A Model of Ethanol Self-Administration in Head-Fixed Mice

(running title: Head-Fixed Ethanol Self-Administration)

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Abstract

Significant advances in neurotechnology, such as the emergence of 2-photon imaging, have enabled unparalleled access to the complex neural circuits that coordinate behavior in rodents. Integration of these techniques would be groundbreaking for the study of animal models of alcohol use disorder (AUD), which is rooted in longitudinal brain adaptations that could be functionally monitored and manipulated at the level of neural circuits from the onset of alcohol use through dependence. However, 2-photon and related methodologies require or are often facilitated by head-fixation, and a lack of head-fixed models have hindered their integration in the study of AUD. Here we present a head-fixed alcohol self-administration model, and find that head-fixed male and female mice will reliably press an active, but not inactive, lever for an oral alcohol reward. The number of alcohol rewards obtained reliably predicted blood alcohol concentrations, at physiologically relevant levels. Furthermore, we demonstrate that mice can extinguish alcohol self-administration when the alcohol reward is omitted, suggesting active lever pressing behavior was alcohol-directed. Following extinction, presentation of alcohol-related cues or a priming reminder of alcohol itself invigorated reinstatement of alcohol seeking, modeling relapse in a manner that mimics decades of work in freely-moving rodent studies. Overall, our head-fixed alcohol self-administration model allows integration of novel technologies that require or are greatly facilitated by head-fixation, improving our ability to study and understand the neural circuits adaptations and computations that underlie AUD.

1. INTRODUCTION

Alcohol use disorder (AUD) is characterized by continuous cycles of positive reinforcement, wherein the pleasant, euphoric effects of alcohol cause an increase in alcohol consumption (1) and negative reinforcement, wherein individuals consume alcohol to avoid withdrawal or alleviate anxiety (2, 3). AUD is a chronic, relapsing neuropsychological condition where excessive alcohol seeking and the inability to stop seeking despite negative consequences are hallmarks of dependence (4). Repeatedly experiencing the positive reinforcing effects of alcohol can increase the likelihood of alcohol-seeking behaviors (5), and contextual associations, such as drug-associated cues, environments or the drug itself, may trigger relapse in individuals with AUD. To adequately replicate this reinforcement of seeking behaviors in preclinical models, researchers often use self-administration paradigms, which reinforce alcohol seeking (e.g., active lever pressing or nose-pokes), the rewarding aspects of alcohol, and perpetuate a cycle that strengthens alcohol-seeking behavior in rodent models (6-8). During self-administration, animals learn an operant task which is subsequently reinforced by drug delivery, often paired with a discrete drug-associated cue, like a tone or a light (9). Following learning, this reinforced behavior is extinguished through drug and cue omission. Once this behavior is adequately extinguished, animals can be tested for reinstatement of seeking behavior to a variety of stimuli, including cues and drug, which models relapse in humans (10). Therefore, preclinical models can effectively parallel the complex cycle of substance use disorders through self-administration (11).

Considering the effectiveness of using operant conditioning as a behavioral model, it can be paired with emerging techniques to elucidate the neurobiological mechanisms of alcohol use disorders. With the advent of biosensor technologies, one such technique that has gained traction in the past decade is two-photon microscopy, which can be used to analyze cellular activity *in vivo* (12, 13). Two-photon calcium imaging, as well as other calcium imaging techniques, can provide a wealth of information regarding specific neural activity patterns, and provides critical data concerning the temporal nature in the development of alcohol use disorders (14). Specifically, two-photon imaging allows researchers to track individual cells across time and analyze ensemble activity (12). Moreover,

77 two-photon imaging allows unparalleled access to the brain through cell-type and circuit specificity
78 using Cre-driver line and/or intersectional viral approaches, allowing researchers to examine vast
79 populations in the brain at unparalleled temporal and spatial resolution. Until more recently, two-photon
80 imaging was constrained by the relatively limited behavioral procedures capable of being tested in
81 head-fixed mice, but creative adaptations have allowed for more sophisticated behavioral paradigms in
82 head-restrained setups. Recent developments have incorporated elements of operant conditioning
83 (e.g., levers) and delivery mechanisms (e.g., sipper tubes for fluid delivery or IV catheterization for IV-
84 drug delivery) with sophisticated two-photon microscopy, allowing for dissection of underlying neural
85 activity (15-19). Applying these approaches to the examination of AUD as the disorder progresses will
86 help us understand how molecular processes may inform behavioral phenotypes.

87 Despite progress in adapting traditional behavioral paradigms to the head-fixed setup, only a
88 couple of reports have used head-fixed behavioral paradigms in alcohol related behaviors (20, 21), and
89 to date, no other research has utilized a head-fixed operant conditioning protocol that features both
90 active and inactive manipulations, and assesses extinction and reinstatement for use with two-photon
91 imaging to examine the spectrum of features of alcohol use disorder. Thus far, no head-fixed alcohol
92 drinking protocols have adequately paralleled the freely moving operant paradigm. Here we present
93 our head-fixed oral operant alcohol self-administration behavioral paradigm adapted from recent Otis
94 lab procedures (18) to model motivated alcohol-seeking behavior.

95 Here we show that head-fixed animals will learn to press an active lever which results in a tone
96 cue followed by an ethanol reward, rapidly discriminate between the active and inactive levers, and
97 achieve physiologically relevant blood alcohol concentrations. Moreover, using an extinction paradigm
98 where cue and ethanol rewards are omitted, we have found that animals will suppress active lever
99 pressing across the course of learning. Notably, when we re-introduce cue or ethanol rewards following
100 extinction learning, we have found that mice will significantly reinstate drug-seeking behaviors.
101 Collectively, these results suggest that we can effectively model alcohol self-administration similar to
102 that of freely moving mice in a head-fixed setup, and establish an effective paradigm for use with high

103 resolution two-photon microscopy to provide extensive insight into the neural mechanisms underlying
104 AUD.
105
106 **2. MATERIALS AND METHODS**
107 **2.1 Animals**
108 Male and female C57BL/6J or wildtype C57BL/6J background mice (8 weeks old/20g minimum at study
109 onset; Jackson Labs or MUSC DLAR Breeding Colony) were group housed pre-operatively and single
110 housed post-operatively under a reversed 12:12-hour light cycle (lights off at 8:00AM) with access to
111 standard Teklad Diet 2918 (Harlan) chow and water *ad libitum*. Experiments were performed during the
112 dark phase and in accordance with the NIH Guide for the Care and Use of Laboratory Animals with
113 approval from the Institutional Animal Care and Use Committee (IACUC) at the Medical University of
114 South Carolina.
115 **2.2 Head-Fixed Surgeries**
116 Mice were anesthetized with isoflurane (0.8-1.5% in oxygen; 1L/min) and placed within a stereotaxic
117 frame (Kopf Instruments) for intracranial surgeries. Ophthalmic ointment (Akorn), topical anesthetic (2%
118 Lidocaine; Akorn), analgesic (Ketorlac, 2mg/kg, IP), and subcutaneous sterile saline (0.9% NaCl in
119 water) treatments were given pre-operatively and intra-operatively for the health and pain management
120 of the animal. Some animals received head-ring implantation only (see below) and utilized for behavior
121 only (n = 5), while some animals reported herein were utilized for other experimentation beyond the
122 scope of this report and experienced additional surgical procedures. Specifically, some mice received
123 a bilateral virus injection of retro-AAV2-cre(rgAAV2-hSyn-Cre) was administered in the nucleus
124 accumbens shell (NAcS; relative to bregma: AP, +1.45mm; ML, ±0.65mm; DV, -4.65mm), followed by
125 a second viral injection of either Halorhodopsin or eYFP (AAV5-ef1a-DIO-eNpHR3.0-eYFP; AAV5-
126 ef1a-DIO-eYFP) into the posterior paraventricular thalamus (PVT; relative to bregma: AP, -1.55mm;
127 ML, -1.13m; DV, -3.30mm; at a 20° angle). Animals utilized for optogenetic experiments (data not
128 reported here) received a custom-made optical fiber was implanted above the pPVT injection site

129 (relative to bregma: AP, -1.55mm; ML, -1.13m; DV, -3.00mm; at a 20° angle). A custom-made head-
130 ring (stainless steel; 5 mm ID, 11 mm OD; Clemson Engineering) was implanted onto the animal skull
131 to allow for head-fixation. Following surgeries animals received antibiotics (Cefazolin, 200mg/kg, sc)
132 and recovered with access to food and water *ad libitum* for 14 days minimum.

133 **2.3 Alcohol Drinking Habituation**

134 Following three to four weeks of recovery from surgery, animals were acclimated to ethanol (EtOH)
135 drinking using a modified Drinking-in-the-Dark (DID) paradigm (22, 23). Three hours into the dark cycle
136 (lights off at 8AM), water bottles were replaced with volumetric sipper tubes containing 15% EtOH (v/v
137 in reverse osmosis water). Animals were permitted to drink for two hours each day over the course of
138 seven days. Animals were weighed each day of home cage drinking and ethanol consumption was
139 normalized to body weight (grams of ethanol consumed per kilogram of body weight; g/kg).

140 **2.4 Head-Fixed Alcohol Operant Self-Administration**

141 *Behavioral Acquisition*

142 Following home cage drinking, mice underwent two days of habituation during which they were
143 head restrained in the operant chambers for 45 minutes with no access to the levers, tone cue, or
144 ethanol reward. Once animals were adequately habituated to head-fixation, animals were trained on
145 an FR1 schedule of reinforcement operant conditioning paradigm. During behavioral acquisition, the
146 levers were placed in front of the mice. Pressing the active lever resulted in an immediate cue
147 presentation (8kHz, 2s), followed by a trace interval (1.6s) and finally an ethanol reward. Mice self-
148 administering alcohol received 15% EtOH droplets (~3-5 μL) in front of their mouth. Reinforced active
149 lever pressing resulted in a 2 second timeout period wherein continuous pressing of the active lever
150 was recorded, but no reward or cue was presented. Pressing of the inactive lever presented neither
151 cue nor reward. Mice underwent alcohol self-administration for 1-hour sessions where mice were
152 allowed to freely consume as much alcohol as desired.

153 *Extinction & Reinstatement Testing*

Animals will learn to extinguish prior alcohol-seeking behavior following 14 days of self-administration. Mice learn to extinguish drug-seeking behavior across the course of 10 days, wherein animals will receive no ethanol or cue reward during lever pressing. Following a minimum of 10 days of extinction, if animals have reached satisfactory lever pressing, i.e., <20% of the last two days of behavioral acquisition (18), they undergo either drug or cue reinstatement. For cue reinstatement, animals received a tone cue (8kHz, 2s) upon pressing the active lever but did not receive an ethanol reward. For drug reinstatement, animals were given access to a volumetric sipper tube filled with 15% ethanol for 30 minutes in their home cage prior to session in the box.

Blood Ethanol Concentration (BEC) Measurements

To determine the degree of intoxication following operant self-administration behavioral sessions, retro-orbital blood samples were collected in heparinized capillary tubes in a subset of animals to measure blood ethanol concentrations (BECs). Blood samples were centrifuged at 10,000x*g* at 4°C for 10 minutes to separate red blood cells and plasma, and plasma samples were extracted and BEC determined using an Analox AM1 Analyzer.

Data Collection and Statistics

Lever pressing data during acquisition was collected using a MATLAB interface connected to an Arduino set up. GraphPad PRISM (version 10) was used to conduct statistical analyses and, where appropriate, either analysis of variance (ANOVA; two-way or three-way) or paired t-tests were used to analyze behavioral data. The following independent variables were analyzed: lever presses (active or inactive), day (acquisition, extinction or reinstatement) and sex (male or female).

3. RESULTS

3.1 Home Cage Ethanol Consumption

We initially habituated animals to ethanol drinking using the Drinking-in-the-Dark (DID) paradigm. Animals were given access to 15% ethanol (v/v) in their home cage via volumetric sipper tubes for 7 days a week prior to head-fixation (**Fig 1B**). Despite previous reports of sex-dependent differences in

180 alcohol consumption, we found that male and female mice drank similar amounts during the home cage
181 access period [female mean 1.99 g/kg ± 0.15 vs male mean 2.07 g/kg ± 0.11 (Fig 1B); main effect of
182 sex, $F_{1,28}$=0.1051, *p*=0.7482]. There was a main effect of day, $F_{3.23,89.8}$=4.317, *p*=0.0057, and Tukey's
183 posthoc analyses showed that drinking on day 2 increased compared to day 1 (*p*=0.0324), but stabilized
184 after that, and there was no sex x day interaction, $F_{6,167}$=1.794, *p*=0.1031. Please note, one drinking
185 value was removed because of spillage.

186 **3.2 Acquisition of Ethanol Self Administration**

187 After home cage DID exposure, mice were trained to press the active lever on an FR1 schedule of
188 reinforcement for delivery of 15% (v/v) ethanol. Over the course of 14 days of acquisition animals
189 learned to reliably discriminate between the active and inactive levers (**Fig 1D**). Analysis using a mixed-
190 effects three-way ANOVA showed a significant main effect of lever (but not day or sex, *p* = 0.5327 and
191 0.1006, respectively), such that overall mice pressed the active lever significantly more than the inactive
192 lever, $F_{1,29}$=215.2, *p*<0.001. Additionally, there was no day x sex interaction (*p*=0.3836) nor a day x sex
193 x lever interaction (*p*=0.2860), suggesting males and females performed comparably overall. However,
194 there was a significant day x lever interaction, $F_{6.884,199.6}$=3.580, *p*=0.0013, suggesting significant
195 separation between the active and inactive levers developed as acquisition went on, and a significant
196 sex x lever interaction, $F_{1,29}$=6.922, *p*=0.0135, suggesting males pressed the active lever slightly more
197 than females. A two-way ANOVA analyzing number of alcohol reinforcers indicated a significant main
198 effect of day $F_{13,377}$=2.610, *p*=0.0017, suggesting that number of alcohol reinforcers increased across
199 acquisition (**Fig 1E, inset**). There was also a main effect of sex, $F_{1,29}$=5.983, *p*=0.0207, suggesting that
200 overall males earned more reinforcers than females (44.61 vs. 36.40, respectively. There was no
201 significant day x sex interaction (*p*=0.1811). Following a subset of self-administration sessions, some
202 animals received retro-orbital eyebleeds to measure blood ethanol concentration (BEC). The data
203 evinced a linear relationship (R^2=0.9162, p=0.0027) between number of alcohol reinforcers earned and
204 BEC, effectively showing that animals become reliably intoxicated with an increasing number of alcohol
205 reinforcers during acquisition (**Fig 1E**).

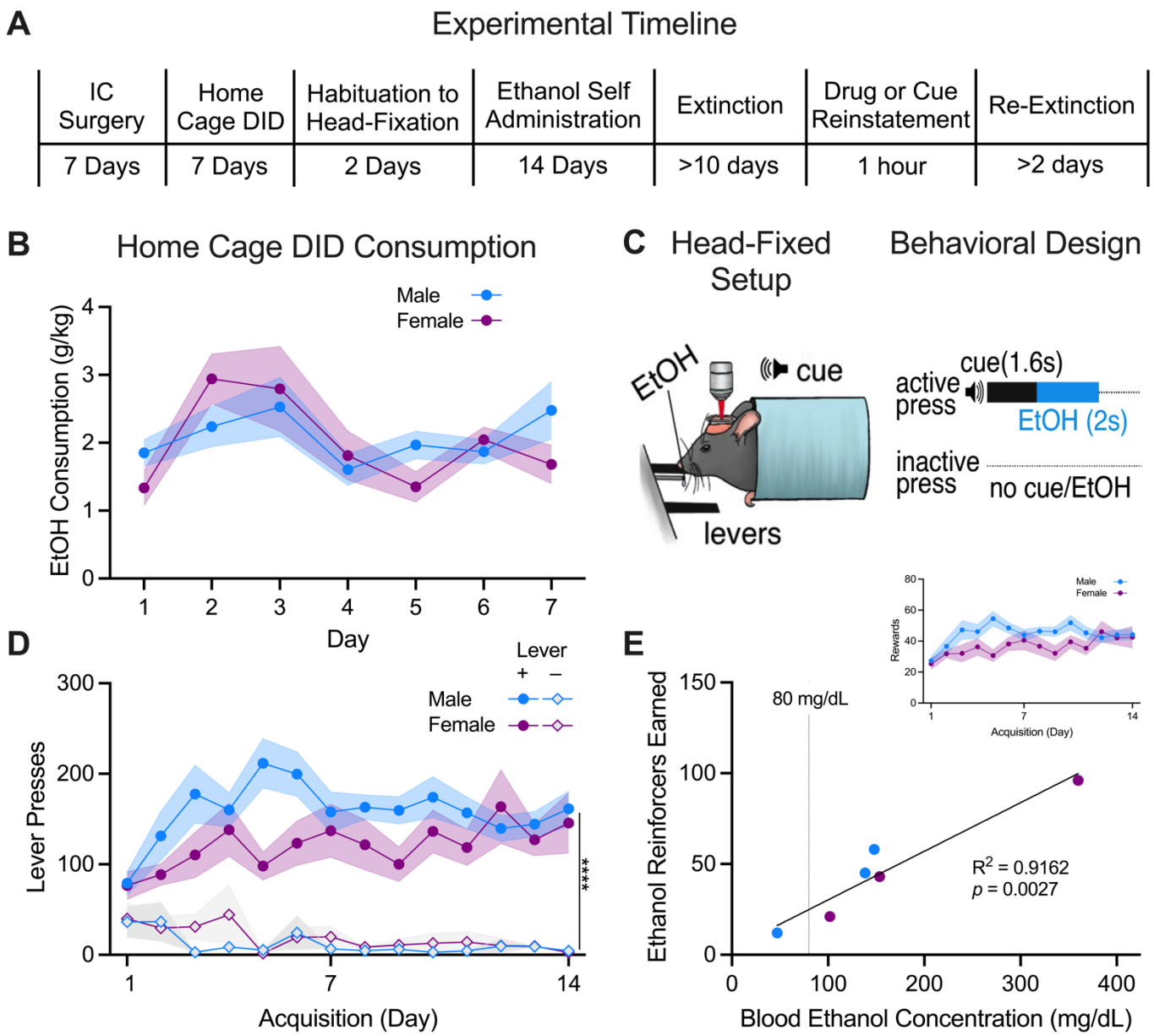

Figure 1. Head-fixed ethanol self-administration paradigm. (**A**) Experimental timeline. (**B**) Home cage Drinking-in-the-Dark (DID) ethanol (15% v/v) consumption in females (purple) and males (blue). (**C**) Schematic of head-fixed operant setup and behavioral design – active lever press results in a 1.6s tone cue, followed by delivery of a 15% (v/v) EtOH reinforcer. (**D**) Lever pressing behavior across 14 days of acquisition on the active and inactive levers for males and females showing significant discrimination between the active and inactive levers, ****p<0.0001. (**E**) Correlation of ethanol reinforcers earned and blood ethanol concentrations achieved and total reinforcers earned (inset).

3.3 Extinction

After acquisition of alcohol self-administration, animals underwent extinction training (n=23; 13 males and 10 females). We have found that animals will sufficiently extinguish active lever pressing from the first day of extinction to the last day (**Fig 2A**). In the early days of extinction, animals will exhibit elevated

219 lever pressing behavior that declines across time. A 2 x 2 RM ANOVA revealed a significant main effect
220 of day, $F_{1,21}$=9.120, p=0.0065, confirming that overall animals significantly reduced lever-pressing
221 behavior from the first day to the last day of extinction (**Fig 2A**). Additionally, a significant main effect
222 of sex, $F_{1,21}$=5.432, p=0.0298 and a significant day x sex interaction, $F_{1,21}$=5.756, p=0.0258, revealed
223 that males pressed the active lever on the first day of extinction significantly more than females
224 (p=0.0017), showing that males might be more prone to extinction burst behavior.

225 **3.4 Reinstatement Tests Following Extinction**

226 Lastly, we have found that head-fixed animals will reinstate ethanol seeking behavior following
227 extinction learning. Here we show that both male and female mice will equivalently reinstate lever-
228 pressing behavior to the tone cue previously paired with the ethanol reward (**Fig 2B**). Specifically, a 2
229 x 2 RM ANOVA revealed a significant main effect of day, $F_{1,18}$=27.90, p<0.0001), such that lever
230 presses were increased after cue presentation in comparison to the previous day's extinction baseline
231 in a sex-independent manner (no main effect of sex, $F_{1,18}$=0.0541, p=0.8187, and no day x sex
232 interaction, $F_{1,18}$=0.0974, p=0.7586). Moreover, these animals will reinstate lever pressing following 30
233 minutes of home cage drinking (HCD) prior to a head-fixed behavioral session. Using a 2 x 2 RM
234 ANOVA we found that, similar to cue-induced reinstatement, both male and female mice will
235 significantly reinstate lever-pressing behavior in response to a drug-priming (**Fig 2C**) as shown by a
236 main effect of day, $F_{1,16}$=22.28, p=0.0002, but no main effect of sex, $F_{1,16}$=2.777, p=0.1151, or a day x
237 sex interaction, $F_{1,16}$=3.377, p=0.0848.

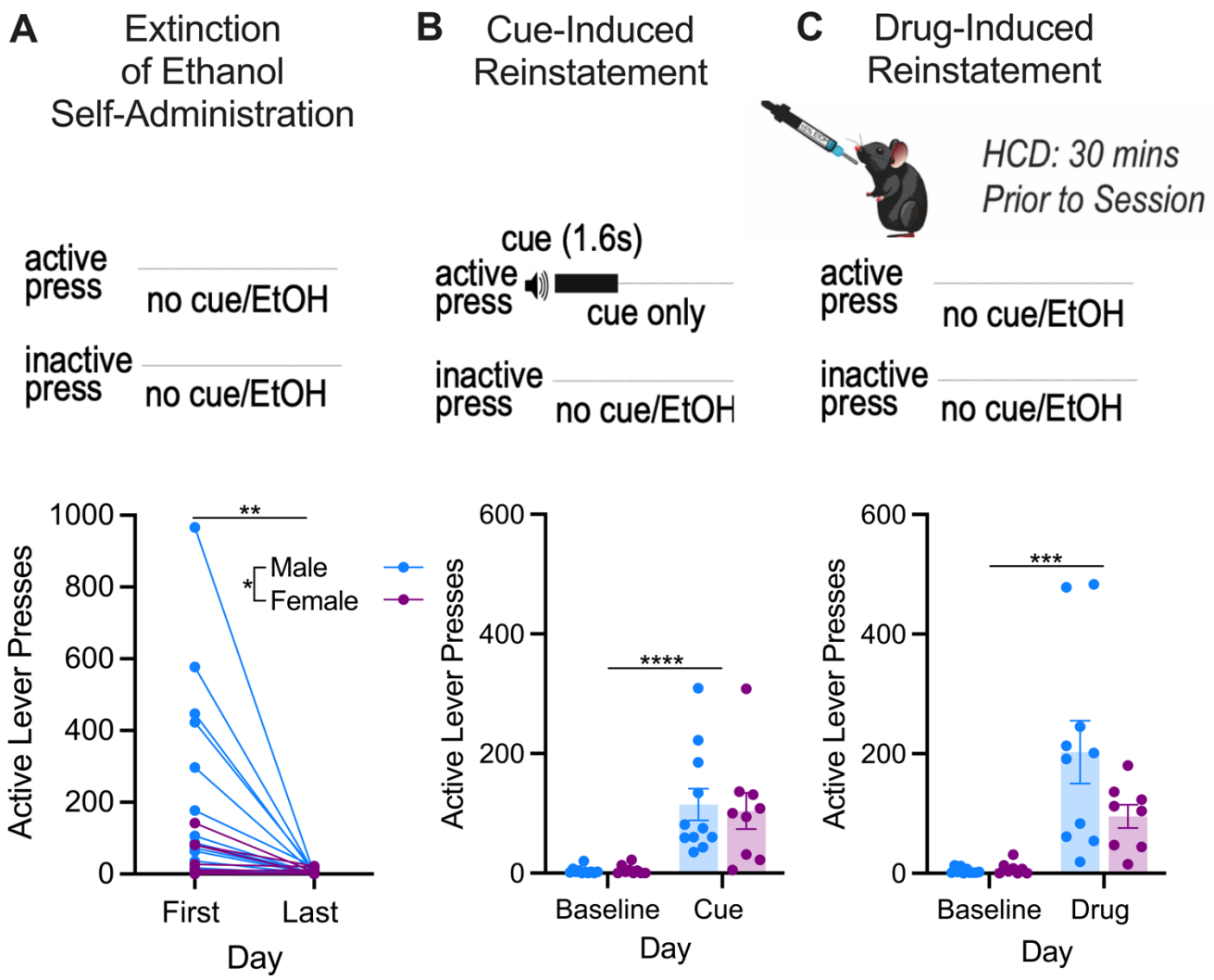

Figure 2. Extinction and reinstatement of operant responding for ethanol in a head-fixed paradigm. (**A**) During extinction learning, responses on the active lever no longer result in delivery of the tone cue or ethanol reward (top panel); males show significantly greater responding on day 1 of extinction compared to females (**p*<0.05), but both male and female mice significantly reduce their lever pressing behavior from the first to last day of extinction (***p*<0.01; bottom graph). (**B**) During cue-induced reinstatement testing, active lever results in delivery of the tone cue but no ethanol reward (top panel); both males and females show significantly greater responding on the cue-test day compared to their extinction baseline (*****p*<0.0001; bottom graph). (**C**) During drug-induced reinstatement testing, mice were given 30-min access to 15% ethanol in their home cage prior to placement in the head-fixed operant set up (top panel); both males and females show significantly greater responding on the drug-test day compared to their extinction baseline (*****p*<0.001; bottom graph).

4. DISCUSSION

Collectively, our findings indicate that animals will self-administer ethanol in a head-fixed operant conditioning paradigm. Moreover, we find that animals will extinguish active lever-pressing behavior in the absence of tone cue and ethanol reward. Interestingly, when animals are re-introduced to a tone cue or ethanol reward, animals will reinstate active lever-pressing behavior. We have found that animals

256 will effectively acquire, extinguish and reinstate alcohol-seeking behaviors while head-fixed effectively
257 establishing the ability to image during behavior with two-photon microscopy, providing for the first time
258 an uniquely analogous behavioral paradigm to that in freely moving mice. These results indicate that
259 our head-fixed ethanol self-administration paradigm provides a robust and novel technique to further
260 dissect the mechanisms underlying alcohol use disorder.

261 ***Head-Fixation in Drug and Reward Self-Administration***

262 Head-fixation during drug self-administration has been a reliable model to study various drugs
263 of abuse, including heroin and cocaine (18). Previous studies have found that animals will effectively
264 acquire lever pressing, extinguish and reinstate during head-fixed heroin and cocaine self-
265 administration (18). Notably, head-fixed animals will learn to discriminate between active and inactive
266 levers, indicating the acquisition of goal-directed behavior that has been illustrated in freely moving
267 models. In addition to drug self-administration, the same operant self-administration, extinction and
268 reinstatement phenomena has been observed in sucrose reward-seeking models (18). Head-fixation
269 during alcohol seeking tasks is emerging as a way to examine circuitry during *in vivo* recordings,
270 providing a wealth of information on how alcohol can alter distinct neurons and ensembles in the brain
271 during alcohol consumption. Other models have utilized aversion-resistance tasks during head-fixation
272 (21) as well as other tasks such as operant licking tasks (20) to examine how animals will respond to
273 various alcohol seeking contexts. However, the literature on head-fixed seeking tasks during alcohol
274 consumption remains limited and research is currently ongoing to understand how various circuits can
275 be studied during alcohol seeking.

276 ***Benefits of Two-Photon Microscopy and Applications to Alcohol Seeking***

277 In recent years, two-photon microscopy has been a paramount tool in unraveling mechanisms
278 underlying substance use disorders, neurodevelopmental disorders, and neurodegenerative disorders,
279 among others (12, 13). Two-photon microscopy utilizes two near-infrared photons to excite
280 fluorophores within a sample simultaneously, allowing for fluorescence emission at a longer wavelength
281 (13). This excitation facilitates deeper tissue penetration and background signal minimization, which

282 improves imaging resolution and clarity. Moreover, two-photon microscopy has reduced phototoxicity
283 and photobleaching, in comparison to other microscopy techniques, which enables prolonged cellular
284 and molecular observations (12). By offering a dynamic, *in vivo* perspective concerning the neural
285 adaptations associated with alcohol use disorder, this technique contributes to our understanding of
286 which brain regions are implicated in AUD, and how this disorder may develop over time. Lastly, two-
287 photon microscopy serves as an invaluable resource for strengthening our understanding of complex
288 neurobiological systems given its capacity to offer insights into physiological and pathological
289 processes at a level of detail previously unattainable. Therefore, using this technique in conjunction
290 with established behavioral methods, such as operant conditioning, offers unprecedented insight into
291 dissecting the behavioral and cellular mechanisms surrounding alcohol use disorder.

292 ***Temporal Tracking of Single Cell and Synaptic Activity in Multiple Regions Through Two-Photon***

293 One of the many applications of two-photon microscopy is the unique ability to temporally
294 monitor and track cells across the course of learning. The ability to track single cell activity across time
295 provide highly compelling and informative information regarding plasticity and migration as various
296 neurobiological conditions develop. Moreover, this temporal tracking technique is highly multifaceted in
297 that it may be used to track single cells, dendritic spines (24, 25), axons (26), and dendrites (27).
298 Overall, this tracking provides pivotal information in dissecting the etiology of substance use disorders
299 and how these disorders alter plasticity and learning across the course of development.

300 Moreover, two-photon microscopy provides researchers with the ability to investigate a myriad
301 of brain regions and circuits at high resolution. Researchers have recorded from a variety of regions
302 including the visual cortex, the motor cortex, hippocampus (28) and the paraventricular thalamus (19).
303 While a vast majority of two-photon microscopy has been centered in the cortex (29) via implantation
304 of cranial windows or gradient refractive index (GRIN) lenses, deeper striatal and brainstem structures
305 remain relatively unexplored. Therefore, there is a growing and expanding drive to extend imaging
306 techniques to be able to effectively record from deeper brain structures to further enhance our
307 understanding of neurocircuitry in the brain.

Potential Stress Responses in Head-Fixed Models

One of the primary concerns with head-fixed behavioral tasks is potential stress responses that may result from head restraint (30). This presents a valid concern as stress responses affect a variety of behaviors, including drug seeking (31). However, longevity of habituation to the head-fixed setup may allow rodents to become more acclimated to this restraint and prevent stress levels from influencing behavior (32). Moreover, previous studies implementing head-fixed drug self-administration have not indicated changes in a behavior as a result of head-restraint (18) and remain sensitive to stress (19). Nevertheless, as technology and equipment improve, there has been increasing integration of elements such as a running wheel or a treadmill into head-fixed setups to track movement and limit potential stress from restraint.

FUTURE DIRECTIONS AND LIMITATIONS

While our head-fixed model has shown robust and consistent results in ethanol self-administration, there are several potential limitations. Furthermore, additions to our current head-fixed set up could be added to track other metrics during operant tasks, such as a camera to measure aspects such as pupil diameter and whisker movement, which could be used to assess states of arousal (33) and object recognition (34).

5. CONCLUSION

Collectively, our results show that head-fixed animals will rapidly and reliably learn to goal-directed behaviors to seek alcohol. Our head-fixed paradigm indicates that animals will learn to discriminate between active and inactive levers, effectively extinguish alcohol seeking behavior and subsequently reinstate to alcohol and alcohol-seeking cues. These results indicate that self-administration protocols can be effectively applied to head-fixed mice during two-photon imaging, revealing unparalleled access to the brain during alcohol seeking behaviors.

334 **ETHICS STATEMENT**

335 All animal experimental procedures were reviewed and approved by the Institutional Animal Care and
336 Use Committee (IACUC) at the Medical University of South Carolina and adhere to NIH guidelines for
337 working with laboratory animals (35).

338

339 **AUTHOR CONTRIBUTIONS**

340 ALW, JMO and JAR designed the experiments and wrote the manuscript. ALW, KTW, SAB, REC,
341 MSB helped conduct the experiments. SAB, REC, MSB, MRM, KMV, JP, EMD, and RIG provided
342 intellectual support and training for self-administration studies. All authors contributed to the article
343 and approved the final submitted version.

344

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350

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