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Assessing loss of control over substance use for sucrose, alcohol and cocaine using the novel seeking under the threat of adversity (STA) task

-Major Project Thesis-

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Laymen's Summary

Substance addiction is a detrimental illness that affects millions of people world-wide. Besides its impact on the affected individuals, substance addiction is also the most expensive brain disorder, costing billions of euros for the health care system and authorities. One of the characteristic symptoms of addiction is loss of control over the use of drugs, which is seen through the patient's inability to control the amount of the drug they consume. It is also seen through their tendency to get involved in risky behaviours to get the drug, such as drunk driving, getting into fights, and even consuming substances that are not intended for consumption (such as drinking mouth wash and sanitary alcohol). In this way, losing control over the behaviour can be devastating to the patient and the people surrounding them.

We aim to better understand loss of control and how it develops in certain people, but not others, by observing the behaviour of rats in a newly designed research model that can capture some of the main features of human addiction. People are aware of the possible consequences of doing drugs, but these consequences do not always occur, and this what our model aims to capture. The Seeking under the threat of adversity (STA) model aims to mimic this behaviour. Rats are trained to voluntarily press on a lever to get a small amount of a drug. Initially, their seeking of the drug is not punished. But after some time, a tone which acts as a warning signal is introduced. If any presses are made during this time, they have a ¼ chance to receive a mild electrical shock. In this way, we hope the rats will feel a similar dilemma to what humans feel in the moment they must decide whether to get a drug or not.

We find that rats tested of the STA model are more likely to lose control for drugs like alcohol and cocaine, but not for food sources like sucrose. We also see that a proportion of the animals are more eager to get the drug compared to others, which is also commonly seen in the humans. And finally, we show that our model can be used for testing treatments for addiction.

This study is important because validating a research model that can better mimic human behaviour can help us get closer to treating and curing addiction, by having a better means of testing treatments. Moreover, it will save a lot of money to the public health sector and hopefully can help decrease the stigma associated with drug consumption in the process.

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Abstract

Substance use disorder (SUD) is a relapsing brain disorder that is characterized by loss of control over substance use, reflected by substance use despite harmful consequences. Various addiction models have been designed to capture loss of control over substance seeking, by associating operant responding for a substance with aversive stimuli such as foot shocks. We recently developed the seeking under the threat of adversity (STA) task to assess adversity in a manner that better resembles the human scenario. In the STA task, active seeking responses, during the presentation of a cue tone, lead to a 25% chance of receiving a foot shock.

The aim of this study was to assess whether rats lose control over substance seeking for sucrose, alcohol, and cocaine in the STA task. Moreover, we aimed to assess whether individual differences in alcohol intake predict loss of control over seeking.

The rats were trained to respond for sucrose and alcohol in an operant task. They were first trained on a fixed ratio 1 (FR1) schedule and then on random interval (RI) schedules of reinforcement, with increasing interval lengths (5 to 120s). Then, the rats were tested for control over substance use in the STA task. The rats in the alcohol batch consumed alcohol in an Intermittent Alcohol Access (IAA) setup for 2 months prior to training. Based on the level of alcohol intake, the rats were divided into low (LD) and high (HD) alcohol drinking rats. The rats in the cocaine batch underwent two sets of 10 STA sessions interspersed with extended sessions of cocaine self-administration to induce cocaine dependence.

A significant and consistent suppression of sucrose seeking was observed over 14 consecutive STA sessions compared to baseline sessions. This denotes that the rats did not develop loss of control over sucrose seeking. For alcohol, we observed differential responding in the STA task for HD and LD animals. Alcohol and cocaine show decreased levels of baseline responding compared to sucrose, but also decreased suppression of responses during the task. This is indicative of loss of control for these substances and is captured by the STA task. Therefore, this model can be a great tool to investigate the neurobiological correlates of SUD, leading to improved treatment strategies in the future.

Keywords: addiction, SUD, loss of control, behavioral testing, operant chambers, sucrose, alcohol, cocaine, glucocorticoid receptor antagonism.

Introduction

Addiction is a chronic relapsing disorder characterized by loss of control over substance use, reflected by compulsive seeking and impulsive consumption of the substance **(Horseman & Meyer, 2019).** SUD (Substance use disorder), as termed by the Diagnostic and Statistical Manual of Mental disorders (DSM-5), is one of the costliest neuropsychiatric disorders of the CNS, and it has a tremendous negative impact on the individuals suffering from it, as well as the society **(Uhl & Grow, 2004).** SUDs can be developed for a multitude of psychoactive substances from legal (alcohol, nicotine) to illicit (methamphetamines, opioids, cocaine, etc.) and they impact over 35 million individuals worldwide. There are concerns about increases in the prevalence of SUDs, especially now due to the COVID-19 pandemic (**UNODC, 2019; 2021).** In the US alone, 17.6 million people were estimated to have an AUD in 2016, with a hospitalization price rate averaging to 1122 dollars per capita **(Gryczynski *et al.*, 2016).** Moreover, a large amount of funds is spent on treatment and recovery for the sufferers, but also prevention and punishment of offenders around the globe **(Horseman & Meyer, 2019).** Thus, there is a strong incentive to prevent or treat SUDs to minimize their impact on society.

Substances of abuse are consumed, initially for their pleasurable effects, mostly brought about by their impact on the reward system **(Belin *et al.*, 2013).** However, a proportion of casual consumers end up developing an SUD. SUD is a complex and heterogeneous disorder that involves the mechanisms of reward, memory, learning, motivation, salience and cognitive control **(Baler & Volkow, 2006).** SUDs also demonstrate a sequence of complex behavioral mechanisms, namely initial intake and exacerbation, habit formation, motivation, loss of control over substance use, and reinstatement. **(Belin *et al.*, 2016; Ahmed, Walker & Koob, 2000)**. However, despite its prevalence and complexity, only 1 in 7 sufferers are receiving the appropriate treatment **(UNODC, 2019).** The treatments available to date are few, and most target substance intake reduction and withdrawal symptoms (e.g. disulfiram and naltrexone for alcohol), relapse prevention (e.g. acamprosate), or abstinence **(Singh, 2021; Koob & Zorrilla, 2010).** Loss of control is one of the core characteristics of SUD, which is expressed as the inability to cut down on substance use, the use of the substance in dangerous situations, the large amount of time spent on substance use, and the continued use of the substance despite negative consequences. The treatments for SUD today, do not typically target loss of control. However, there are a few potential treatment strategies being tested at the moment, such as the use of glucocorticoid

antagonisms (e.g. CORT113176), which was previously shown to reverse the compulsive seeking of alcohol in both rodents and humans **(Vendruscolo *et al.*, 2015).**

Another important factor to consider is that not all individuals consuming drugs become addicted. Worldwide, over 35 million individuals suffer from SUDs, out of ±270 million said to have consumed substances of abuse in 2020 alone **(UNODC, 2019; 2021).** In these individuals, the initial casual consumption escalates, leading to abuse and loss of control thus turning into addiction **(Lesscher & Vanderschuren, 2012)**. There are indications that the maladaptive control over the behavior and the development of compulsive seeking could be predicted via the initial intake level of alcohol **(Spoelder *et al.*, 2015).** The loss of control can be seen via the extreme lengths affected individuals will go to procure and use the substance, neglecting their social and professional responsibilities, and even their own well-being in the process (e.g. drinking mouthwash and aftershave to receive alcohol) **(Leon *et al.*, 2007).** A lot has been deciphered about the neural circuitry and major areas involved in addiction **(figure 1)**, but the emphasis is on reward motivation, relapse, and withdrawal, and less on loss of control, despite it being one of the main leading factors of addiction development, as outlined in the previous section. Thus, deciphering the neural pathways affected by loss of control, could not only help us identify the main risk factors for developing a SUD, but could also lead to the development of better treatments in the future, aimed to target the initial stages of addiction and prevent the compulsive seeking patient's experience.

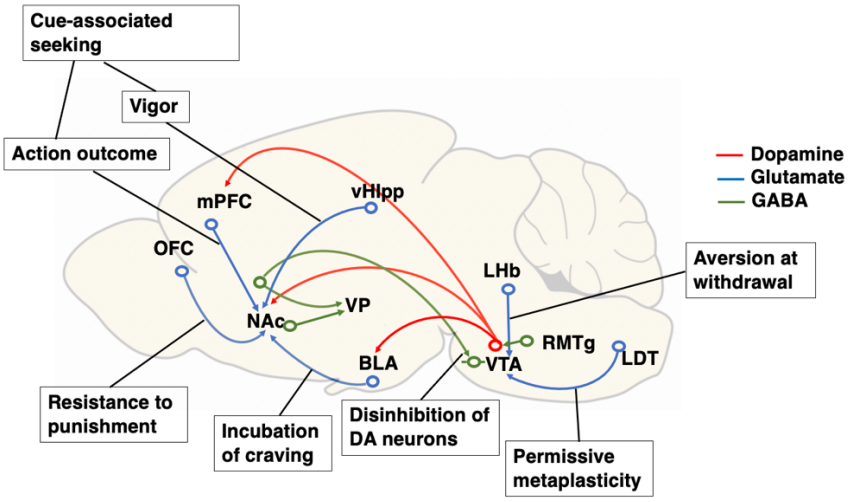

Figure 1. Neural circuits of addiction. The figure depicts the main circuitry involved in addictive behavior, their main associated cerebral structures, and their active role in addiction development and maintenance. Most of the connections identified are excitatory glutamatergic, followed by GABAergic via the projections from the NAc to the VTA. (adapted from **(Lüscher, 2016)**

There are various models in place to test loss of control in animals. In the last few decades, various aversion setups have been developed. These include the coupling of behavioral operant testing with quinine alterations, foot shocks, histamine, and lithium chloride **(Woolverton *et al.*, 2012; Hopf & Lesscher, 2014).** The similarities between them are the association of substance use with aversive stimuli e.g. through quinine adulteration, presentation of an electric footshock, or in the form delayed but prologued physical discomfort through the administration of histamine or lithium chloride **(Woolverton *et al.*, 2012; Hopf & Lesscher, 2014; Vanderschuren *et al.*, 2017; Minnaard *et al.*, 2020; Vanderschuren & Ahmed, 2021).** These have proven to be good translational models for the loss of control subpart of addiction, and have led to insightful knowledge in the field, especially when looking at the behavior of motivation for reward collection. We recently developed a novel model, namely the Seeking under the Threat of Adversity (STA) model, to capture loss of control and motivation over the process of drug-seeking. The STA model aims to better mimic the process of loss of control seen in humans. Direct, constant punishment after each use is not representative of what is observed in individuals suffering from SUD. Compulsive seeking and impulsive intake of substances of abuse are intrinsic to addiction, but individuals are warned about the aversive consequences of continued use of psychoactive substances. Moreover, these consequences do not occur with every use but are rather spread out and varied in the level of adversity (from embarrassment to self-injury, social and familial troubles, and even fatalities) **(Minnaard *et al.*, 2020).** The STA model aims to include the warning signal together with the probability of an aversive stimulus taking place if the substance is sought. The STA model includes a 25% chance of footshock when active lever presses are made during the presentation of a tone that functions as a warning signal **(Minnaard *et al.*, 2020).** With this approach, the animals make an association between the aversive stimuli and the seeking of the substance and can avoid the punishment altogether if they abstain. Once validated, this model allows for new pharmacological testing and neurobiological research to better understand the processes that underlie loss of control over substance use and to ultimately allow for better treatment of the disorder.

The aim of this study was to assess the ability of the STA model to capture loss of control over substance use. A second aim is to pinpoint the difference in neural circuitry between those individuals that do develop a SUD and those that remain casual users, which is the vast majority **(UNODC, 2020)**. A further, final aim was to use the STA task to test glucocorticoid antagonism as a possible treatment for loss of control in animals. For this

study, three experiments were performed to determine whether rats develop loss of control over sucrose, alcohol, and cocaine-seeking respectively. Sucrose is a natural reward and therefore serves as a control for the model, expecting no loss of control to be seen. Alcohol and cocaine are substances of abuse, both being two of the most consumed drugs, legal and illicit respectively and therefore expected to show loss of control during the testing. These are important steps in the discovery of better translational models and can potentially get us closer to the development of improved treatments for addiction, which could greatly aid the search for appropriate treatments and reduce the economic burden associated with addiction.

Methods

Subjects

52 adult male lister hooded rats (Charles River) weighing 200-250 grams at the beginning of the experiment were included in this study. The animals were divided into 3 cohorts as follows:

- Cohort A: 10 animals undergoing behavioral testing with sucrose
- Cohort B: 30 animals undergoing behavioral testing and pharmacological interventions with alcohol
- Cohort C: 12 animals undergoing behavioral testing with cocaine.

All animals were acclimatized to the cages for 1 week prior to the start of the experiments. The cocaine and sucrose cohorts were socially housed (2/cage). The alcohol cohort was individually housed to allow for measurements of individual home cage alcohol consumption levels. The animals were housed in Macrolon type III (single housing) and IV (social housing) cages respectively. Tissue paper and a wooden block were used per animal/per cage for enrichment. The humidity and temperature conditions were kept constant (21±2°C, and 50-70% humidity). They were all housed under a reversed 12h dark-light cycle (lights ON at 7 pm, light OFF at 7 am). Water and lab chow were available *ab libitum* during the duration of the entire study. The experiments were carried out in the dark cycle (between 9:00 am and 3:00 pm) 5 days a week.

5 out of the 12 animals in the cocaine cohort have been lost during training and testing because of catheter breakage due to inflammation around the backmount following surgery (see next section). Their responses have been recorded but not included in the final data analysis.

Surgery

Surgery was performed on the 12 rats undergoing the cocaine-seeking design. The rats were anesthetized with a mix of ketamine hydrochloride (75mg/kg, i.p.) and dexmedomidine (0.25mg/mg, i.p.). The animals were implanted with a catheter (Med Associates Inc.) in the right jugular vein and tunneled towards the back where the backmount was sutured. The backmount could be locked via a magnetic metallic cap (Med Associates Inc.). All equipment was thoroughly sterilized before use. Following the surgery, the animals were given gentamycin and atipamezole to aid wake up from anesthesia. The animals were further treated for 5 days with carprofen for analgesia (s.c.) and for 7 days with gentamycin to prevent infection (s.c). The animals were given a 7-day recovery period following surgery. The intravenous catheters were flushed daily with 0.1ml physiological saline, or with a lock solution containing heparin, taurolodine, and citrate (TauroLock, TauroPharm, Ghmb, Germany) for the weekend to maintain catheter patency.

Apparatus

The training and testing took place in Operant Chambers (29,5x24x25 cm, Med Associates Inc., USA) **(figure 2)**. Each chamber is equipped with two retractable levers on one side of the cage. A white cue light is also present above each lever. Between the two levers, there is a nose-poke opening, giving access to a recessed liquid dipper and food receptacle. On the outside of the chamber, a sucrose dispenser, and a retractable dipper can be found, which connect to the nose-poke opening for reward collection. On the opposite side of the chamber a white house light, a ventilation fan, and a tone generator are present. The floor of the chambers is a metal grid, connected to a shock generator. The chambers are sound and light attenuating **(Vanderschuren & Everitt, 2004; Minnaard *et al.*, 2020).**

In addition to the main setup, for the cocaine study, Silastic tubing engulfed by a mental spring was present, that could be connected to the animal's i.v. catheter via the backmount, using a magnetic mechanism. The tubing was supported and adjusted via a counter-weight arm placed on the outside of the box. A Razel Infusion pump was connected to the tubing and operated automatically, leading to a 0.1 ml liquid reward (0.125mg cocaine diluted in saline), delivered over a period of 5.6 seconds whenever the animal reached the response requirement **(based on Veeneman *et al.*, 2012; Limpens *et al.*, 2014).**

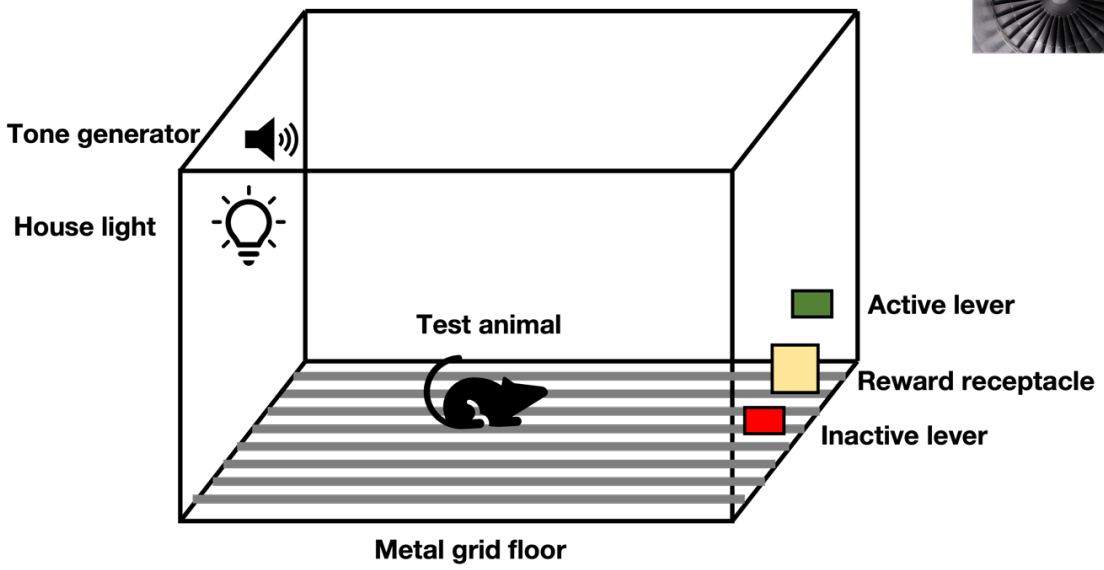

Figure 2 Schematic diagram of an operant chamber. The figure depicts the main components of an operant chamber. The floor is made from a mental grid connected to a shock generator (used to deliver mild foot-shock during the punishment sessions). The animal can move freely around the cage. On the right side, the active and inactive levers can be seen (in green and red respectively). Pressing on the active lever leads to a reward, whereas pressing on the inactive lever does not. The reward receptacle is found between the two levers (in yellow); it consists of an opening where the reward can be collected. Reward collection is recorded via an infrared beam which gets activated when the animal nose-pokes through the opening. On the left side, there is a house light, which stays on to denote the start of a trial, and a tone generator, used in the STA sessions as warning signals for probabilistic punishment. On the outside of the chamber, there is a fan, ensuring proper airflow during the experimental session.

Procedure

Alcohol consumption: The animals were initially allowed to consume alcohol in their homecage using an Intermediate Alcohol Access (IAA) schedule. Animals were given a choice between two bottles, one filled with water and the other filled with alcohol (20% v/v), 3 times a week (Monday, Wednesday, and Friday). Access to alcohol was available for 7h during the 1st month, and then increased to 24h access during the 2nd month **(figure 3A).** This allowed for the assessment of individual consumption levels for the animals, which has been previously shown to be predictive of loss of control **(Spoelder *et al.*, 2015).** Following this period, the animals were divided into high, medium, and low drinking animals (HD, MD, LD). This was done by ranking the rats based on their weekly alcohol intake levels and the sum-rank score across the weeks of the IAA schedule. In this manner, a tertile split was performed based on the alcohol intake, and 8 animals were selected for each group (the top 8 animals highest in EtOH consumption were placed in the HD group, the lowest 8 animals in EtOH consumption were placed in the LD group, and the 8 animals ranking medium in consumption were placed in the MD group). 6 animals were left out of the group division, as

they were considered intermediate in their consumption and placed between HD-MD and MD-LD. These animals have only been included in the overall analysis of the cohort but not further included when looking at the comparison between the alcohol drinking groups.

The animals were trained to respond to sucrose, alcohol, and cocaine in 30 min or 140 min (for cocaine) operant sessions taking place daily for 5 days a week. Initially, the rats were trained on a FR1 schedule of reinforcement, which aimed for the animals to acquire the substance seeking and collection behaviors. For sucrose and alcohol, two levers were presented, one active and one inactive. Pressing on the active lever led to a reward (dipper ascending to the nose-poke whole for collection or sucrose pellet dropping in the food receptacle), whereas pressing on the inactive lever did not. For cocaine, only one lever (the active lever) was extended at the beginning of each trial and each press led to one cocaine infusion (0.1 ml of 0.125mg cocaine diluted in saline). This was done to acquire the seeking response. Once more than 70% of the responses were active, indicating the rats acquired the task and learned about the task contingencies, the animals were moved to a random interval (RI) training schedule progressively increasing in length from 5 to 120 seconds (5,15,30,60,120) **(figure 3A and 3B).** The random interval period was aimed to promote substance seeking, as no active lever press during this time led to a reward. A subsequent press following the RI, led to one reward (a sucrose pellet in the nose-poke receptacle the raising of the dipper for alcohol collection, or the presentation of the 'taking' lever for cocaine, leading to a cocaine infusion). Starting with RI5, a random interval time was selected per trial. The interval lengths were randomly assigned but averaged out to 5s, such that the animals cannot predict the exact time they had to wait for the reward, such that they learned that they should press continuously to get rewarded. For alcohol and sucrose, the active and inactive levers were extended during the RI. For cocaine, only one lever was extended during the RI (the seeking lever); if this lever was pressed during the RI, it led to the presentation of the 'taking' lever at the end of the RI, ultimately leading to a cocaine infusion (done to replace the act of 'reward collection' seen for sucrose and alcohol).

The same mechanisms were applied for all the increasing RI schedules. The rats were trained on each RI schedule for 3-4 days or until constant seeking and reward collection was observed. For RI120, the animals were trained until there was less than 20% variation in the seeking and reward collection responses for the last three consecutive days of training for both the entire cohort and individual animals. Exceptions were made for animals where the individual variation was just slightly outside of the 20% margin (up to 25% at most), and

only if this was due to a response that was not in line with the responding seen throughout the entire RI120 training period. However, this was only accepted if the overall cohort responding was still within the 20% margin.

Subsequently, the rats were trained in the STA task. This was largely similar to the RI120 schedule of reinforcement, except that each interval lasted at least 30 seconds. This is because a 30s tone interval was implemented at the end of the random interval in the STA task, which acts as a warning signal. Rats were required to make a lever press during tone presentation to receive a reward; however, pressing during tone also leads to a 25% chance of receiving a mild 0.25mA footshock **(figure 3B and 3C) (Minnaard *et al.*, 2020)**. For the sucrose and alcohol studies, a 0.25mA shock was used, which **Minnaard *et al.*, 2020** showed was sufficient to decrease seeking of the substance, whilst leaving room for the animal to overcome the negative consequences of punishment. For cocaine, the intensity of the shocks administered in the STA session started at 0.25mA and was increased up to 0.35m using incremental 0.05mA increases over the first 5 days of testing to assess the appropriate intensity that elicits suppression in the seeking behavior. This titration method was used based on the work of **Marchant *et al.*, 2019.** The intensity of 0.35mA proved sufficient to elicit suppression in animals consuming cocaine and was used for the rest of the study.

For sucrose and alcohol: Following 14-18 sessions of the STA model, the animals were retrained for 2-3 days on the RI120 and were subsequently tested in an adjusted STA protocol for 6 days. This alternating STA task is based on the regular STA task but includes both tone and no-tone trials with a 50% chance that each trial is either one of the two **(figure 3D)**.

For Cocaine: Following the initial set of 10 STA sessions, the rats were exposed to 7 extended cocaine self-administration sessions **(Ahmed, Walker & Koob, 2000; Vanderschuren & Everitt, 2004)(figure 3A).** These extended sessions consisted of 6 hours of FR1 training, with a maximum reward capacity of 80 rewards. The sessions were interspersed with RI120 training sessions, such that after the animals received 2 consecutive extended sessions, followed by one day on RI120 training. Once the extended sessions were completed, the animals were given 3 days of further RI120 training, and then started the next and final round of 10 STA sessions.

The programmes used were created by A.M Minnaard, M. Spoelder and N. Papavoine and updated as needed. They were controlled by MedPC software.

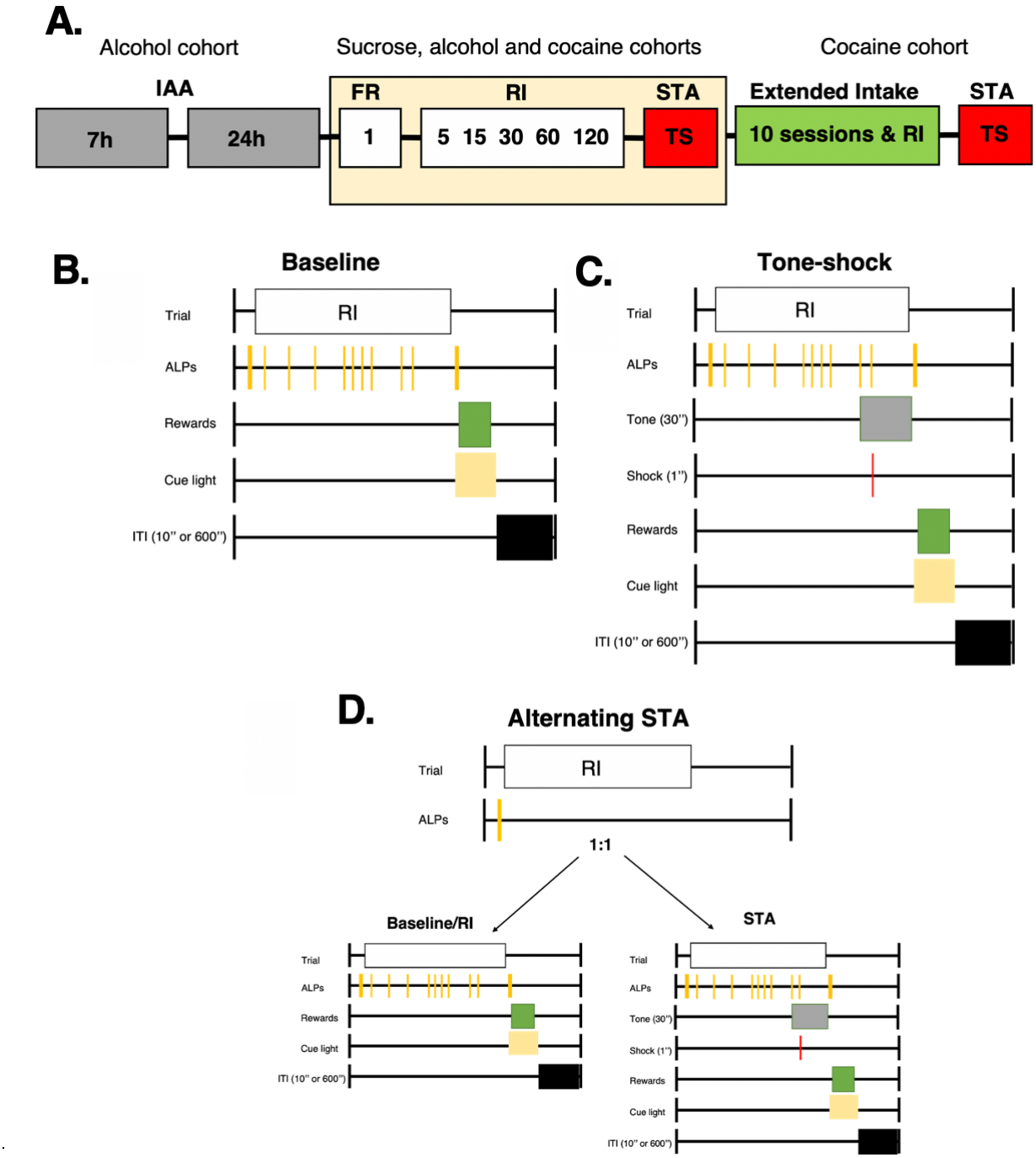

Figure 3 Schematic diagram of the experimental setup and timeline. A. Depicts the timeline of the study for the sucrose, alcohol, and cocaine cohorts. The alcohol cohorts started with IAA for 7 hours three times a week for the first month and progressed to a schedule of 24 hours three days a week for the second month. All cohorts underwent Random Interval (RI) training. Fixed Ratio 1 (FR1) training was done for ± 7 days, or until stable responding was seen (less than 20% variation/ inactive responses). The proceeding RI sessions (5,15,30,60,120) continued for 3-4 days until stable responding was seen (80-120% variation in responses). The STA tone-shock session then took place for 10 to 18 days. The cocaine cohort continued for another 7 sessions of extended cocaine intake in an FR1 manner. Finally, 10 sessions of STA sessions were performed again. B-D. Depict the events of one trial for the base sessions (RI120)(B), the STA task(C), and the alternating STA task(D). Adapted from **(Minnaard et al., 2020)**

Pharmacological Intervention

Following the STA and alternating STA testing, we ran an additional set of STA sessions for the HD and LD rats in the alcohol cohort. The rats were administered the glucocorticoid antagonist CORT 113176, which is one of the substances previously studied as a possible treatment for AUD. This compound was shown to restore control over alcohol use, while also proving promising in terms of decreasing alcohol physiological markers of alcohol consumption **(Vendruscolo *et al.*, 2015).** Cort113176 was diluted in sesame oil to the appropriate concentration with an injection volume of 2ml/kg **(Pineau *et al.*, 2016).** The animals received doses of 0 mg/kg, 60 mg/kg, and 100 mg/kg, administered during 3 consecutive days, following a Latin square design. The animals received an I.P. injection and were returned to their cages for 90 min for the CORT113176 to be taken up, before being tested on the alternating STA setup. The animals had 1-2 days break between test sessions, to recover from the treatment.

Data analysis and statistics

<u>For the alcohol cohort:</u> The alcohol and water bottles were weighed daily before and after the IAA sessions. The difference was recorded to calculate the intake of both liquids. The formulas used were as follows:

To calculate alcohol intake in ml: *(difference bottle weight in grams)/ (0.8+(0.2*0.789))*

To calculate alcohol intake g/kg:*((alcohol intake in ml)*(0.2*0.789))/(bodyweight in kg)*

To calculate the preference (%):

(alcohol intake in ml/((alcohol intake in ml)+(water intake in ml))*100

<u>For all three cohorts:</u>

To assess the seeking behavior of the animals the suppression and suppression ratio were calculated. The suppression is based on the seeking responses in the STA sessions compared with the 80 to 120% range of the seeking responses during baseline. Once the responses are below the 80% mark of the seeking responses at baseline, it is considered that the animals show suppression. This is displayed on a 0 to 1 scale, with 0.8 denoting the 80% border. The following formula was used to calculate suppression:

Presses during tone shock session/ presses during baseline (last 3 days of RI)

The suppression ratio denotes the difference between the seeking responses at baseline compared to the seeking responses during the STA testing sessions. As such, the suppression ratio can be used to display the degree of suppression in seeking responses and help identify the animals that do not show loss of control versus the animals that show loss of control over substance use. It also offers the ability to compare the seeking behavior between the three substances, to identify whether animals tend to lose control more for substances of abuse (alcohol and cocaine) compared to natural rewards (sucrose). This ratio is denoted from a scale of 0 to 1, with 0 denoting no suppression and 1 denoting total suppression. The following formula was used to calculate the suppression ratio:

(presses during baseline (last 3 days of RI) – presses during the tone-shock session)/ (presses during baseline (last 3 days of RI) + presses during the tone-shock session)

It is expected that all animals show some level of suppression, disregarding the type of substance due to the implementation of the punishment (i.e. the mild footshock). Nonetheless, showing suppression does not equal loss of control. To quantify loss of control, the data was thoroughly analyzed in terms of suppression ratio for the cohorts. As previously mentioned, the suppression ratio can help identify the degree of loss of control over substance use that the individual animals and the three cohorts show. We observed that there is a different suppression ratio average between the three cohorts (i.e., sucrose, alcohol, and cocaine) and were able to distinguish rats that display low versus high suppression across the substances. Individual animals were placed into high and low suppression groups. A pronounced divide was found between two distinguishable groups, with no overlapping data points for all substances. This was notable given the small sample sizes worked with. This divide occurred across a suppression ratio line of 0.6. Due to the discernible divide between the low and high suppression groups, the high suppression group is assumed to contain animals that do not show loss of control, and the low suppression group is assumed to be animals that do show loss of control. This point of 0.6 suppression ratio was then further used to divide the animals into low and high suppression groups (denoting loss of control or normal behavior respectively) and the seeking data was further analyzed in this way. However, this value only applies to our cohort and is not an absolute value.

For all the STA sessions two-way repeated-measures ANOVA were used with time as the within factor. For the alcohol cohort division into LD and HD groups, the time was used at the within factor and the group was used as the in-between factor. For the cocaine cohort,

the non-factorial Wilcoxon matched-pairs signed-rank test was used to assess the difference in between the time points.

For all the alternative STA data two-way repeated-measures ANOVA were used also with time as the within factor. For the alcohol cohort, only the animals in the LD and HD groups were considered. As such, for this cohort, the alcohol intake group was used as the in-between factor.

For the suppression group divisions data two-way repeated measures ANOVA were also used with time as the within factor. Here, however, the in-between factor used was the suppression group (high/low) the animals belonged to.

For the pharmacological intervention setup using glucocorticoid antagonism data, two-way ANOVA measures were also used. In this setup, only the HD and LD groups belonging to the alcohol cohort were tested. For this, the concentration of the compound (0, 60, or 100mg/kg) was used as the within factor. The alcohol intake group was used at the in-between factor.

To keep the data constant between the substances, only the first 10 days of STA were considered in the analysis. Moreover, days 1-4 of the STA testing are considered training days, where the STA task is learned. All the days are displayed throughout this paper, but unless stated otherwise, the main data analyzed for all three cohorts was that between days 4-10 of the STA testing compared to baseline responding.

The data is represented as mean and SEM, unless stated otherwise. The data was analyzed and presented using Microsoft Excel and PowerPoint, GraphPad (Prism), and SPSS. A statistical significance of $p<0.05$ was used for all the analyzed data. If the data did not meet the Mauchly's sphericity test requirement for the ANOVA analysis, then the Greenhouse-Geisser correction was used to correct for the degrees of freedom. All the animals were included, unless unable to finish the experiment. No outliers were taken out due to the small cohort size and importance for individual variances in the study. *** denotes $p<0.001$, ** denotes $p<0.01$, * denotes $p<0.05$

Results

The STA task was used to determine whether rats display loss of control over substance seeking. To quantify the seeking behavior in the STA task in the face of adversity, we looked at the suppression ratio displayed by the animals, reflecting the suppression relative to baseline training sessions, where no punishment was present (see methods). It was expected that all animals show some level of suppression, regardless of the type of substance, but that this would be more pronounced for sucrose with alcohol and cocaine, which are substances of abuse.

Suppression of substance seeking in the STA task

Seeking for sucrose

Analysis of the responding in the STA task for the sucrose cohort revealed a significant effect of time in the ALP responding during RI from baseline to the testing sessions ($F_{time}(1.445,13.003)=51.407$;$p<0.001$). This difference in responding is not observed anymore when the analysis is performed only for the session days 4-10 ($F_{time}(2.209,19.884)=3.299$;$p=0.054$)**(figure 4A).** Moreover, post-hoc analysis denotes that there is a difference still seen between sessions 4 and 5 ($p=0.021$), but remains constant for the remainder (e.g., for sessions 5 and 6; $p=0.645$), denoting that once the task has been acquired, the responding remains constant. Similar findings are seen for reward collection, with a significant time effect seen for baseline and the testing sessions ($F_{time}(7,63) = 10.915$;$p<0.001$), but not for the testing sessions alone ($F_{time}(6,54)=0.608$;$p=0.723$) (Figure 4B). Post-hoc analysis between testing sessions reflects no significant difference in responding between consecutive days (e.g., $p=0.325$ for sessions 4 and 5). The recoded ALPs and shocks show no significant differences between testing sessions 4-10 ($F_{time}(6,54)=77.624$; $p=0.497$ and $F_{time}(2.587,23.286)=0.669$;$p=0.559$ respectively) **(figure 4B and 4C).** No significant differences are recorded between consecutive testing sessions. Overall, all animals show high suppression from baseline. The responses recorded are below the 80%, margin, which is the lower cut-off considered for stable responding during baseline **(figure 4E).** There is also a high suppression ratio displayed (0.74), which does not show a significant difference as a factor of time during the testing days 4-10 ($F(6,48)=2,225$; $p=0.570$), further showing that once the task has been gained, there is no significant alteration in behavior and the responding is low **(figure4F).** Thus, once the STA task is gained, the

animals in the sucrose cohort show constant suppression of behavior at the cohort level, which is indicative of no loss of control over the substance used.

Seeking for alcohol

Analysis of the responding in the STA task for the alcohol cohort revealed a significant effect of time in the ALP responding during RI from baseline to the testing sessions (Ftime(1.390, 40.321)=47.069; p <0.001). This time effect is still observed when the analysis is performed only for the session days 4-10 (Ftime(4.273,128.912)=4.659; p=0.001)**(figure 4A).** Post-hoc analysis shows that there is a significant difference in recorded responses between the remainer of some of the testing days (e.g., for sessions 8 to 4,5,6,7 and 9; p=<0.001, p=007, p=0.008, p=0.002, p= 0.012 respectively), which fluctuate between increases and decreases in responding, denoting that for alcohol there is still some instability in responding over the testing days. For reward collection, there is a significant difference between baseline and sessions 4-10. (Ftime(7,203)=35.145; p<0.001), but not between the sessions 4-10 alone (Ftime(6,174)=2.029; p=0.064) **(Figure 4B).** Post-hoc analysis between testing sessions reflects no significant difference in responding between consecutive days. The recoded ALPs and shocks also show a significant time difference between testing sessions 4-10 (Ftime(4.273, 123.912)= 4.659; p=0.001 and Ftime(3.878; 112.463)=4.509; p= 002 respectively) **(figure 4C and 4D).** As revealed by the post-hoc analysis, these differences can be seen between a few testing sessions (e.g., session 5 to 7, 8 and 10 for the ALPs during tone p=0.004; p=0.004 and p<0.001 respectively; and sessions 5 to 7, 8, and 10 for shocks p = 0.001, p=0.003 and p <0.001 respectively). Overall, all animals show moderate suppression from baseline **(figure 4E).** There is a low suppression ratio displayed (0.59), which also shows a significant difference as a factor of time during the testing days 4-10 Ftime(3.457,100.262)=3.746; p=0.002) **(figure 4F).** The responding in the STA task for alcohol animals shows significant differences in seeking between consecutive days of testing, denoting that even once the task has been gained, the animals still show an unpredictable behavior, which may be indicative of loss of control.

Seeking for cocaine

Analysis of the responding in the STA task for the cocaine cohort revealed a significant effect of time in the ALPs made during RI from baseline compared to the testing sessions (Ftime(4.492,20.954)=12.230; p<0.001). This difference in responding is not observed anymore when the analysis is performed only for the session days 4-10

(Ftime(3.123,18.739)=0.511; p=0.687)**(figure 4A).** Moreover, post-hoc analysis denotes that there is no significant fluctuation in responding between the remained of the testing days (e.g., for sessions 5 and 6; p=0.966), denoting that once the task has been acquired, the responding remains constant. For rewards, there is no significant difference between baseline and session days 4-10 (Ftime(3.044, 18.265)=1.574; p=0.230), as well between the sessions alone (Ftime(2.207; 11.036)=1,254; p=0.308) (Figure 4B). Post-hoc analysis between testing sessions reflects no significant difference in responding between consecutive days (e.g., p=0.325 for sessions 4 and 5). The recoded ALPs and shocks show no significant differences between testing sessions 4-10 (Ftime(6,36)=0.379; p=0.887 and Ftime(2.207; 11,036)=1.254; p=0.308 respectively) **(figure 4C and 4D).** No significant differences are recorded between consecutive testing sessions. Overall, all animals show moderate suppression from baseline, most animals responding below the 80%, margin, which is the lower cut-off considered for stable responding during baseline **(figure 4E).** There is a low suppression ratio displayed **(0.55),** which does not show a significant difference as a factor of time during the testing days 4-10 (Ftime(3.173,15.863)=1.054; p=0.399) **(figure4F).** The animals in the cocaine cohort show a lower suppression from baseline compared to both alcohol and sucrose, despite no significant changes in responding being observed during testing. This may be indicative of loss of control for cocaine as a substance if all animals show a similar behavior.

The STA task can successfully lead to suppression of behavior in the STA task using a mild footshock. The suppression remains constant at the cohort level throughout the testing days for sucrose and cocaine, but not alcohol. However, assessing the whole cohort is not necessarily indicative of loss of control, as the variation displayed by the animals in the cohort groups is high **(Figure4A).** Therefore, to further assess the degree of loss of control displayed, an analysis of the seeking responses and suppression level displayed by individual animals was made.

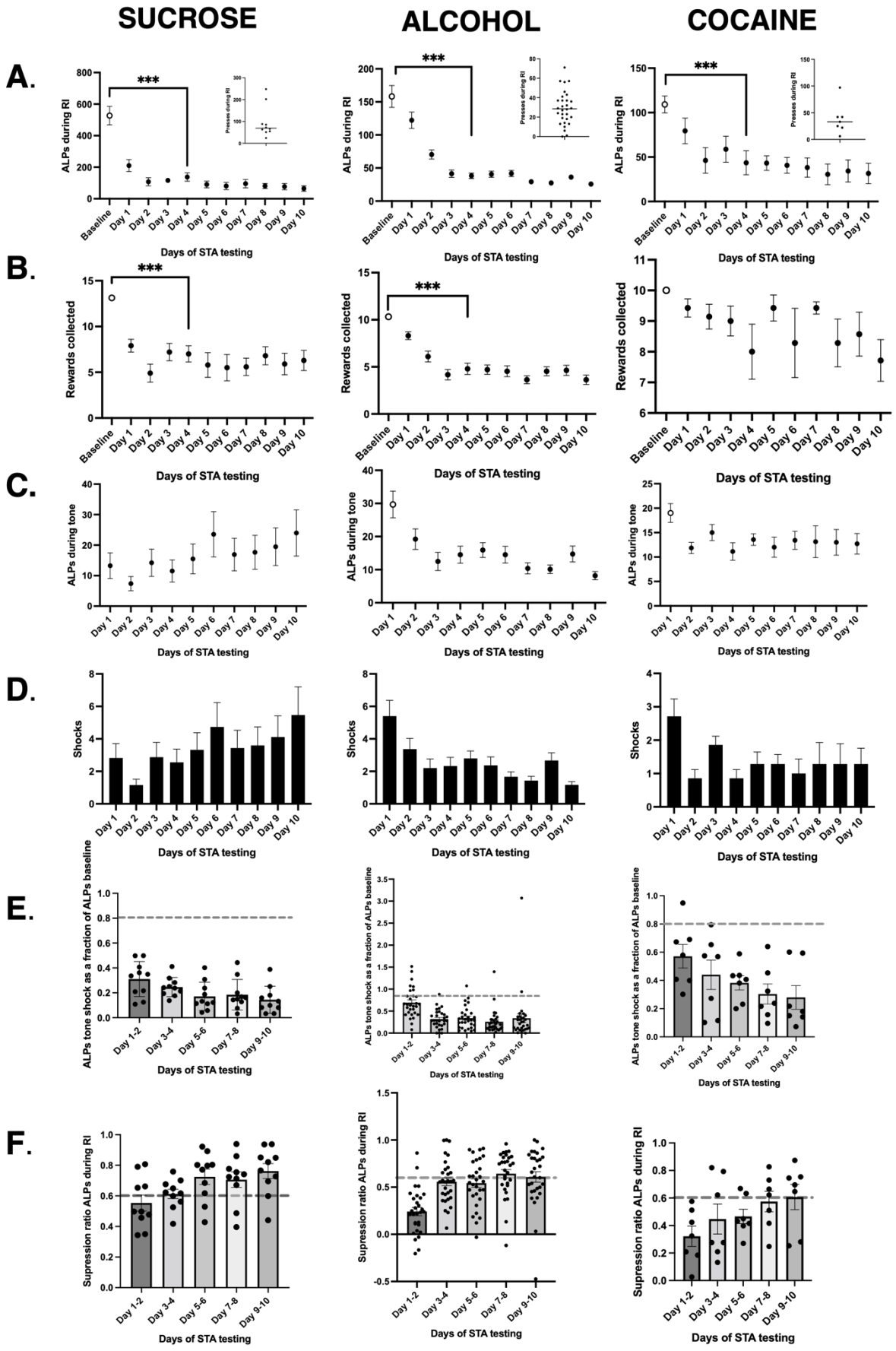
SUCROSE
ALCOHOL
COCAINE
A.
B.
C.
D.
E.
F.
***
ALPs during RI
Presses during RI
Rewards collected
ALPs during tone
Shocks
ALPs tone shock as a fraction of ALPs baseline
Supression ratio ALPs during RI
Baseline
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 1-2
Day 3-4
Day 5-6
Day 7-8
Day 9-10
Days of STA testing

Figure 4. Seeking behavior in the STA task. Summary of the behavioral data for the STA task for sucrose, alcohol, and cocaine, which are presented side by side. A. Active presses made during RI for the sessions of STA testing compared to baseline. On the right corner of each graph, there is a depiction of the individual variation in the seeking of animals at day 7. B. Rewards collected for the sessions of STA testing compared to baseline. C. Presses made during the warning tone signal for each STA session. D. Shocks received for each STA session. E. 2-day averages of the suppression displayed by the animals in the STA task F. 2-day averages of the suppression ratio displayed by the animals in the STA task. E-F Individual animals' behavior can be seen as dots on the bar graphs. The dotted gray line denotes the upper limit for suppressed behaviour(E)and the limit for high and low supression behavior displayed by the animals (F). *** *denotes* $p<0.001$, ** *denotes* $p<0.01$, * *denotes* $p<0.05$

Individual suppression of substance seeking in the STA task

Our data showed high variation in seeking responses between individual animals, and this variation was analyzed. This was done by assessing the suppression ratio displayed by individual animals responding each substance (methods). The data displayed showed the formation of two distinct suppression ratio subgroups for all three reinforcers. Moreover, it was displayed that for sucrose, alcohol, and cocaine; the point of no overlap between the subgroups of the suppression ratio occurred at value of 0.6. As a result, the animals were divided into high and low suppression groups based on whether their average suppression in active responses made during the testing was above or below 0.6. The two groups for each substance were then further analyzed in their responding during the RI, responding during the warning tone and rewards **(figure 5).**

For sucrose, analysis of the suppression ratio for the two groups was performed for sessions 4 to 10 (figure 5A, B). There was no significant effect of time or time and group interaction ($F_{time}(6,48)=2.225$; $p=0.057$; $F_{timexgroup}(6,48)=0.806$; $p=0.570$). However, there is a significant effect of group ($F_{group}(1,8) =1.009$;$p<0.001$), which was also reflected by the post-hoc analysis (LOC: M=0.471, STD=0.023; No LOC: M=0.771, STD=0.023; $p<0.001$). Thus, there is a significant difference between the responding of the two suppression subgroups for sucrose, although it is not time-dependent.

For alcohol, analysis of the suppression ratio for the two groups was performed for sessions 4 to 10 **(figure 5A, B).** There was no significant time and group interaction ($F_{timexgroup}(3.440,96.320)=0.856$; $p=0.480$). However, there was a significant effect of time and group found ($F_{time}(3.440,96.320)=3.693$; $p=0.011$; $F_{group}(1,28) =49.366$; $p<0.001$). This was also reflected by the post-hoc analysis between the LOC and no LOC groups (LOC: M=0.395, STV=0.039; No LOC: M=0.767, STV=0.036; $p<0.001$). Thus, there is a significant difference in the seeking responses between the subgroups for alcohol.

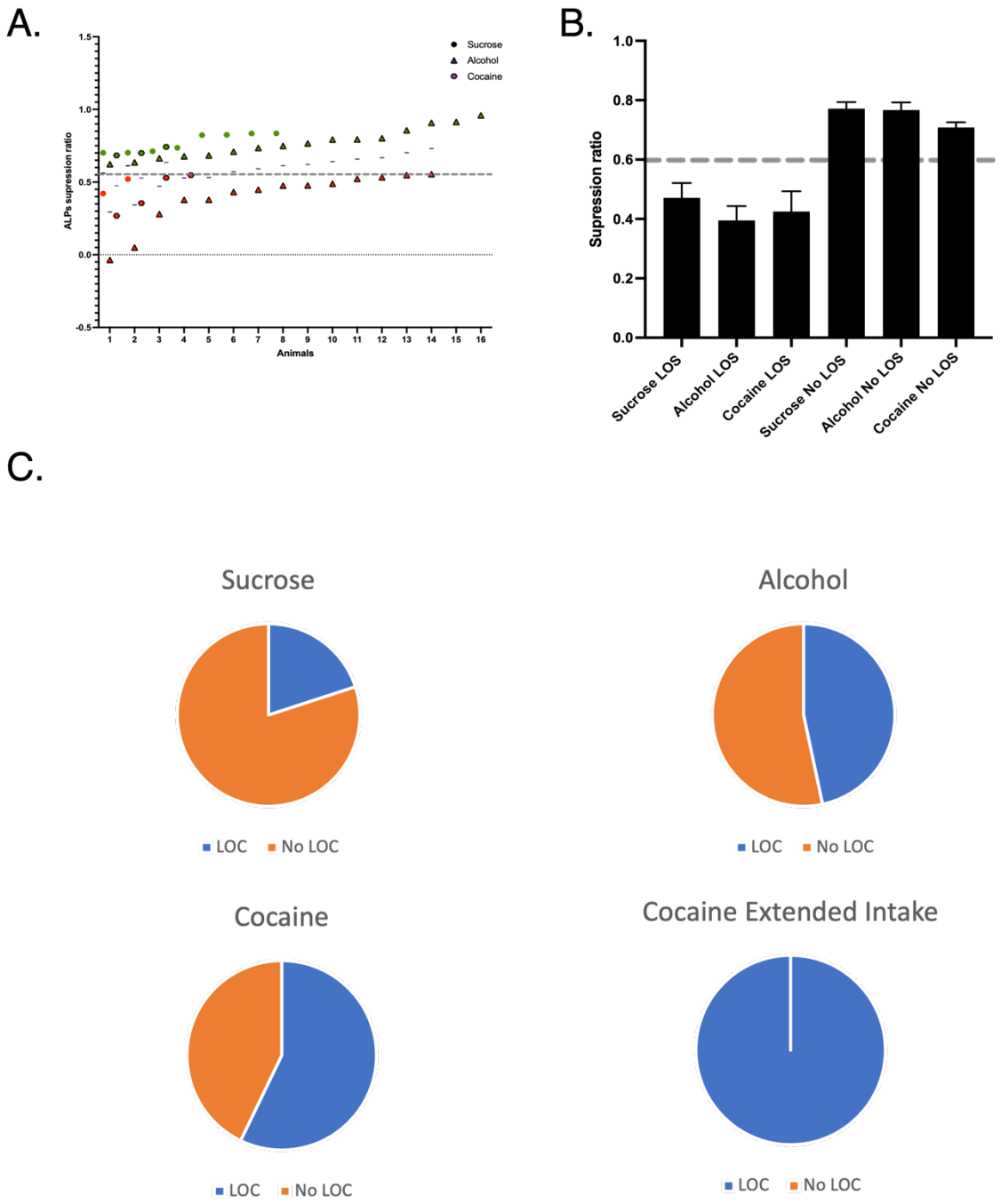

Figure 5. Individual suppression levels and classification into suppression subgroups. A. Division of the three cohorts into the high and low suppression groups, with all the individual animals being represented. Red denotes all animals belonging to the LOC subgroup, whereas green denotes all animals belonging to the no LOC subgroup. B. Representation of the average suppression ratio displayed by the low and high suppression subgroups of the three cohorts, seen side by side. C. Visual representation of the number of animals belonging to the low and high suppression subgroups for sucrose, alcohol, cocaine, and cocaine following extended exposure.

For cocaine, analysis of the suppression ratio for the two groups was performed for sessions 4 to 10 **(figure 5A, B).** There was no significant effect of time or time and group

interaction (Ftime(3.173,15.863)=1.054; p=0.399; Ftimexgroup (3.173,15.86)=1.245; p=0.328). However, there is a significant effect of group (Fgroup(1,5) =11.927;p=0.018), which was also reflected by the post-hoc analysis (LOC: M=0.425, STV=0.054; No LOC: M=0.708, STV=0.062; p<0.001). Thus, there is a significant difference between the responding of the two suppression subgroups for cocaine, although it is not time-dependent.

The overall suppression ratio displayed by the three cohorts shows a significant effect of time in relation to the substance consumed Ftime(4.156,186.592)=3.551;p=0.007), but not in relation to the group type (Ftime(4.241,182.871)=0.670;p=0.622). There is no overall significant effect of the substance (Fsubstance(2,44)=1.537; p=0.226). There is also no significant interaction of time and substance or time and group (Ftimexsubstance(8.312,182.871)=1.190; p=306; Ftimexgroup(4.241, 186.592)=1.661, p=0.157). However, there is a highly significant difference between the LOC and no LOC groups (Fgroup(1,44)=5.078; p<0.001). It can be stated that the suppression subgroup has an overall significant effect on the seeking behavior, which is not dependent on the type of substance used. Therefore, assessing the behavior of animals based on their level of suppression of the seeking behavior can be a well-suited characteristic for assessing loss of control in animal.

Moreover, it can also be observed that the prevalence of animals showing high and low suppression within each cohort is related to reinforcer used. For the sucrose cohort, 80% of the animals belong to the high suppression subgroup, indicative of no loss of control. For the alcohol cohort, there was an almost equal division of rats across the two low and high suppression subgroups (46% and 54% respectively), and for cocaine there were more animals recorded in the low suppression group than the high suppression subgroup (57%). Furthermore, extended exposure to cocaine intake leads to 100% of the animals denoting that for cocaine extended intake leads to even less pronounced suppression of cocaine seeking, illustrating loss of control over cocaine seeking after extended cocaine exposure (figure 5C). Thus, denoting that there is a higher degree of loss of control displayed by animals responding for alcohol and cocaine.

Finally, the responding of the animals in the STA task was reanalyzed using the suppression subgroup as an in-between factor **(Figure 6A-D).** The statistical analysis displayed here only shows the days 4 to 10 of the testing. For sucrose, there are significant differences between the LOC and No LOC groups for presses during RI

(Fgroup(1,8)=48.058; p<0.001), and rewards (Fgroup(1,8)=10.913; p=0.011), ALPs during tone (Fgroup(1,8)=11.324; p=0.010) and shocks (Fgroup(1,5)=3.427; p=0.123).For alcohol, there are significant differences between the LOC and No LOC groups for presses during RI (Fgroup(1,28)=12,042; p=0.002), and rewards (Fgroup(1,28)=16.557; p<0.001), ALPs during tone shocks (Fgroup(1,28)=11.654; p=0.002) and shocks (Fgroup(1,28)=9.019; p=0.006). For cocaine, there are significant differences between the LOC and No LOC groups for presses during RI (Fgroup(1,5)=17.516; p=0.009), and rewards (Fgroup(1,5)=16.557; p<0.001, but not for ALPs or shocks (Fgroup(1,5)=5.186; p=0.072 & Fgroup(1,5)=3.427; p=0.123).

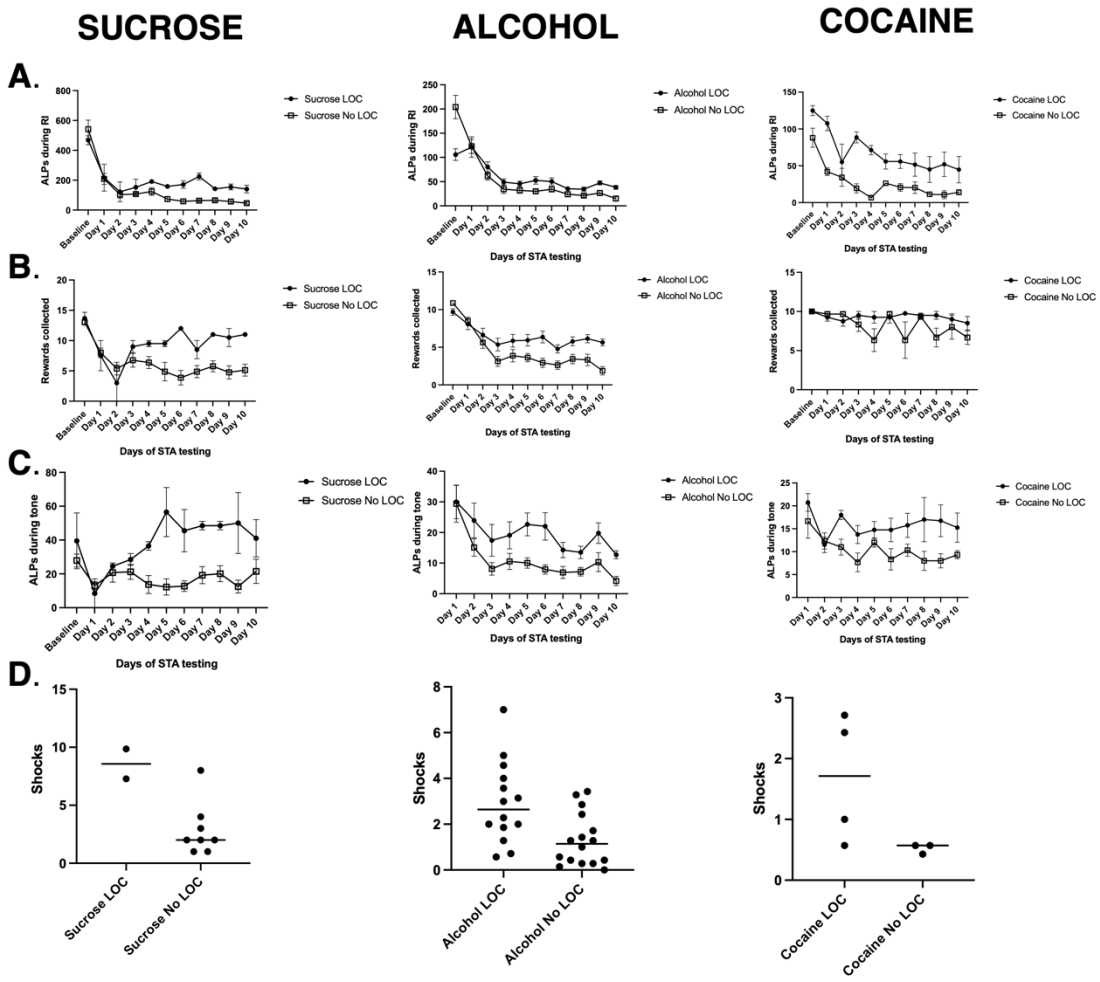

Figure 6. Seeking behavior based on the level of suppression displayed during the STA task. Representation of seeking behavior of the LOC and No LOC subgroups for sucrose, alcohol, and cocaine, seen side by side. A. Seeking behavior during the RI for each session of the STA testing. B. Rewards collected for each session of the STA testing C. ALPs made during the warning tone period for each session of the STA testing. D. Average shocks received by the individual animals in the LOC and No LOC subgroups over the course of sessions 4-10. Individual dots represent the days of testing.

Therefore, there are distinct significant subgroups of animals differing in their degree of loss of control, irrespective of the reinforcer used. These groups respond significantly

different to the seeking in the STA task, denoting that can be further used to identify loss of control, at least at the discrete cohort level.

Initial alcohol intake and extended cocaine intake predictive of loss of control behavior

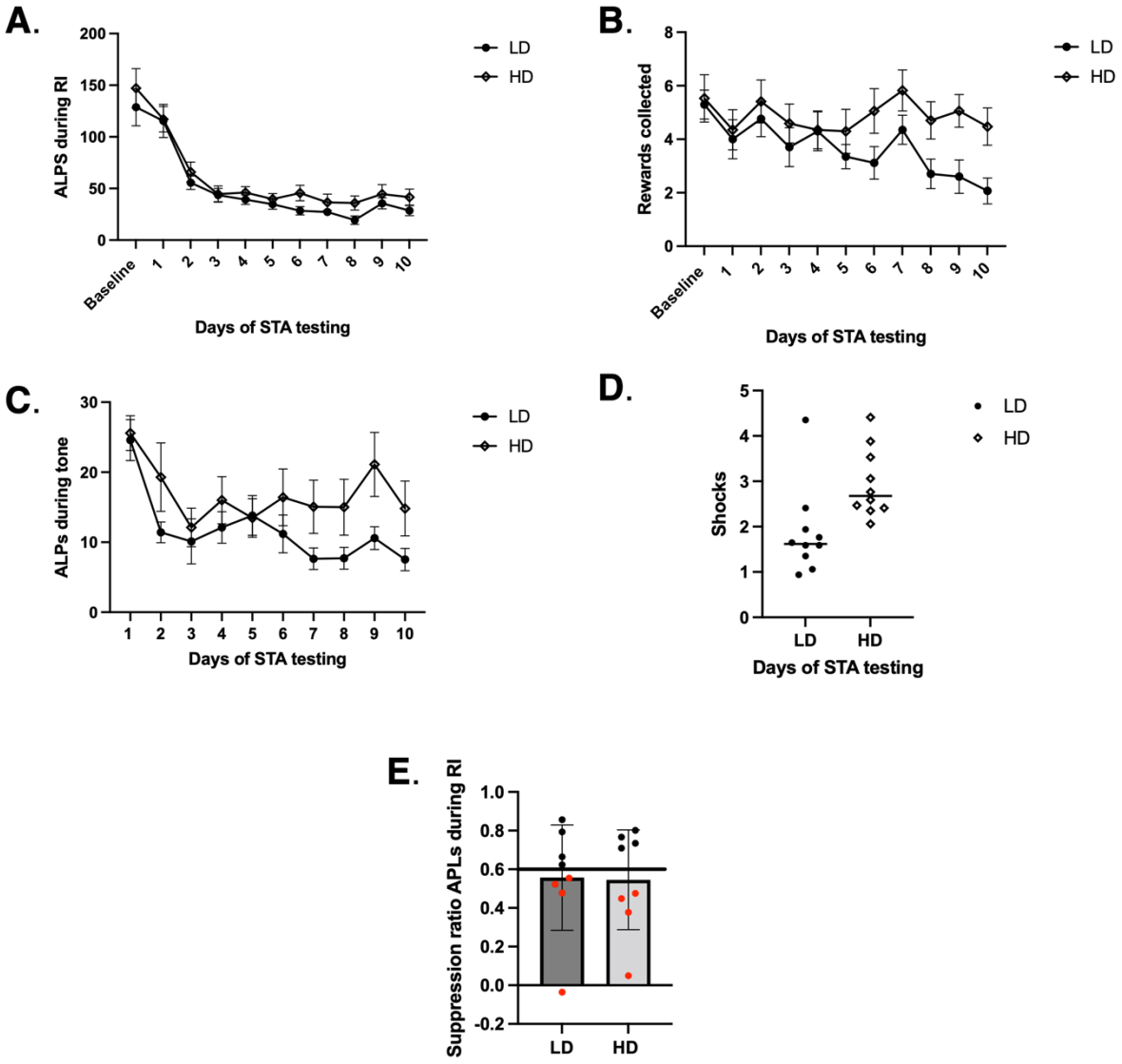

Figure 7. Seeking responses recorded for the HD and LD subgroups of the alcohol cohort A. Presses made during the RI for each session of the STA testing. B. Rewards collected for each session of the STA testing C. ALPs made during the warning tone period for each session of the STA testing. D. Shocks received by the two subgroups during the testing days E. Suppression ratio displayed by the animals throughout the testing period (sessions 4-10). Red dots denote the animals belonging to the LOC subgroup (low suppression subgroup).

Voluntary alcohol intake and extended cocaine intake were assessed as predictive factors for the development of loss of control over substance use in the STA task.

Voluntary alcohol intake

Analysis of the STA data for the alcohol cohort, taking into account the LD and HD subgroups of the alcohol cohort **(Appendix)**, revealed that the HD animals show more alcohol seeking in the face of adversity compared to the LD group for seeking during RI, reward collection, and foot-shocks recieved **(Figure 7 A-C).** However, this is especially pronounced when looking at the data for the ALPs made during the warning tone period **(Figure 7C).** However, repeated measure ANOVA analysis has revealed that there is no group effect and no group and time interaction observed during the analysis for the ALPs during tone ($F_{group}(1,14)=1.630$; $p=0.222$; $F_{groupxtime}(3.256,45.579)=0.450$; $p=0.733$). This is observed for all timepoints between testing day 4-10.

The alcohol intake subgroups were further analysed based on their average suppression ratio, but this showed no statistical difference. Moreover, when the alcohol intake and suppression level subgroups were combined, it was observed that an equal number of HD and LD animals belong to the low and high suppression group. Thus, further denoting that initial alcohol intake is not predictive of loss of control over alcohol in our cohort **(Figure 7E).**

Extended cocaine intake

The cocaine cohort underwent 7 sessions of extended cocaine intake, followed by an additional 10 sessions of STA testing to assess the impact of extended intake on the degree of loss of control. The analysis of the two sets of STA testing shows that extended cocaine intake does increase the seeking responses in the task, indicative of loss of control.

For the presses seen during RI, there is a significant increase in responding from the first set (44.6±14.7) to the second set (60.9±18.6) of STA sessions ($p=0.028$) **(Figure 8A).** This is not seen for reward collection (Set 1: 8.72±0.64; Set 2: 8.48±0.73; $p=0.343$) **(Figure 8B)**. However, responding has decreased during the second set for ALPs made during tone (Set1: 13.49±2.21; Set2: 11.77±1.73; $p=0.008$) and shocks (Set1: 1.37±0.55; Set2: 0.99±0.46; $p=0.011$) **(Figure 8C-D)**. Moreover, the suppression from baseline significantly increased (Set1: 0.39±0.13; Set2: 0.67±0.24; $p=0.037$) and the suppression ratio has significantly decreased (Set1: 0.48±0.13; Set2: 0.33±0.075; $p=0.037$) **(Figure 8E-F)**. Thus, the cohorts' recorded responses are closer to their baseline levels of responding in the second set of STA sessions, which is indicative of loss of control in the face of adversity.

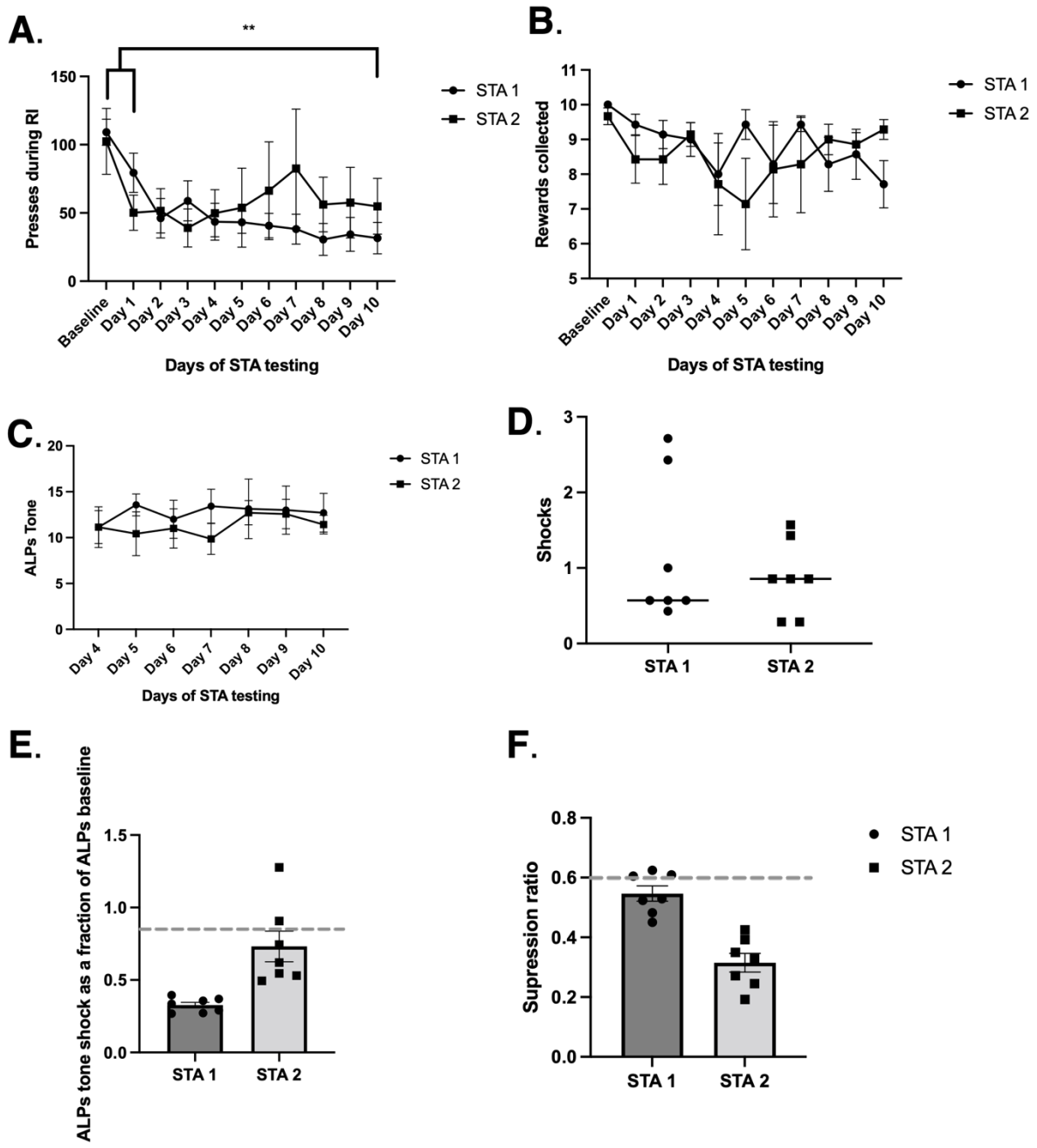

Figure 8. Seeking behavior displayed by the cocaine cohort following extended cocaine intake. Overview of responding in the STA session for the cocaine cohort during the initial set of STA testing compared to the later set of STA testing following extended cocaine intake. Individual animals are represented as black dots. A. Seeking behavior during the RI for each session of the STA testing. B. Rewards collected for each session of the STA testing C. ALPs made during the warning tone period for each session of the STA testing. D. Average shocks received by the animals in the two sets of STA testing. E. Average suppression displayed by the animals in the two sets of STA testing. F. Average suppression ratio displayed by the animals in the two sets of STA testing.

Suppression of substance seeking in the alternative STA task

The alternating STA model includes the addition of a 50/50 division over the trials of tone and no-tone conditions. The task can still capture the seeking behavior similarly to the STA task **(Figure 9A-D).** This variant of the STA task was designed as a potential improvement to assess whether animals learn the association between the tone, punishment, and reward on a trial-to-trial basis. If the task is learned, and the animals show a higher degree of loss of control over substance use, more seeking responses should be recorded during tone trials compared to no tone trials. This is what we observed in our data **(Figure 9E).** A ratio was calculated between ALPs made during the tone vs. no tone trials per session to reflect the difference in responses. If the association between the tone, punishment and reward is not made, it would be reflected by a value of 1. For the sucrose cohort, it is observed that more presses are made during the no tone interval, whereas for alcohol more presses are made during tone for both the LD and HD subgroups, with the HD responding more than the LD during the tone trials. This suggests that the animals acquire the alternating STA task, and that there is a difference in responding under the threat of adversity between sucrose and alcohol.

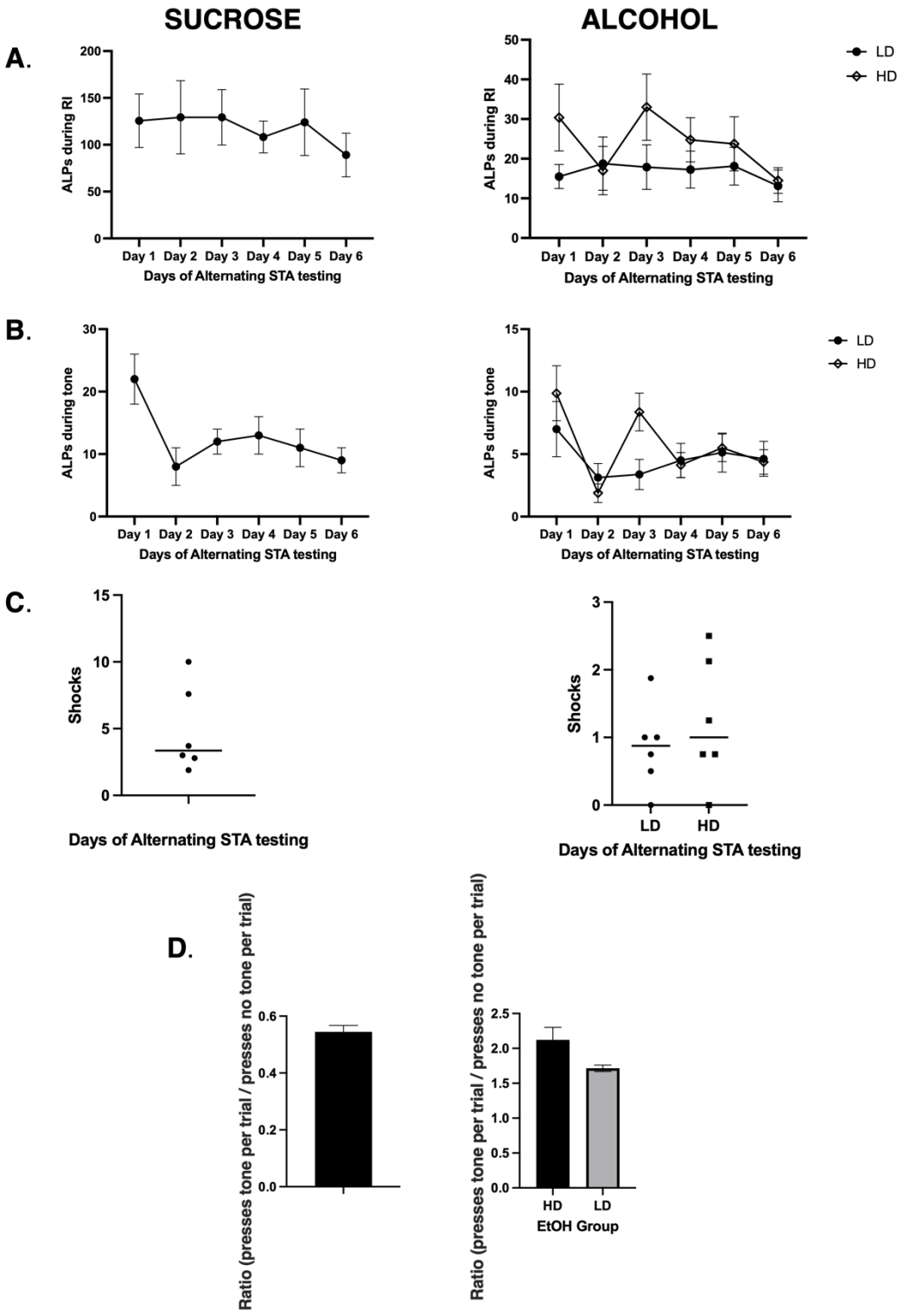

Figure 9. Seeking behavior in the alternating STA model. Seeking responses during the alternating STA for the sucrose and alcohol cohorts are presented side by side. A. Seeking behavior during the RI for each session of the alternating STA testing. B. Rewards collected for each session of the alternating STA testing C. ALPs made during the warning tone trials for each session of the alternating STA testing. D. ALPs made during the no tone trials for each session of the alternating STA testing. E. Average shocks received by the animals in the alternating STA. The days of testing

are represented as black dots. F. The average ratio of presses made per tone vs. no tone trial during the alternating STA setup for responding to sucrose and alcohol.

Glucocorticoid antagonism as possible AUD treatment

Glucocorticoid antagonism has been shown to be an effective way of reverting loss of control for the consumption of alcohol. Studies show decreased motivation for seeking the drug following treatment, and also a decrease in markers associated with alcohol abuse, making it an ideal candidate for further research **(Vendruscolo *et al.*, 2015).** In this study, we also determined the effect of the compound CORT113176 on responding in the STA task for our HD and LD animals.

There appears to be an effect for the higher dose in decreasing the seeking behavior of 100mg/kg, especially for the seeking responses made by the high drinkers during the RI and reward collection **(Figure 10 A-B).** This effect is especially pronounced in the HD animals for ALPs made during the no tone trials (Fdose(1.876,26.262)=1.020, p=0.373); Fdosexgroup(1.876,26.262)=0.711; p=0.500) and tone trials (Fdose(1.269,17.764)=0.430, p=0.568); Fdosexgroup(1.269,17.764)=1.163; p=0.311), as well as shocks (Fdose(6.125,18.897)=1.222, p=0.310); Fdosexgroup(6.125,18.897)=0.474; p=0.556) **(Figure 10 C-E).** Moreover, the ratio between the seeking response made during tone and no tone also decreases as a result of the 100 mg/kg dose, for both HD and LD. This denotes that following the 100mg/kg dose of CORT113176, animals are more aversion sensitive and thus less prone to seek the reward during tone, possibly denoting regained control over substance use. These finding have not reached clinical significance, however, the pattern emerging from a small cohort is promising and could prove clinically relevant once a larger cohort has been tested on the STA task.

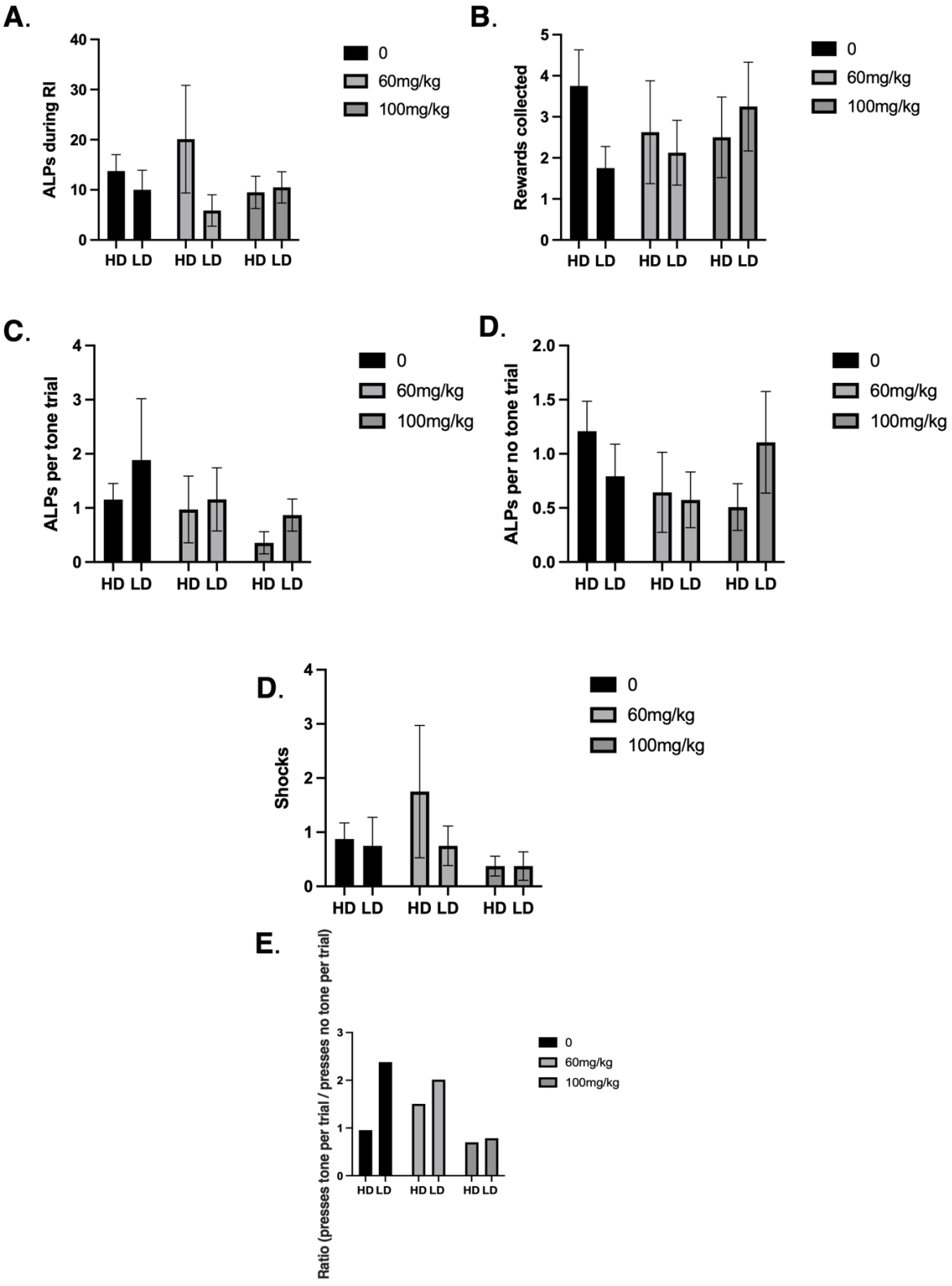

Figure 10. The effect of the glucocorticoid antagonist CORT113176 effect on substance seeking for alcohol A representation of the responding in the STA task while under the effects of CORT113176 for the LD and HD subgroups of the alcohol cohort. A. Seeking behavior during the RI for the conditions of 0, 30 and 60mg/kg CORT113176. B. Rewards collected for the conditions of 0, 30 and 60mg/kg CORT113176. C. ALPs made during the warning tone trials for the conditions of 0, 30 and 60mg/kg CORT113176. D. ALPs made during the no tone trials for the conditions of 0, 30 and

60mg/kg CORT113176. E. Average shocks received by the animals for the conditions of 0, 30 and 60mg/kg CORT113176 F. The average ratio of presses made per tone vs. per no tone trial during the alternating STA setup for the conditions of 0, 30 and 60mg/kg CORT113176. *Note: Graph F does not contain any error bars, as the cohort was too small, and no significant variations could be added based on the division performed*

DISCUSSION

The main aim of this study was to reassess the ability of the novel STA task to observe loss of control in animals. Secondary aims were to assess individual variances and subgroup performances of the animals, with an emphasis on initial alcohol intake as a predictor of compulsive seeking in the task. Finally, we aimed to combine the task with pharmacological interventions directed at the reversal of loss of control. Our results show that the STA task can induce suppression for reward seeking for sucrose, alcohol, and cocaine. The suppression level differs for the three substances tested, denoting loss of control over substance use being predominantly seen for alcohol and cocaine. The STA task can also be used to distinguish between subgroups of animals which differ in their level of control over behaviour, bringing us a step closer to revealing the intricate neural mechanisms of loss of control. Moreover, the STA task captured some reduction in reward seeking in combination with the administration of the glucocorticoid antagonist, CORT 113176, showing that the task could be used in the future for testing of new treatment compounds. Overall, we show that the STA task is a great tool to use to monitor SUD development and possible SUD treatment effects at both the cohort and individual level.

Loss of control over seeking in the STA task

To assess the seeking behaviour of the animals, the STA task has included the RI interval which can monitor the seeking of the animal, as well as couple it to a warned punishment, while decoupling the punishment from the reward. In this way what is punished is the seeking behaviour. Moreover, due to the small units of substance consumed by the animals (1 sucrose pellet, 0.1 ml, 20% v/v alcohol, 0.1 ml containing 0.125mg cocaine infusion), combined with the time of the RI (30s to 480s) and the inter trial interval (ITI)(10 min), the effects of the substance are negligible. Thus, allowing the monitoring of the seeking behaviour, while controlling substance intake and possible other interactions.

However, the responding of the animals is dependent on the type of reinforcer that is being consumed. Despite sucrose being the preferred substance in many setups, when given the choice, responding for a natural reward such as sucrose is expected to decrease to a higher

level than responding to for a substance of abuse that can alter the functioning of the natural reward system **(Belin *et al.*, 2013; Augier *et al.*, 2018).** This has been shown numerous times in setups where responding for sucrose in the face of adversity was lower compared to responding for substances such as alcohol and cocaine **(Vanderschuren & Everitt, 2004; Pelloux, Everitt & Dickinson, 2007; Spoelder *et al.*, 2015; Limpens *et al.*, 2014; Minnaard, 2020).** This is also reflected by our data. The animals in our cohorts that respond for sucrose, alcohol and cocaine all show suppression of behaviour, but this is more pronounced for sucrose compared to cocaine and alcohol, denoting that the STA task can lead to differentiation in behaviour based on the reinforcer used.

Moreover, the setup can induce suppression while using a mild punishment of 0.25mA. This intensity is not meant to harm the animal, but rather cause a slight discomfort that can be overcome in time if the animal is compulsive enough in their seeking (while giving it the choice to abstain). Previous studies have been using higher intensities to reach suppression of behaviour **(Spoelder *et al.*, 2017; Sutton *et al.*, 2021). Minnaard et. al, 2020** identified the intensity of 0.25mA as being ideal to show this behavior for sucrose and alcohol. For the cocaine setup, no intensity titration was previously tried with the STA task **(Marchant *et al.*, 2019).** Due to time restrains, the titration was included in the main STA task study. Previous data defines a mild footshock as able to induce suppression in the animals at 0.4-0.5 mA for cocaine **(Limpens *et al.*, 2014; Vanderschuren & Everitt, 2004).** However, due to the promising results on the STA task for alcohol at 0.25mA, here the same intensity was chosen as a starting point and gradually increased up to a point where a decrease in responding was detected, which was 0.35mA. Future studies can titrate the intensity separately, but in this study the 0.35mA was sufficient to induce suppression over the course of the testing, without fully suppressing the behavior. Thus, the STA task also allows for intensity titration of the footshocks, which can be useful, especially when looking at varying degrees of loss of control.

A further observation in our study was the variation of the animals withing the cohort, pinpointing to subgroups showing differing levels of loss of control. This prompted us to investigate individual variation for the three cohorts in responding between baseline and the test sessions, as it will be outlined in the next section.

Overall, the STA task, can induce suppression in the animals and there is differential responding observed over the testing days for sucrose, alcohol, and cocaine, with sucrose

responding being significantly lower. Thus, the STA task is a great model to further induce suppression and assess the development of loss of control behaviour for various substances of abuse.

Individual variation in responding during the task indicative of loss of control over substance use

Individual variation has been observed for the three cohorts responding for sucrose, alcohol, and cocaine. This variation can be especially seen when looking at individual rats responding in each session of the STA testing, denoting that there may be animals more prone to develop loss of control then others. This can be expected, as SUD development is related to a variety of genetic and environmental factors **(Belin *et al.*, 2016)**. Moreover, the lifetime prevalence of developing AUD, even for a socially acceptable and widespread substance such as alcohol, is 8.5% **(Grant *et al.*, 2015)**. Therefore, it is predictable to have subpopulations responding differently for each reinforcer, while still observing an effect of the substance.

To assess these individual variations, the suppression ratio was used **(Methods)**, as it represents the ratio between baseline and responding for each test session, making it a suitable variable to compare the seeking behavior seen between the reinforcers. The suppression ratio in our data showed different averages for each substance, with sucrose showing the highest suppression, followed by alcohol and cocaine. However, further analysis of the data showed the formation of two distinct groups for each substance. When looking at the responses throughout the testing days, a significant increase in seeking behavior is recorded for the animals in the low suppression group for all substances. Moreover, the proportion of animals belonging to the low and high suppression groups also differs by substance (Low suppression: 20% for sucrose, 47% for alcohol and 57% for cocaine).

Therefore, the STA task can capture individual variations and subgroups between the cohorts tested, which can be used to further assess the different degrees of loss of control, ideally leading to the determination an absolute characteristic value of the behavior.

Impact of prior exposure and increased substance consumption on loss of control over substance use

Prior espouse to the substance and increased consumption have both been shown to play a role in the development of loss of control over substance use **(Vanderschuren & Everitt, 2004; Spoelder *et al.*, 2015).**

Prior alcohol exposure

Previous studies have shown that during initial alcohol exposure groups can be formed based on their preference for alcohol. The higher ranking groups ('HD') have been shown to display an increase in compulsive seeking behaviour when tested on setups involving aversive stimuli such a quinine alternations and footshocks. **(Spoelder *et al.*, 2015, 2017).** However, this is not seen for our cohort. There is a pattern emerging, where HD animals seek relatively more than LD animals, but this does not reach any statistical significance. This could be due to the animals only being given small amounts of alcohol over short periods of testing times. However, initial intake may not be a good predictor of loss of control, as other studies have revealed **(Augier *et al.*, 2018). Augier *et al.*, 2018** has found that a small population of their cohort preferred alcohol over sucrose when given the choice; however, they did not find any correlation with the initial preference for the substance. Thus, the initial intake may not be a reliable predictor of loss of control. Moreover, the prior exposure is relative to the cohort being tested. Some animals may be high responding in one group and lower in others. Unless a mass-scale study can be done, to further assess this, a definite alcohol intake delimitation should be given to discern the animals that are considered HD.

Extended cocaine intake

Extended cocaine intake setups have been previously used and show that animals do show an increase in compulsive behaviour as a result of prolonged cocaine consumption **(Vanderschuren & Everitt, 2004; Limpens *et al.*, 2014).** Our data also supports these findings, showing that there is an increase in responding between the two sessions, especially in terms of suppression over the seeking behaviour.

However, we observed a decrease in the shocks received and ALPs made during tone, making it uncertain whether the animals are compulsively seeking for the substance or showing a level of impulsivity when it comes to the lever pressing. In following setup, the

STA task could be performed together with the alternating STA task to see how the animals behave in tone and no tone trials occurring in the same session.

Nonetheless, our data further shows that extended cocaine consumption can induce an increased level of compulsive seeking even in a small cohort (7 animals), which showed significant variance in responding between suppression groups. As such, it can be stated that increased consumption does have a positive effect on seeking behaviour, whether that could be defined as loss of control or not.

Despite the HD and LD subgroups in our cohort not showing differential responding for alcohol in the STA task, it can be assessed whether initial cocaine intake could be predictive of the loss of control behaviour. To further assess the effect of prior exposure on loss of control, a correlation could be made of the responding during FR1 compared to the responding in the STA task for the cocaine cohort. Alternatively, the alcohol cohort could also be given extended sessions of alcohol intake, to assess whether these characteristics are substance related or a characteristic of SUD.

The alternating STA

The alternating STA is a variant of the STA model that can be used to assess the acquisition of the task in the animals. Due to the inclusion of the trial-to-trial changes between STA and baseline trials, it allows to form a session-based analysis of the seeking responses recorded during the trials where there is a chance of adversity compared to those where there is not. Decrease in seeking during the tone in alternating STA task can be a good indication that the animals still maintain control over their behavior. It is expected that a preference for responding during non-aversive trials would be increased for sucrose compared to alcohol. This is reflected by our data. The alcohol animals press almost twice as much during the tone trials compared to sucrose, denoting that they are more likely to risk being punished to receive the reward. The overall responding, however, is not significantly higher, denoting that possibly the animals responding for alcohol may have an altered decision-making ability, or increased impulsivity compared to sucrose animals. This could be a possibility, as the use of substances of abuse can not only alter the reward pathways in the midbrain (leading to incorrect reward-prediction), but also can lead to changes in the prefrontal cortex functioning, altering high-functions such as motivation, impulse control and decision making **(Jentsch & Taylor, 1999; Kalivas & Volkow, 2005).** We cannot state if this was the case for this study. However, ideally the animals showing the increase behavior

during tone could be further assessed using tasks such as the 5-Choice Serial Reaction Task Time, to assess whether their decision making, and impulsivity levels are altered **(Esteves et al., 2021).** Overall, the alternating STA task is a useful addition to the existing STA model and should be used in parallel to assess whether animals acquire the task.

Glucocorticoid antagonism and its role in reverting loss of control over alcohol use

One of the main potentials of the STA model is to be used as a preclinical model for testing pharmacological compounds and novel treatment options for the reversal of loss of control. A promising proposed treatment for the reversal of loss of control is the use of glucocorticoid receptor antagonist antagonists. The glucocorticoid receptors are bound by glucocorticoid hormones, and as a result can alter gene transcription **(Deroche-Gamonet *et al.*, 2003).** Glucocorticoid hormones and an altered expression in the glucocorticoid receptor expression have been previously related with an increased tendency for individuals to develop a cocaine addiction and compulsive alcohol drinking in rats **(Goeders, 2002; Vendruscolo *et al.*, 2012).** Therefore, inactivating the receptors or using a receptor antagonist to decrease the activation of the glucocorticoid receptors in the brain by the glucocorticoid hormones may decrease the tendency for substance abuse. This has been previously shown to be case. A study from 2003 showed that inactivation of the glucocorticoid receptor in the rodent brain can significantly reduce cocaine self-administration **(Deroche-Gamonet *et al.*, 2003).** Similar results have been found for alcohol using glucocorticoid antagonists such as mifepristone and CORT113176 **(Vendruscolo *et al.*, 2015).**

In this study, we used two doses of the glucocorticoid antagonist CORT113176 previously shown to decrease compulsive alcohol consumption to assess if we would see a decrease in the substance seeking behaviour for the animals in the alcohol cohort **(Vendruscolo *et al.*, 2015).** Our data shows that there is difference in responding, although statistical significance has not been reached due to small number of animals left at the end of the experiment. Nonetheless, these results are very promising, as a small effect is observed, further showing that the STA task is sensitive enough to capture this alteration in behaviour.

Future uses of the STA model

The STA model proves to observe differences in behavior between both substances and individual animals, thus making it a good model to be further used in SUD research. The novel STA task is a great translational tool, as in includes many of the subtle human behavior associated with SUD, such as making the choice of whether to seek a substance, while being

aware of the consequences. Moreover, it is also sensitive enough to be able to distinguish varying levels of the loss of control behavior for animals, allowing for the formation of subgroups that can be better analyzed. And finally, it allows for easy manipulation, by manipulating the intensity of punishment received, as well as the testing times.

Given its sensitivity to individual variances, the STA task would be a great model to combine with an array of neurobiological setups. Firstly, in vivo neuronal manipulation could be done in the operant chamber while the animal is performing the task, where ideally the activity in areas related to addiction development could be monitored for altered activity, such as the prelimbic and orbitofrontal cortex **(Schoenbaum & Shaham, 2008; Minnaard *et al.*, 2020).** Moreover, subgroups of varying levels of loss of control could be compared in their activity in neuronal connectivity within the prefrontal cortex and midbrain, allowing for better deciphering the loss of control mechanisms. Alternatively, an exciting opportunity would be to monitor other behaviours and see whether they can be predictive of loss of control. For instance, anxiety and addiction have a high comorbidity **(Castle, 2008).** A setup could be done to monitor anxiolytic levels of the animals before substance administration. The behaviour of the animals in the STA task could then be assessed. Similarly, substance abuse is linked to altered functioning in the prefrontal cortex, which could be combined with a social play deprivation setup**.** Social play is vital for healthy cognitive, social and emotional development, and its deprivation has been correlated to a decreased connectivity in the prefrontal cortex **(Omrani *et al.*, 2020)**. A setup could be designed where animals undergo social play deprivation and are then administered the substance of abuse and tested on the STA task. This could give us a deeper insight into the development of SUD and loss of control, which will ultimately lead to more efficient treatment for the affected patients.

Conclusion

The STA model proves to be robust enough to trigger suppression even at mild levels of adversity. Moreover, it is also sensitive enough to further explore individual differences between animals responding for substances of abuse. This can be further used for probing various neural correlates of loss of control. In conclusion, the STA task is a great tool with potential for the in-depth study of loss of control over substance use, as well as for the development of more efficient SUD treatment options.

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Conflict of interest

The author declares no conflict of interest.

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APPENDIX

Appendix 1

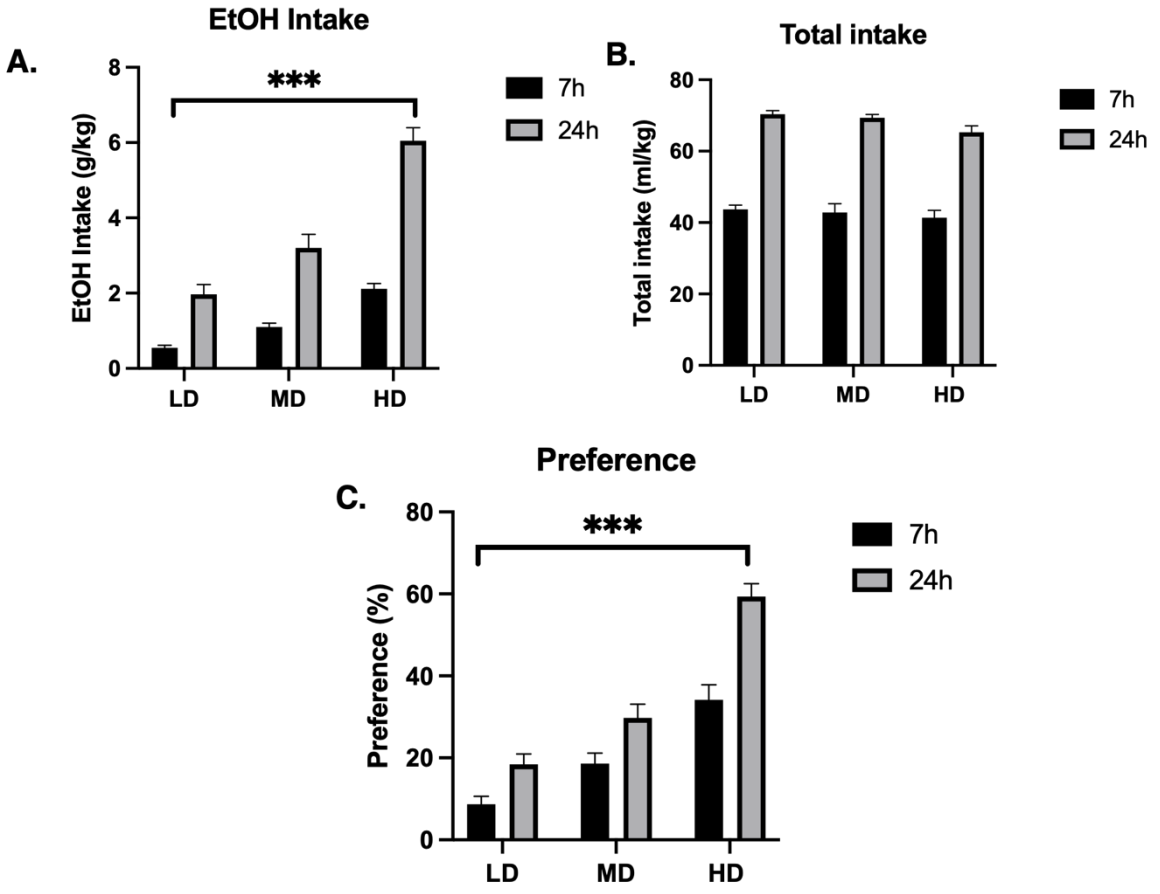

Figure 11. IAA based division into drinking subgroups A. EtOH intake averages for the LD, MD and HD subgroups for the 7h and 24h IAA B. Total Intake averages for the LD, MD and HD subgroups for the 7h and 24h IAA. C. Preference averages for the LD, MD and HD subgroups for the 7h and 24h.

Voluntary alcohol intake was assessed in the beginning of the study. Following the 2-month setup, the cohort was divided into three groups based on their alcohol intake, namely low drinkers, medium drinkers, and high drinkers (LD, MD and HD) (See methods). Three main characteristics were analyzed for the three groups, the alcohol intake, their preference for alcohol and the overall total intake of liquid. There is a significant difference in the effect of time and group, as well as a significant interaction effect between the time and group for both EtOH intake (**Ftime(1,21)=105,537; p<0.001 ; Fgroup(2,21) = 77,516;p<0.001 ; Fgroupxtime(2,21) = 8,290; p=0.002**) and EtOH preference **(Ftime(1,21) = 68,827; p<0.001; Fgroup(2,21) = 70,163; p<0.001; Fgroupxtime(2,21) = 5,408; p=0.013).** However, there is only a significant effect of time, but not of group in the total liquid intake **(Ftime(1,20). = ; p<0.001; Fgroup(2,20) = 2,087; p=0.150; Fgroupxtime(2,20) = 0.035;**

p=0.966). Thus, a clear increase in EtOH consumption can be seen over the course of the 8 weeks, with clear divisions between animals preferring the substance, which is not due to an alteration of overall liquid intake.