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Prefrontal sigma-1 receptors in alcohol use disorder and cognitive functioning

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PREFRONTAL SIGMA-1 RECEPTORS IN ALCOHOL USE DISORDER AND COGNITIVE FUNCTIONING

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SEAN TANINO

ABSTRACT

Introduction: Alcohol Use Disorder (AUD) is a chronic relapsing condition characterized by compulsive, uncontrolled consumption of alcohol. AUD is also characterized by impairments in decision making, driven by dysregulation of prefronto-cortical regions, including the anterior cingulate cortex (ACC) and medial prefrontal cortex (mPFC). Little is known about the neurotransmitter systems involved in both the excessive, compulsive drinking and the cognitive deficits observed in alcohol addiction. The Sigma 1 Receptor (Sig-1R) system has been suggested as a novel target for the treatment of addictive disorders, due to its ability to modulate the rewarding and reinforcing effects of multiple drugs of abuse, including alcohol.

Objective: The goal of the present study was to examine the role of Sig-1Rs in prefronto-cortical regions in alcohol addiction-relevant behaviors, including alcohol self-administration, motivation to work to obtain alcohol, compulsive alcohol seeking, and cognitive flexibility.

Methods: Male Wistar rats were microinfused with a viral vector containing either a Sig-1R knockdown shRNA (Sig-1R-knockdown) or GFP-Control in prefrontal regions, which encompassed the mPFC and the ACC. Rats were trained to self-administer alcohol under

increasing fixed-ratio (FR) schedules of reinforcement, as well as a progressive-ratio (PR) schedule of reinforcement, a measure of motivation to work to obtain alcohol. Animals were then trained to seek and take alcohol on a chained-schedule of reinforcement; in addition, to test compulsive alcohol seeking in the face of aversive consequences, a footshock punishment was introduced following the completion of a seeking response cycle. To test whether prefrontal Sig-1R-knockdown affected anxiety-like behavior, rats were subject to a light/dark box test, which involves allowing rats to move freely between a light and a dark compartment. The latency to leave the dark compartment and the amount of time spent in the light compartment were used as measures of anxiety-like behavior. A secondary aim of the study was to start investigating the effects of prefrontal Sig-1R-knockdown on cognitive flexibility. Alcohol naïve rats were tested in an operant attentional set-shifting paradigm, where rats were initially trained to lever press for a reward using a visual-cue strategy. Rats were then subsequently trained to lever press for a reward using a spatial response strategy. Thus, during this "set-shift," rats were required to extinguish the use of the visual-cue and return to using the spatial response strategy for obtaining a reward. Finally, the spatial strategy rule was inverted in a reversal task. In both the attentional set-shifting and reversal tasks, a greater number of previously reinforced errors (e.g. perseverative responding) was considered to reflect lower cognitive flexibility.

Results: Prefrontal Sig-1R-knockdown resulted in significantly higher responding for alcohol under high-effort conditions (FR3 and FR5) and higher motivation for alcohol (PR). In the compulsive alcohol seeking task, while GFP-Control animals decreased

seeking lever responses after the addition of the aversive footshock consequence, Sig-1R-knockdown animals showed no significant changes in lever responses. In the light/dark test, Sig-1R-knockdown animals displayed decreased latencies to enter the light compartment, possibly indicating lower anxiety-like behavior as compared to the GFP-Controls. In an attentional set-shifting task, Sig-1R-knockdown animals committed a greater number of perseverative errors during the shift to response discrimination strategy than GFP-Controls, indicating some deficits in cognitive flexibility.

Conclusion: Prefrontal Sig-1R-knockdown resulted in greater alcohol responding, motivation, and compulsive seeking behavior. Additionally, prefrontal Sig-1R-knockdown reduced cognitive flexibility in an operant attentional set-shifting task in alcohol-naïve rats. Results from these experiments support a key role for prefrontal Sig-1Rs in alcohol addiction and cognitive flexibility.

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LIST OF ABBREVIATIONS

ACC .....Anterior Cingulate Cortex

AUD ..... Alcohol Use Disorder

FR.....Fixed Ratio

GFP .....Green Fluorescent Protein

mPFC ..... Medial Prefrontal Cortex

NMDA ..... N-Methyl-D-Aspartate Receptor

NAcc .....Nucleus Accumbens

OCD ..... Obsessive Compulsive Disorder

OFC.....Orbitofrontal Cortex

PFC .....Prefrontal Cortex

PR..... Progressive Ratio

PS .....Pregnenolone

RI.....Random Interval

Sig-1R .....Sigma 1 Receptor

Sig-2R .....Sigma 2 Receptor

INTRODUCTION

Alcohol Use Disorder

Alcohol Use Disorder (AUD), or alcohol addiction, is a chronic relapsing condition characterized by compulsive, uncontrolled consumption of alcohol and by the emergence of negative emotional states during periods of withdrawal (Koob and Le Moal, 1997; American Psychiatric Association, 2013). In a given year, in the United States, 8.5% of adults will have an AUD (Hasin and Grant, 2015). This number rises to 30.3% across the lifetime (Hasin and Grant, 2015). The neurobiological basis for the transition to an AUD consists of three phases: Binge/Intoxication, Withdrawal/Negative Affect, and Preoccupation/Anticipation (Koob and Le Moal, 1997; Koob and Volkow, 2016). The development of alcohol addiction can be thought of as a cycling spiral of these phases, which ultimately results in the dysregulation of the brain's reward, stress, and decision-making systems (Koob and Le Moal, 1997).

In the early stages of the addiction cycle, the motivation to drink alcohol stems from the positively reinforcing and rewarding effects of alcohol consumption (Koob et al., 1994; Lewis, 1996; Koob and Zorrilla, 2010). After repeated cycles of overconsumption of alcohol, abstinence results in a withdrawal state characterized by the emergence of a negative affective state (e.g. dysphoria, anxiety, irritability). This causes a shift in the motivation to drink alcohol from alcohol's positive reinforcing effects to its negative reinforcing effects (i.e. alcohol is consumed to alleviate the aversive withdrawal state) (Hershon, 1977; Koob, 2015). Additionally, excessive alcohol consumption is

responsible for parallel executive functioning deficits which combine with dysregulated reward and stress systems to cause compulsive alcohol consumption (Koob and Volkow, 2016).

Alcohol Addiction as a Disorder of Decision Making: Role of Prefronto-Cortical Regions of the Brain

Addictions are becoming increasingly recognized as disorders of decision-making, motivation, and compulsion, rather than merely as pathological pursuits of pleasure (Gould, 2010; Koob and Volkow, 2016). The preoccupation/anticipation stage of the addiction cycle comprises deficits in executive control over pathological behaviors (Koob and Volkow, 2016). This "self-control" is mediated by prefronto-cortical brain regions, which is thought to become dysregulated by chronic drug and alcohol use (Koob and Volkow, 2016).

The prefronto-cortical region is composed of a collection of brain structures, including the medial prefrontal cortex (mPFC) and anterior cingulate cortex (ACC), which are involved in mediating cognitive functions including decision-making, inhibition of response, motivation, and planning (Abernathy et al., 2010). These regions are known to be dysregulated in AUD, and result in deficits of inhibitory control, cognitive performance, and decision making (Tedstone and Coyle, 2004; Sabia et al., 2011). Furthermore, these deficits are hypothesized to contribute to relapse and sustain addiction (Dias et al., 1996; Robbins, 1996).

Overpowering motivation to consume drugs and alcohol, along with a decreased ability to inhibit this desire, are main drivers of addiction (Kalivas and Volkow, 2005). The underlying mechanisms are thought to involve an imbalance of prefrontal systems. On one side, prefrontal areas are hyperresponsive to alcohol cues, causing increases in motivation for alcohol (Koob and Volkow, 2016). On the other side, there is an overall hypoactivity of prefrontal circuits encharged with inhibiting alcohol seeking and taking behaviors (Koob and Volkow, 2016). Individuals with AUD display increased activity in the mPFC and ACC in response to alcohol-associated visual cues, suggesting a role for these neural structures in the cue-induced craving (Tapert et al., 2003; Grusser et al., 2004). Indeed, greater cue-activation of these brain regions was found to be associated with higher rates of relapse in subjects who were previously abstinent from alcohol (Grusser et al., 2004). Furthermore, silencing the ACC in AUD patients through repeated transcranial magnetic stimulation reduced the cravings for alcohol (De Ridder et al., 2011).

A loss of inhibitory control over drug-taking behavior is often observed as continuing to seek and take drugs in the presence of aversive consequences (Piazza and Deroche-Gamonet, 2013). In AUD patients, this is observed as continuing to drink alcohol despite a multitude of health, psychological, and social consequences associated with drinking (American Psychiatric Association, 2013). In laboratory animals, dependent subjects will continue to perform tasks to obtain alcohol/drugs even when aversive shocks are randomly delivered upon response (Pelloux et al., 2007; Marchant et al., 2013). This compulsive reward seeking behavior has been associated to prefrontal

regions of the brain as evidenced by pharmacological inactivation of the mPFC blocking compulsive responding during aversive footshocks (Latagliata et al., 2010).

Another major domain of executive functioning disrupted by alcohol consumption is cognitive flexibility. Cognitive flexibility refers to the ability of an individual to switch between mental processes in order to elicit appropriate behavioral responses, especially in the face of changing schedules of reinforcement (Floresco et al., 2008). The ACC and mPFC are required for intact cognitive flexibility (Carter et al., 2000) and exposure to chronic, intermittent access to alcohol causes deficits in cognitive flexibility tasks, such as attentional set-shifting (Badanich et al., 2011; Kroener et al., 2012; Trantham-Davidson et al., 2014). Such deficits in cognitive flexibility and related functions likely hinder the acquisition of adaptive behaviors that support abstinence, consequently promoting relapse.

The Sigma 1 Receptor System

Extensive research has implicated the Sigma 1 Receptor (Sig-1R) system in multiple psychiatric diseases. Sig-1Rs are intracellular molecular chaperones located at the interface between the endoplasmic reticulum and the mitochondria, where they regulate calcium signaling (Martin et al., 1976; Gundlach et al., 1986; Su et al., 1988; Bowen et al., 1990; Walker et al., 1990; de Costa et al., 1992; Huang et al., 1998; Nakazato et al., 1999; Hayashi and Su, 2007). When activated, Sig-1Rs move to various parts of the cell where they interact with ion channels, receptors and kinases, and are able to modulate multiple neurotransmitter systems including dopaminergic and glutamatergic

systems (Weatherspoon et al., 1996; Ault et al., 1998; Ault and Werling, 1999; Gronier and Debonnel, 1999; Skuza and Kolasiewicz, 2001; Su and Hayashi, 2003). Endogenous ligands for Sig-1R continue to be investigated; previous studies have suggested that neurosteroids and N, N-dimethyltryptamine are potential endogenous ligands for Sig-1R (Chen et al., 2006a; Li et al., 2006; Fontanilla et al., 2009). Sig-1Rs are widely distributed across the central nervous system, but are found in high concentrations in regions such as the prefrontal cortices, amygdala, hippocampus and brainstem (Bouchard and Quirion, 1997; Alonso et al., 2000).

Sig-1Rs and Addiction

In recent years Sig-1Rs have been investigated as a promising target for the treatment of substance use disorders. Previous studies have demonstrated Sig-1Rs involvement in the mechanisms of cocaine, methamphetamine, and alcohol reward and reinforcement (Matsumoto et al., 2002; Romieu et al., 2004; Sabino et al., 2009c). Studies from our laboratory found systemic Sig-1R antagonists reduce alcohol consumption and the motivation to obtain alcohol in alcohol-dependent as well as alcohol-preferring rats (Sabino et al., 2009b; Sabino et al., 2009c). Conversely, Sig-1R agonists have been shown to induce binge-like drinking behavior in alcohol-preferring rats (Sabino et al., 2011). Furthermore, the action of Sig-1R appears to be specific to models of excessive drinking. Indeed, antagonism of Sig-1R does not affect alcohol self-administration in outbred, non-dependent rats, nor the intake of sweet solutions (Sabino et al., 2009b; Sabino et al., 2009c). The latter suggests that modulation of the Sig-1R

system does not reduce motivation for natural rewards. Previous studies have also shown elevated levels of Sig-1R expression in alcohol preferring mice when compared to alcohol adverse strains of rodents (Phan et al., 2002). Additionally, other groups have identified relationships between variations in the Sig-1R gene and alcoholism in humans (Miyatake et al., 2004).

Sig-1R and Glutamatergic Signaling in Prefronto-Cortical Regions: **Implications for AUDs**

Effects of chronic alcohol consumption on executive functioning and inhibitory control have been linked to alcohol-induced changes in glutamatergic signaling and altered neural connections in the prefrontal cortices. For example, chronic alcohol use has been shown to negatively impact the integrity and development of neurons and dendrites, as well as increase mature spine density in the prefrontal cortex (PFC) (Kalivas and Brady, 2012; Kroener et al., 2012). These modifications are associated with increased expression and activation of glutamatergic N-Methyl-D-Aspartate (NMDA) receptors (Kroener et al., 2012; Kim et al., 2015). Sig-1Rs have also been identified as regulators of neural plasticity, as Sig-1R knockdown reduces dendritic spine formation (Tsai et al., 2009). Sig-1Rs have also been found to increase glutamatergic NMDA signaling in various regions of the central nervous system. In the spinal cord, activation of Sig-1Rs increases phosphorylation of NMDA subunits in the dorsal horn, leading to increases in NMDA induced pain behavior (Kim et al., 2008; Roh et al., 2010; Yoon et al., 2010). In the hippocampus, neurosteroid dehydroepiandrosterone sulfate (DHEAS) interactions

with Sig-1R increased NMDA-mediated calcium efflux and phosphorylation of NMDA subunits (Chen et al., 2006b; Li et al., 2006). Sig-1R agonists are also known to increase the expression of NMDA subunits in regions such as the hippocampus and amygdala, while Sig-1R antagonists have been shown to prevent NMDA induced toxicity in the hippocampus following introduction of methamphetamine (Wang et al., 2007; Smith et al., 2010).

Even though there is extensive evidence that Sig-1Rs modulate glutamatergic signaling, it is not yet known whether Sig-1Rs are involved in the prefrontal cognitive dysfunction associated with AUD.

Study Objective

The first goal of the study was to evaluate the role of prefronto-cortical Sig-1Rs in alcohol drinking. Thus, we examined the effects of viral-mediated knockdown of Sig-1Rs in prefrontal regions, including the mPFC and ACC, on alcohol self-administration, motivation to obtain alcohol, and compulsive alcohol seeking in rats. To determine if any effects on alcohol-related behaviors were due to changes in emotional state, we also examined anxiety-like behavior in a light/dark box test.

The second goal of the study was to perform a preliminary study into the role of prefrontal Sig-1Rs in cognitive flexibility, independent of alcohol exposure. Alcohol naïve rats with viral-mediated knockdown of Sig-1Rs were used to determine the effects of prefrontal Sig-1R knockdown on cognitive flexibility. The purpose of this preliminary

study was to acquire initial data for future studies investigating the role of prefrontal Sig-1Rs in the cognitive deficits shown by animals chronically exposed to alcohol.

METHODS

Intracranial Surgery

Animals were anesthetized with 3-5% isoflurane. Rats were stereotaxically injected with an adeno-associated viral vector (AAV) containing either GFP (GFP-Control, AAV-SC-CMV-GFP) or a Sig-1R shRNA (Sig-1R-knockdown, Sig-1R shRNA AAV). Using a 10 µl Hamilton syringe, with a fixed needle, 1 µl of virus was injected bilaterally into the prefrontal region (AP: +0.4, ML: +/- 0.5, DV: -2.9) at a rate of 0.2 µl/min. The needle was kept in place for 5 min, to reduce backflow.

Operant Chambers

The operant chambers (30.5 cm x 24.1 cm x 29.2 cm, Med Associates, Inc., St. Albans, VT) were located in sound-attenuating, ventilated cubicles. Syringe pumps delivered fluid to two stainless steel drinking cups mounted 2 cm above the grid floor in the middle of one wall of the chamber (Blasio et al., 2015). Two retractable levers were located to either side of the drinking cups. A cue light was located above each lever. A single houselight was located on the wall opposite the cue lights and levers. Solution delivery and recording of operant responses were controlled by computers.

Experiment 1: Examining the Role of the Sig-1R System in Alcohol Reinforcement

Alcohol solution

10 % (*w/v*) alcohol solutions were prepared using 95% ethyl-alcohol and tap water.

Alcohol Self Administration

Operant Alcohol Self Administration: Fixed Ratio (FR)

A group of male Wistar rats (n=21, GFP-Control n=11, Sig-1R-knockdown n=10, Charles River Laboratories) weighing between 300-325 g upon arrival, were triple housed and provided access to water and food ad libitum. Prior to operant self-administration training, group-housed animals were allowed two bottle choice access to 10% alcohol (*w/v*) and water in their home cages for 1 day. The following day, animals were allowed one overnight operant training session, where subjects were trained to press a lever to receive 0.1 ml of water under a fixed ratio 1 (FR1) schedule of reinforcement. The total number of lever responses was recorded. The following two days, animals underwent two additional operant training sessions, 2 hours and 1 hour in length, where. subjects were allowed to press a lever to receive 0.1 ml of 10% alcohol (*w/v*) under an FR1 schedule of reinforcement.

Daily, 30-minute FR1 self-administration sessions were then started, with access to two levers, one delivering 0.1 ml of 10% alcohol (*w/v*) and the other 0.1 ml of water. The first 10 days of FR1 alcohol self-administration were analyzed as 'acquisition.'

Animals were maintained on FR1 sessions until responding was stable (<20%). Animals then underwent 7 days of daily 30-minute sessions under an FR3, and then an FR5 schedule of reinforcement. Data from the final 5 days under each schedule were analyzed.

Progressive Ratio Self Administration of Alcohol

Animals were then moved to a progressive ratio (PR) schedule of reinforcement to measure motivation to work to obtain alcohol. Under a PR schedule, the number of responses required to produce successive alcohol deliveries increased exponentially (response ratio=$4 \times (e^{\text{no. of reinforcer} \times 0.1})-3.8$) as in (Sabino et al., 2009b; Sabino et al., 2011). The session began upon completion of the first ratio with a maximum duration of 2 hours. To avoid unintended starts to the PR sessions, the first reinforcement required three responses. The session was ended if an animal did not complete a ratio for 15 minutes. Breakpoint, defined as the last ratio completed by a subject, was used as a measure of motivation for alcohol. Lever pressing on the inactive lever was recorded but had no consequences. The dependent measures for these sessions included: breakpoint, total responses (both reinforced and non-reinforced), and total reinforced responses. Sessions were repeated daily for 8 consecutive days, data from the final 5 days under each schedule were analyzed.

Compulsive alcohol seeking procedure

The protocol for the compulsive alcohol seeking procedure was developed from previous experiments (Giuliano et al., 2018). Prior to start, rats were re-acclimated to daily, 30-minute FR1 schedule operant sessions.

Taking phase

Following re-acclimation, animals began "FR1-taking lever" training. At the beginning of each session, a single lever (termed "taking lever") was randomly assigned and inserted into the operant chamber. Rats were trained to press the taking lever on an FR1 reinforcement schedule. Each response resulted in a 5-second illumination of a cue-light (positioned above the taking lever) and delivery of 0.1 ml of 10% alcohol (*w/v*). Sessions continued for 7 days and each lasted 1 hour.

Seeking-taking phase

In the "seeking-taking phase", each cycle began with the insertion of a single lever (termed "seeking lever"). The seeking lever was the lever opposite the taking lever, the latter of which was retracted. Seeking lever responses were never directly reinforced, but instead resulted in the extension of the taking lever and simultaneous retraction of the seeking lever under a random interval (RI) schedule, which increased incrementally every 3 days (RI 5-15-30-60 sec). Under a RI schedule of reinforcement (i.e. presentation of the taking lever) occur randomly in time within a given interval, absent any response requirement. The RI schedule reduces the levels of predictability for an outcome

following response. If presented, responses on the taking lever were reinforced under an FR1 schedule and resulted in a 5-second illumination of the cue-light, delivery of 0.1 ml of 10% alcohol (*w/v*), extinction of the houselight, and a 2-minute timeout period where both levers were retracted. Animals continued the seeking-taking phase under RI-60 until stable (<20%).

Seeking-taking-punishment phase

Following the seeking-taking phase, animals began the "punishment phase" of the experiment. A cycle in the punishment phase was identical to the seeking-taking phase, except that mild foot shocks were delivered upon completion of approximately 30% of trials instead of presentation of the taking lever (i.e. following responding on the seeking lever). Each session consisted of 25 cycles: 17 cycles were reinforced with the presentation of the taking lever and the delivery of 0.1 ml of 10% alcohol (*w/v*) upon taking lever response, while 8 were randomly punished via a 0.5-second foot shock and no taking lever presentation. Although the cycles were randomly punished during the session, the first cycle of each session was always reinforced, and no more than 2 consecutive cycles delivered a foot shock punishment. The intensity of the shocks was increased progressively every second or third session from 0.25 mA to 0.50 mA, to 0.75 mA, and finally to 1.00 mA. Compulsive alcohol seeking was measured by the rats' resistance to the punishment phase, i.e. when lever responding did not decrease from baseline levels of trials completed under the no shock, seeking-taking phase.

Light/Dark Box Anxiety Test

The light/dark test was conducted as previously described in (Sabino et al., 2009a).The light/dark apparatus consisted of a Plexiglas rectangular box divided into two compartments by a black Plexiglas partition. The partition had a small door. The smaller of the two compartments (33%, 14.5 cm x 27 cm x 26.5 cm) was dark, while the larger compartment (67%, 28.5 cm x 27 cm x 26.5 cm) was illuminated with a 75W light source suspended above (60 lux). To assess initial emergence behavior, rats were placed in the center of the dark compartment, facing away from the partition.

The latency to enter the light compartment and the percent time spent in each compartment were later measured from the video recorded during the 10-min sessions. Video data was scored by experimenters blind to the condition of the animal. An entry into the dark or light regions of the apparatus was defined as the animal moving all four paws into either side of the chamber (Bourin and Hascoet, 2003).

Experiment 2: The Role of Sig-1R in Cognitive Flexibility

Operant attentional set-shifting procedure was modified from Floresco et al. (2008).

Supersaccharin Solution

In all phases of the experiment, 0.1 ml of 'supersaccharin' solution (1.5% glucose, 0.8% sucrose, *w/v*) was used as a reinforcer.

Pre-training

A separate group of male Wistar rats (n=23, Charles River Laboratories) weighing between 300-325 g upon arrival, were triple housed and provided access to water and food *ad libitum*. After 5 days of habituation to their home cage, animals were food restricted to 85% of their body weight. Two days after the start of food restriction, animals began training sessions. Each session began with the illumination of a houselight and presentation of a single lever (right or left, alternating across training sessions), which remained extended for the duration of the session. Lever responses were reinforced under an FR1 schedule of reinforcement. Rats underwent two or more overnight sessions until a criterion of at least 100 presses on each lever was reached. Rats reached overnight criterion after 2-4 days, then daily 30-min sessions were run, where criterion was 50 presses per lever in the 30-minute period.

As soon as each rat reached criterion in pre-training, they were fed *ad libitum* and then underwent intracranial surgery as described above (GFP-Control n=11, Sig-1R-knockdown n=12). Animals were allowed to recover for one week with food and water ad libitum after surgery before being placed back on food restriction.

After one week of recovery following surgery, animals continued to timed lever pre-training sessions. These sessions consisted of 90 trials, each beginning with the illumination of the houselight and left or right lever extended (alternating across trials). Animals had 10 seconds to respond for a reward delivery followed by retraction of the lever and a 10-second timeout period. If the animal did not respond within 10 seconds, the lever was retracted and an omission was recorded. Each session lasted 30 minutes.

Criterion to proceed to the next phase of the experiment was less than 10 omissions per session.

Side Bias Determination

One session was run to determine a side-bias for the animals. The session consisted of 7 'cycles,' where a complete cycle for the side-bias procedure consisted of a response on both levers. This procedure was identical to previous timed training, except that in each trial the two levers were presented simultaneously. In the first trial, response on either lever resulted in reward delivery, retraction of both levers, and a 10-second timeout. In subsequent trials, the reward was delivered only if the rat responded on the lever opposite of the one chosen initially in the first trial of the cycle. If the rat responded on the same lever chosen initially, both levers retracted with no reward delivery, followed by extinguishing of the houselight and a 10-second timeout. This continued until the animal responded on the lever opposite of the one chosen initially. After the animal responded on both levers, a new cycle began.

If an animal disproportionally pressed one lever over the other (greater than 2:1 ratio), that lever was considered the animals side bias. If the cumulative presses on either lever were relatively equal, the initial lever selected on 4 out of 7 cycles was considered the side bias.

Visual-Cue Discrimination Training

For this discrimination, rats were required to respond to a visual-cue (i.e. a cue-light located above each lever) to obtain the reward. The first trial began with illumination of one of the two cue-lights. After 3 seconds, the houselight was illuminated and both levers extended. A response on the lever with the cue-light illuminated above it (correct response) resulted in reward delivery and the end of the trial. At the end of each trial, the levers retracted and the houselight was extinguished, followed by a 10-second timeout period. If no response was made within 10 seconds, the trial ended and was recorded as an omission. An incorrect response (response on the lever opposite to the visual-cue) resulted in trial ending with no reward. The location of the cue-light randomly alternated across trials. Sessions were repeated until all animals received a minimum of 30 trials and met the criterion of 8 consecutive correct responses, or after 120 trials. If an animal failed to reach criterion, the experiment was repeated daily until it was met.

Shift to Response Discrimination (Set-Shift)

The acquisition of response discrimination required the rat to cease use of the visual response strategy, and instead utilize a spatial response strategy to obtain the reward. Trials were identical to those in the visual-cue discrimination training. In these sessions, a correct response, required the animal to respond on the lever opposite its side bias, independent of the illuminated stimulus light. The first trial began with a 3-second illumination of one of the two cue-lights, followed by illumination of the houselight and extension of both levers. A response on the correct lever resulted in reward delivery and

the end of the trial. A response on the incorrect lever or an omission (failure to respond within 10 seconds) resulted in the trial ending with no reward. Trials continued until the animal responded on the correct lever for 10 consecutive trials, or the number of trials reached the maximum number of 120. If an animal failed to reach criterion, the experiment was repeated daily until criterion was met.

Reversal of Response Discrimination

In this phase of the experiment, animals were required to reverse the previous rule. Trials were identical to the previous sessions, except that the correct lever was the opposite of the one rewarded in the response discrimination sessions. The session continued until the animal responded on the correct lever for 10 consecutive trials, or the number of trials reached the maximum number of 120. If an animal failed to reach criterion, the experiment was repeated daily until criterion was met.

Statistical Analysis

Acquisition, FR1, FR3, FR5, and compulsive alcohol seeking data were analyzed by factorial ANOVAs followed by Student's t-test. Light/dark box and set-shifting data were analyzed with Student's t-tests. Statistical significance level was set at $p \leq 0.05$. The software/graphic packages used were SigmaPlot 11.0, Statistica 7.0, and Origin 8.5.

RESULTS

Effects on Prefrontal Sig-1R-knockdown on Alcohol Self-Administration

Acquisition (first 10 days of FR1)

No differences in acquisition of alcohol self-administration were observed as a result of Sig-1R-knockdown (Virus, $F(1,19) = 2.12$, n.s.; Fig. 1). Alcohol self-administration increased over time similarly in both groups (Day, $F(9,171) = 2.21$, $p<0.05$ (Day*Virus, $F(9,171) = 1.05$, not significant (n.s.)). There were no differences in water responding between groups (Virus, $F(1,19) = 0.758$, n.s.), and responses for water decreased over time similarly in both groups, presumably as lever discrimination improved (Day, $F(9,171) = 3.05$, $p<0.05$ (Day*Virus, $F(9,171) = 1.09$, n.s.).

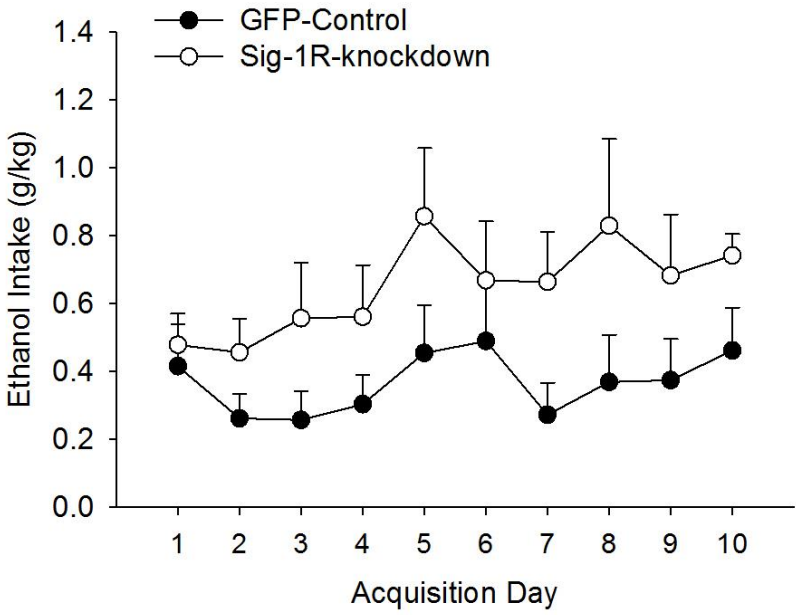

Figure 1: Acquisition of alcohol self-administration. Sig-1R-knockdown effects on daily intake of alcohol during the first 10 sessions of operant FR1 alcohol self-administration.

FR1, FR3, FR5 schedules of reinforcement

For each of the subsequent schedules of reinforcement, the first 2 days were considered habituation and were not analyzed, while the following 5 days were compared between groups. As the amount of effort required to obtain alcohol increased from FR1 to FR3 and FR5, prefrontal Sig-1R-knockdown significantly affected the responding for alcohol (Virus: FR3: $F(1,19) = 7.87$, $p=0.01$; FR5: $F(1,19) = 5.27$, $p<0.05$; Fig. 2A). Sig-1R-knockdown did not affect responding for water (Virus: FR3: $F(1,19) = 2.209$, n.s.; FR5: $F(1,19) = 3.063$, n.s.; Fig. 2B).

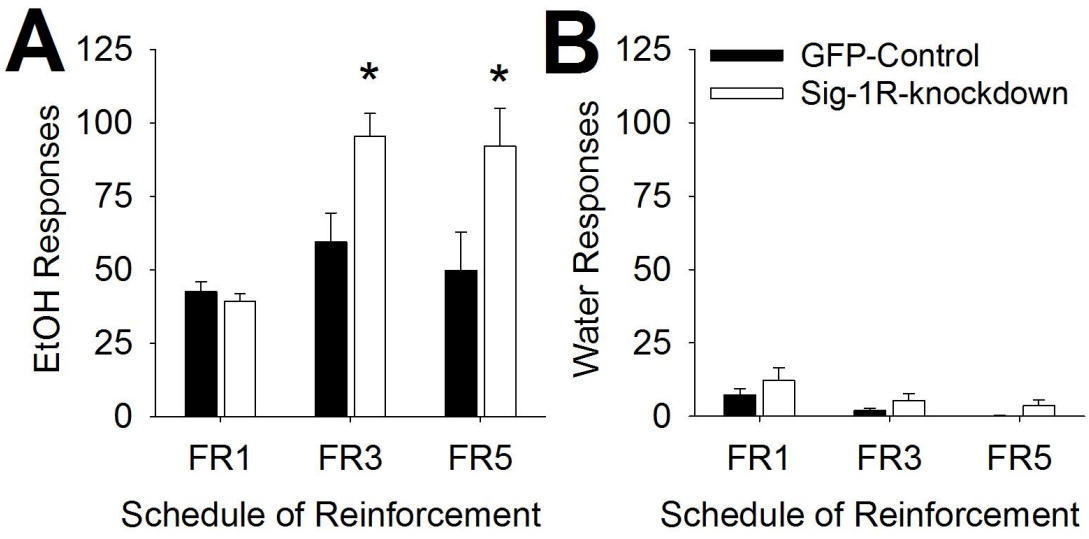

Figure 2: Higher responding for alcohol in Sig-1R-knockdown animals. (A) Responding for alcohol under increasing schedules of reinforcement; fixed ratio (FR) 1, 3, and 5 (average of last 5 days for each schedule). (B) Water responses under the different reinforcement schedules (average of last 5 days for each schedule).* $p<0.05$ vs. GFP-Control.

PR schedule of reinforcement

In sessions conducted on a PR schedule of reinforcement, prefrontal Sig-1R-knockdown also significantly affected the responding for alcohol (Virus: $F(1,19)= 4.72$, $p<0.05$), but not water (Virus: $F(1,19) = 0.864$, n.s.). Sig-1R-knockdown animals showed a higher breakpoint than GFP-Controls during the 5 days (Fig. 3A, $t(19) = -2.23$, $p<0.05$). Active responses were also greater in Sig-1R-knockdown animals ($t(19) = -2.17$, $p<0.05$; Fig. 3B). No differences in inactive lever responses were observed between groups ($t(19) = -0.93$, n.s.; Fig. 3B).

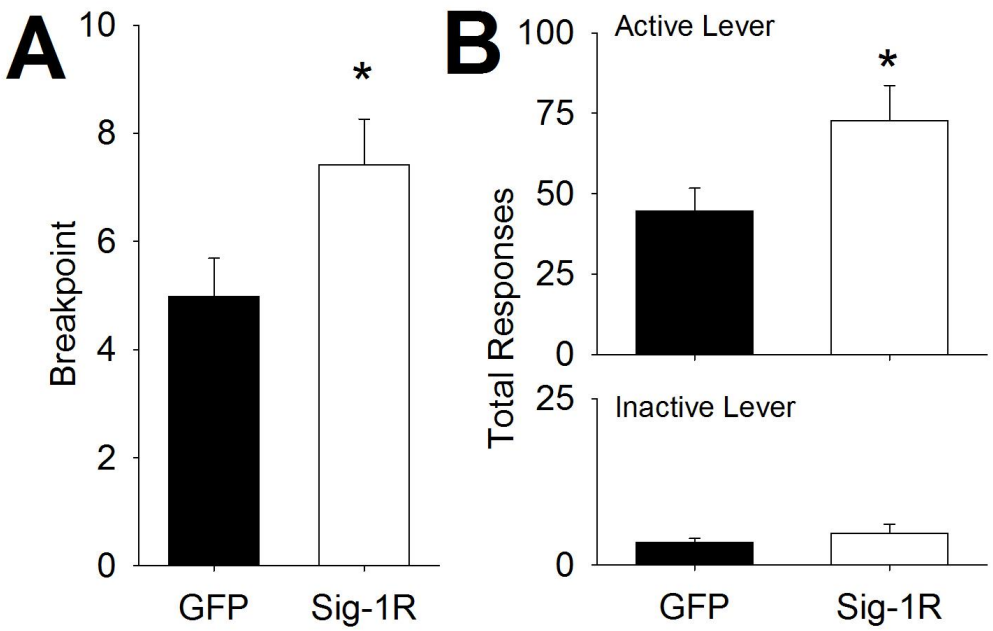

Figure 3: Motivation for alcohol is greater in Sig-1R-knockdown animals. (A) PR breakpoint for alcohol. (B) Total number of responses on the active (alcohol) lever and the inactive lever. * $p<0.05$ vs. GFP-Control.

Compulsive Alcohol Seeking

Prefrontal Sig-1R-knockdown differentially affected responding for alcohol following increasing intensities of footshock in the seeking-taking experiment (Shock*Virus, $F(3,57) = 3.35$, $p<0.05$). Indeed, GFP-Control animals, but not Sig-1R-knockdown, showed suppression of alcohol-seeking behavior in the presence of footshock. The number of responses for GFP-Control animals relative to their pre-shock baseline values were significantly decreased at shock intensities of 0.5, 0.75 and 1.00 mA, but not 0.25 mA (0.5 mA, $t(10) = -2.85$, $p<0.05$; 0.75 mA, $t(10) = -3.11$, $p<0.05$; 1.00 mA, $t(10) = -3.53$, $p<0.01$; Fig. 4). In contrast, Sig-1R-knockdown animals displayed no change from baseline response values with increasing shock intensity (0.25 mA, $t(9) = -0.82$, n.s.; 0.5 mA, $t(9) = -0.77$, n.s.; 0.75 mA, $t(9) = -0.48$, n.s.; 1.00 mA, $t(9) = 0.20$, n.s.; Fig. 4). The percent of trials completed was also significantly lower in GFP-Controls compared to Sig-1R-knockdown animals at the highest shock intensity tested (1.00 mA; $t(19) = -2.26$, $p<0.05$; Fig. 4).

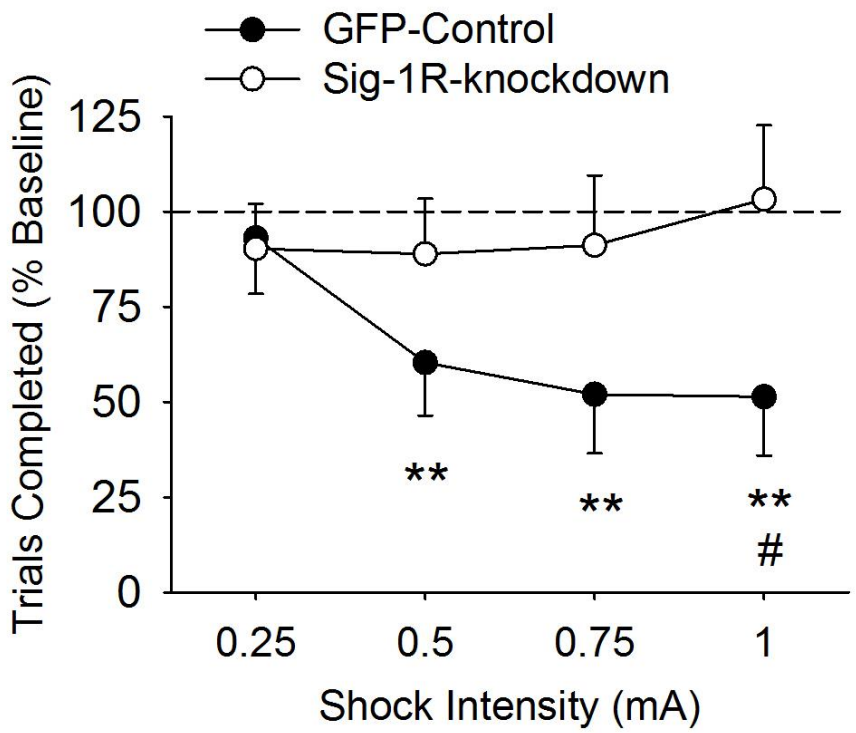

Figure 4: Compulsive alcohol seeking in Sig-1R-knockdown animals. GFP-Controls completed significantly fewer trials relative to their baseline values. GFP-Controls and Sig-1R-knockdown animals differed in percent of trials completed relative to baseline at 1.00 mA shock. ** $p<0.01$ vs. 0 mA shock (pre-shock); # $p<0.05$ vs. GFP-Controls.

Light/Dark Box Test

In the light/dark test, Sig-1R-knockdown animals exhibited lower latencies to enter the light side of the apparatus in comparison to GFP-Controls ($t(17) = 2.52$, $p<0.05$; Fig. 5A). However, despite this initial delay in entering the light portion of the chamber, both groups spent similar amounts of time overall in the light portion of the apparatus ($t(17) = 0.73$, n.s.; Fig. 5B). Light-dark transitions were also no different between the two groups ($t(17) = 0.08$, n.s.; Fig. 5C).

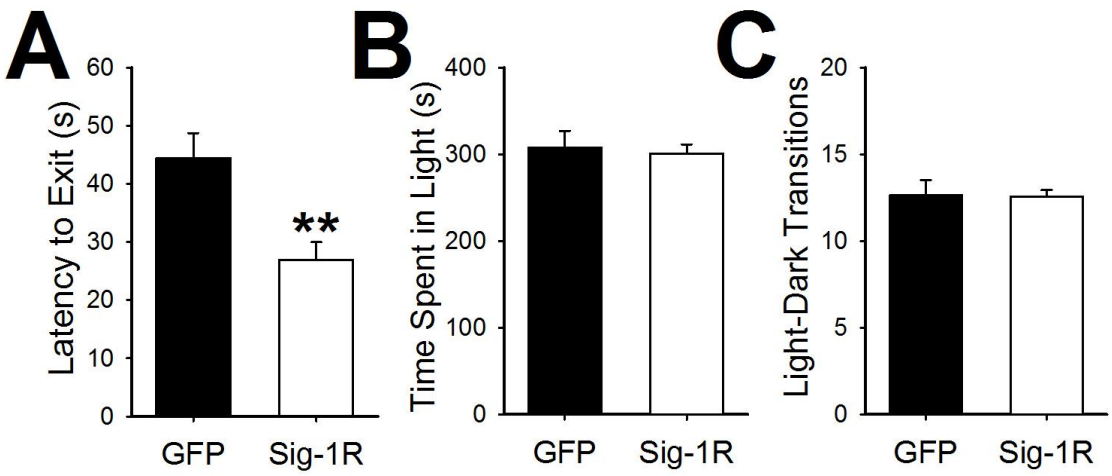

Figure 5: Light/dark box anxiety test. Effects of Sig-1R-knockdown on (A) Latency to first enter the light compartment. (B) Overall time spent in light compartment. (C) Light-dark transitions.** $p<0.01$ vs. GFP-Controls.

Attentional Set Shifting

During visual-cue training, there were no significant differences between GFP-Control and Sig-1R-knockdown animals in the number of trials to reach criterion ($t(21) = 0.443$, n.s.; Fig. 6).

In the attentional set-shifting task, there were no significant differences in trials to achieve criterion between GFP-Control and Sig-1R-knockdown animals ($t(21) = -1.38$, n.s.; Fig. 7A). However, Sig-1R-knockdown animals performed a greater number of perseverative errors compared to GFP-Controls ($t(21) = -2.751$, $p<0.05$; Fig. 7B). There were no significant differences between GFP-Control and Sig-1R-knockdown animals with regards to regressive or never-reinforced errors (Regressive, $t(21) = 0.862$, n.s.; Never-Reinforced, $t(21) = 0.40$, n.s.; Fig. 7B).

In the reversal task, Sig-1R-knockdown animals took a greater number of trials to reach criterion compared to GFP-Controls ($t(21) = -2.54$, $p<0.05$; Fig. 8A). The number of perseverative and regressive errors did not, however, differ between groups (Perseverative, $t(21) = -0.66$, n.s.; Regressive, $t(21) = -1.11$, n.s.; Fig. 8B).

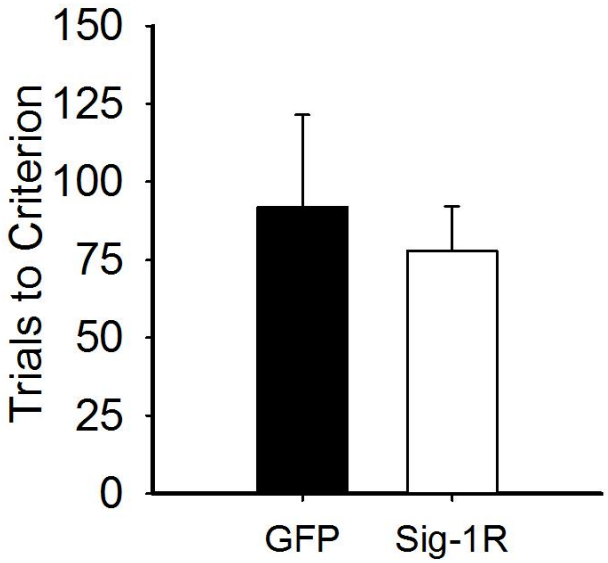

Figure 6: Visual-cue learning. Trials to criterion in the visual-cue discrimination learning task.

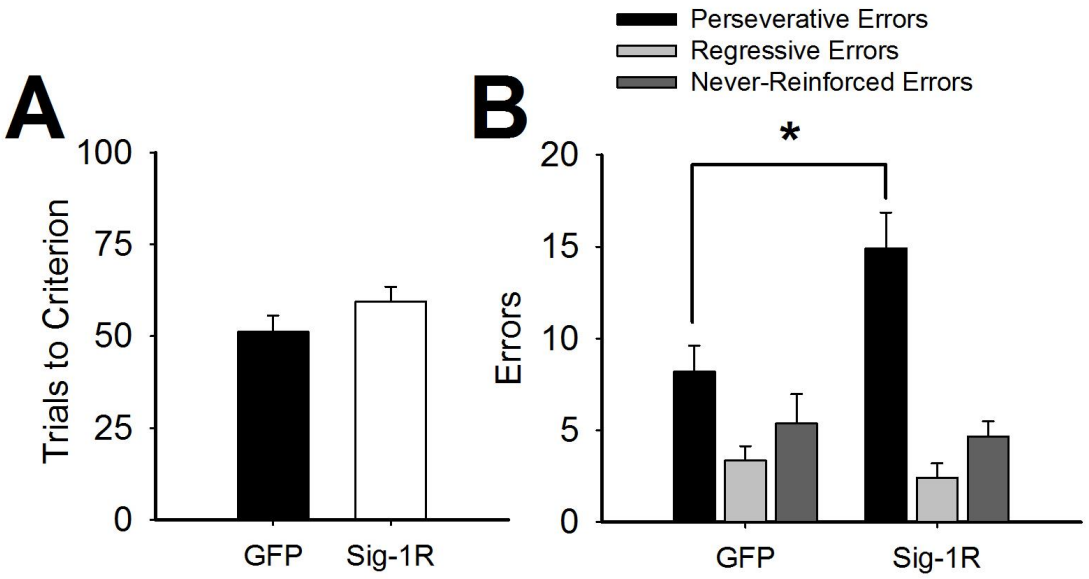

Figure 7: Response discrimination attentional set-shifting performance. (A) Number of trials to criterion. (B) Number of perseverative, regressive, and never-reinforced errors. * $p<0.05$ vs. GFP-Controls.

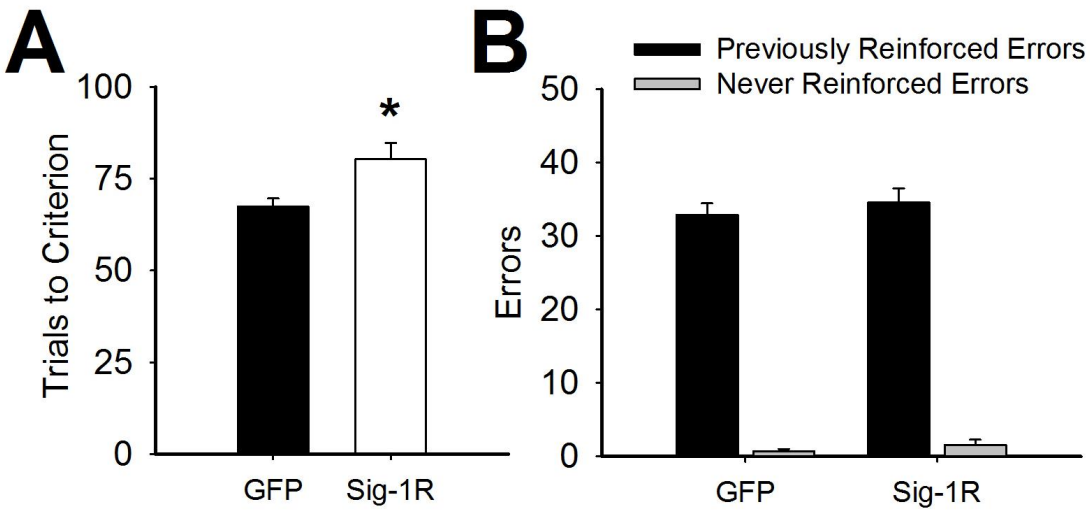

Figure 8: Reversal performance in a discrimination set-shifting task. (A) Number of trials to criterion. (B) Number of previously reinforced and never reinforced errors. * $p<0.05$ vs. GFP-Controls**.**

DISCUSSION

The present study evaluated the role of Sig-1Rs in prefronto-cortical regions on the behavioral phenotypes associated with alcohol addiction in rats, including motivation to self-administer alcohol, compulsive alcohol seeking behavior, anxiety, and cognitive flexibility.

Alcohol Self-Administration

In this study, prefrontal Sig-1R-knockdown in rats did not affect alcohol self-administration under an FR1 schedule of reinforcement. Previous studies, also using outbred, non-dependent Wistar rats found similar results, in that systemic injections of Sig-1R antagonists did not affect FR1 responding for alcohol (Sabino et al., 2009b). Together, these studies demonstrate that Sig-1Rs may not influence alcohol under this schedule of reinforcement. On the other hand, in models of excessive drinking, Sig-1R ligands have been shown to have a bi-directional effect on FR1 alcohol self-administration (Sabino et al., 2009c). Sig-1R agonists increased, while Sig-1R antagonists decreased alcohol administration in alcohol preferring and alcohol-dependent rats (Sabino et al., 2009b). Thus, Sig-1Rs may be recruited in excessive drinking or dependent animals to affect FR1 responding for alcohol, while in nondependent animals, and when the work requirement is low, the Sig-1R system may be less involved.

Operant conditioning performance depends on the workload needed to acquire the reinforcement (i.e. schedule of reinforcement) and is mediated largely by dopamine

signaling in the mesocorticolimbic pathway. Although the Sig-1R system has previously been suggested to modulate dopamine levels in various regions of the brain including the mesocorticolimbic pathway (Ault et al., 1998; Meurs et al., 2007; Navarro et al., 2010), the lack of effect in an FR1 schedule, suggests that Sig-1Rs are likely not involved in the modulation of prefrontal dopaminergic signaling under shallow reinforcement of alcohol in non-dependent animals.

Motivation for Alcohol

When the workload required to obtain alcohol increased (i.e. from FR1 to FR3 and FR5), Sig-1R-knockdown animals showed higher responding for alcohol compared to GFP-Controls. Rats with prefrontal Sig-1R-knockdown also displayed higher motivation for alcohol, as measured by a higher breakpoint in a PR schedule of reinforcement. Importantly, there were no differences between groups in lever pressing behavior for water (FR3 and FR5) or inactive lever pressing (PR), ruling out a general increase in behavioral performance. FR responding requires animals to complete a defined number of responses before each delivery of the alcohol reward. When this response requirement was increased from FR1 to FR3 and FR5, the animal must perform a greater number of responses (i.e. work harder) to obtain the desired number of reinforcers. Our data suggests that under conditions of greater effort, prefrontal Sig-1R-knockdown begins playing a role. In studies using alcohol preferring animals, Sig-1R receptor antagonism decreased, while Sig-1R agonism increased, motivation to work to obtain alcohol (Sabino et al., 2009b; Sabino et al., 2011).

The increased drive or motivation for drugs and alcohol seen in addiction has been linked to pathologies in prefrontal-accumbens circuits (Kalivas et al., 2005). Prefrontal regions are important in effort-related behaviors, as inactivation of ACC or mPFC areas causes disruptions in effort-related decision making (Walton et al., 2003; Rogers et al., 2004). Prefronto-cortical projections to the NAcc specifically mediate motivation for drugs and alcohol, as drug-related adaptations in the NAcc, including reductions in basal extracellular glutamate and an increased probability of glutamatergic release (Baker et al., 2003), are thought to contribute to heightened motivation for drugs and to relapse. Prefrontal Sig-1R in the motivation for alcohol may, therefore, occur through the modulation of glutamate transmission in the prefronto-cortical to NAcc pathway (Ault and Werling, 1997; Dong et al., 2007).

Compulsive Alcohol Seeking

The present study also showed the involvement of Sig-1Rs of the prefronto-cortical regions in compulsive alcohol seeking behavior. Following the presentation of aversive shocks, Sig-1R-knockdown animals, unlike control animals, maintained high levels of alcohol seeking behavior despite the risk of punishment.

This result seems opposite to that of a previous study which found that systemic Sig-1R antagonism blocks compulsive eating behavior (Cottone et al., 2012). As the role of Sig-1R in compulsive seeking behavior continues to be explored, it is difficult to know for certain why the results differ between studies, but a possible explanation for the

discrepancymay be = related to the different rewards used in the two studies (food vs. alcohol).

Prefronto-cortical regions are known to be involved in compulsive behaviors (Carli et al., 2006). Hypoactivity of prefrontal cortices, which act as regulatory structures for other regions of the brain, are indeed thought to be drivers for the development of compulsive seeking behaviors (Chen et al., 2013). The present study showed maintained seeking behavior of Sig-1R-knockdown animals even in the face of aversive stimuli. Previous studies have suggested that loss of function in the prefronto-cortical regions leads to poor decision making and elevated sensitivity to reward, features often associated with compulsive seeking behavior (Volkow et al., 2008; Koob and Volkow, 2010).

Anxiety-like Behavior

Sig-1R-knockdown animals displayed decreased latency to first exit the dark compartment of a light-dark box test compared to controls, which would be suggestive or an anxiolytic effect. However, no differences in total time spent in the light compartment were observed, which likely indicates that the role of Sig-1Rs of the prefrontal cortex in anxiety-like behavior at most is not a major one.

Indeed, previous studies have suggested a mixed role for Sig-1Rs in mediating anxiety-like behaviors. A study of Sig-1R knockout mice found no differences from control mice in anxiety-like behavior (Sabino et al., 2009a). However, in another study, Sig-1R antagonists were found to block the anxiolytic effects of fluvoxamine (a selective

serotonin reuptake inhibitor) used in treatment of obsessive compulsive disorder (OCD) (Egashira et al., 2007).

Sig-1R-knockdown in prefrontal regions, therefore, had an anxiolytic effect in rats. It can be hypothesized that this effects may have contributed to the compulsive seeking observed following foot shock. However, since the putative anxiolytic effect was only present in the first minute of the test, we consider this explanation unlikely.

Cognitive Flexibility

The set-shifting experiment examined the role of Sig-1Rs in prefrontal regions of the brain in cognitive flexibility, assessed through error analysis. Perseverative errors measured an animal's ability to inhibit the use of previously reinforced, but now incorrect rules for obtaining a reward (Floresco et al., 2008). Regressive errors assessed the animal's ability to maintain use of the novel strategy (Floresco et al., 2008). Never-reinforced errors assessed the animal's ability to eliminate irrelevant strategies (Floresco et al., 2008).

In the present study, there were no differences between Sig-1R-knockdown and control rats during the training and acquisition of the visual-cue strategy. This is consistent with the results of other studies of set-shifting behavior, which have shown that mPFC and ACC inactivation have little or no impact on learning of novel response strategies (Ragozzino et al., 1999; Birrell and Brown, 2000; Floresco et al., 2008). On the other end, during the set-shifting task, our results showed that Sig-1R-knockdown animals performed a significantly greater number of perseverative errors than GFP-

Controls, indicating a reduced ability to cease use of the previously learned rule. The number of regressive and never-reinforced errors committed by Sig-1R-knockdown animals was not significantly different from GFP-Controls. Perseverative errors, or perseveration of expired rules, specifically are used as an indicator of deficits in cognitive flexibility in the set shifting task (Birrell and Brown, 2000). The increase in perseverative errors following Sig-1R-knockdown in the present study suggests a role for Sig-1Rs in mediating the extinction of expired rules in cognitive flexibility.

The role of prefronto-cortical regions in cognitive flexibility is well known (Carli et al., 2006). Inactivation of the mPFC, or the circuit between downstream structures including the thalamus or NAcc, resulted in deficits in shifting between rules, exhibited as perseverative errors (Ragozzino et al., 1999; Block et al., 2007). Neural remodeling of prefrontal cortices has been hypothesized to underlie set-shifting deficits (Liston et al., 2006). Retraction and poor development of dendrites of the mPFC was found to be correlated with poor performance in set-shifting and increased perseveration (Liston et al., 2006). Sig-1R-knockdown has been shown to impede dendritic branching and extension in other regions of brain such as the hippocampus(Tsai et al., 2009)(Tsai et al., 2009), which has been attributed to mitochondrial dysfunction and accumulation of free radicals induced by Sig-1Rs (Tsai et al., 2009). Studies have also linked deficits in dendritic development to Sig-1R-induced alterations of glutamatergic signaling (Sha et al., 2013; Staples et al., 2015). Along with results from the present study, we can speculate that Sig-1R mediation of cognitive flexibility may be through maintenance of dendritic development.

While our preliminary studies focused on the physiological role of Sig-1R in cognitive flexibility, future studies will be critical in investigating its effects on cognitive flexibility in the context of excessive alcohol consumption. Indeed, chronic intermittent exposure to alcohol is known to cause both remodeling of dendrites in the mPFC and impairments in cognitive flexibility (Holmes et al., 2012). Further studies have suggested that such remodeling is a result of alcohols perturbation of glutamatergic signaling via the NMDA receptor system (Staples et al., 2015). This is a similar mechanism of impairments in neuronal development seen following Sig-1R-knockdown (Sha et al., 2013). We hypothesize that Sig-1R represents a potential link between the effects of alcohol use on cognitive flexibility.

In the reversal experiment, there were no significant differences between the groups with regard to types of error committed. The present study supports previous literature with regards to reversal learning, which suggested that the mPFC and ACC mediate cross-modal set-shifting, while the orbitofrontal cortex (OFC) mediates intra-modal reversal learning (Ragozzino et al., 1999; Chudasama and Robbins, 2003; McAlonan and Brown, 2003) (Birrell and Brown, 2000; Liston et al., 2006).

Potential Mechanisms of Sig-1R-knockdown Phenotype

The experiments presented here suggest an important role for Sig-1R in alcohol addiction. Specifically, animals with prefrontal Sig-1R knockdown showed increases in addictive-like behaviors, including increased motivation for alcohol and persistent compulsive responding despite aversive consequences. Furthermore, Sig-1R-knockdown

animals displayed deficits in cognitive flexibility; an aspect of executive functioning that has been tightly linked with addiction. AUDs can be thought of largely as disorders of decision-making, and prefrontal dysfunction underlies many of their symptoms. The studies presented here offer multiple avenues of research that are worth further pursuing to elucidate the role of Sig-1Rs in addiction and how this receptor system might be best targeted for therapeutic purposes.

Modification of glutamatergic signaling by Sig-1R is one potential mechanism underlying the overall phenotype of Sig-1R-knockdown animals. Increases in glutamatergic signaling in the ACC and NAcc have been correlated with cravings and subsequent alcohol seeking behavior (Bauer et al., 2013). Although some studies have suggested that Sig-1R agonists increase glutamatergic signaling (Guitart et al., 2000; Dong et al., 2007; Martina et al., 2007), others have indicated an inhibitory role for Sig-1R in glutamatergic signaling (Monnet, 2005; Katnik et al., 2006; Shen et al., 2008). In a study of Sig-1R agonists, activation of Sig-1R decreased glutamate concentration in the extracellular space (Monnet, 2005; Katnik et al., 2006; Shen et al., 2008). In this scenario, a decrease in Sig-1R could lead to subsequent increases in glutamate signaling, and the accompanying compulsive seeking behavior.

An important point of general discussion is related to the apparent discrepancy between some of the results obtained here and previously published findings. A number of factors may explain these differences. First, are the effects of systemic Sig-1R antagonists versus localized Sig-1R-knockdown, which may explain many of the discrepancies between the results obtained. Sig-1Rs are expressed in many regions of the

central nervous system including the amygdala and hippocampus (Alonso et al., 2000). As there is still much to be discovered about Sig-1R systems in other brain regions, it is difficult to know for certain that Sig-1Rs in other regions of the brain do not impact alcohol seeking behaviors.

It is also possible that Sig-1R-knockdown results in compensatory mechanisms of other receptor systems, specifically that of the Sigma-2 receptor (Sig-2R) system. Certain cell types such as cancerous cells have been shown to express both Sig-1R and Sig-2R (Vilner et al., 1995). An interesting finding in cancer biology is that Sig-1R and Sig-2R may have opposite effects. In tumors and self-reliant cells, Sig-1R *antagonists* were found to induce apoptosis and cancer cell death (Spruce et al., 2004), while Sig-2R *agonists* were found to induce apoptosis of breast tumor cells and have antineoplastic properties (Crawford and Bowen, 2002). While the opposing mechanisms of Sigma receptors are still not fully understood, the results from cancer studies opens the door for a potential compensatory mechanism involving compensatory action from Sig-2R in response to Sig-1R-knockdown.

CONCLUSION

The present study suggests a role for Sig-1R in prefrontal regions in mediating various behaviors commonly associated with alcohol addiction. Future research will be needed to understand the role of Sig-1R in individual prefrontal regions (e.g. prelimbic, infralimbic, and anterior cingulate cortex) and to investigate the effects of Sig-1R-knockdown on the reduced cognitive flexibility during alcohol withdrawal. Further experiments will also be necessary to elucidate the mechanisms by which Sig-1R mediates these behaviors.

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