of the dark cycle, at 2200h, water bottles were removed from the animals' home cages, and mice received either a bottle containing 20% ethanol in tap water, or a new bottle containing tap water. For days 1–3,

ethanol bottles remained in place for 2 h. On days 4 and 5, ethanol bottles remained in place for 4h. Ethanol consumption was measured for each night and grams of ethanol per kilogram body weight was determined using the animal weights from 1830h.

DNA methylation studies

The mouse *Htr3a* promoter methylation level was quantified exactly as described in our previous study (Barker et al., 2012). Briefly, genomic DNA was extracted from the mouse brain tissue and the mouse trunk blood using the QIAamp DNA Micro Kit (QIAGEN, Valencia, CA) and the PAXgene Blood DNA kit (PreAnalytiX, Hombrechtikon, Switzerland), respectively. Bisulfite conversion of DNA was then performed using reagents included in the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Finally, methylation levels of eight CpG sites around the *Htr3a* transcription start site (TSS) (from 96 bp upstream to 151 bp downstream of the TSS) were assessed via the Sequenom MassARRAY EpiTYPER method (Sequenom, San Diego, CA). The methylation calls were performed by the EpiTyper software v1.0 (Sequenom), which generates quantitative results or methylation levels (methyl CpG/total CpG) for each CpG site.

5HT3A expression

Assessment of 5HT3A protein expression was performed in a separate cohort of animals. Mice were trained through the PA and PIT procedures as described above and were sacrificed via rapid decapitation at least 24 hours after training. Tissue was immediately frozen on dry ice and dissected while frozen. Sonicated tissues were run on 10–20% tris-glycine gels and transferred to nitrocellulose membranes for blotting with anti-5HT3A (1:500; Abcam; Cambridge, MA), and anti- GAPDH (1:10,000; Advanced Immunochemicals, Inc., Long Beach, CA) antibodies. Membranes were then incubated with IRDye700 anti-Rb IgG and IRDye800 (1:1000) anti-Ms IgG for detection using the Odyssey imaging system (LICOR; Lincoln, NE). Bands were quantified using Odyssey software and normalized to GAPDH to account for variation in protein loading. Two bands were excluded because levels of protein were too low to quantitate.

Statistical Analysis

Analysis of data was performed in JMP. High and Low PA and High and Low PIT groups were assigned by median split. ANOVA or t-tests were used to compare the effects of these factors as appropriate. Significant interactions and main effects were followed by Tukey's HSD. Results were considered significant if the probability of error was less than 5%.

Results

Individual differences in food-associated (cue reactivity) behavior

To assess individual differences in cue reactivity, mice were screened in approach and PIT paradigms. High PA mice showed higher rates of cue-elicited approach to the magazine than low PA mice. ANOVA revealed a main effect of PA (High vs. Low) on normalized responding (magazine entries during Cue On vs. Cue Off periods; $F_{1,62} = 53.642$, $p < .001$), but no effect of PIT ($F_{1,62} = .091$, $p > .7$; Fig 1a). No interaction was seen indicating that

while High and Low PA animals show significantly different approach behavior, PIT is not related.

PA status was also not predictive of PIT, a measure of stimulus control over an instrumental action. High PIT mice were those that showed greater increases in lever presses during cue presentation. ANOVA revealed a main effect of PIT (High vs. Low) on normalized responding (lever presses during Cue On vs Cue Off periods; $F_{1,62}=41.82$, $p<.001$), but no effect of PA ($F_{1,62}= .462$, $p=.5$). This indicates that High and Low PIT animals show significantly different degrees of transfer, but that PA status does not predict PIT.

Individual differences in food PA did not predict differences in reinforcement across acquisition

No differences in estimated ethanol consumption were observed across training for ethanol habit testing. rmANOVA of g ethanol per kg body weight revealed no differences in estimated consumption based on PA or PIT status in mice trained for the contingency degradation test ($ps>.6$) or for the outcome devaluation test ($ps>0.49$). A main effect of day of training was observed in mice trained for contingency degradation ($p=.049$) which was not significant in mice trained for the outcome devaluation test ($p=.099$).

Individual differences in food PA predict the formation of habitual and compulsive ethanol seeking

To determine the relationship between cue reactivity and inflexible ethanol seeking, mice were screened through PA and assigned to High and Low PA groups based on a median split, then trained to perform an action (nosepoke) for a 10% ethanol reinforcer using a 'moderate' training paradigm which enabled identification of sensitive populations. The expression of habitual or goal-directed behavior was determined using either a devaluation test or a contingency degradation paradigm.

In the devaluation test, an ANOVA revealed an interaction effect between PA (High vs. Low) and Devaluation (Valued vs. Devalued) on normalized responding during the 5 min extinction test (response rate during test as a percentage of the last training session; $F_{1,23}=4.21$, $p=.05$) but no effect of PIT or PIT $\times$ Devaluation interaction ($ps>.3$). Post hoc analyses indicated an effect of Devaluation in Low PA animals ($p<.01$), but not in High PA mice ($p>.2$), indicating that High PA ($n=11$ valued, 12 devalued) mice are insensitive to outcome devaluation at this time point (habitual) while Low PA ($n=10$ valued, 10 devalued) mice remain goal-directed and reduce responding for the devalued reinforcer (Fig 1b). Importantly, individual differences in PA were not related to sensitivity to outcome devaluation. To confirm devaluation of the ethanol reinforcer, consumption was measured after the 5 min test in extinction. While devaluation was predictive of ethanol consumption ($p<.05$), no main effect or interaction with PA status was observed ($p>.9$, $p>.2$, respectively).

In the contingency degradation test, repeated measures (rm)ANOVA revealed an interaction effect between PA (High vs. Low; between-subjects) and Degradation (Degraded vs. Nondegraded; within-subjects) on response rate ($F_{1,27}=4.07$, $p=.04$) but no effect of PIT or PIT $\times$ Degradation interaction ($ps>.5$). Post hoc analyses indicate an effect of Degradation

in Low PA animals ($n=16$; $p<.01$), but no significant difference between Degraded and Nondegraded conditions in High PA mice ($n=15$; $p>.25$), indicating that High PA mice are insensitive to degradation of the action-outcome relationship at this time point (habitual) while Low PA mice remain goal-directed and change response strategy (Fig 1c).

To determine whether the relationship between PA status and habit formation was specific to ethanol reward, we investigated habitual sucrose seeking using the contingency degradation paradigm ($n=16$ low PA, 15 high PA). No relationship existed between PA status and habitual sucrose seeking in a contingency degradation test by rmANOVA ($F_{1,29}=0.09$, $p>.7$; Fig 1d) or in a pairwise correlation between normalized PA and normalized contingency degradation responding ($p>.7$; data not shown), suggesting that this relationship does not generalize to all reinforcers. Importantly, this test was performed at a time point where we see significant individual variation in responding in a contingency degradation test. This time point was selected because we expected that elevated individual differences would enable us to observe any relationship between PA and propensity toward development of habitual sucrose seeking. Shorter training protocols have been employed by our lab to bias behavior toward the expression of goal-directed behavior, while more extended training protocols promote the development of habitual behavior (Quinn et al., 2007). Consistent with our previous findings, we saw high levels of individual variability but individual differences in habitual sucrose seeking were not explained by individual differences in PA, suggesting that these individual differences in the rate of sucrose habit formation are not predicted by PA.

To investigate the relationship between cue reactivity and compulsive ethanol seeking, we investigated reward seeking after association with an adverse consequence. Specifically, we used a modified CPP paradigm where animals learn to associate one chamber with ethanol, and the other with a saline injection. Latency to enter the reward-paired chamber was assessed before and after association of the reward-paired chamber with footshock. Rapid entry to the reward-paired chamber was interpreted as compulsive-like behavior, or reward seeking despite an adverse association. Importantly, rmANOVA ($PIT \times PA$) revealed no differences in preference scores based on PA status ($F_{1,20}=1.5$, $p=.15$), though a main effect of PIT status was observed ($F_{1,20}=3.15$, $p<.01$). Additionally, PA status did not predict differences in locomotor activity in the acclimation session ($ps>.6$), or during conditioning ($ps>.6$). Together, these data suggest that any differences observed in latency to enter the reward paired chamber after footshock were not due to baseline differences in locomotor activity or in initial preference for the reward-paired chamber. rmANOVA ($Shock \times PIT \times PA$) on latencies to enter each chamber indicated a main effect of Shock and $Shock \times PA$ interaction on latency to enter the shock/ethanol-paired chamber ($F_{1,16}=6.4815$, $p=.02$). Post-hoc analyses indicate that Low PA ($n=10$) mice show greater latencies to enter the paired chamber after the shock than before ($p<.01$) while High PA ($n=10$) mice enter at the same rate ($p=.2$; Fig 1e). No differences in pre-shock latency were seen between High and Low PA mice ($ps>.4$).

Cue reactivity was not predictive of differences in alcohol consumption

rmANOVA (day $\times$ PA $\times$ PIT) revealed no effect of day, PA or PIT status on g/kg ethanol consumption ($n=28$; 7 per group of PA $\times$ ethanol exposure; $p>.5$) in a drinking in the dark paradigm, confirming that individual differences in cue-mediated behavior were predictive only of inflexible alcohol-seeking behavior, but not predictive of actual alcohol consumption in a subacute paradigm (Fig. 1f). Mice consumed an average of 6.32 g ethanol/kg body weight (SEM = .6). This level of consumption is expected to relate to approximately .5mg/ml based on published literature (Rhodes et al., 2006).

PA status is predictive of methylation and expression of *Htr3a* in the amygdala

To determine innate differences in epigenetic regulation of 5HT3A, methylation of the promoter region of the gene encoding the requisite subunit of 5HT3 was assessed. Mean methylation levels were calculated across the promoter region of *Htr3a*. An ANOVA indicated a main effect of PA status on mean methylation only in the amygdala ($F_{1,24}=4.33$, $p<.05$; Fig 2a) showing that high PA mice ($n=14$) showed lower methylation than low PA mice ($n=12$; one mouse was excluded because of missing methylation scores and one additional mouse was a statistical outlier with a score greater than three standard deviations from the mean). No main effect of PA was seen on methylation in blood ($p>.9$), or other brain regions that were investigated including dmPFC ($p>.2$), where main effects of ethanol consumption on DNA methylation were observed (Barker, et al., 2012). All animals were included these analyses ($n=14$ for high and low PA). Notably, an interaction was observed between ethanol consumption and PA status on mean methylation in the blood ($F_{1,24}=3.8$, $p=.05$). Post hoc analyses indicate that PA predicts methylation status in the ethanol treated group ($t=2.26$, $p<.05$) but not in mice receiving water ($p>.3$). These data suggest that there are both baseline differences in methylation based on PA status, as observed in the amygdala, as well as PA mediated differences in the effects of exposure to ethanol, as observed in the blood (Fig 2c). rmANOVA of methylation at individual CpG sites in the amygdala revealed a main effect of PA ($F_{1,15}= 33.26$, $p=.0189$), consistent with our observation that methylation across the promoter region is higher in low PA animals (Fig 2b). In addition, we saw no effect of PA status or ethanol exposure on *Htr3a* methylation in the dorsolateral striatum ($p=.11$, $p=.51$, respectively), the dorsomedial stratum ($p=.53$, $p=.25$), the ventral striatum ($p=.67$, $p=.66$) or the vmPFC ($p=.60$, $p=.95$).

To examine whether differential DNA methylation levels reflected differences in protein expression, 5HT3A protein levels were assessed in a separate group of mice ($n=8$). Normalized 5HT3A protein expression was calculated by normalizing to GAPDH to account for loading, and then comparing to mean expression. The relationship between 5HT3A expression and PA was determined by performing a pairwise correlation ($r^2=.699$, $p<.01$; Fig 2d).

Pharmacological manipulation of 5HT3 signaling can alter compulsive-like ethanol seeking in a phenotype-dependent manner

To investigate whether differences in 5HT3 expression were causally related to the expression of inflexible behavior, we used pharmacological tools to investigate the role of 5HT3 signaling in compulsive reward seeking. We first tested the hypothesis that 5HT3

agonism with mCPBG would promote compulsive ethanol-seeking by administering mCPBG prior to pre- and post-shock test sessions. Importantly, no drugs were on board during training. Latencies were square root transformed as appropriate to maintain homogeneity of variance to enable the use of parametric statistics. rmANOVA indicated a shock $\times$ drug interaction ($F_{1,42}=4.69$, $p<.05$) on latency to enter the reward paired chamber. A trend toward an interaction between approach status and drug was observed ($F_{1,42}=2.96$, $p<.1$), consistent with the anticipated differences in high and low PA mice. Post hoc analyses indicated that latency to enter the reward-paired chamber increased significantly in saline treated animals ($p=.01$), but no differences were seen in mice receiving the 5HT3 agonist prior to testing ($p>.5$), indicative of increased compulsive-like ethanol seeking in both high and low PA animals relative to saline controls (Fig 3a).

To determine whether decreased 5HT3 signaling could reduce compulsive-like ethanol seeking, we used a modified paradigm in which we saw high levels of compulsive-like ethanol seeking in both high and low PA animals. We then administered the 5HT3 antagonist ondansetron at multiple doses during test sessions. Change in latency to enter the reward-paired chamber was calculated by subtracting pre-shock latencies from post-shock latencies. Data were then translated to generate non-negative data and square root transformed to maintain homogeneity of variance. A total of 53 low PA (14 saline, 11 .001mg/kg, 19 .01 mg/kg and 9 .05 mg/kg) and 54 high PA (17 saline, 11 .001mg/kg, 13 .01mg/kg and 13 .05 mg/kg) animals were included in analyses. Between subjects ANOVA indicated an interaction between PA status and dose ($F_{3,103}=4.65$, $p=.05$) on change in latency. Importantly, the dose that maximally reduced compulsive-like ethanol seeking was lower in low PA animals than high PA animals, consistent with differential 5HT3 expression (Fig 3b).

To confirm specificity of this relationship to ethanol, we assessed compulsive-like sucrose seeking ($n=16$ low PA, 17 high PA). Importantly, we saw that PA status was predictive of compulsive-like sucrose seeking; rmANOVA revealed an interaction between approach status and shock ($F_{1,29}=4.408$, $p<.05$). Only low PA mice showed a trend toward increased latency after shock ($p=.07$). High PA mice entered the reward-paired chamber at the same latency ($p>.3$; Fig 3c). Despite this relationship, no effect was seen of ondansetron on behavior ($p>.5$), suggesting that this relationship may not be mediated by individual differences in 5HT3 signaling.

Discussion

Our data demonstrate that individual differences in Pavlovian approach behavior can predict the development of habitual and compulsive-like ethanol seeking, and further that PA status is predictive of *Ht3ra* methylation in the amygdala. Specifically, we found that high PA mice rapidly form inflexible ethanol-seeking behaviors and become insensitive to changes in outcome value (outcome devaluation; Fig 1b) or the relationship between actions and their outcome (contingency degradation; Fig 1c). In both paradigms, high PA mice continued responding after disruption, consistent with the expression of inflexible habits, while low PA mice were able to reduce responding, indicative of goal-directed behavior. These data suggest that mice that greatly use cues to guide their behavior are at elevated risk for the

development of stimulus-response mediated ethanol-, but not sucrose- (Fig 1d), seeking behavior. Importantly, PIT behavior was not predictive of the formation of ethanol-seeking habits by either measure, suggesting that these behaviors are distinct from other measures of inflexible ethanol-seeking behaviors including extinction and cue-induced reinstatement (Barker et al., 2012a). PA status was not predictive of degree of Pavlovian-to-instrumental transfer, suggesting that while these behaviors are both indices of cue-driven behavior, innate propensity toward PA and PIT may be driven by separate neurocircuitry. Indeed, other reports have suggested that PA and PIT are not correlated and are differentially sensitive to changes in contingency and reward magnitude (van den Bos et al., 2004). Our own findings have shown that PIT, but not PA, is predictive of resistance to extinction that is mediated innate differences by prefrontal plasticity (Barker et al., 2012a). Notably, estimates of ethanol consumption during the habit training paradigm are not different based on PA status. Importantly, these are relatively low levels of ethanol consumption and it is possible that differences may emerge under conditions where large volumes of ethanol are consumed. Further, blood alcohol concentrations were not measured. While no differences in ethanol consumption were seen, it remains possible that differences in the metabolism of ethanol and blood ethanol levels achieved based on PA status may underlie these effects.

Compulsive-like ethanol seeking was determined with a novel paradigm that investigates the competition between ethanol-reward seeking and avoidance of adverse (i.e., negative) consequences. We hypothesized that compulsive reward seeking might be differentially expressed in mice that have distinct propensities for habitual behavior (i.e., low vs. high PA mice). Specifically, we investigated how latency to enter an ethanol-paired chamber changed after administration of a footshock in the reward-paired chamber. Indeed, we found that low PA mice, that exhibit goal-directed ethanol seeking after moderate training, increased their latency to enter the reward-paired chamber significantly more than high PA mice, suggesting that the low PA mice exhibit flexible ethanol seeking (Fig 1e). High PA mice continued to enter the ethanol-paired chamber with low latencies. While we interpret this data to mean that high PA mice are entering in low latencies *despite* the association of the reward-paired chamber with footshock, an alternative interpretation is that high PA mice are increasingly motivated by pain or stress to seek the ethanol reward. Interestingly, unlike the habitual behavior, PA status was similarly predictive of compulsive-like food seeking. Together, these data indicate that mice whose food seeking is highly invigorated by reward-paired cues rapidly form habitual and compulsive-like ethanol seeking.

Using this model to assess innate neurobiological differences that may predate risk for the formation of inflexible ethanol seeking, we found that *Htr3a* methylation is decreased in the amygdala (but not other brain regions, including subregions of the PFC, or blood) of high PA (higher risk) mice as compared to low PA mice (Fig 2a; 2b). Though the pattern of methylation in blood was opposite that observed in the amygdala, it is not surprising to us that differential regulation was observed within distinct brain regions and the periphery as we did not expect similar expression patterns in all tissues. Notably, baseline differences that were seen in the blood of water exposed animals, which were opposite that seen in the amygdala were no longer present after exposure to ethanol, which we believe highlights the importance of identifying risk factors prior to the exposure to ethanol. Differences in DNA

methylation are thought to inversely predict expression of Htr3a mRNA, and ultimately protein, consistent with the observed elevation in 5HT3A expression in the amygdala of high PA mice (Fig 2d). These data implicated a possible connection between 5HT3 signaling and the expression of habitual and compulsive-like ethanol seeking. Importantly, methylation studies were performed in animals that had received subchronic exposure to ethanol. It is unclear from these studies how chronic ethanol exposure and physiological dependence would impact expression of 5HT3A in the amygdala.

The amygdala has been repeatedly identified as a key neurobiological substrate in PA and PIT (e.g., Cardinal et al., 2002; Everitt et al., 2006; Chang et al., 2012). Importantly, both the central and basolateral nuclei of the amygdala have similarly been shown to mediate the development of behavior that is insensitive to outcome devaluation (Balleine et al., 2003; Lingawi and Balleine, 2012), potentially through interactions with striatal circuitry, though bidirectional connectivity with the prefrontal cortex is likely also critically involved in amygdalar regulation of cue-driven reward seeking (Everitt et al., 2008; Peters et al., 2009). It is perhaps, then, unsurprising that individual differences in amygdala neurotransmitter systems were observed based on PA status that may mediate the propensity to develop stimulus-driven, outcome insensitive behaviors.

To determine a causal relationship, we employed pharmacological techniques to manipulate 5HT3 signaling. Using a modified version of our compulsive ethanol-seeking task where both high and low PA mice showed no increase in latency to enter the ethanol-paired chamber after footshock, we investigated the ability of 5HT3 antagonism to decrease compulsive ethanol seeking. Antagonism of 5HT3 reduced compulsive-like behavior in a dose-dependent manner. Interestingly, the dose that maximally increased latency to enter the ethanol-paired chamber after shock was dependent upon PA status. Low PA mice were responsive to a lower dose of the 5HT3 antagonist ondansetron than high PA mice (Fig 3a), consistent with decreased expression of 5HT3A in the amygdala of low PA mice compared to high PA mice. Interestingly, HT3 antagonism did not alter the expression of compulsive-like food-seeking behavior, suggesting that the role of 5HT3 in this behavior may be specific to ethanol (Fig 3b). We further demonstrated that compulsive-like ethanol seeking could be bidirectionally modulated through 5HT3 agonism. Enhanced 5HT3 signaling resulted in rapid entry to the ethanol-paired chamber despite association with adverse consequences (Fig 3c). We argue that epigenetic differences in amygdala 5HT3A expression are causally related to flexible ethanol seeking because modulations of 5HT3 signaling bidirectionally alter the expression of compulsive-like ethanol seeking.

As alcohol consumption itself can be altered through manipulations of amygdala 5HT3 signaling (Lynch et al., 2011), these innate differences may be crucial in understanding and identifying individual differences in successful alcoholism treatment with 5HT3 antagonists. Indeed, our data suggest that animals with elevated amygdalar 5HT3 require a larger dose of the antagonist to reduce compulsive-like ethanol seeking. In addition to dosage-related treatment questions, our data would suggest that 5HT3 expression may not be related to all forms of inflexible alcohol seeking, such as cue-induced reinstatement, and may only be implicated for particular components of alcoholism. As these studies do not investigate a role for 5HT3 signaling in ethanol dependence but rather highlight its role in the loss of

flexible behavior that supports addictive behavior, future research in rodents could be used to model whether individuals that are responsive to 5HT3 antagonism to reduce compulsive reward seeking would also show increased efficacy for other alcoholism-related behaviors, including alcohol drinking, compulsive self-administration, the transition to dependence and relapse.

In summary, we have seen that individual differences in cue reactivity measured by PA are predictive of the formation of habitual and compulsive-like alcohol seeking. Using animal models we identified innate differences in epigenetic regulation of 5HT3A expression in the amygdala, and used pharmacological tools to determine a mechanistic role of 5HT3 signaling in compulsive-like alcohol seeking. We suggest that these data emphasize the need for investigation of behavioral screens in humans that may be indicative of individual differences in risk status, and furthermore, the ability of treatment strategies such as 5HT3 antagonism to reduce addiction-related behavior. This information may help us move toward a better understanding and implementation of individualized treatment studies in addiction, which have proven to be highly effective in the treatment of other disorders. In contrast to individualized epigenetic/genetic-based pharmacotherapies for somatic illnesses, our findings indicate that behavioral endophenotypes, i.e., PA status, that reflect brain epigenetic status could provide a unique and efficacious therapeutic strategy for highly heterogeneous psychiatric disorders.

Acknowledgments

This research was supported by National Institutes of Health Grants AA012870 (JRT), AA017776 (JRT), AA020135 (JMB), DA031745 (MMT), DA022891 (HZ) and the Connecticut Department of Mental Health and Addiction Services.

References

- Balleine BW, Dickinson A. Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. *Neuropharmacology*. 1998; 37:407–419. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9704982>. [PubMed: 9704982]
- Balleine BW, Killcross AS, Dickinson A. The effect of lesions of the basolateral amygdala on instrumental conditioning. *J Neurosci*. 2003; 23:666–675. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12533626>. [PubMed: 12533626]
- Barker JM, Torregrossa MM, Taylor JR. Low prefrontal PSA-NCAM confers risk for alcoholism-related behavior. *Nat Neurosci*. 2012a; 15:1356–1358. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22922785>. [PubMed: 22922785]
- Barker JM, Zhang Y, Wang F, Taylor JR, Zhang H. Ethanol-Induced Htr3a Promoter Methylation Changes in Mouse Blood and Brain. *Alcohol Clin Exp Res*. 2012b Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22834954>.
- Becker. Alcohol Dependence, Withdrawal, and Relapse. *NIAAA Publ.*; 2008.
- Berridge KC, Robinson TE. Parsing reward. *Trends Neurosci*. 2003; 26:507–513. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12948663>. [PubMed: 12948663]
- Cardinal RN, Parkinson JA, Hall J, Everitt BJ. Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev*. 2002; 26:321–352. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12034134>. [PubMed: 12034134]
- Chang SE, Wheeler DS, Holland PC. Roles of nucleus accumbens and basolateral amygdala in autoshaped lever pressing. *Neurobiol Learn Mem*. 2012; 97:441–451. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22469749>. [PubMed: 22469749]

- Dickinson A, Wood N, Smith JW. Alcohol seeking by rats: Action or habit? 2002;331–348.
- Enoch M-A. The influence of gene-environment interactions on the development of alcoholism and drug dependence. *Curr Psychiatry Rep.* 2012; 14:150–158. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22367454>. [PubMed: 22367454]
- Everitt BJ, Belin D, Economidou D, Pelloux Y, Dalley JW, Robbins TW. Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. *Philos Trans R Soc Lond B Biol Sci.* 2008; 363:3125–3135. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2607322&tool=pmcentrez&rendertype=abstract>. [PubMed: 18640910]
- Everitt BJ, Cardinal RN, Parkinson JA, Robbins TW. Appetitive Behavior. *Ann N Y Acad Sci.* 2006; 985:233–250. Available at: <http://doi.wiley.com/10.1111/j.1749-6632.2003.tb07085.x>. [PubMed: 12724162]
- Fligel SB, Akil H, Robinson TE. Individual differences in the attribution of incentive salience to reward-related cues: Implications for addiction. *Neuropharmacology.* 2009; 56(Suppl 1):139–148. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2635343&tool=pmcentrez&rendertype=abstract>. [PubMed: 18619474]
- Heilig M, Goldman D, Berrettini W, O'Brien CP. Pharmacogenetic approaches to the treatment of alcohol addiction. *Nat Rev Neurosci.* 2011; 12:670–684. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3408029&tool=pmcentrez&rendertype=abstract>. [PubMed: 22011682]
- Johnson BA. Serotonergic agents and alcoholism treatment: rebirth of the subtype concept--an hypothesis. *Alcohol Clin Exp Res.* 2000; 24:1597–1601. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11045870>. [PubMed: 11045870]
- Kareken DA, Liang T, Wetherill L, Dziedzic M, Bragulat V, Cox C, Talavage T, O'Connor SJ, Foroud T. A polymorphism in GABRA2 is associated with the medial frontal response to alcohol cues in an fMRI study. *Alcohol Clin Exp Res.* 2010; 34:2169–2178. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20698837>. [PubMed: 20698837]
- Lingawi NW, Balleine BW. Amygdala central nucleus interacts with dorsolateral striatum to regulate the acquisition of habits. *J Neurosci.* 2012; 32:1073–1081. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22262905>. [PubMed: 22262905]
- Lovinger DM. 5-HT₃ receptors and the neural actions of alcohols: an increasingly exciting topic. *Neurochem Int.* 1999; 35:125–130. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10405996>. [PubMed: 10405996]
- Lynch WJ, Bond C, Breslin FJ, Johnson BA. Severity of drinking as a predictor of efficacy of the combination of ondansetron and topiramate in rat models of ethanol consumption and relapse. *Psychopharmacology (Berl).* 2011; 217:3–12. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21424693>. [PubMed: 21424693]
- McMahon FJ, Insel TR. Pharmacogenomics and personalized medicine in neuropsychiatry. *Neuron.* 2012; 74:773–776. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22681682>. [PubMed: 22681682]
- Peters J, Kalivas PW, Quirk GJ. Extinction circuits for fear and addiction overlap in prefrontal cortex. *Learn Mem.* 2009; 16:279–288. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19380710>. [PubMed: 19380710]
- Quinn JJ, Hitchcott PK, Umeda Ea, Arnold AP, Taylor JR. Sex chromosome complement regulates habit formation. *Nat Neurosci.* 2007; 10:1398–1400. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17952068>. [PubMed: 17952068]
- Robison AJ, Nestler EJ. Transcriptional and epigenetic mechanisms of addiction. *Nat Rev Neurosci.* 2011; 12:623–637. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3272277&tool=pmcentrez&rendertype=abstract>. [PubMed: 21989194]
- Saunders BT, Robinson TE. Individual variation in resisting temptation: Implications for addiction. *Neurosci Biobehav Rev.* 2013 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23438893>.
- Van den Bos R, van der Harst J, Vijftigschild N, Spruijt B, van Luijtelaar G, Maes R. On the relationship between anticipatory behaviour in a Pavlovian paradigm and Pavlovian-to-

- Instrumental Transfer in rats (*Rattus norvegicus*). *Behav Brain Res*. 2004; 153:397–408. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15265635>. [PubMed: 15265635]
- Yaakob N, Malone DT, Exintaris B, Irving HR. Heterogeneity amongst 5-HT₃ receptor subunits: is this significant? *Curr Mol Med*. 2011; 11:57–68. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21189117>. [PubMed: 21189117]
- Zhang H, Herman AI, Kranzler HR, Anton RF, Zhao H, Zheng W, Gelernter J. Array-Based Profiling of DNA Methylation Changes Associated with Alcohol Dependence. *Alcohol Clin Exp Res*. 2012 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22924764>.

Figure 1.

a. PIT training for food. Mice first learned that a cue (tone) predicted the delivery of a food reinforcer (PA). Mice were assigned to high and low PA groups that were significantly different based on a median split of normalized approach scores. Mice were then trained to perform an instrumental response (lever press) for the same reinforcer. PIT was assessed in a 6-min test in extinction. Responding during Cue On and Cue Off intervals was measured. PA and PIT status were not related. **b.** Ethanol habit. High PA mice form ethanol habits more rapidly than Low PA mice. At a moderate training time point, Low PA mice remained goal-directed at a time point when high PA mice were habitual. **b.** Low PA mice reduce responding after outcome devaluation with LiCl-induced conditioned taste aversion (i.e., goal-directed) while high PA mice continued to respond for the

devalued ethanol reinforcer at high rates (i.e., habitual). **c.** Low PA mice reduce responding when the action-outcome relationship is degraded (i.e., goal-directed) while high PA mice were insensitive to the changes in contingency (i.e., habitual). **d.** PA status was not predictive of the formation of habitual sucrose seeking by contingency degradation measures. **e.** High PA mice show compulsive-like ethanol-seeking behavior. Low PA mice show increased latency to enter an ethanol-paired chamber after pairing with a shock ($p=.01$), whereas High PA mice do not differ in their latency to enter the ethanol-paired chamber after footshock. **f.** PA status was not predictive of ethanol consumption. In a drinking in the dark procedure, high and low PA mice consumed equivalent amounts of alcohol, showed here as total grams of ethanol per kg of body weight across five days of drinking in the dark. Graphs show mean $\pm$ SEM. * $p<.05$, ** $p<.01$, *** $p<.001$

Figure 2.

a. *Htr3a* promoter region methylation was assessed in mice screened through PA and exposed to ethanol in a DID paradigm. We saw that PA status predicted methylation of *Htr3a* in the amygdala such that low PA mice have a higher mean methylation status of CpG sites across the promoter region than high PA mice. **b.** Individual differences in PA status also predicted methylation of individual CpG sites across the promoter region of *Htr3a*. **c.** PA status was predictive of methylation of *htr3a* in the blood only in control animals. No differences were seen in *htr3a* methylation in blood after ethanol exposure in the DID paradigm. **d.** PA scores significantly correlated with normalized 5HT3A expression in the amygdala, such that lower PA is related to decreased expression of 5HT3, consistent with epigenetic regulation (shown by median split in insert). Bar graphs show mean $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$

Figure 3.

a. Based on the differential epigenetic regulation, the ability of 5HT3 antagonism to prevent compulsive ethanol seeking was assessed. The ability of decreased 5HT3 signaling to increase latency to enter the reward-paired chamber is mediated by PA status; higher doses of ondansetron were required to attenuate compulsive-like ethanol seeking in high PA mice than in low PA mice, consistent with differences in amygdalar 5HT3 expression. **b.** Activation of the 5HT3 receptor with the agonist mCPBG injected i.p. at 5mg/kg resulted in increased compulsive-like behavior, that is, no change in latency was observed after association of the reward-paired chamber with footshock. **c.** Antagonism of the 5HT3 receptor with ondansetron (.05mg/kg) did not impact latency to enter a sucrose paired chamber after a footshock, suggesting that the ability of a 5HT3 receptor to reduce compulsive-like behavior may be specific to ethanol seeking. Bar graphs show mean $\pm$ SEM. * $p < .05$, ** $p < .01$, *** $p < .001$