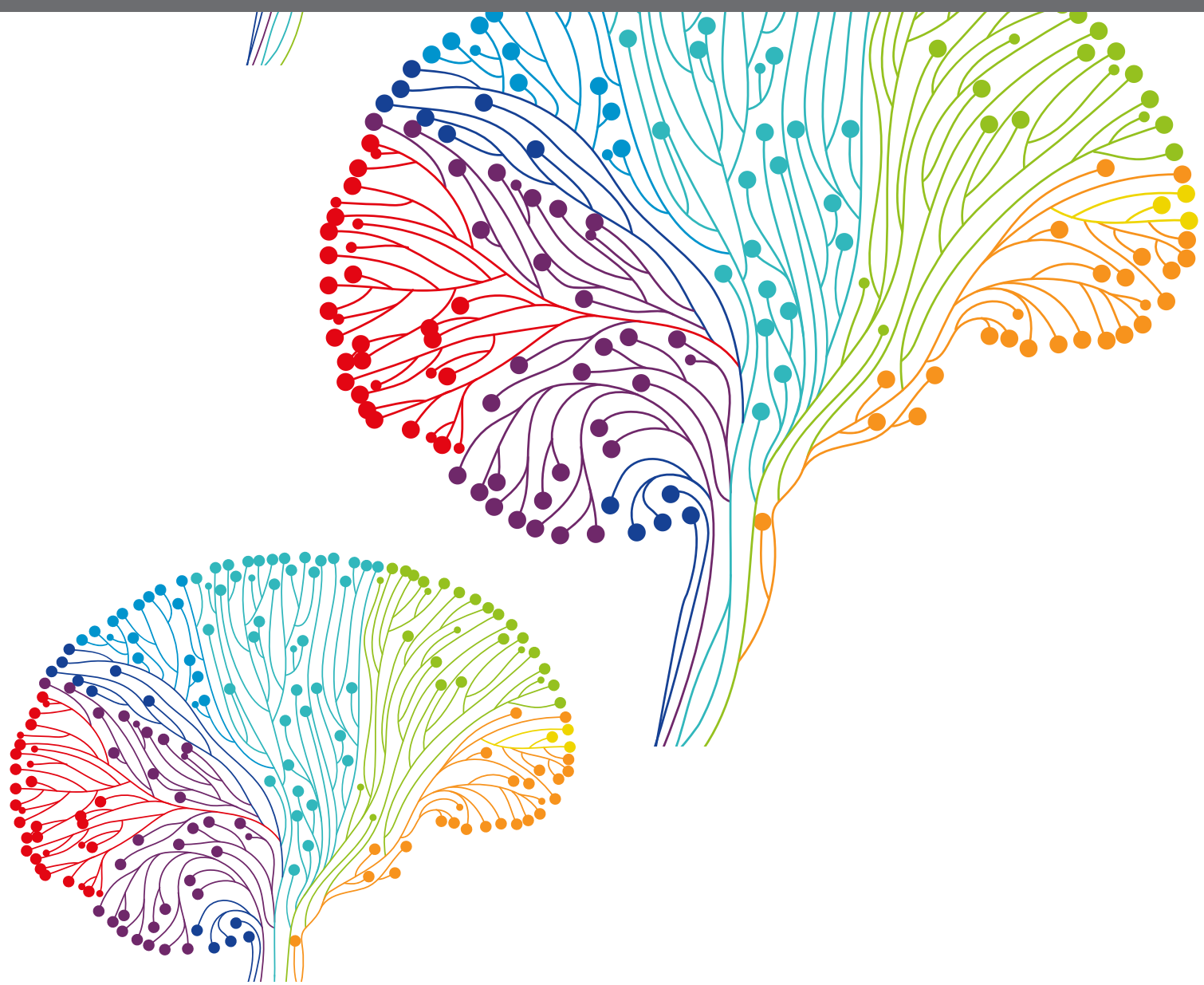
MOTIVATION AND REWARD - EDITORS' PICK 2022

EDITED BY: John D. Salamone and Liana Fattore
PUBLISHED IN: Frontiers in Behavioral Neuroscience



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ISSN 1664-8714
ISBN 978-2-88976-807-3
DOI 10.3389/978-2-88976-807-3

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MOTIVATION AND REWARD - EDITORS' PICK 2022

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Citation: Salamone, J. D., Fattore, L., eds. (2022). Motivation and Reward - Editors' Pick 2022. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-807-3

Table of Contents

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 03 February 2021
doi: 10.3389/fnbeh.2020.617418

Administration of *N*-acetylcysteine Plus Acetylsalicylic Acid Markedly Inhibits Nicotine Reinstatement Following Chronic Oral Nicotine Intake in Female Rats

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Background: Nicotine is the major addictive component of cigarette smoke and the prime culprit of the failure to quit smoking. Common elements perpetuating the use of addictive drugs are (i) cues associated with the setting in which drug was used and (ii) relapse/reinstatement mediated by an increased glutamatergic tone (iii) associated with drug-induced neuroinflammation and oxidative stress.

Aims: The present study assessed the effect of the coadministration of the antioxidant *N*-acetylcysteine (NAC) plus the anti-inflammatory acetylsalicylic acid (ASA) on oral nicotine reinstatement intake following a post-deprivation re-access in female rats that had chronically and voluntarily consumed a nicotine solution orally. The nicotine-induced oxidative stress and neuroinflammation in the hippocampus and its effects on the glutamate transporters GLT-1 and XCT mRNA levels in prefrontal cortex were also analyzed.

Results: The oral coadministration of NAC (40 mg/kg/day) and ASA (15 mg/kg/day) inhibited by 85% of the oral nicotine reinstatement intake compared to control (vehicle), showing an additive effect of both drugs. Acetylsalicylic acid and *N*-acetylcysteine normalized hippocampal oxidative stress and blunted the hippocampal neuroinflammation observed upon oral nicotine reinstatement. Nicotine downregulated GLT-1 and xCT gene expression in the prefrontal cortex, an effect reversed by *N*-acetylcysteine, while acetylsalicylic acid reversed the nicotine-induced downregulation of GLT-1 gene expression. The inhibitory effect of *N*-acetylcysteine on chronic nicotine intake was blocked by the administration of sulfasalazine, an inhibitor of the xCT transporter.

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: 14 October 2020
Accepted: 31 December 2020
Published: 03 February 2021

Citation:
Quintanilla ME, Morales P, Ezquer F, Ezquer M, Herrera-Marschitz M and Israel Y (2021) Administration of N-acetylcysteine Plus Acetylsalicylic Acid Markedly Inhibits Nicotine Reinstatement Following Chronic Oral Nicotine Intake in Female Rats. Front. Behav. Neurosci. 14:617418. doi: 10.3389/fnbeh.2020.617418

Conclusion: Nicotine reinstatement, following post-deprivation of chronic oral nicotine intake, downregulates the mRNA levels of GLT-1 and xCT transporters, an effect reversed by the coadministration of *N*-acetylcysteine and acetylsalicylic acid, leading to a marked inhibition of nicotine intake. The combination of these drugs may constitute a valuable adjunct in the treatment of nicotine-dependent behaviors.

Keywords: acetylsalicylic acid, *N*-acetylcysteine, nicotine, oxidative stress, reinstatement

INTRODUCTION

Nicotine is a neuroactive alkaloid responsible for the development and maintenance of tobacco addiction (Stolerman and Jarvis, 1995; Pontieri et al., 1996; Merlo Pich et al., 1999). While tobacco is mostly smoked, oral intake of nicotine is well recognized. Tobacco chewing (Gajalakshmi and Kanimozhi, 2015) and nicotine gums (Kostygina et al., 2016) have become public health concerns in Asia and the United States.

Globally, 20% of the population 15+ years of age are current smokers (WHO, 2019). Current smoking cessation pharmacotherapies have largely targeted the nicotinic acetylcholine receptors (nAChRs), as the activation of these receptors mediates the early rewarding effects of tobacco (Coe et al., 2005; Rollema et al., 2007; Polosa and Benowitz, 2011). However, the molecular mechanisms underlying the vulnerability to smoking relapse are multifaceted and the risk of relapse persists even in individuals receiving replacement therapy (Medioni et al., 2005; Etter and Stapleton, 2006).

Recent studies suggest a likely role of astrocytes and microglia in nicotine addiction. Cue-induced reinstatement of nicotine self-administration and chronic nicotine exposure in rats is associated with increased tumor necrosis factor-alpha (TNFα) levels in the prefrontal cortex and nucleus accumbens (Royal et al., 2018; Namba et al., 2020). Consistent with the above, studies in the authors' laboratory showed that both chronic nicotine intake and nicotine-conditioned place preference were associated with changes in markers of glial inflammation, including an increased length and thickness of astrocytic processes and increased microglial density (cell number/area) in the rat hippocampus (Quintanilla et al., 2018, 2019). Recent studies in mice also showed that chronic nicotine administration and nicotine withdrawal symptoms were associated with changes in microglial morphology and microglial-mediated release of pro-inflammatory cytokines: TNF-α, and IL-1β in striatal tissues (Adeluyi et al., 2019; Saravia et al., 2019). Adeluyi et al. (2019) also showed that microglial activation was associated with increases in reactive oxygen species (ROS) levels in the nucleus accumbens.

Nicotine exposure has also been shown to induce oxidative stress in brain regions responsible for learning and memory in rodents (Bhagwat et al., 1998; Gumustekin et al., 2003; Toledano et al., 2010; Budzynska et al., 2013; Biala et al., 2018). Nicotine induces oxidative stress through several mechanisms. *Per se*, dopamine released by nicotine during the stimulation of the mesolimbic dopamine system is deaminated by monoamine oxidase, generating hydrogen peroxide (Cadet and Brannock, 1998). In addition, dopamine released into the extracellular pH is rapidly auto-oxidized to superoxide radicals and hydrogen peroxide (Cunha-Oliveira et al., 2013). The above findings indicate (a) a nicotine-induced activation of microglia as associated with ROS production and (b) TNF-α increases, known to activate NADPH oxidase (Kim et al., 2007; Basuroy et al., 2009) and generate hydrogen peroxide and mitochondrial superoxide ions (Kastl et al., 2014). Combined, these studies suggest that oxidative stress and neuroinflammation are self-perpetuated in a vicious-like cycle, making relapse a protracted event (Berrios-Carcamo et al., 2020).

The hippocampus appears as the most relevant region to study the induction of brain oxidative stress and neuroinflammation by nicotine given that large increases in ROSs and increases of TNF-α and IL-1β were shown in the hippocampus following chronic nicotine administration to rats (Toledano et al., 2010; Motaghinejad et al., 2020). Recently, Bade et al. (2017) using non-invasive imaging techniques monitored the nicotine-induced activation of specific subregions of the rat brain. They reported that of all central nervous system reward regions, the hippocampus was the one most activated by the 1-week nicotine infusion, while resonance imaging in the nucleus accumbens, amygdala, and prefrontal cortex were less affected.

Oxidative stress (and associated neuroinflammation), as induced by chronic nicotine intake, inhibits astrocyte glutamate transporters that regulate glutamate homeostasis (Berrios-Carcamo et al., 2020). Previous research has established that alterations of glutamate homeostasis in the prefrontal cortex and nucleus accumbens contribute to the reinstatement of drug-seeking behavior (Kalivas, 2009). For several drugs, including cocaine, ethanol, and heroin, it has been shown that reinstatement of drug seeking by conditioned cues is associated with glutamate overflow in the nucleus accumbens (Baker et al., 2003; LaLumiere and Kalivas, 2008; Gass et al., 2011). For nicotine, this glutamate overflow at the tripartite synapse was associated with a reduced ability to lower glutamate levels from the synaptic cleft, due to reduced levels of the glial glutamate transporter GLT-1 in the nucleus accumbens and striatum (Knackstedt et al., 2009; Gipson et al., 2013; Alasmari et al., 2017). Further, nicotine self-administration decreased the levels of the xCT cystine/glutamate transporter, in the hippocampus and nucleus accumbens in rats (Knackstedt et al., 2009; Alasmari et al., 2017). The xCT transporter exchanges extracellular cystine for intracellular glutamate and normally is the main source of extracellular glutamate (McBean, 2002).

The prefrontal cortex is an important brain region since upon addictive drug seeking and reinstatement there is a large increase in both prefrontal firing activity and glutamate release in the nucleus accumbens derived from prefrontal afferents neurons (Kalivas, 2009). It is noted that the nucleus accumbens

is primarily a relay from the hippocampus and prefrontal glutamatergic structures onto medium spiny neurons (Britt et al., 2012; Scofield and Kalivas, 2014).

While the prefrontal cortex is an important region in the glutamatergic decision-making system, little is known about the effects of nicotine exposure on the expression of the glutamate transporter-1 (GLT-1) and xCT in the prefrontal cortex. Two previous sub-chronic studies did not reveal changes in GLT-1 or xCT in the prefrontal cortex of rats receiving nicotine via self-administration or via minipumps for 21 days (Knackstedt et al., 2009) or in mice exposed phasically (not continuously) to e-cigarette vapor containing nicotine (Alasmari et al., 2017). However, no studies have assessed whether changes occur in GLT-1 or xCT in the prefrontal cortex of rats that have consumed nicotine continuously for long periods (equivalent to years for chronic smokers), an area addressed in the present work.

The likely mechanism of inhibition of the two glutamate transporters GLT-1 and xCT is an oxidation of their cysteine residues (Trotti et al., 1997, 1998; Berrios-Carcamo et al., 2020). Additionally, TNFα leads to the downregulation of GLT-1 gene expression (Szymocha et al., 2000; Wang et al., 2003; Sitcheran et al., 2005). Noteworthily, a dysfunction of either one of the two transporters increases postsynaptic glutamate tone, namely, (i) a dysfunctional GLT-1 prevents the glial glutamate removal at the tripartite synapse while (ii) a dysfunctional xCT transporter, being unable to activate glutamate efflux, reduces the inhibition of the inhibitory mGlu2/3R (Xi et al., 2002). Both effects lead to higher glutamate levels at the tripartite synapse, primarily during cued and drug-induced reinstatement, thus driving a drug-seeking behavior (Moran et al., 2005).

The above findings suggest that both antioxidants and anti-inflammatory agents, via the restoration of normal glutamate homeostasis, would reduce the cue-induced reinstatement of nicotine-seeking behavior. *N*-acetylcysteine, an antioxidant and a prodrug in the generation of cysteine, has been reported to inhibit cue-induced nicotine seeking, an effect that was accompanied by normalization of the reduced GLT-1 expression induced by nicotine self-administration in the nucleus accumbens (Namba et al., 2020). The finding that *N*-acetylcysteine also activates the xCT cystine/glutamate transporter (Baker et al., 2003; Knackstedt et al., 2009; Alasmari et al., 2017) suggests that *N*-acetylcysteine could normalize the nicotine-induced overflow of glutamate seen in the nucleus accumbens during cue-induced nicotine reinstatement (Gipson et al., 2013), both by increasing the xCT cystine-glutamate transporter levels and importantly by increasing cystine levels a substrate for the antiporter, thus activating the inhibitory presynaptic metabotropic mGlu2/3 receptor.

In line with the existence of a self-perpetuating oxidative stress–neuroinflammation relationship, it was recently reported that the combined administration of the antioxidant *N*-acetylcysteine plus the anti-inflammatory acetylsalicylic acid inhibited chronic alcohol intake and alcohol reinstatement to a greater extent than that induced by each agent alone (Israel et al., 2019). A number of studies have shown that aspirin activates the synthesis of the peroxisome proliferator-activated receptor gamma (PPAR-γ) known to have both anti-inflammatory effects (Jiang et al., 1998; Ricote et al., 1999; Pascual et al., 2007) and to activate brain GLT-1 transcription, increasing GLT-1 protein levels (Romera et al., 2007). Six PPAR response elements (PPREs) exist in the GLT-1 gene (Romera et al., 2007). Indeed, acetylsalicylic acid was found to markedly increase the levels of GLT-1 in the prefrontal cortex (Israel et al., 2019).

Given the above, we hypothesize that should a prolonged nicotine exposure result in oxidative stress and neuroinflammation in the hippocampus and a reduction of GLT-1 and xCT in the prefrontal cortex, the coadministration of acetylsalicylic acid that increases GLT-1, added to the effect of *N*-acetylcysteine which increases the levels and activity of the cystine glutamate transporter xCT, would provide a greater inhibitory effect on the reinstatement of oral post-deprivation nicotine intake, than that induced by *N*-acetylcysteine and acetylsalicylic acid administered separately. To further test the hypothesis, we analyzed if the coadministration effects of both drugs were accompanied by increases in prefrontal cortex mRNA levels of GLT-1 and xCT. Finally, we evaluated whether blocking the xCT cystine/glutamate antiporter by the inhibitor sulfasalazine blunted the inhibition of oral nicotine intake exerted by *N*-acetylcysteine.

MATERIALS AND METHODS

Two-month-old UChB female rats (Quintanilla et al., 2006) were used in the experiments. The rationale for using female rats in this study is that female rats show a higher nicotine-seeking behavior compared to males (Donny et al., 2000; Torres et al., 2009). Additionally, females maintain stable body weights (within 10%) over time—of value in long-term studies. Animals were maintained on a 12-h light/dark cycle (lights off at 7:00 PM) and were regularly fed with a soy protein, peanut meal rodent diet (Cisternas, Santiago, Chile). Given that rats do not readily voluntarily consume nicotine orally, the animals were pretreated with an intraperitoneal dose of nicotine (0.6 mg/kg/day), for 2 weeks, which promoted a subsequent escalation of oral nicotine intake voluntarily when offered the choice of water and nicotine solution using a two-bottle paradigm (Quintanilla et al., 2018, 2019). Details of this procedure are described below. All animal procedures were approved by the Committee for Experiments with Laboratory Animals at the Medical Faculty of the University of Chile (Protocol CBA# 0994 FMUCH).

Drugs

(−)-Nicotine hydrogen tartrate was obtained from Sigma-Aldrich, St. Louis, MO, United States. The nicotine solution for the initial intraperitoneal administration was prepared by dissolving nicotine hydrogen tartrate in saline and adjusted to pH 7.2–7.4 with NaOH (0.1 N) and injected in a volume of 5.0 mL/kg/day, at a dose of 0.6 mg/kg/day (Bashiri et al., 2016) by the intraperitoneal route (Al Shoyaib et al., 2019). The intraperitoneal administration was carried out by holding the rat in supine position with its head tilted lower than the posterior part of the body, and the needle was inserted in the lower right quadrant of the abdomen (at ∼10° angle) with care to avoid

accidental penetration of the viscera (Al Shoyaib et al., 2019). The nicotine solution concentrations for oral intake, calculated as free base, were 5, 10, 30, 50, or 60 mg/L (w/v) and were prepared by dissolving nicotine hydrogen tartrate in distilled water every day. *N*-acetylcysteine (NAC) (Sigma, St. Louis, MO, United States) was dissolved in water, adjusted with NaOH to pH 7.2, and administered in a volume of 5.0 mL/kg/day at a dose of 40 mg/kg/day, using the intragastric route of administration by gavage (Conybeare and Leslie, 1988). Briefly, the intragastric administration by oral gavage was carried out using a steel curved needle 70 mm long, 5-gauge, with a tip "bulbed" of 4 mm. For the oral administration the rat was held firmly by the skin of the neck and back so that the head is kept immobile and in line with the back. The needle attached to a syringe was passed into the mouth and after locating the entry to the esophagus is pushed gently into the stomach to initiate the discharge (Conybeare and Leslie, 1988). Acetylsalicylic acid (ASA) (Sigma-Aldrich, St. Louis, MO, United States), was dissolved in water and adjusted with NaOH to pH 7.2 and administered in a volume of 5.0 mL/kg/day at a dose of 15 mg/kg/day, by the intragastric route using oral gavage, as indicated above. This ASA dose is considerably lower than that used as chronic treatment for arthritis in humans (Colebatch et al., 2012). Doses of ASA of 15–30 mg/kg were shown not to generate gastric irritation in rats (Wallace et al., 2004). When the combination of NAC + ASA was administered, both drugs were dissolved in water, adjusted with NaOH to pH 7.2, and delivered in a volume of 5 mL/kg/day by oral gavage (Israel et al., 2019). Sulfasalazine, an xCT transporter inhibitor, was administered intraperitoneally (Al Shoyaib et al., 2019) to achieve a better bioavailability (Bernabucci et al., 2012). Sulfasalazine stock solution was prepared dissolving sulfasalazine in DMSO (80 mg/ml), as previously reported (Bernabucci et al., 2012). Fresh sulfasalazine (referred as SZ) was prepared every day by dilution of the stock solution in isotonic saline and administered intraperitoneally at a dose of 8 mg/kg/day delivered as 5 ml/kg/day (Bernabucci et al., 2012). As reviewed earlier, this dose of sulfasalazine does not have an anti-inflammatory effect (Quintanilla et al., 2020).

Brain Tissue Samples

We selected the hippocampus to study oxidative stress and neuroinflammation produced by nicotine since, as indicated above, it has been shown that nicotine induces both (i) a greater production of oxidative stress in the hippocampus than in other regions and (ii) an increase of TNF−α and IL-1β in this region (Toledano et al., 2010; Motaghinejad et al., 2020). In addition, the hippocampus is an area involved in the associations of nicotine's rewarding effects with specific contextual cues (Dols et al., 2000). We dissected the whole left hippocampus for GSSG/GSH assays, while the right brain hemisphere was fixed in 4% paraformaldehyde in phosphate-buffered saline (0.1 M PBS) to obtain coronal sections of the hippocampus (20 μm thick) to evaluate astrocyte and microglial reactivity, focusing on the stratum *radiatum* of the CA1 region, between Bregma −3.14 to −4.16 mm (Paxinos and Watson, 1998) according to our previous studies (Ezquer et al., 2019; Israel et al., 2019) and to Gomez et al. (2018).

Since, as indicated, the prefrontal cortex is an important region in the glutamatergic decision-making system (Kalivas, 2009); while little is known about the effects of nicotine exposure on GLT-1 and xCT expression, we examined the mRNA levels of both GLT-1 and xCT in the prefrontal cortex of rats following the reinstatement of oral consumption of a nicotine solution post-deprivation. The medial prefrontal cortex, including cingulate and pre- and infralimbic regions, between Bregma +3.2 and +1.7 mm (Paxinos and Watson, 1998), was selected for quantification of the mRNA levels of GLT-1 and the xCT cystine/glutamate antiporter.

Determination of Astrocyte and Microglial Immunoreactivity

Immunofluorescence against the astrocyte marker, glial fibrillary acidic protein (GFAP), and the microglial marker ionized−calcium–binding adaptor molecule 1 (Iba−1) were evaluated in coronal cryo−sections of the hippocampus (30 μm thick) as previously reported (Ezquer et al., 2019). Nuclei were counterstained with DAPI. Microphotographs were taken from the *stratum radiatum* of the hippocampus using a confocal microscope (Olympus FV10i). The area analyzed for each stack was 0.04 mm^2, and the thickness (*Z* axis) was measured for each case. The total length and thickness of GFAP-positive astrocyte primary processes and the density of Iba−1–positive microglial cells were assessed using FIJI image analysis software[1] as previously reported (Ezquer et al., 2019).

Glutathione Determination in the Hippocampus

Brain oxidative stress was determined by assessing the ratio of oxidized (GSSG) to reduced (GSH) glutathione in the hippocampus as previously described (Ezquer et al., 2019). Glutathione reductase (G3664), NADPH (N1630), and DTNB (5,50−dithiobis−2−nitrobenzoic acid), used for the determination of glutathione, were purchased from Sigma-Aldrich.

Quantification of mRNA Levels of GLT−1 and xCT Cystine/Glutamate Exchanger in the Prefrontal Cortex

Two hours after recording the nicotine relapse (day 128), the animals were anesthetized with chloral hydrate (280 mg/kg, i.p.) and euthanized to obtain prefrontal cortex samples. Total RNA from the prefrontal cortex was purified using Trizol (Invitrogen, Grand Island, NY, United States). One microgram of total RNA was used to perform reverse transcription with MMLV reverse transcriptase (Invitrogen) and oligo dT primers. Real-time PCR reactions were performed to amplify the glutamate transporters GLT1 and xCT using a Light-Cycler 1.5 thermocycler (Roche, Indianapolis, IN, United States). The primers used for qPCR amplifications were designed by the authors using the following sequence: GLT-1 sense 5′-CCTCATGAGGATGCTGA AGA-3′ GLT-1 antisense 5′-TCCAGGAAGGCATCCAGGC

[1]http://fiji.sc/Fiji

TG-3′ and xCT sense 5′-CCTGGCATTTGGACGCTACAT-3′ xCT antisense 5′-TCAGAATTGCTGTGAGCTTGCA-3′. To ensure that amplicons were generated from mRNA and not from genomic DNA, controls without reverse transcriptase during the reverse transcription reaction were included. Relative quantifications were performed by the ΔΔCT method. The mRNA level of each target gene was normalized against the mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample.

Induction of Voluntary Oral Consumption of a Nicotine Solution

The voluntary oral consumption of a nicotine solution was induced as was previously described (Quintanilla et al., 2018, 2019). Briefly, twenty-eight female naïve UChB rats were intraperitoneally administered 0.6 mg/kg/day of nicotine hydrogen tartrate (Bashiri et al., 2016), for 14 consecutive days. On day 15, 24 h after nicotine intraperitoneal injections were discontinued, all rats were given continuous (24 h/day) two-bottle free choice access between water and a nicotine solution. The concentration of the nicotine solution, calculated as free base, was 5 mg/L (w/v) on the initial 3 days and was increased to 10 mg/L from days 4 to 6, to 30 mg/L from days 7 to 9, and to 50 mg/L from days 10 to 15. Finally, from day 16 onward, the nicotine concentration was raised to 60 mg/L and was kept constant for 4 months. Oral nicotine and water intakes were recorded daily, and the bottle positions were alternated every day to avoid the development of a side preference. Oral voluntary nicotine intake was expressed as milligrams of nicotine consumed per kilogram body weight per day (mg/kg/day), and water intake was expressed as ml of water consumed per kilogram body weight per day (mL/kg/day).

Experiment 1A. Effect of the Oral Administration of NAC, ASA, or NAC + ASA on the Oral Voluntary Consumption of a Nicotine Solution

Once a sustained voluntary nicotine intake was achieved, as described above, the 28 female UChB rats were given continuous (24 h/day) free-choice access to a nicotine solution (60 mg/L; w/v) and water, for 112 days. On day 84, rats were randomly assigned to four groups (n = 7 rats/group), which received for 11 consecutive days one of the following treatments: (1) Vehicle group: rats were given water, by oral gavage; (2) NAC group: rats were given *N*-acetylcysteine (NAC) (40 mg/kg/day), by oral gavage; (3) ASA group: rats were given ASA (15 mg/kg/day), by oral gavage; and (4) NAC + ASA group: rats were administered the combination of NAC (40 mg/kg/day) plus ASA (15 mg/kg/day), by oral gavage. It is important to note that access to oral nicotine-solution intake was not interrupted during the 11 days while NAC, ASA, or NAC + ASA was administered, nor was it interrupted for the following additional 17 days posttreatment (washout period). Voluntary oral nicotine consumption was recorded daily and expressed as milligrams of voluntary nicotine consumed orally per kilogram body weight per day (mg/kg/day). Water intake was expressed as ml of water consumed per kilogram body weight per day (ml/kg/day).

Experiment 1B. Effect of Oral Administration of NAC, ASA, or NAC + ASA on the Reinstatement of the Oral Nicotine Consumption Post-deprivation

After 113 days of voluntary chronic oral nicotine intake, the animals of experiment 1A were deprived of nicotine, but not of water, for 14 days, and thereafter allowed re-access to a nicotine solution (60 mg/l; w/v) for 2 days. On the last 9 days of nicotine deprivation, rats were divided into four groups (n = 7) that received a daily dose of the following: (1) Vehicle group: rats were administered water, by oral gavage (Conybeare and Leslie, 1988); (2) NAC group: rats were administered *N*-acetylcysteine (40 mg/kg/day) by oral gavage; (3) ASA group: rats were administered ASA (15 mg/kg/day) by oral gavage; and (4) NAC + ASA group: rats were administered a combination of NAC (40 mg/kg/day) plus ASA (15 mg/kg/day) by oral gavage. The last treatment dose was administered 24 h prior to the reinstallation of 60 mg/l (w/v) nicotine solution (on day 126), for two continuous days (days 127 and 128). Once the experiment had ended, after recording the voluntary oral nicotine intakes at the second day (day 128) of nicotine re-access, animals were anesthetized with chloral hydrate (280 mg/kg, i.p.), perfused intracardially with 100 ml of PBS (pH 7.4), and euthanized to obtain brain samples for determination of the GSSG/GSH ratio, to assess astrocyte and microglial immunoreactivity in the hippocampus and for quantification of mRNA levels of the GLT-1 and xCT cystine/glutamate exchanger in the prefrontal cortex. The rat estrous cycle was not monitored since neither the estrous cycle nor sex differences have been reported to influence the reinstatement of extinguished nicotine-seeking behavior in male and female rats (Feltenstein et al., 2012).

Experiment 2. Effect of the Inhibition of the xCT-Cystine/Glutamate Exchanger on Oral Voluntary Consumption of a Nicotine Solution

To study the participation of the xCT cystine/glutamate antiporter in the inhibitory effect of *N*-acetylcysteine on chronic nicotine intake, we administered sulfasalazine (SZ), a potent inhibitor of this transporter (Gout et al., 2001). For this study, we used a new group of twenty adult female UChB rats, which after being induced to voluntarily consume a nicotine solution orally as described above were given access to nicotine (60 mg/L; w/v) and water for 91 days. On day 83, rats were divided into four groups (n = 5 rats/group), which received for two consecutive days the following treatments: (1) Vehicle/vehicle group: rats were administered saline (NaCl 0.9%) via i.p. (5 mL/kg), as was described above, 15 min before water administration (5 ml/kg) by oral gavage, as was described above; (2) sulfasalazine/NAC group: rats were administered sulfasalazine, an inhibitor of xCT-cystine/glutamate exchanger, at a dose of 8 mg/kg/day (i.p.)

(Bernabucci et al., 2012), 15 min before NAC administration (40 mg/kg/day) by oral gavage; (3) sulfasalazine/vehicle group: rats were administered SZ at a dose of 8 mg/kg/day via i.p., as was described above, 15 min before water administration (5 ml/kg/day), by oral gavage; and (4) vehicle/NAC group: rats were administered saline (NaCl 0.9%) via i.p. (5 mL/kg) 15 min before the administration of a dose of 40 mg/kg of NAC by oral gavage. Oral voluntary nicotine intake was recorded daily and expressed as milligrams of nicotine consumed per kilogram body weight per day (mg/kg/day).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (San Diego, CA, United States). Data are expressed as means ± SEM. The normal distribution of data for all experiments was first tested using the Shapiro–Wilk test. For normally distributed data, one-way analysis of variance (ANOVA) (**Figures 2**, **4B**, **5**–**7**, and **8A**) or two-way (treatment × day) ANOVA (**Figures 3**, **4A**, **8B**) was used followed by a Tukey or Fisher *post hoc* test. A level of $P < 0.05$ was considered for statistical significance. To facilitate text reading, full statistical ANOVA analyses were presented as shown in the figure legends.

RESULTS

Figure 1 shows the experimental timeline indicating the time at which (i) intraperitoneal nicotine was administered; (ii) free choice of oral nicotine and water was allowed; (iii) NAC, ASA, and NAC + ASA were administered to animals chronically consuming oral nicotine; (iv) nicotine deprivation was carried out; and (v) NAC, ASA, and NAC + ASA were administered prior to reinstatement of oral nicotine intake.

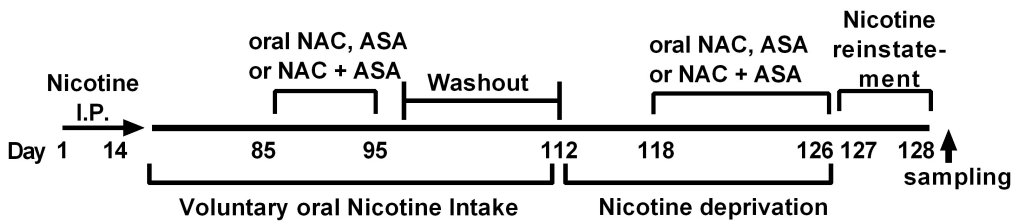

FIGURE 1 | Experimental time-line. The timeline indicates the times at which (i) intraperitoneal nicotine was administered; (ii) free choice of oral nicotine and water was allowed; (iii) NAC, ASA, and NAC + ASA were administered to animals chronically consuming oral nicotine; (iv) nicotine deprivation was carried out; (v) administration of NAC, ASA, and NAC + ASA prior reinstatement of oral nicotine intake and reinstatement; (vi) brain tissue sampling was done.

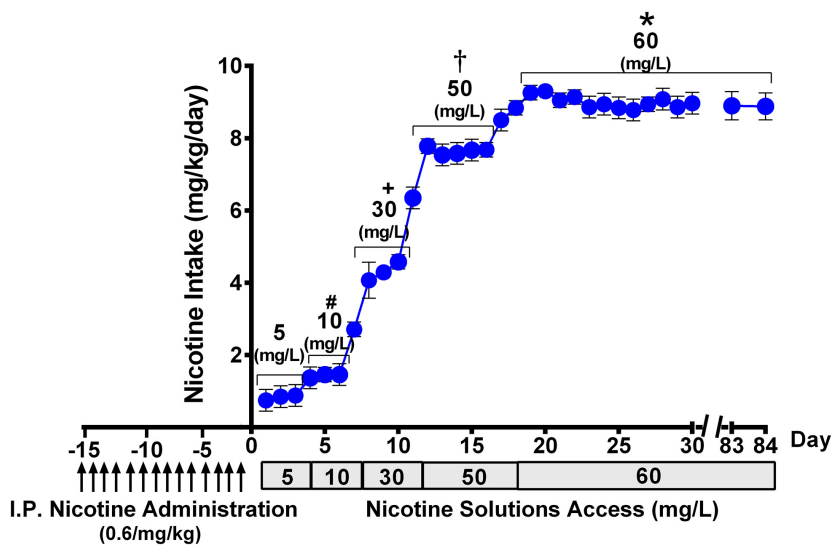

FIGURE 2 | Progressive escalation of voluntary oral nicotine consumption (mean ± SEM) in rats allowed continuous free access to water and a nicotine solution of increasing concentrations, after their pretreatment with an intraperitoneal daily dose of nicotine (0.6 mg/kg), for 14 days. One-way ANOVA of all voluntary oral nicotine intake data indicated a significant effect of the concentration of the nicotine solution offered to the rats [$F_{concentration(4,117)} = 977.3$, $p < 0.0001$]. Tukey *post hoc* test revealed that when rats were allowed access to nicotine, 60 mg/L displayed higher levels of voluntary oral nicotine intake than when they were allowed access to nicotine 50, 30, 10, or 5 mg/L (*$p < 0.0001$), and when rats were allowed access to nicotine 50 mg/L displayed higher levels of oral nicotine intake than when they were allowed access to nicotine 30, 10, or 5 mg/L (†$p < 0.0001$). Further, when rats were allowed access to nicotine, 30 mg/L displayed higher levels of oral nicotine intake than when they were allowed access to nicotine 10 or 5 mg/L (+$p < 0.0001$), while when rats were allowed access to nicotine 10 mg/L displayed higher levels of oral nicotine intake than when they were allowed access to nicotine 5 mg/L (#$p < 0.0001$).

Figure 2 shows that following animal pretreatment with an intraperitoneal dose of nicotine (0.6 mg/kg/day) for 14 days, a progressive escalation of oral nicotine consumption was seen in animals that were allowed free choice of water and nicotine solution, the concentration of which was increased every 3–5 days. Following 16 days of oral access to a nicotine solution of 60 mg/L and water, the rats achieved a stable oral nicotine consumption of 8.5 ± 0.1 mg of nicotine/kg of body weight (mean ± SEM). The nicotine concentration of 60 mg/L was kept constant throughout the experiment, excluding the deprivation period. The oral voluntary nicotine intake described in this study was similar to that reported for female rats selectively bred for their high nicotine preference (Nesil et al., 2013) and in naive female rats belonging to the P (high alcohol-preferring) line (Sari et al., 2016). In those studies, animals were given free-choice access of an oral nicotine solution prepared in water adulterated with sucrose, unlike the free-choice access of nicotine dissolved in distilled water that was used in the present work.

Figure 3 (left) shows that the voluntary nicotine intake of control rats (vehicle-treated) that continued to consume nicotine orally following three consecutive months of intake (8.5 ± 0.1 mg/kg/day; mean ± SEM, n = 7) was inhibited by 32% by the oral daily administration of *N*-acetylcysteine (NAC, 40 mg/kg/day) (5.8 ± 0.1 mg/kg/day; mean ± SEM; n = 7), by 26% by the oral administration of ASA (15 mg/kg/day) (6.3 ± 0.1 mg/kg/day; mean ± SEM, n = 7/group), and by 45%

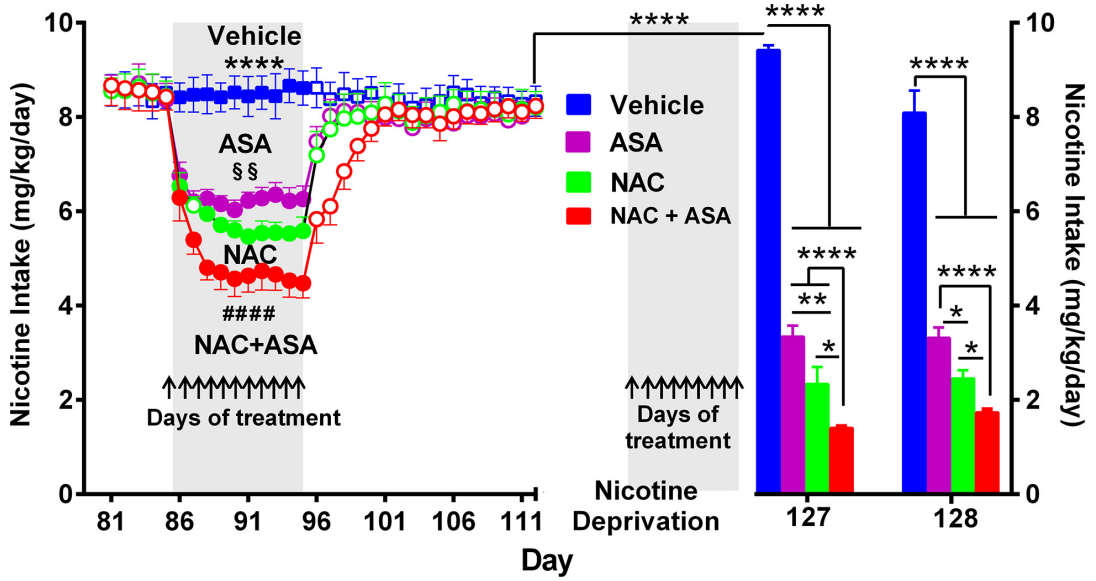

FIGURE 3 | The coadministration of *N*-acetylcysteine (NAC; 40 mg/kg/day p.o.) plus acetylsalicylic acid (ASA; 15 mg/kg/day p.o.) induced a significantly greater inhibition of both chronic oral nicotine intake **(left)** and post-deprivation reinstatement **(right)** in rats that had chronically consumed nicotine. **(Left)** following 12 weeks of chronic voluntary oral consumption of the nicotine solution, animals were treated with either *N*-acetylcysteine, acetylsalicylic acid, *N*-acetylcysteine + acetylsalicylic acid, or vehicle by oral gavage, for 11 consecutive days. Two-way ANOVA (treatment × day) of nicotine intake data revealed a significant effect of treatment [$F_{treatment(3,239)}$ = 1839, $p < 0.0001$], day [$F_{day(11,239)}$ = 28.44, $p < 0.0001$], and treatment x × day interaction [$F_{interaction(33,239)}$ = 17.07, $p < 0.0001$]. Tukey *post hoc* analysis indicated that the groups of rats treated *N*-acetylcysteine (NAC; green circles), acetylsalicylic acid (ASA; pink circles), and *N*-acetylcysteine + acetylsalicylic acid (NAC + ASA; red circles) showed reduced voluntary oral nicotine intake versus that of the control group treated with vehicle (Blue squares) (****$p < 0.0001$). *N*-Acetylcysteine + acetylsalicylic acid treatment induced a significantly greater reduction of chronic voluntary oral nicotine intake compared with those induced by *N*-acetylcysteine and acetylsalicylic acid alone (####$p < 0.0001$ NAC + ASA compared with NAC and ASA group). In addition, *N*-acetylcysteine induced a significantly higher reduction of oral nicotine intake compared with that induced by acetylsalicylic acid (§§$p < 0.01$ NAC compared with ASA group). **(Right)** following 112 days of voluntary oral consumption of the nicotine solution, animals were treated with either *N*-acetylcysteine, acetylsalicylic acid, *N*-acetylcysteine + acetylsalicylic acid, or vehicle by oral gavage, on the last 9 days (arrows) of a 2-week nicotine deprivation period. Bars show daily oral consumption of the nicotine solution during the 2-day of nicotine re-access, which was carried out 24 and 48 h after treatments were discontinued. Student *t*-test revealed that on the first day (127), the reinstatement of oral nicotine intake of the vehicle group that had consumed oral nicotine for 112 days and was vehicle treated during the nicotine deprivation period (blue bar) was significantly higher compared to the oral nicotine intake of the same group prior to the 14-day deprivation period (mean of nicotine intake of the last eleven baseline days) (empty blue squares) (****$p < 0.0001$), indicating an increased nicotine seeking behavior. Two-way ANOVA (treatment × day) of the oral nicotine intake data, during the first (day 127) and second (day 128) days of nicotine re-access, indicates significant effect of treatment [$F_{treatment(3,38)}$ = 335.6, $p < 0.0001$] but not of day. Fisher *post hoc* analysis indicated that, compared with the control group that had consumed oral nicotine for 112 days and was vehicle treated during the nicotine deprivation period (blue bar), the groups treated with *N*-acetylcysteine (NAC; green bar), acetylsalicylic acid (ASA; pink bar), or *N*-acetylcysteine + acetylsalicylic acid (NAC + ASA; red bar) showed a significant reduction of the post-deprivation reinstatement of oral consumption of the nicotine solution (****$p < 0.0001$ vehicle group versus ASA, NAC, and NAC + ASA) on the first and second days of nicotine re-access (days 127 and 128). *N*-Acetylcysteine + acetylsalicylic acid treatment induced a significantly higher reduction of the reinstatement of oral consumption of the nicotine solution than that induced by acetylsalicylic acid or *N*-acetylcysteine on the first day (****$p < 0.0001$ NAC + ASA versus ASA; *$p < 0.05$ NAC + ASA versus NAC) and the second day (****$p < 0.0001$ NAC + ASA versus ASA; *$p < 0.05$ NAC + ASA versus NAC) of re-access. In addition, *N*-acetylcysteine induced a significant higher reduction of the reinstatement of oral consumption of the nicotine solution than that induced by acetylsalicylic acid (*$p < 0.05$ NAC versus ASA) on the 2 days of re-access.

by the oral administration of *N*-acetylcysteine + acetylsalicylic acid (4.6 ± 0.1 mg/kg/day; mean ± SEM, $n = 7$), from day 88 to 95. Importantly, the inhibition of oral consumption of the nicotine solution achieved by the combined administration of *N*-acetylcysteine plus ASA was significantly higher than that shown by rats receiving either *N*-acetylcysteine or ASA alone ($p < 0.0001$). Noteworthily, these effects of the antioxidant *N*-acetylcysteine and the anti-inflammatory ASA are seen, while the oral nicotine intake pro-oxidant and pro-inflammatory effects of nicotine continued (*vide infra versus the inhibition on post deprivation reinstatement*). **Figure 3** (right) shows the effect on nicotine seeking behavior (relapse/reinstatement) akin to the deprivation followed by the re-access nicotine model (O'Dell and Koob, 2007; Bagdas et al., 2019). Specifically, we assessed the oral consumption of nicotine during the first 24 h of re-access to nicotine (day 127) which followed 14 days of deprivation (blue bar) in rats that had a prior consumption of oral nicotine for 112 days. Water was available throughout the experiment. Results indicate a significant increase of the oral nicotine intake ($p < 0.0001$) in rats of the control group treated with vehicle (9.42 ± 0.1 mg of nicotine/kg/day, $n = 7$; blue bar) compared to the oral nicotine intake of the same group prior to the 14-day deprivation period (8.5 ± 0.1 mg/kg/day; empty blue squares). This increase in oral nicotine intake following 2 weeks of nicotine deprivation referred to as the nicotine deprivation effect (O'Dell and Koob, 2007) reveals a nicotine-seeking behavior in female rats, in line with studies indicating that female rats show a cue-induced nicotine-seeking reinstatement response (Feltenstein et al., 2012; Wang et al., 2014). The increase in the oral voluntary nicotine intake over baseline was temporary, since on the second day of re-access (day 128) the oral nicotine intake of the control group treated with vehicle (8.10 ± 0.5 mg nicotine/kg/day, $n = 7$; blue bar) was not different compared to the nicotine intake of the same group prior to the 14-day deprivation period. To study the effect of the NAC, ASA, and NAC + ASA treatment on the reinstatement of oral consumption of the nicotine solution, the animals were orally administered either (i) *N*-acetylcysteine (40 mg/kg/day), (ii) ASA (15 mg/kg/day), (iii) *N*-acetylcysteine + ASA, or (iv) vehicle, during the last 9 days of the 2-week nicotine deprivation period (indicated by arrows), carried out after 112 days of continuous oral nicotine consumption (see **Figure 1**). The bars indicate the oral consumption of the nicotine solution on days 127 and 128 (24 and 48 h after discontinuing the NAC, ASA, and NAC + ASA treatments) following the re-access of the nicotine solution (60 mg/l). On day 127 (the first day of re-access of the nicotine solution), the reinstatement of oral consumption of the nicotine solution of the control group (vehicle treated) (9.42 ± 0.1 mg of nicotine/kg/day, $n = 7$; day 127) was inhibited by 75% by *N*-acetylcysteine (NAC) administration (2.3 ± 0.4 mg of nicotine/kg/day; mean ± SEM; $n = 7$; day 127) ($p < 0.0001$) and by 65% by ASA administration (3.5 ± 0.2 mg of nicotine/kg/day; mean ± SEM, $n = 7$; day 127) ($p < 0.0001$). Importantly, the reinstatement of oral consumption of the nicotine solution of the vehicle-treated group was inhibited by 85% by the combined administration of *N*-acetylcysteine plus ASA (NAC + ASA)

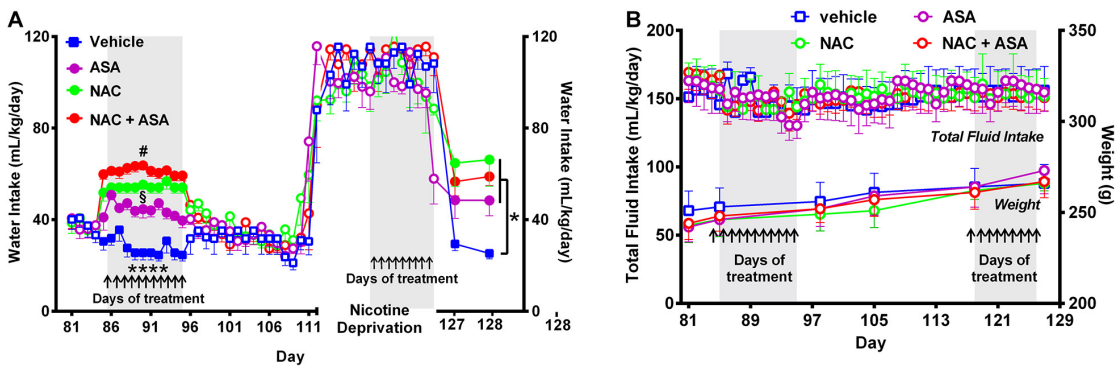

FIGURE 4 | Administration of *N*-acetylcysteine (NAC; 40 mg/kg/day p.o.), acetylsalicylic acid (ASA; 15 mg/kg/day p.o), or *N*-acetylcysteine + acetylsalicylic acid treatment increased oral water intake and did not change fluid intake nor body weight. **(A)** Shows the changes in oral water consumption at the times of chronic voluntary oral nicotine intake, nicotine deprivation, and reinstatement of oral nicotine intake. **(A)** Left, Two-way ANOVA (treatment × day) of oral water intake data following repeated treatment with NAC, ASA, or NAC + ASA or vehicle by oral gavage (day 85–95) at the times of chronic oral nicotine intake revealed significant effect of treatment [$F_{treatment(3,205)} = 219.7$, $p < 0.0001$], but not of day. Fisher *post hoc* analysis indicated that animals treated with *N*-acetylcysteine (NAC), acetylsalicylic acid (ASA), or *N*-acetylcysteine + acetylsalicylic acid (NAC + ASA) consumed greater amounts of water as compared to that shown by the control group (vehicle treated) (****$p < 0.0001$ vehicle group versus NAC, ASA or NAC + ASA). Further, *N*-acetylcysteine + acetylsalicylic acid treatment induced a significantly greater increase of oral water intake compared with that induced by *N*-acetylcysteine or acetylsalicylic acid alone (#$p < 0.05$ NAC + ASA versus NAC and ASA), while *N*-acetylcysteine induced a greater increase of water intake compared with that induced by acetylsalicylic acid (§$p < 0.05$ NAC versus ASA). **(A)** Right; following 112 days of chronic free-choice nicotine/water intake, a 14-day nicotine deprivation period followed. On the last 9 days (arrows) of the nicotine derivation period, animals received NAC, ASA, or NAC + ASA. Water was always available throughout the experiment. Two-way ANOVA (treatment × day) performed on water intake data obtained on the first (day 127) and second (day 128) days of nicotine re-access post deprivation revealed a significant effect of treatment [$F_{treatment(3,36)} = 7.79$, $p < 0.0004$], but not of day. Fisher *post hoc* test indicated that *N*-acetylcysteine (NAC), acetylsalicylic acid (ASA), and *N*-acetylcysteine + acetylsalicylic acid (NAC + ASA) increase oral water intake compared with that of the control group (*$p < 0.05$). **(B)** The administration of *N*-acetylcysteine, acetylsalicylic acid, or the combination of *N*-acetylcysteine + acetylsalicylic acid did not affect oral total fluid intake [ANOVA: $F_{(3,184)} = 1.44$, *p*: N.S.[, or body weight- [ANOVA: $F_{(3,20)} = 0.40$, *p*: N.S.], compared with vehicle administration.

(1.4 ± 0.04 mg of nicotine /kg/day; mean ± SEM, n = 7; red bar) ($p < 0.0001$; day 127). On day 128, the inhibition of the reinstatement of oral consumption of the nicotine solution induced by the administration of NAC, ASA, or NAC + ASA was not significantly different from that obtained on day 127.

Figure 4A shows that NAC, ASA, or NAC + ASA treatment increased the oral consumption of water at the times that they reduced both the oral chronic nicotine intake and the reinstatement of oral consumption of the nicotine solution (**Figure 4A**, left and right, respectively), such that the total oral intake of fluidics was not modified by the NAC, ASA, or NAC + ASA treatment (**Figure 4B**). These results, along with the finding that these treatments also did not affect total body weight (**Figure 4B**), indicate that the changes in oral nicotine consumption by the NAC, ASA, or NAC + ASA treatments do not reflect a non-specific animal malaise.

Figure 5 shows the hippocampal oxidative stress, determined as the ratio of oxidized/reduced glutathione (GSSG/GSH), in rats that had voluntarily consumed nicotine solution orally during 112 days, were deprived of the nicotine solution for 14 days, and were allowed oral nicotine consumption re-access (60 mg nicotine/l) for 2 days. Rats that had shown reinstatement of oral consumption of the nicotine solution and were vehicle-treated (nicotine-vehicle, blue bar) showed a 100% increase ($P < 0.01$) in the GSSG/GSH ratio versus that of control rats that had consumed only water and were water-treated (naïve, white bar). The nicotine-induced oxidative stress was fully normalized by administration of either (a) NAC, (b) ASA, or (c) coadministration of NAC + ASA ($p < 0.01$) during the last 9 days of a 14-day period of nicotine solution deprivation. The marked reduction of the oxidative stress (GSSG/GSH) induced by the anti-inflammatory ASA drug (15 mg/kg/day) is noteworthy.

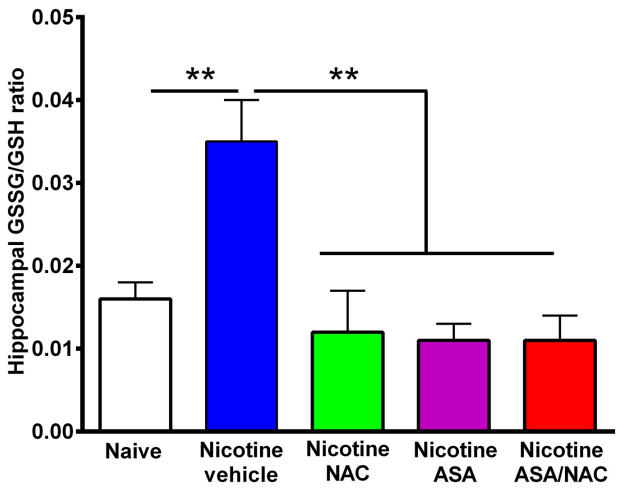

FIGURE 5 | The marked increase in oxidative stress detected in the hippocampus of animals following the post-deprivation reinstatement of oral consumption of nicotine solution was reversed by the administration of *N*-acetylcysteine (NAC; 40 mg/kg), acetylsalicylic acid (ASA; 15 mg/kg/day p.o), or *N*-acetylcysteine + acetylsalicylic acid treatment. One-way ANOVA of all the oxidized/reduced glutathione (GSSG/GSH) ratios data shown indicates a significant effect of treatment [ANOVA: $F_{4,23} = 9.227$, $p < 0.0001$]. Tukey *post hoc* test revealed that the oxidative stress, determined as the ratio of oxidized/reduced glutathione (GSSG/GSH), was markedly increased in the hippocampus of rats following the reinstatement of oral consumption of the nicotine solution and that had been treated with vehicle (blue bar) compared with that of naïve rats drinking only water and were vehicle-treated (white bar) (**$p < 0.01$). In addition, *N*-acetylcysteine, acetylsalicylic acid, or *N*-acetylcysteine + acetylsalicylic acid fully normalized the nicotine-induced increase in GSSG/GSH ratio [Tukey *post hoc* **$p < 0.01$ naïve rats drinking only water (white bar) compared with nicotine drinking rats treated with *N*-acetylcysteine (green bar), acetylsalicylic acid (purple bar), or *N*-acetylcysteine + acetylsalicylic acid (red bar)].

Figure 6 top, center, and bottom panels show the hippocampal neuroinflammation observed in rats that had voluntarily consumed nicotine solution orally during 112 days, were deprived of the nicotine solution for 14 days, and were allowed oral nicotine consumption re-access (60 mg nicotine/l) for 2 days. Neuroinflammation was seen as an increase in the length ($p < 0.0001$) and thickness ($p < 0.001$) of the primary astrocytic processes in GAFP-positive cells of rats that had shown reinstatement of oral consumption of the nicotine solution and were vehicle-treated (nicotine-vehicle group, blue bars) versus that of control rats that had consumed only water and were water-treated (naïve rats, white bar) (top and bottom panel A and B). In addition, **Figure 6** center panel and bottom panel D indicate that these animals (nicotine-vehicle group, blue bars) also showed a significant increase ($p < 0.0001$) of the density (cells per cubic millimeter) of microglial cells determined by Iba-1 immunoreactivity compared to that of control rats that had consumed only water and were vehicle-treated (naïve rats, white bar). The treatment of the animals during the last 9 days of the 14-day deprivation period of the nicotine solution with either (a) *N*-acetylcysteine (NAC, green bar), (b) acetylsalicylic acid (ASA, pink bar), or (c) coadministration of both drugs (NAC + ASA, red bar) significantly reduced ($p < 0.05$ to $p < 0.0001$) both astrocyte morphology and microglial tissue density—changes most clearly seen in microglial density ($p < 0.001$). The marked anti-inflammatory effect of the antioxidant *N*-acetylcysteine (40 mg/kg/day) on neuroinflammation is noteworthy.

Figure 7 shows that rats that had shown a reinstatement of oral consumption of the nicotine solution and were vehicle-treated (nicotine-vehicle group, blue bars) showed a downregulation of GLT-1 gene expression (mRNA levels) in the prefrontal cortex compared to that of control rats that had consumed only water and were vehicle-treated (naïve rats, white bar) ($p < 0.05$). The treatment of the animals during the last 9 days of the 14-day deprivation period of the nicotine solution with either (a) *N*-acetylcysteine (NAC, green bar), (b) acetylsalicylic acid (ASA, pink bar), or (c) coadministration of both drugs (NAC + ASA, red bar) restored the GLT-1 mRNA level in the prefrontal cortex ($p < 0.01$).

Figure 8A shows that vehicle-treated rats that had voluntarily consumed nicotine solution orally during 112 days, were deprived of the nicotine solution for 14 days, and were allowed oral nicotine consumption re-access (60 mg nicotine/l) for 2 days (nicotine-vehicle, blue bar) showed a downregulation of xCT transporter gene expression (mRNA level) in the prefrontal cortex compared with that of rats drinking only water (naïve group, white bar) ($P < 0.05$). The treatment

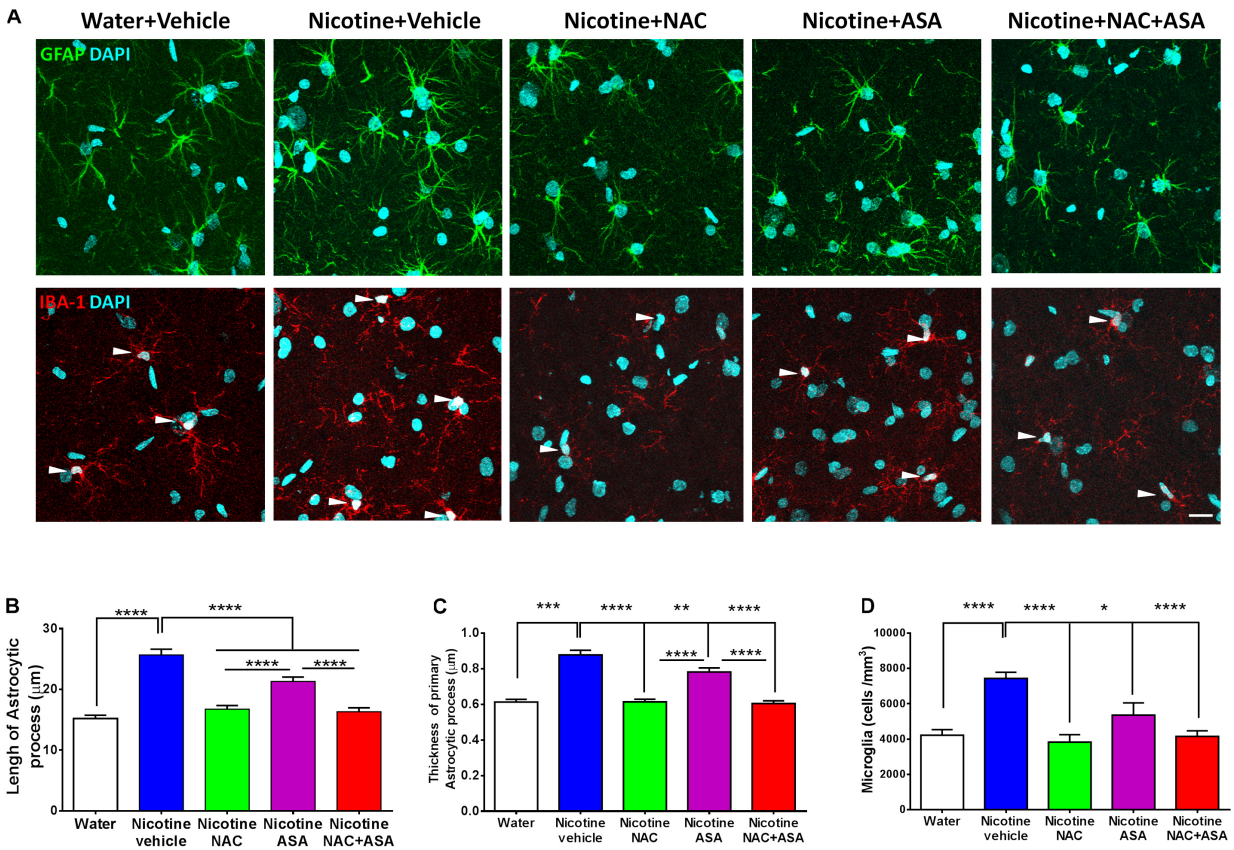

FIGURE 6 | Neuroinflammation detected in the hippocampus of animals following the post-deprivation reinstatement of oral consumption of nicotine solution was inhibited by the administration of *N*-acetylcysteine (NAC; 40 mg/kg), acetylsalicylic acid (ASA; 15 mg/kg/day p.o), or *N*-acetylcysteine + acetylsalicylic acid. **(A)** Representative images of GFAP positive astrocytes (top) and IBA1- positive microglia (center). **(B)** Quantitation of length and **(C)** thickness of GFAP-positive astrocyte primary processes and **(D)** Quantitation of the density of IBA1-positive microglial cells. The top panel shows astrocyte immunofluorescence (GFAP immunoreactivity, green; DAPI, blue). The center panel shows microglia immunofluorescence (IBA-1 immunoreactivity, red; DAPI, blue, depicted by white arrow heads). Scale bar 25 µm. One-way ANOVA of data of primary astrocytic processes of rats in the hippocampus of rats following the reinstatement of oral consumption of the nicotine solution and that had been treated with vehicle (nicotine-vehicle, blue bar) indicates a significant increase of length [$F_{(4,943)} = 39.76$, **** $p < 0.0001$; *post hoc* ****$p < 0.0001$], a significant increase of thickness [$F_{(4,489)} = 42.10$, **** $p < 0.0001$; *post hoc* ***P < 0.001], and a significant increase of microglial density [$F_{(4,39)} = 11.14$, ****$p < 0.0001$; Tukey's *post hoc* ****$p < 0.0001$] compared with that of naïve rats drinking only water and were vehicle-treated (white bar). The administration of either *N*-acetylcysteine (nicotine + NAC), acetylsalicylic acid (nicotine + ASA), or coadministration of *N*-acetylcysteine plus acetylsalicylic acid (nicotine + NAC + ASA) fully normalized nicotine-induced increase in length (Tukey *post hoc*: NAC, ****$p < 0.0001$; ASA, ****$p < 0.0001$; NAC + ASA, ****$p < 0.0001$); the nicotine-induced increase in thickness (Tukey *post hoc*: NAC, ****$p < 0.0001$; ASA, **$p < 0.01$; NAC + ASA, ****$p < 0.0001$); and the nicotine-induced increase in microglial density (Tukey *post hoc*: NAC, ****$p < 0.0001$; ASA, *$p < 0.05$; NAC + ASA, ****$p < 0.0001$).

with *N*-acetylcysteine (nicotine-NAC) or the combination of *N*-acetylcysteine + ASA (nicotine-NAC + ASA) normalized the expression of the xCT transporter gene expression ($P < 0.05$). The treatment with only ASA did not increase the xCT transporter mRNA level. To determine whether the xCT cystine-glutamate transporter plays a role in the mechanism by which *N*-acetylcysteine inhibits nicotine intake, we determined the effect of sulfasalazine (SZ), an inhibitor of the xCT transporter, on the *N*-acetylcysteine-induced reduction of the oral voluntary consumption of a nicotine solution. Results in **Figure 8B** show that the oral voluntary consumption of a nicotine solution for 83 consecutive days of the control group (vehicle/vehicle group) (7.9 ± 0.1 mg/kg/day; mean ± SEM, $n = 5$) was significantly inhibited by 39.2% ($p < 0.0001$) by the oral administration of NAC (40 mg/kg/day) for a 2-day period (4.8 ± 0.1 mg/kg/day; mean ± SEM; $n = 5$). Pretreatment of animals with sulfasalazine (SZ) (8 mg/kg/day i.p), 15 min before the administration of each of the two *N*-acetylcysteine doses (SZ/NAC group) fully blocked the *N*-acetylcysteine-induced inhibition of chronic voluntary oral consumption of a nicotine solution.

DISCUSSION

Although rats do not spontaneously ingest nicotine orally, they voluntarily consume nicotine solutions if they became dependent on nicotine following a repeated intraperitoneal administration. The present preclinical study shows that the combined administration of *N*-acetylcysteine (40 mg/kg/day) plus ASA (15 mg/kg/day) reduced by 85% the oral nicotine

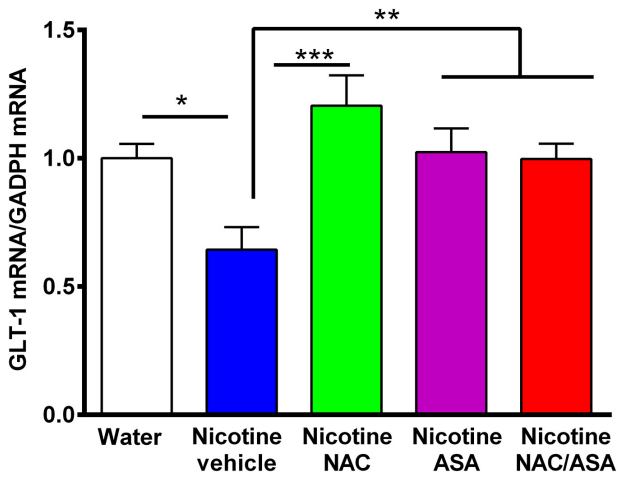

FIGURE 7 | Down-regulation of GLT-1 mRNA detected in the prefrontal cortex of animals following the post-deprivation reinstatement of oral consumption of nicotine solution was reversed by the administration of *N*-acetylcysteine (NAC; 40 mg/kg), acetylsalicylic acid (ASA; 15 mg/kg/day p.o) or *N*-acetylcysteine + acetylsalicylic acid. One-way ANOVA revealed downregulation of GLT-1 mRNA level in the prefrontal cortex of rats following reinstatement of oral consumption of the nicotine solution, and the treatment with vehicle (nicotine-vehicle, blue bar) compared with that of naïve rats drinking only water and were vehicle-treated (naïve, white bar) [One-way ANOVA $F_{(423)} = 4.961$, $^{**}p < 0.01$; Fisher *post hoc*: $^{*}p < 0.05$ water group versus nicotine vehicle group, $n = 7$ rats per group], whereas treatment with *N*-acetylcysteine (nicotine-NAC), acetylsalicylic acid (nicotine-ASA), or the combination of both drugs (nicotine-NAC + ASA) normalized GLT-1 mRNA level ($^{***}p < 0.001$ nicotine-vehicle compared with nicotine-NAC; $^{*}P < 0.05$ nicotine-vehicle compared with nicotine-ASA and NAC + ASA group).

intake after chronic intake and restatement, a significantly higher inhibition than that generated by each agent alone. The dose of *N*-acetylcysteine (40 mg/kg/day administered orally) used in the present study is within the range used in other studies. An intraperitoneal dose of *N*-acetylcysteine of 30 mg/kg was shown to reduce nicotine self-administration in rats (Ramirez-Nino et al., 2013). The ASA dose used in the present study (15 mg/kg/day) was chosen since doses in the range of 15–30 mg/kg were shown not to generate gastric irritation in rats (Wallace et al., 2004).

In the relapse-like reinstatement condition of oral nicotine intake post-deprivation in rats that had consumed nicotine chronically, the study showed a marked increase in the ratio of oxidized/reduced glutathione (GSSG/GSH) in the hippocampus, an indicator of oxidative stress, which was associated with hippocampal neuroinflammation, as seen both by a greater length and thickness of primary astrocytic processes and by an increase in microglial density. In mice, microglial morphology changes in striatal tissues have been reported following chronic treatment with nicotine and undergoing withdrawn, which were associated with the release of pro-inflammatory cytokines and increase in ROS (Adeluyi et al., 2019; Saravia et al., 2019). Increased TNFα levels have been reported in the nucleus accumbens of rats during cue-induced nicotine seeking (Namba et al., 2020), and an increased TNFα gene expression has also been described in the prefrontal cortex of nicotine- and cigarette smoke-exposed rats (Lau et al., 2012). It is well established that through the activation and nuclear translocation of the transcription factor NF-κB, TNFα induces the synthesis of various pro-inflammatory cytokines, including IL-6 (Shimizu et al., 1990; Kunsch and Rosen, 1993; Ping et al., 1996). The report that the induction of the nuclear transcription of NF-κB by injecting a viral vector expressing an analog of the enzyme IκB kinase (IKK) facilitated cue-induced nicotine seeking reinstatement suggests that the NF-κB pathway and neuroinflammation mediate the conditioned nicotine seeking (Namba et al., 2020).

Two previous sub-chronic nicotine studies did not reveal changes in glutamate transporter-1 (GLT-1) or xCT in the prefrontal cortex of rats receiving nicotine via self-administration or via minipumps for 21 days (Knackstedt et al., 2009) or in mice exposed phasically (not continuous) to e-cigarette vapor containing nicotine (Alasmari et al., 2017). Given the pivotal role of glutamate and the prefrontal cortex in cocaine-seeking behavior, we sought to investigate whether a chronic nicotine intake reduced the mRNA levels of GLT-1 and xCT in the prefrontal cortex of rats that had been subjected to nicotine reinstatement *following 112 days* of continuous nicotine intake (equivalent to many years of nicotine use in a chronic smoker) and a subsequent 2-week deprivation period.

The present study demonstrated that in a reinstatement condition of chronic oral nicotine intake, rats showed a downregulation of both GLT-1 and xCT gene expression in the prefrontal cortex, a region that via the glutamatergic system informs the nucleus accumbens and mediates cue-induced reinstatement and the drug seeking behavior (McFarland et al., 2003; LaLumiere and Kalivas, 2008; Gipson et al., 2013; Smith et al., 2017). Downregulation of GLT-1 and xCT protein expression has been observed in the nucleus accumbens of rats self-administering nicotine while the restoration of GLT-1 expression was associated with a decrease in nicotine self-administration (Knackstedt et al., 2009; Sari et al., 2016). Further, a significant reduction in GLT-1 protein expression has been shown after cue-induced nicotine reinstatement in the nucleus accumbens (Namba et al., 2020). Noteworthily, several studies show that both brain oxidative stress and neuroinflammation downregulates GLT-1. Oxidative stress (ROS) inhibits glutamate transport by the direct inhibition of GLT-1 activity (Trotti et al., 1997, 1998) and also by the formation of adducts of glutamate transporters with lipoperoxidation products like 4-hydroxynonenal and 4-hydroxyhexenal (Schaur et al., 2015), while the pro-inflammatory cytokine TNFα leads to the downregulation of the GLT-1 mRNA level (Szymocha et al., 2000; Wang et al., 2003; Sitcheran et al., 2005). Taken together, these studies support the growing view that conditions present in an oxidative-stress/neuroinflammation self-perpetuating cycle (Berrios-Carcamo et al., 2020) disrupt glutamate homeostasis by impairing glutamate transport in areas involved in the brain reward system, increasing the relapse-promoting effect of drug-related cues, thus sustaining drug craving and subsequent drug consumption (Kalivas, 2009).

Administration of *N*-acetylcysteine combined with an appropriate psychotherapy has been shown to be clinically

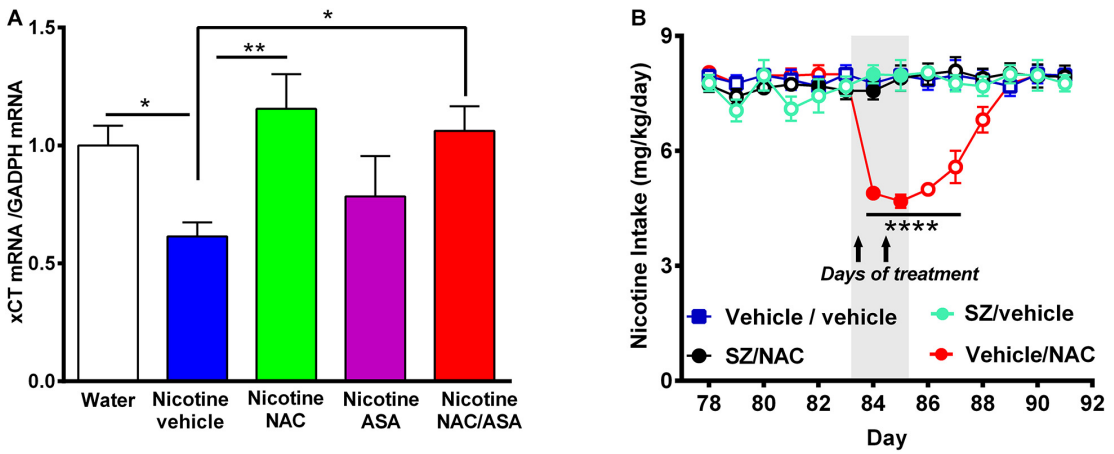

FIGURE 8 | (A) Downregulation of xCT-cystine/glutamate exchanger mRNA detected in the prefrontal cortex of animals following the post-deprivation reinstatement of oral consumption of nicotine solution was reversed by the administration of *N*-acetylcysteine or the combination of *N*-acetylcysteine + acetylsalicylic acid. **(B)** In chronically oral nicotine-consuming rats, the inhibition of oral nicotine intake induced by *N*-acetylcysteine (NAC 40 mg/kg) was fully prevented by the xCT inhibitor sulfasalazine (SZ). **(A)** Rats that had shown reinstatement of oral consumption of the nicotine solution and were vehicle-treated (nicotine-vehicle, blue bar) showed downregulation of xCT transporter mRNA level in the prefrontal cortex compared with that of naïve rats drinking only water and were vehicle-treated (naïve, white bar) [One-way ANOVA $F_{(4,19)} = 3.151$, $^{*}p < 0.05$; Fisher *post hoc*: $^{*}p < 0.05$ water group versus nicotine vehicle group, $n = 7$ rats per group]. Treatment with *N*-acetylcysteine (nicotine-NAC) or the combination of both drugs (nicotine-NAC + ASA) normalized the expression of the xCT transporter ($^{**}p < 0.01$ nicotine-vehicle compared with nicotine-NAC, $^{*}P < 0.05$ nicotine vehicle compared with the nicotine-NAC + ASA group). **(B)** Two-way ANOVA (treatment × day) of all voluntary nicotine oral intake data shown in this Figure revealed a significant effect of *N*-acetylcysteine treatment (vehicle-NAC) [$F_{(3,111)} = 60.13$, $^{***}p < 0.0001$], day [$F_{(6,111)} = 2.622$, ***p < 0.02], and also treatment × day interaction [$F_{(18,111)} = 8.513$, $^{***}p < 0.0001$]. Fisher *post hoc* indicated that *N*-acetylcysteine treatment induced a marked reduction of the chronic voluntary consumption of a nicotine solution orally from day 84 to 87 ($^{****}p < 0.0001$ vehicle/vehicle compared with vehicle/NAC), while pretreatment with sulfasalazine, an inhibitor of xCT transporter prevented the reduction of the chronic voluntary consumption of a nicotine solution orally induced by *N*-acetylcysteine.

effective in the treatment of the tobacco use disorder (Prado et al., 2015), while several preclinical studies have shown that *N*-acetylcysteine inhibits cue-induced nicotine reinstatement (Ramirez-Nino et al., 2013; Powell et al., 2019; Goenaga et al., 2020; Moro et al., 2020; Namba et al., 2020). Noteworthily, such an effect of *N*-acetylcysteine was blocked by microinjection of a GLT-1 antisense morpholino into the nucleus accumbens (Namba et al., 2020).

The likely mechanism by which *N*-acetylcysteine reduces oxidative stress was investigated by Zhou et al. (2018) who showed that in rats subjected to traumatic brain injury, the administration of *N*-acetylcysteine amide (NACA), a precursor of *N*-acetylcysteine, attenuated oxidative stress via the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) signal pathway. The Nrf2 system activates the transcription of a number of antioxidant enzymes, and additionally, it upregulates the activity of the promoter of the xCT cystine-glutamate transporter gene (Shih et al., 2003; Habib et al., 2015). Thus, *N*-acetylcysteine could reduce the glutamatergic tone *both* by increasing xCT cystine-glutamate antiporter levels and by increasing the levels of cystine as co-substrate for the antiporter, a dual effect leading to the activation of the inhibitory metabotropic mGlu2/3 receptor.

An added xCT co-substrate effect of *N*-acetylcysteine is based on its property of generating both cysteine and cystine (in reducing GSSG into GSH, cysteine becomes oxidized into cystine), thereby increasing the cystine availability for the cystine/glutamate xCT exchanger. Such an added co-substrate effect of the *N*-acetylcysteine enhances the extrusion of glutamate into the extracellular space (Baker et al., 2003) and stimulates the auto-receptor mGlu2/3 negatively, reducing the release of synaptic glutamate (Schoepp et al., 1999). Such a dual effect can explain why *N*-acetylcysteine showed an additive behavioral effect inhibiting the nicotine reinstatement of oral intake over that of ASA but did not show an additive effect on cellular parameters. The relevance of a high activity of the xCT transporter was further shown by the observation that sulfasalazine fully prevented the inhibitory effect of *N*-acetylcysteine on chronic nicotine intake, when administered by itself. This finding is consistent with early studies of Baker et al. (2003) and in line with the fact that the *N*-acetylcysteine metabolite additionally acts on the xCT transporter enhancing glutamate efflux into the extracellular space, likely stimulating the presynaptic mGlu2/3 auto-receptors and negatively modulating synaptic glutamate release (Moussawi and Kalivas, 2010).

The effect of ASA on nicotine intake was associated with a reduction of oxidative stress and neuroinflammation and with an increase in GLT-1 gene expression. A number of studies have shown that ASA activates the synthesis of peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Yiqin et al., 2009; Tang et al., 2014) known to have both strong anti-inflammatory effects (Jiang et al., 1998; Pascual et al., 2007) and also activates brain GLT-1 transcription (Romera et al., 2007), increasing GLT-1 protein levels and glutamate uptake.

Overall, it is suggested that the additive inhibitory effects of ASA and *N*-acetylcysteine on chronic nicotine intake and

relapse is due to the fact that both drugs are acting primarily by different mechanisms, suggesting that the coadministration of ASA and *N*-acetylcysteine may be considered for the treatment of nicotine-dependent behaviors.

SIGNIFICANCE

Common elements contributing to the perpetuation of addictive drugs use are (i) cues associated with the setting in which these drugs were used, leading to relapse/reinstatement, mediated from an increased glutamatergic tone which is (ii) associated with drug-induced oxidative stress and neuroinflammation. The present study showed that the coadministration of the antioxidant *N*-acetylcysteine (NAC) plus the anti-inflammatory ASA inhibited by 85% the oral nicotine reinstatement intake seen after a post-deprivation re-access in rats that had chronically consumed a nicotine solution. *N*-acetylcysteine and ASA administration normalized hippocampal oxidative stress and neuroinflammation showing an additive inhibitory effect on oral nicotine reinstatement behavior. Both NAC and ASA activated the synaptic glutamate transporter (GLT-1) gene expression, an effect compounded by an increase in cystine/glutamate xCT transporter gene expression and activity by NAC administration. Thus, acting via different mechanisms, these combined effects are expected to reduce the glutamate tone. Should the present preclinical data be transferable to humans, two well-known available medications may be repurposed as adjuncts in the treatment of nicotine dependent behaviors.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be available upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for Experiments with Laboratory Animals at the Medical Faculty of the University of Chile (Protocol CBA# 0994 FMUCH). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MQ: conception and design, collection of data, data analysis, manuscript writing, and final approval of the manuscript. FE: conception and design, collection of data, data analysis, financial support, manuscript writing, and final approval of the manuscript. PM: design, collection of data, data analysis, and final approval of the manuscript. ME: collection of data, data analysis, and final approval of the manuscript. MH-M: conception and final approval of the manuscript. YI: conception and design, financial support, manuscript draft, and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Studies were supported by the FONDECYT grants # 1180042, 1190562, and 1200287.

ACKNOWLEDGMENTS

We thank Mr. Juan Santibañez, Mr. Robel Vasquez, and Ms. Carmen Almeyda for technical support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 01 April 2021
doi: 10.3389/fnbeh.2021.639372

Development of a Classical Conditioning Task for Humans Examining Phasic Heart Rate Responses to Signaled Appetitive Stimuli: A Pilot Study

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OPEN ACCESS

Edited by:
Ricardo Marcos Pautassi, Medical Research Institute Mercedes and Martín Ferreyra (INIMEC), Argentina

Reviewed by:
Mauricio R. Papini, Texas Christian University, United States
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *08 December 2020*
Accepted: *09 March 2021*
Published: *01 April 2021*

Citation:
Sayão A, Alves H, Furukawa E, Schultz Wenk T, Cagy M, Gutierrez-Arango S, Tripp G and Caparelli-Dáquer E (2021) Development of a Classical Conditioning Task for Humans Examining Phasic Heart Rate Responses to Signaled Appetitive Stimuli: A Pilot Study. Front. Behav. Neurosci. 15:639372. doi: 10.3389/fnbeh.2021.639372

Cardiac responses to appetitive stimuli have been studied as indices of motivational states and attentional processes, the former being associated with cardiac acceleration and latter deceleration. Very few studies have examined heart rate changes in appetitive classical conditioning in humans. The current study describes the development and pilot testing of a classical conditioning task to assess cardiac responses to appetitive stimuli and cues that reliably precede them. Data from 18 adults were examined. They were shown initially neutral visual stimuli (putative CS) on a computer screen followed by pictures of high-caloric food (US). Phasic cardiac deceleration to food images was observed, consistent with an orienting response to motivationally significant stimuli. Similar responses were observed to non-appetitive stimuli when they were preceded by the cue associated with the food images, suggesting that attentional processes were engaged by conditioned stimuli. These autonomic changes provide significant information about classical conditioning effects in humans.

Keywords: appetitive conditioning, cardiac deceleration, orienting response, attention, motivation

HIGHLIGHTS

- A classical conditioning task assessed cardiac responses to appetitive US and CS
- Phasic cardiac deceleration to food images was observed
- Phasic cardiac deceleration to the US likely represents an orienting response
- Cardiac deceleration to the neutral stimuli was observed when preceded by CS+

INTRODUCTION

Cardiac responses have been studied as indices of both motivational/emotional states and attentional processes (Hunt and Campbell, 1997; Bradley, 2009). Heart rate has been shown to accelerate in the presence of rewarding stimuli, such as monetary incentives during a motor task (Fowles et al., 1982), a plate of preferred food being available to look at, smell and then eat

(Nederkoorn et al., 2000), repeated presentations of positive food images (Kuoppa et al., 2016), and anticipating the opportunity to perform a task to earn money (Rakover and Levita, 1973). Such acceleration is thought to reflect autonomic arousal and a heightened emotional state triggered by the presence of motivationally significant stimuli and mediated by sympathetic nervous system activation (Fowles et al., 1982; Richter, 2012). In contrast, deceleration of heart rate is observed in response to sensory stimuli (Graham and Clifton, 1966), and this is exaggerated when the stimuli are novel (Tuber et al., 1985; Gianaros and Quigley, 2001; Bradley, 2009) and have motivational or emotional significance (Abercrombie et al., 2008; Bradley et al., 2012). Such deceleration responses are thought to indicate cardiac orienting responses (Binder et al., 2005; Bradley, 2009), reflective of the organisms' attention to environmental stimuli and mediated by the parasympathetic system (Graham and Clifton, 1966; Barry, 1983).

To further examine the relationship between cardiac responses and behavior, recent human experimental studies have measured heart rate changes in response to reward or feedback during learning and decision making tasks (Somsen et al., 2000; Drobes et al., 2001; Crone et al., 2004; Groen et al., 2007; Luman et al., 2007; Kube et al., 2016; Kastner et al., 2017). The majority of these studies show cardiac deceleration to anticipation or receipt of incentives, pleasant stimuli, or positive feedback. In some studies, the deceleration occurred after the presentation of cues predicting reward/feedback (Kube et al., 2016; Kastner et al., 2017), in others immediately before (Jennings et al., 1992; Crone et al., 2004; Veen et al., 2004; Groen et al., 2007; Luman et al., 2007) and/or after (Somsen et al., 2000; Drobes et al., 2001; Luman et al., 2007) the reward/feedback onset. These responses have variously been hypothesized to represent reward/feedback expectancy, processing or evaluation. However, it is difficult to determine the extent to which these heart rate changes represent a motivational response. Heart rate changes have also been observed in response to task difficulty (Eubanks et al., 2002), attentional demands (Somsen et al., 2000), and motor response preparation (Rakover and Levita, 1973).

In these human studies, the involvement of the central nervous system is inferred in the observed cardiac deceleration responses. In parallel with the peripheral indications of attention, the central nervous system shows changes related to orientation. The central states, and corresponding orienting responses, however, are influenced by a number of factors, such as the context in which stimuli are presented, sensory features of stimuli, ongoing motor activity and affective state (Gianaros and Quigley, 2001). In addition to the direct autonomic control of cardiac functions, the basal state of the autonomic nervous system as well as its responses to stimuli affect the central nervous system inputs to heart rate changes. Similar challenges in identifying the origins of physiological responses have been encountered in human neuroimaging studies that examined the neural correlates of reward and feedback. To measure reward-related blood-oxygen-level-dependent (BOLD) responses unconfounded by motor responses or complex cognitive processes, researchers have employed classical conditioning paradigms (Bray and O'Doherty, 2007; Prévost et al., 2013; Furukawa et al., 2014, 2020; Klucken et al., 2016).

Classical conditioning refers to a process where previously neutral stimuli acquire motivational significance [becomes a conditioned stimulus (CS)] when paired repeatedly with a pleasant/rewarding stimulus or aversive stimulus [i.e., unconditioned stimulus (US)]. The CS then comes to elicit physiological and behavioral responses [conditioned responses (CR)] (Cartoni et al., 2016). The CR may be similar to the response [unconditioned response (UR)] elicited by appetitive or aversive stimuli (US), but this is not necessarily the case (Bourne, 1990). In appetitive conditioning, the US used is a pleasant/rewarding stimulus, a CS+ signals the occurrence of an appetitive US. To date, there have been very few studies examining heart rate changes in appetitive classical conditioning in humans. One study demonstrated cardiac acceleration to a tone after being repeatedly paired with delivery of a glucose solution to infants (Clifton, 1974). Another study with adult subjects reported cardiac deceleration in response to an abstract image that was repeatedly paired with display and receipt of preferred snacks (Prévost et al., 2012). Variation in the direction of heart rate change, as a CR, may reflect differences in cognitive development or autonomic system maturity. The acceleration in infants may reflect autonomic arousal triggered by CS+. Cardiac deceleration in adults may indicate cognitive awareness that reward will follow the CS+, leading to an orienting response.

A number of animal studies have measured phasic heart rate changes during appetitive classical conditioning paradigms (e.g., Harris and Brady, 1974; Cohen and Obrist, 1975; Randall et al., 1985; Kalin et al., 1996; Hunt and Campbell, 1997). The direction of heart rate changes in these studies has been mixed. Several studies showed cardiac acceleration in response to CS+ (Goldstein et al., 1970; Powell and Milligan, 1975; Randall et al., 1985; Boivin, 1986; McLaughlin and Powell, 1999). This acceleration was interpreted as a CR (Randall et al., 1985; Hunt and Campbell, 1997). Other studies showed cardiac deceleration in response to appetitive CS (Powell and Milligan, 1975; Campbell and Ampuero, 1985; Hunt and Campbell, 1997; McLaughlin and Powell, 1999), which was interpreted as a learned attentional/orienting response (Powell and Kazis, 1976; Campbell and Ampuero, 1985; Kapp et al., 1992). These varied outcomes have been attributed to paradigm differences, including reward type, as well as species differences in how sympathetic and parasympathetic nervous systems are activated (Hunt and Campbell, 1997; Roberts and Clarke, 2019).

Clearly, further human studies are needed to clarify the nature of cardiac responses to appetitive US and CS+ during classical conditioning. Procedural constraints have likely limited research to date, especially difficulties surrounding the use of food or liquid outcomes and deprivation requirements to ensure their continued salience during conditioning (Freeman et al., 2015; Manglani et al., 2017; Schad et al., 2019a). An appetitive classical conditioning paradigm that does not require food/liquid consumption would expand the opportunities to study heart rate changes to appetitive US and CS across a wide range of populations.

In this pilot study, we developed an appetitive classical conditioning task to assess (1) phasic heart rate responses to an US during a training and a subsequent test phase, and (2) phasic heart rate responses to the CS during the test phase when conditioning was expected to have taken place. In the task, participants were shown initially neutral visual stimuli (intended CS+) on a computer screen followed by pictures of high-caloric food (US). Pictures of food have been used as positive stimuli that elicit cardiac changes (Beaver et al., 2006; Pursey et al., 2014; Kuoppa et al., 2016).

We hypothesized heart rate changes in response to the US during the training phase. If the food pictures evoke a positive motivational state, heart rate acceleration would be expected. Pleasant and rewarding stimuli are associated with increases in sympathetic activity (Pribram and McGuinness, 1975; Braesicke et al., 2005; Critchley, 2009; Rudebeck et al., 2014) as well as behavioral activation (Schad et al., 2019b; Watson et al., 2019; da Costa et al., 2020). On the other hand, if the heart rate changes in response to food pictures are reflective of attentional/orienting responses, then deceleration would be expected.

During the test phase, after establishment of the CS-US association (conditioning), we hypothesized that the phasic heart rate changes would be observed in response to the CS. If the CS has acquired the motivational significance of the US, we would expect an acceleration of heart rate. Heart rate changes to the US may not be as pronounced during this phase. In animals, striatal dopamine responses to CS after conditioning are accompanied by diminished responses to US (Schultz et al., 1997; Pan et al., 2005). If the CS triggers the engagement of attentional processes, the heart rate deceleration to the CS would be expected. It is unclear whether the deceleration after the US onset would continue to be observed. Responses reflective of reward prediction errors may be observed when the US is not consistent with the CS preceded it (Kamin, 1967; Rescorla and Wagner, 1972).

MATERIALS AND METHODS

The study was approved by the ethics committee of the teaching hospital of the State University of Rio de Janeiro (UERJ) in Rio de Janeiro, Brazil. All participants were volunteers who provided written informed consent. At the beginning of each session, participants were informed that their heart rate would be recorded at rest and while they viewed some pictures.

Subjects

Participants were 19 students attending UERJ, aged between 22 and 46 years. The inclusion criteria were normal or corrected vision, and no history of: psychiatric disorders; neurological diseases; cardiovascular diseases, including thoracic surgery or current use of cardiovascular medication; diabetes; obesity, defined as a BMI over 30 kg/m^2. This information, together with demographic data, were collected via questionnaire. Following preliminary data analysis, one participant was excluded as an outlier, i.e., having significantly slower heart rate, compared to other participants. The final sample included data from eighteen participants[1] (**Table 1**).

TABLE 1 | Participant characteristics.

N = 18	Mean	*SD*	Range
Age (years)	31.17	7.11	21–46
Weight (kg)	72.82	13.45	49–102
Height (cm)	168.88	8.59	154–185
Males *n* (%)		8 (44.4%)	

Experimental Task

The appetitive classical conditioning task (**Figure 1**) was programmed using Delphi. Task stimuli were displayed on a 19-inch monitor screen (Dell 1901N). Each trial began with a fixation cross presented for 1 s followed by a 0.5 s black screen. One of two initially neutral stimuli (two Japanese hiragana characters unfamiliar to participants; Cue 1 or 2) was presented on a computer screen for 4 s followed by a blank screen for 0.5 s. After this, an appetitive unconditioned stimulus (US) or a neutral stimulus (NS) was presented for 4 s. The US were pictures of high-caloric food (e.g., brownies, pizza). These pictures have previously been rated by adults, of similar demographic backgrounds to the current participants, as significantly more likable than images of everyday objects (Furukawa et al., unpublished). The NS was an image of a gray square. Each US and NS was followed by a variable inter-trial-interval (3, 5, or 7 s), to reduce the temporal predictability of the next trial. No motor responses were required during the task.

The training and test phases of the task were programmed separately. During both phases, Cue 1 (CS+ after conditioning) was followed by the US 66% of the time and the NS 33% of the time. Cue 2 (CS- after conditioning) was always followed by the neutral stimulus. The training phase included 16 trials of Cue 1 followed by a US, 8 trials of Cue 1 followed by the neutral stimulus, and 24 trials of Cue 2 followed by the neutral stimulus. The test phase was implemented after completion of the training phase. This phase included six trials of Cue 1 followed by a US, three trials of Cue 1 followed by the neutral stimulus, and nine trials of Cue 2 followed by the neutral stimulus.

The number of presentations of Cue 1 and 2 was the same within each phase to ensure similar levels of habituation to the stimuli. The presentation order of the cues and US (22 different food images) and neutral stimuli were randomly scheduled and unique for each participant.

ECG Recording

Prior to the experiment, two disposable adhesive electrocardiogram (ECG) electrodes were placed on participants' chest; one electrode on the left side of the sternum around the 5th intercostal space, and the other electrode near the right

[1] A total of 23 trials from eight participants were excluded due to unclear recordings (undefined peaks). Details available upon request.

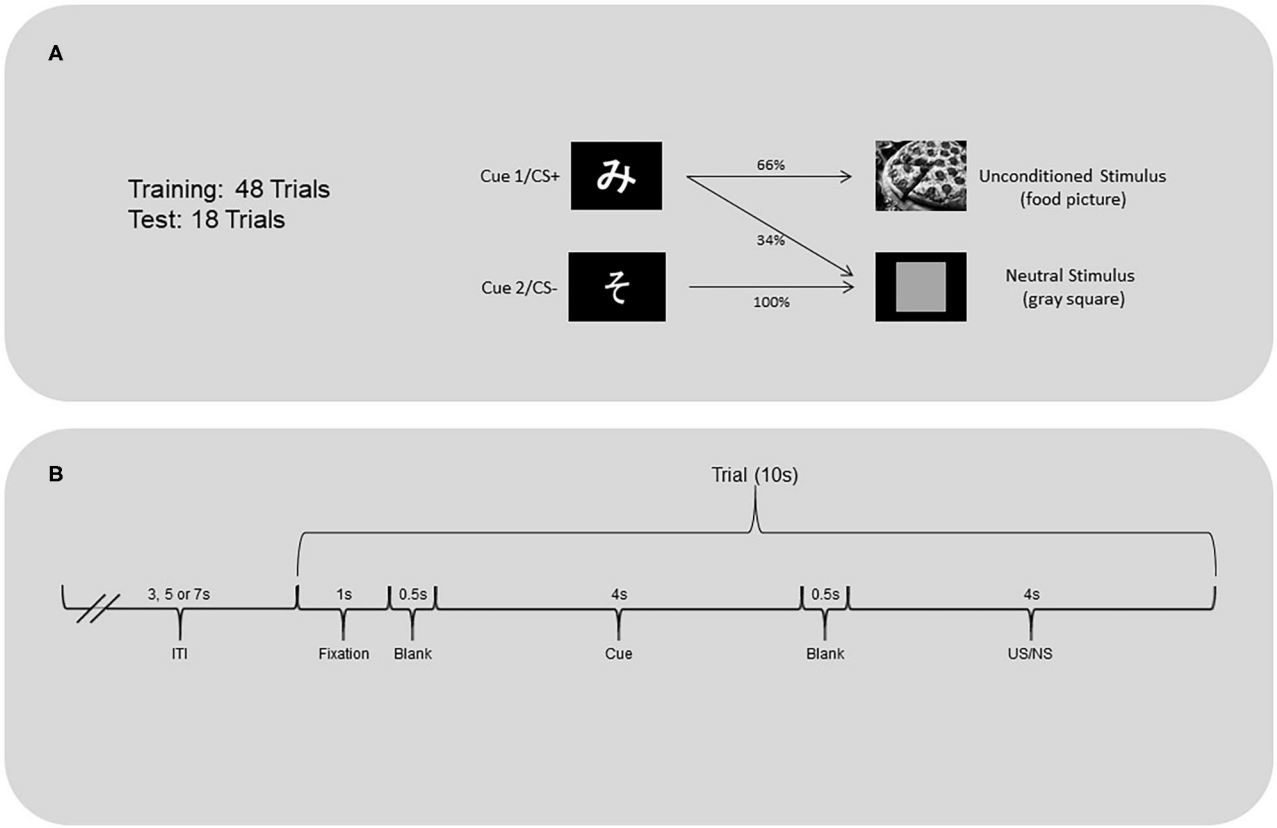

FIGURE 1 | (A) Classical conditioning task and **(B)** trial timeline.

sternal border, around the 2nd intercostal space. The ECG signals were recorded on a Windows-based computer, with a Labview script, biopotential amplifier, sampling rate of 400 Hz, notch filter (60 Hz), high-pass filter cutoff set at 0.3 Hz, and low pass filter cutoff at 25 Hz. Before the experimental task began, baseline cardiac activity was recorded for 5 min at rest. The ECG recording was restarted and synchronized with the start of the experimental task. Testing was carried out in a quiet room. The experimenter was present for the duration of the experiment.

Data Analysis

Data was extracted into and processed in Matlab. Phasic heart rate responses were estimated using R-R intervals. The R-R interval is the time between successive heartbeats.

To address normal heart rate variability within and across participants, the mean of the four R-R intervals preceding the start of each trial (before presentation of fixation point) was calculated to provide a measure of pre-trial R-R interval[2] (**Figure 1**). The changes from this pre-trial R-R interval, i.e., "heart index" (iH), were then obtained by dividing each target R-R interval by the pre-trial R-R interval + the target R-R interval. For example, the interval between the first R peak and second R peak immediately following the US, divided by the pre-trial R-R interval plus the interval of the first and second R peaks, was the first post-US iH. An iH = 0.5 would indicate no change from the pre-trial heart rate. An iH value >0.5 would indicate cardiac deceleration (larger interval length), while iH value smaller than 0.5 would indicate cardiac acceleration (smaller interval length). The absolute values of iH indicated the magnitude of the phasic heart rate changes.

The dependent variables were heart rate responses to the US/NS as well as to the cues that predicted them. The iH values were calculated for the first, second, third, and fourth R-R intervals following the US/NS presentation (interval +1, +2, +3, +4 after US/NS in **Figure 2**). As heart rate changes may be observed in anticipation of the stimulus presentation (Groen et al., 2007; Poli et al., 2007), the iH values were also calculated for a period before the US/NS presentation. The interval between the two R peaks immediately before the US/NS is the first pre-US/NS iH and so on (intervals −1, −2, −3, −4 before US in **Figure 2**). Similar procedures were followed to examine

[2]Four intervals were used as most participants' heart rate responses consistently had at least four intervals during the ITI. There was no significant difference in the resulting iHs when using two, three, or four intervals to calculate the pre-trial R-R interval. In addition, the pre-trial means were compared for the 3, 5, and 7 s ITI trials; no significant difference was observed.

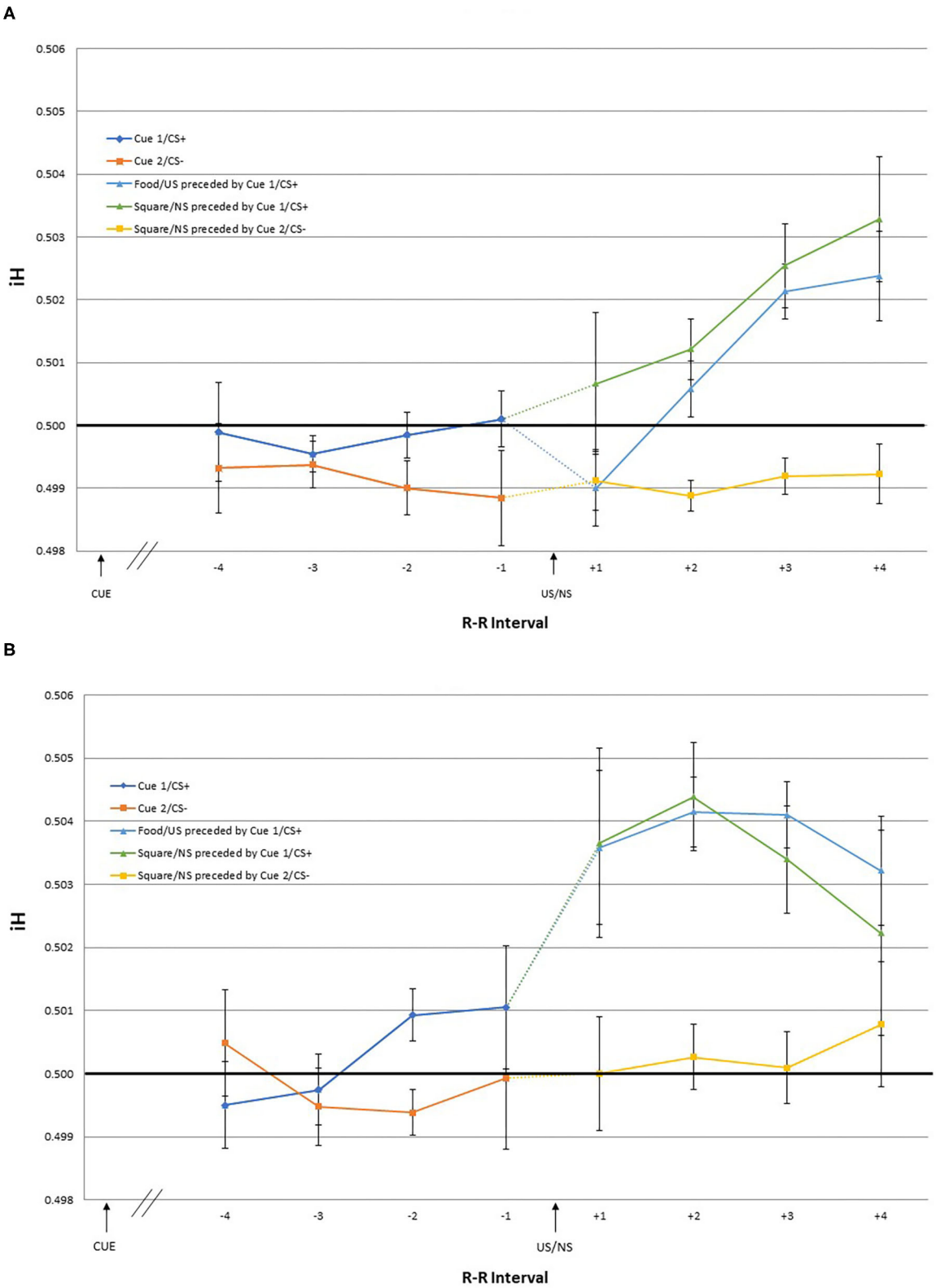

FIGURE 2 | The mean iH values and within-subject standard errors for the first through fourth interval before US onset [separated by preceding cue type: Cue 1/CS+ (blue) and Cue 2/CS– (orange)], and the first through fourth interval after US onset [separated by the cue and US type; Food/US preceded by Cue 1/CS+ (light blue), Square/NS preceded by Cue 2/CS+ (green), and Square/NS preceded by Cue 2/CS– (yellow)] during the **(A)** training and **(B)** test phase.

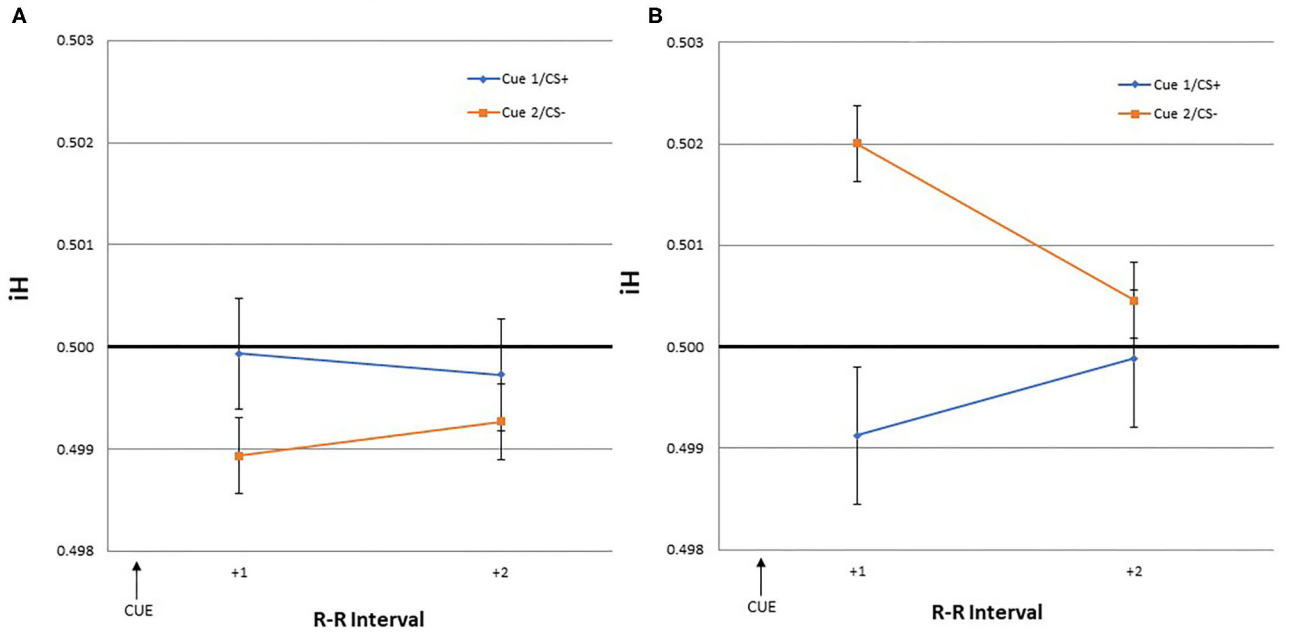

FIGURE 3 | The mean iH values and within-subject standard errors for the first and second interval after a cue onset [Cue 1/CS+ (blue) or Cue 2/CS– (orange)] during the **(A)** training and **(B)** test phase.

heart rate responses to cues, the iH values for the two intervals following Cue 1 and 2 were evaluated (interval +1, +2 after cue in **Figure 3**)[3].

For each of the post-cue and pre-US/NS intervals, average iH values were obtained for each participant separately for two trial types: those following a Cue 1/CS+ presentation, and those following a Cue 2/CS– presentation. For each of the post-US/NS intervals, average iH values were obtained for three trial types: those following US preceded by Cue 1/CS+, those following the NS preceded by Cue 1/CS+, and those following the NS preceded by Cue 2/CS–.

Two-way repeated measures linear effects of time, US/NS and cue type on the iH values were examined using GLM. To explore the timing of the heart rate changes, one-sample tests were conducted to examine whether the iH value for each interval was significantly different from 0.5. All statistical analyses were undertaken with SPSS.

As tonic heart rate is reported to change in the presence of rewarding stimuli (Rakover and Levita, 1973; Fowles et al., 1982; Blanchard et al., 2000; Nederkoorn et al., 2000; Starcke et al., 2018), we undertook a series of exploratory analyses to test for these effects in our data. Tonic heart rate changes (bpm; beats per minute) and heart rate variability (RMSSD; the root mean square of successive differences between heart beats) during the rest period before the experimental task began and during the training and test phase were examined.

[3]In most participants, there were 4–6 R-R intervals between the cue and US/NS. Thus, for some participants, the four pre-US/NS intervals and two post-cue intervals overlapped.

RESULTS

The participants' ratings of the food pictures, on a 4-point scale, completed after the experimental task, showed a similar level of likability ($M = 3.28$, $SD = 0.75$) as previously identified ($M = 2.78$, $SD = 0.43$) (Furukawa et al., unpublished).

Motivational vs. Orienting Effects of US on Cardiac Responses

Heart rate changes after US (vs. neutral stimulus [NS]) were evaluated to examine whether the changes are reflective of motivational state (cardiac acceleration) or orienting responses (cardiac deceleration). This was done separately for the training and test phases.

Two-way repeated measures linear effects of time (four intervals), US/NS type (US, NS preceded by Cue 1/CS+, and NS preceded by Cue 2/CS-) and the interaction, were examined using GLM. During the training phase, there was a significant time*US/NS type interaction [$F_{(1,17)} = 6.58$, $p = 0.020$] and a significant main effect of time [$F_{(1,17)} = 8.87$, $p = 0.008$]. *Post-hoc* pairwise comparisons indicated that the mean iH values for the +1 and +2 intervals were significantly different from those of +3 and +4 intervals. Visual inspection of the data suggest that the significant effect of time is driven by heart rate changes following the US and NS preceded by Cue 1/CS+, while the iH values following the NS preceded by Cue 2/ CS– remained stable across the four intervals. During the test phase, no significant effects were observed.

Given the significant interaction effect observed in the GLM during the training phase, one-sample t-tests were conducted to examine for which intervals iH values were different from

pre-trial R-R interval (i.e., different from 0.5) following the presentation of US and NS. Following the US, the significant effects were observed for +4 [$t_{(17)} = 2.16$, $p = 0.046$] interval, indicating cardiac deceleration. Differential results were obtained for the iH values following the NS depending on the preceding cue type. When the NS was preceded by Cue 1/CS+, the iH values after the NS were also significantly different from the pre-trial R-R interval for the +4 [$t_{(17)} = 2.35$, $p = 0.031$] interval, indicating deceleration. In contrast, when the NS was preceded by Cue 2/CS–, the iH values were not significantly different from the pre-trial heart rate.

While no significant effect was observed in the GLM for the test phase, to explore whether the pattern of heart rate changes observed during the test phase were consistent with that of the training phase, one-sample *t*-tests were conducted also for the iH values for the intervals during the test phase. One-sample *t*-tests showed that following the US presentation, the iH values were significantly different from the pre-trial R-R interval for the +2 [$t_{(17)} = 2.34$, $p = 0.032$], and +3 [$t_{(17)} = 2.29$, $p = 0.035$] intervals, indicating cardiac deceleration. Following the NS preceded by Cue 1/CS+, the iH values showed a trend toward deceleration for the +1 [$t_{(17)} = 1.87$, $p = 0.079$] and +2 [$t_{(17)} = 1.93$, $p = 0.070$] intervals. As in the training phase, the iH values following the NS preceded by Cue 2/CS– were not significantly different from the pre-trial R-R interval.

Analysis of the pre-US heart rate, during both the training and test phases, showed that no effect of time or preceding CS type, or the interaction. The iH values immediately prior to the US or NS presentation following Cue 1/CS+ (anticipation of a positive outcome) were not significantly different from the pre-trial R-R interval across all four intervals. Similarly, the iH values immediately prior to the NS presentation following Cue 2/CS– (anticipation of a negative outcome) were not significantly different from the pre-trial across all four intervals.

Cardiac Responses to the Cues

The iH values for the two intervals following Cue 1/CS+ and Cue 2/CS– were evaluated during both the training and test phases. This was done to assess if similar cardiac responses to the US would occur in response to the cues predicting the US.

GLM yielded no significant effect of time, CS type, or the interaction. During the training and test phase, none of the iH values for the two intervals following the cue stimuli were significantly different from the pre-trial R-R interval.

Tonic Heart Rate and Heart Rate Variability at Rest and During the Task

Repeated measures ANOVA indicated that bpm and RMSSD during the rest, training, and test phases were not different from each other, indicating no significant changes in the tonic heart rate or heart rate variability.

DISCUSSION

The current study describes the development and pilot testing of an appetitive classical conditioning task to assess heart rate responses to appetitive stimuli and cues preceding them in humans. Cardiac deceleration to US (food images with high likability ratings) was observed, consistent with an orienting response to motivationally significant stimuli. Similar cardiac responses were observed to the neutral stimuli (gray square) when participants were expecting the food images, i.e., following the cue typically associated with these images. Tonic heart rate and heart rate variability were not influenced by the task.

Significant changes in the heart rate responses following the US onset were observed during the training phase. Cardiac deceleration was observed for the fourth R-R interval, but not for the first through third intervals, following the stimulus presentation. Heart rate responses during the four intervals following the US onset did not change significantly during the test phase. Exploratory analyses showed that cardiac deceleration occurred earlier during the test phase, i.e., for the second and third R-R intervals, with effect beginning in the first interval. The effects of the cue might be seen in the latency of these cardiac responses from the US onset for the training vs. test phases. The cue proceeding the food images had developed predictive power and become a CS+, i.e., participants expected the food images following the cue presentation, which led to earlier heart rate changes.

Consistent with this hypothesis, the participants showed an orienting response to the neutral stimulus when it was preceded by the purported CS+, but not when it was preceded by the CS–. This may be taken as evidence that the cue had come to serve as a predictor of food pictures, a CS+, and therefore participants attended to the US regardless of its value. Alternatively, the uncertainty surrounding the CS+ maintained the salience of the stimuli that followed. The heart rate responses may be reflective of the prediction error (Kamin, 1967; Rescorla and Wagner, 1972), as the CS+ was predictive of the US only 66% of the time, whereas for the CS– was always associated with the neutral stimulus.

We hypothesized that increased sympathetic activity, and therefore heart rate acceleration, might be observed in response to the US (Pribram and McGuinness, 1975; Braesicke et al., 2005; Critchley, 2009). Increased autonomic arousal has been shown to be associated with positive motivational states in the presence of rewarding stimuli (Fowles et al., 1982; Richter, 2012). The results of the current study however showed clear cardiac deceleration to the presented appetitive stimuli. Food images were used as the US. The parasympathetic nervous system is activated when organisms digest food, which is accompanied by heart rate deceleration (Woods, 1972). This could explain the cardiac deceleration to the food images observed. Interestingly, other studies using non-classical conditioning tasks, such as learning and decision-making tasks, show phasic cardiac deceleration to non-food appetitive stimuli, such as monetary incentives, positive feedback, and pleasant pictures (Tuber et al., 1985; Somsen et al., 2000; Drobes et al., 2001). Heart rate deceleration is also seen in response to a range of aversive stimuli (Somsen et al., 2000; Drobes et al., 2001; Veen et al., 2004; Kuoppa et al., 2016; Kastner et al., 2017). These findings suggest that heart rate deceleration to the US observed in the current study is likely an attentional/orienting response. Heart rate deceleration assists

organisms to attend selectively to stimuli important for survival (Hunt and Campbell, 1997).

Expectations created by the CS+ influenced heart rate responses to the US and the neutral stimuli in the current study. During the test phase, heart rate changes were observed with shorter latency from the US onset. When the neutral stimulus followed the CS+, cardiac deceleration was also observed. Together these results offer some evidence that the CS+ was eliciting the cardiac deceleration. Heart rate changes after feedback have also been suggested to reflect expectation errors (Somsen et al., 2000; Luman et al., 2007). The CS+ established an expectation that a positive stimulus would follow. However, the CS+ was not always followed by US. Under such conditions of uncertainty, the US itself provides meaningful feedback, eliciting orienting responses. In humans, cardiac responses may be linked to the cognitive processes of attending as well as to the valence of the stimulus (McLaughlin and Powell, 1999). It is interesting that the only human classical conditioning study demonstrating cardiac acceleration to a CS+ was with infants, whose cognitive skills are less developed (Clifton, 1974). In animal studies, the direction of the heart rate changes to the CS+ is mixed (Hunt and Campbell, 1997), with some reporting bi-phasic responses with initial orienting deceleration to the CS+ onset then cardiac acceleration closer to the time of US delivery (Holland, 1979).

The current task, together with the data processing and analysis methods, provide reliable measurements of heart rate responses during appetitive classical conditioning in humans. These reflect important developments toward a new methodology. Differential effects of US vs. neutral stimuli on heart rate were observed, and the data reflected awareness of the purported CS+ vs. CS–. As the task was simple and no behavioral responses were required, these cardiac responses were likely elicited by these stimuli, unconfounded by task difficulty (Richter, 2010; Fairclough and Ewing, 2017) or action/action preparation (Cooke et al., 2014; Rösler and Gamer, 2019). The data offer new information on adult human responses to appetitive stimuli and cues that predict them.

Despite the promise of this new methodology and the interesting findings obtained, the current study is not without limitations. As a pilot study, the sample size is relatively small, possibly impacting statistical significance of findings. The total number of trials may need to be expanded in future studies, during both training and testing to better detect effects. More trials/stronger associations would help clarify the importance of these trends. As presentation of trial type was randomized, each participant received trials in a unique order. This prevented us from examining the time course of the heart rate changes within the training and test phases. Moving forward we would recommend using the same trial order presentation across participants. It would also be helpful to present the US repeatedly without cues to examine whether the shorter latency between the US onset and the heart rate responses reflect participants becoming desensitized after the repeated US presentations; however, such desensitization would likely be associated with reduced heart rate changes. We would also consider including longer intervals, i.e., more heart beats, before and after presentation of the US to further clarify the nature of cardiac changes and their timing. There is some evidence that anticipatory heart rate changes are bi- or tri-phasic, responses following the CS changing as the time gets closer to the US onset (Rakover and Levita, 1973; Bohlin and Kjellberg, 1979). While food images are often used experimentally in human studies, we question if they served as strong appetitive stimuli in this study. Furthermore, we did not control for participants' hunger levels. In non-human studies, animals are usually food/liquid deprived, with mixed findings attributed to differing stimulus saliency (Hunt and Campbell, 1997; McLaughlin and Powell, 1999). The use of consumable stimuli and/or food/liquid restriction, as well as other types of appetitive stimuli, in future studies with the current task should be considered. If similar results are obtained using other appetitive stimuli, it would support the suggestion that the heart rate responses seen in the current study are reflective of appetitive conditioning rather than other forms of associative learning. If using consumable US, however, the effects of the act of consuming the food or drink on heart rate responses would need to be carefully considered. In any studies, in addition to the motivational and attentional processes, other factors that could affect heart rate, such as motor responses, stimulus type, presentation and timing, should be taken into account in hypothesis generation, task development and the interpretation of results. A final consideration for future studies is the extent to which the CS+ is predictive of the US. A task containing only CS+ trials may lead to different tonic heart rates during the rest, training, and test phases and show gradual heart rate acceleration with time.

Evaluating and monitoring autonomic changes during appetitive classical conditioning is important to understanding how predictive cues exert control over human behaviors. In this pilot study, a cue that typically preceded food images led to cardiac orienting responses. These data suggest that attentional processes can be engaged or disengaged by conditioned stimuli. Heart rate changes are relatively easily measured physiological responses that can be assessed across settings and populations. The study also generated helpful future considerations. Further studies employing larger samples of typically developing adults and children would clarify whether these responses are consistent across the life span. Individuals with clinical conditions may demonstrate different cardiac response patterns to cues and appetitive stimuli. If such differences can be relatively easily identified, i.e., using the current task and analytical methods, this information can be used to better understand the underlying nature of these disorders and potentially their management.

DATA AVAILABILITY STATEMENT

The data supporting the conclusion of this study are available upon reasonable request to the corresponding author.

ETHICS STATEMENT

This study was reviewed and approved by Comissão de Ética em Pesquisa (Coep)—UERJ. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, HA, and EC-D: participated in study conceptualization, data collection, and data analysis. EF and TSW: participated in study conceptualization and data analysis. MC: participated in experimental task development. SG-A: participated in data analysis. GT: participated in study conceptualization. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a joint research agreement between OKinawa Institute of Science and Technology Graduate University and UERJ.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 01 April 2021
doi: 10.3389/fnbeh.2021.646337

Disruption of Cigarette Smoking Addiction After Dorsal Striatum Damage

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: 26 December 2020
Accepted: 08 March 2021
Published: 01 April 2021

Citation:
Jing C, Jing C, Zheng L, Hong G, Zheng J, Yu L, Song N, Zhang T, Ma Q and Fang J (2021) Disruption of Cigarette Smoking Addiction After Dorsal Striatum Damage. Front. Behav. Neurosci. 15:646337. doi: 10.3389/fnbeh.2021.646337

Studies have shown that addictive behavior is associated with many brain regions, such as the insula, globus pallidus, amygdala, nucleus accumbens, and midbrain dopamine system, but only a few studies have explored the role of the dorsal striatum in addictive behavior. In June 2020, we started contacting 608 patients who were hospitalized between January 2017 and December 2019, and we recruited 11 smoking addicts with dorsal striatum damage and 20 controls with brain damage that did not involve the dorsal striatum (the damaged areas included the frontal lobe, temporal lobe, parietal lobe, brain stem, thalamus, internal capsule, and so on). All study participants had brain damage due to acute cerebral infarction. Disruption of smoking addiction was found to be significantly associated with the dorsal striatum (Phi = 0.794770, $P = 0.000015$). Our findings suggested that patients in the dorsal striatum group were more likely to discontinue smoking than those in the non-dorsal striatum group. The characteristics of this interruption is that smoking can be quit more easily and quickly without recurrence and that the impulse to smoke is reduced. These results suggest that the dorsal striatum is a key area for addiction to smoking.

Keywords: cigarette smoking, addiction, disruption, dorsal striatum, behavioral

INTRODUCTION

Addiction is a pathological process that involves learning and memory. Potentially addictive drugs activate the brain's reward system, adaptively changing the structure and function of the nerves in this part of the brain. Research on addiction, which has increased in the past 10 years, indicates that many areas of the brain are involved in the addiction pathway, including the insula, globus pallidus, amygdala, nucleus accumbens, ventral striatum, frontostriatal, and the midbrain dopamine system (Volkow et al., 2006; Naqvi et al., 2007; Wang et al., 2007; Koob and Volkow, 2010; Hefzy et al., 2011; Yuan et al., 2018).

Recent evidence indicates that the dorsal striatum plays an important role in addiction. Several studies have shown that dorsal striatum activity increases in response to drug cues relative to neutral cues in drug users (Vollstädt-Klein et al., 2010; Claus et al., 2011; Schacht et al., 2011). A study by McClernon et al. had 18 adult smokers undergo fMRI scanning following two conditions: smoking as usual and a 24-h abstinence period. After abstinence, greater fMRI activity was observed in response to smoking cues compared to control cues in the dorsal striatum. The same effect was also observed in the parietal, frontal, occipital, and central

cortical regions, and the thalami (McClernon et al., 2009). Vollstadt-Klein and colleagues also reported that dorsal striatum activity in response to drug cues was positively correlated with drug craving in heavy drinkers (Vollstädt-Klein et al., 2010). A study by Janes et al. reported that nicotine-dependent smokers who failed to quit smoking showed greater cue-induced activity in the dorsal striatum, among other regions, compared to smokers who remained abstinent (Janes et al., 2010). Zhou et al. found that heavy cannabis users selectively exhibited dorsal striatal reactivity (Zhou et al., 2019). Another study found that the volume of the putamen was positively correlated with the duration of abstinence in former regular users of alcohol who were abstinent for a long time (Korponay et al., 2017). A clinical trial showed that damage to only the dorsal striatum can cause disruption of smoking addiction, and when basal ganglia damage is combined with insula damage, the disruption increases (Gaznick et al., 2014). Furthermore, dorsal striatum connectivity with the cingulo-insular network was found to be associated with smoking cessation (Sweitzer et al., 2016).

Against this background, because smoking is one of the most common addictions in China, and there are few clinical studies that have investigated the relationship between the dorsal striatum and smoking addiction, our research has tried to understand the role of the dorsal striatum in the pathway of smoking addiction. As acute cerebral infarction is one of the most important causes of brain damage treated in a neurology department, we recruited patients who had an acute cerebral infarction, of whom, 11 only had damage to the dorsal striatum and 20 had brain damage that did not involve the dorsal striatum. Participants answered a series of questions about their smoking history, and their results were compared to investigate the relationship between the dorsal striatum and smoking addiction.

MATERIALS AND METHODS

All of the study participants were recruited between January 2017 and December 2019 from the First Affiliated Hospital of Xiamen University, Xiamen, China. All the procedures were reviewed and approved by the First Affiliated Hospital of Xiamen University, and all the subjects provided signed informed consent prior to participating in the study. We reviewed the patients' electronic records to ensure they met the following inclusion criteria: (1) they suffered an acute cerebral infarction; (2) their lesions could be visualized using MRI; (3) they had a smoking history; (4) they did not suffer from amnesia or severe aphasia; and (5) they were not addicted to drugs other than nicotine at the time of lesion onset, per their medical records.

Subject Selection

Starting in June 2020, we attempted to contact 608 patients who met these inclusion criteria to determine their smoking history: 78 patients could not be contacted (because their telephone numbers had changed, they died, or some other reason), and 157 patients reported they smoked at some time, but quit a number of years before lesion onset. Of the 373 remaining patients, 198 reported that they smoked more than 10 cigarettes per day for more than 10 years at the time of lesion onset. Among these 198 patients, 11 had only dorsal striatum damage and 20 had non-dorsal striatum damage (The damaged regions included the thalamus, internal capsule, caudate nucleus, and other regions in the brain, and some patients had damage in more than one region). Each of these 31 patients was asked to complete the Fagerström Test for Nicotine Dependence (FTND), which is the most valid and commonly used scale for measuring nicotine addiction (Fagerström, 1978). Scores on the test range from 0 to 10, with the higher scores indicating stronger smoking dependence. Nicotine addiction was categorized as low nicotine dependence (0–4 points), moderate nicotine dependence (5–6 points), and high nicotine dependence (7–10 points). All 31 of the patients scored 7–10 points, which means they were highly nicotine dependent. These 31 patients finally served as the subjects in this study, and were included in the statistical analysis. A flow chart of patient inclusion is shown in **Figure 1**.

Measures

We obtained relevant information about the subjects through electronic records and interviews, including their sex, current age, age at lesion onset, years of smoking at lesion onset, and number of cigarettes smoked per day at lesion onset. Additional smoking measures are described in section Behavioral Classification.

Statistical Analysis

Group differences in demographic characteristics and detailed smoking data were analyzed by SPSS Version 20.0 (IBM SPSS Statistics, Armonk, NY). Specifically, the means of the dorsal striatum group and non-dorsal striatum group were compared at baseline on the following variables: sex composition (i.e., number of males and females) current age, age at lesion onset, years of smoking at lesion onset, and number of cigarettes smoked per day at lesion onset. An independent two-sample *t*-test was used to analyze continuous variables, and Fisher's exact probability test was used for proportions. $P < 0.05$ was considered to be statistically significant. The effect sizes are reported as Phi coefficients (0.10 indicates a small effect, 0.3 indicates a medium effect, and 0.5 indicates a large effect).

Behavioral Classification

The 31 patients were interviewed in order to determine how their smoking behavior changed after lesion onset (Information was obtained from relatives when necessary). All the patients were asked whether or not they had smoked in the past 6 months. Patients who reported they smoked during the past 6 months were classified as "non-quitters." Those who reported they did not smoke during the past 6 months were classified as "quitters." According to the classification method of Naqvi et al. (2007), all of the "quitters" were asked some further questions in order to understand their experience of quitting smoking in relation to the onset of their lesions. The questions were as follows: (1) "How soon after your brain injury did you quit smoking?"; (2) "How difficult was it to quit smoking after your brain injury (on a scale of 1–7, with one being very easy and seven being very difficult, the score is based on the subjective feelings of the patient)?"; (3) "How many times have you started smoking again since your

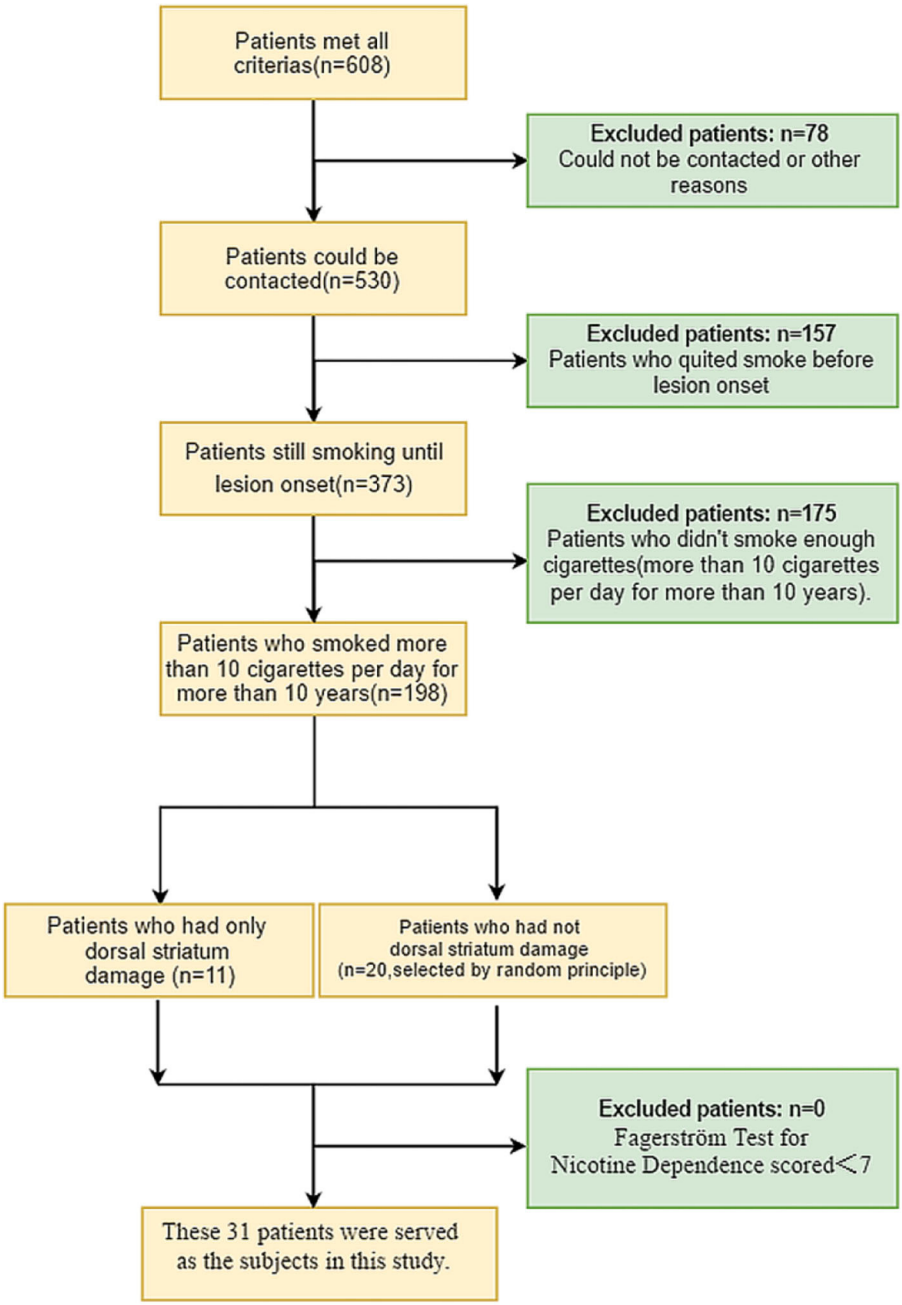

FIGURE 1 | Flow chart of patient inclusion.

brain injury?"; and (4) "Have you experienced any impulse to smoke again since you quit smoking?" Patients who reported they quit smoking <1 day after their brain injury, who rated their difficulty of quitting as <3, who reported they did not start smoking again since their brain injury, and reported that they felt no impulse to smoke again since quitting were classified as having a "disruption of smoking addiction." The remaining patients were classified as having "no disruption of smoking addiction."

MRI Acquisition

A 3.0 T MRI system (Ingenia, Philips Medical Systems, Netherlands) was used for all data collection. The head coil had a 16-channel phased-array. Other imaging parameters were as follows: T1-weighted images (TR = 250 ms; TE = 2.3 ms; slices = 21; thickness = 6 mm; gap = 1 mm; FA = 75°; matrix = 256 × 163; FOV = 230 × 180 mm. NSA = 2. The sequence took 1 min and 23 s); T2-weighted images (TR = 2866 ms;

TE = 120 ms; slices = 21; thickness = 6 mm; gap = 1 mm; FA = 90°;matrix = 358 × 299; FOV = 230 mm × 200 mm. NSA = 1.5. The sequence took 1 min and 37 s); Fluid attenuated inversion recovery (FLAIR) sequence (TR = 10000 ms; TE = 125 ms; slices = 21; thickness = 6 mm; gap = 1 mm; FA = 75°; acquisition matrix = 308 × 200; FOV = 230 × 200 mm. NSA = 1. Inversion recovery delay time = 2450 ms. The sequence took 2 min); Diffusion weighted imaging sequence with b-values = 1000 s/mm^2 (TR = 4234 ms; TE = 78 ms; slices = 21; thickness = 6 mm; gap = 1 mm; FA = 90°; FOV = 230 × 230 mm; matrix = 152 × 122; voxel size = 1.5 × 1.9 × 6.0 mm^3. NSA = 1. The sequence took 1 min and 54 s).

RESULTS

Among the 11 cigarette smokers who had suffered only dorsal striatum damage, five had right dorsal striatum damage and six patients had left dorsal striatum damage (**Figure 2**). The means of the dorsal striatum group and non-dorsal striatum group did not differ significantly for sex composition, current age, age at lesion onset, years of smoking at lesion onset, or number of cigarettes smoked per day at lesion onset ($p > 0.05$) (**Tables 1**, **2**).

Based on the criteria described in section Behavioral Classification, 14 of the 31 patients were "non-quitters," and 12 of the patients were "quitters" who quit smoking after lesion onset and met all four criteria for "having a disruption of smoking addiction." The five remaining patients were "quitters" who failed to meet all four of these criteria, so they were classified as having "no disruption of smoking addiction" (**Figure 3A**). More details are provided in **Figures 3B,C**. In **Table 3**, the percentage of quitters with disruption of smoking addiction in the dorsal striatum group was 83.3%, which was much higher than 16.7% in the non-dorsal striatum group. Our findings showed that the likelihood of having a disruption of smoking addiction after a lesion in either the right or the left dorsal striatum was significantly higher than the likelihood of having a disruption of smoking addiction after a non-dorsal striatum lesion (Phi = 0.794770, $P = 0.000015$). When we examined the right and left dorsal striatum separately, we found that the likelihood of having a disruption of smoking addiction was significantly higher after a right dorsal striatum lesion than it was after a non-dorsal striatum lesion (Phi = 0.774597, $P = 0.001412$), and it was also significantly higher after a left dorsal striatum lesion than it was after a non-dorsal striatum lesion (Phi = 0.726641,

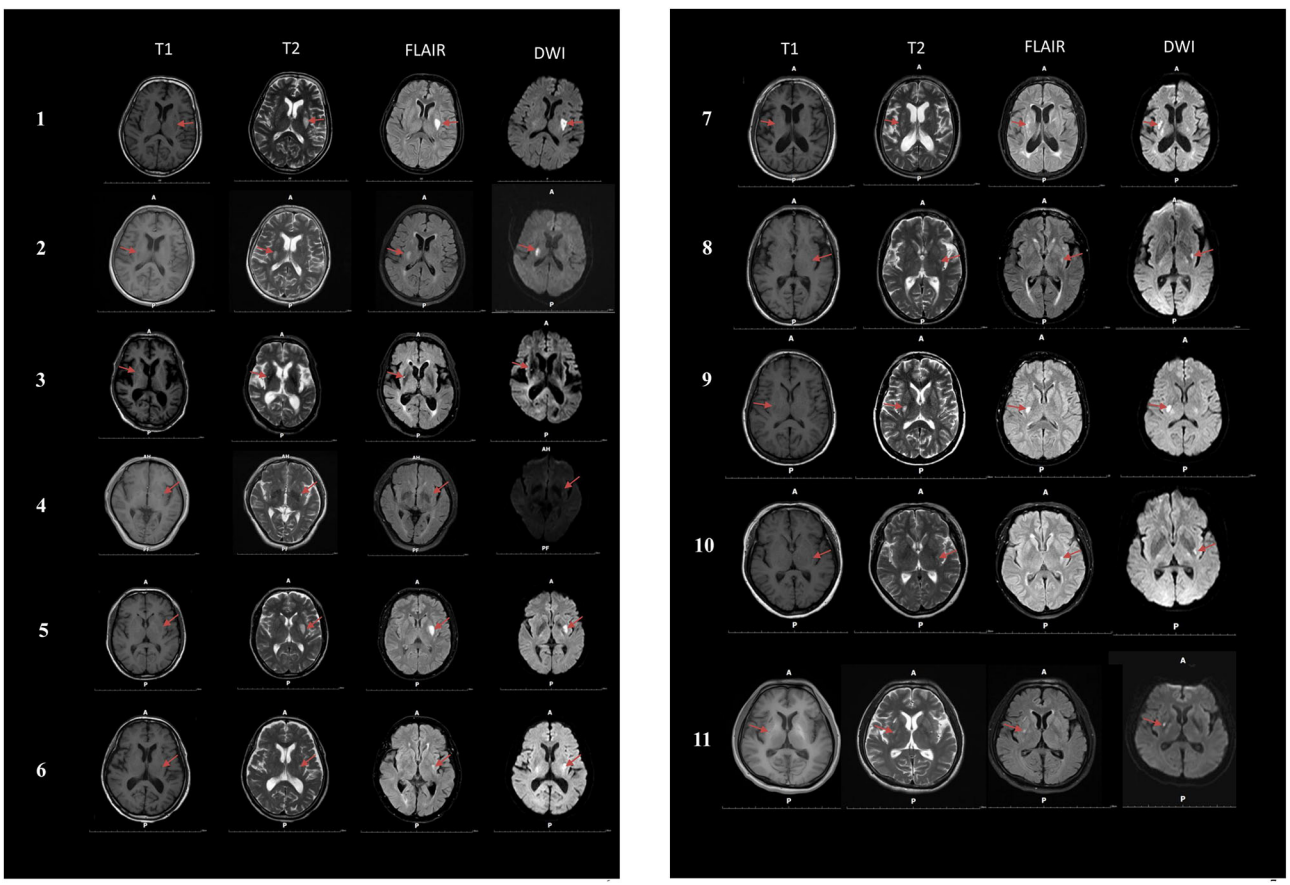

FIGURE 2 | Magnetic resonance imaging (MRI) was performed on 11 smokers with dorsal striatum damage. T1, T1-weighted images; T2, T2-weighted images; FLAIR, Fluid attenuated inversion recovery; DWI, Diffusion weighted imaging.

TABLE 1 | Detailed information about patients who acquired dorsal striatum damage.

Number	Region	Gender	Age	Age at lesion onset	Years smoking at lesion onset	Cigarettes/day at lesion onset
1	Left	Male	71	68	25	20
2	Right	Female	62	61	19	15
3	Right	Male	71	70	22	22
4	Left	Male	63	60	35	30
5	Left	Male	55	53	24	18
6	Left	Male	77	75	35	30
7	Right	Male	47	44	10	12
8	Left	Male	79	78	33	15
9	Right	Female	59	58	12	10
10	Left	Female	56	55	14	10
11	Right	Male	54	51	17	20

Left, left dorsal striatum; Right, right dorsal striatum.

TABLE 2 | Characteristics of the dorsal striatum group and the non-dorsal striatum group.

	Dorsal striatum ($n = 11$)	Non-dorsal striatum ($n = 20$)	*t*	*P*-value
Females (number)	4.00 (36.36%)	9.00 (45.00%)	–	0.718
Age (years)	63.09 ± 10.23	64.10 ± 10.58	−0.257	0.80
Age at lesion onset (years)	61.18 ± 10.55	61.85 ± 10.05	−0.174	0.86
Years smoking at lesion onset	22.36 ± 9.01	23.50 ± 8.90	−0.488	0.63
Cigarettes smoked per day at lesion onset	18.36 ± 7.08	18.70 ± 7.76	−0.119	0.91

$P = 0.000608$). There were two patients who had a disruption of smoking addiction after suffering brain damage that did not involve the dorsal striatum. When examined their lesions, each of them had damage in a unique set of regions. This raises the possibility that certain patients may have a disruption of smoking addiction as a general effect of suffering brain injury.

DISCUSSION

A review of the literature shows that few studies have examined the disruption of addiction after brain injury and most of those that have are case reports (**Table 4**). A study by Naqvi et al. of 69 patients which is one of the few studies to find a direct relationship between insula damage and disruption of smoking addiction. The results suggest that the insula is a critical neural substrate in the addiction to smoking (Naqvi et al., 2007). Recent evidence indicates that damage to the basal ganglia alone can cause disruption of smoking addiction, and when basal ganglia damage is combined with insula damage, the disruption increases (Gaznick et al., 2014).

The ventral striatum has classically been considered to play an important role in addiction. Many studies have implicated the ventral striatum in the anticipation and immediate response to rewards (Hariri et al., 2006; Luijten et al., 2017), and that it is associated with abstinence-induced craving (David et al., 2005; Franklin et al., 2007; Wang et al., 2007). One study also found that 4h of abstinence can significantly increase craving and reduce regional cerebral blood flow in the ventral striatum (Franklin et al., 2018).Our findings indicate the dorsal striatum may also be involved in the addiction pathway. Our results indicate that smokers who acquired dorsal striatum damage were very likely to quit smoking easily and immediately and to remain abstinent. Moreover, smokers with dorsal striatum damage were far less likely to experience conscious urges to smoke after quitting. Based on the research literature, the dorsal striatum may be involved in addiction through the following pathways.

First, many studies have demonstrated dopamine release in response to cigarette smoking (Brody et al., 2006; Scott et al., 2007) and that nicotine intake is an important factor in dopamine release from smoking (Cumming et al., 2003; Marenco et al., 2004; Brody et al., 2006; Scott et al., 2007; Takahashi et al., 2008). Belin et al. found that the interactions between the ventral striatum and the dorsal striatum mediated by dopaminergic transmission play an important role in drug addiction (Belin and Everitt, 2008). In addition, Volkow et al.'s human neuroimaging studies also observed cocaine cue-induced increases in dopamine release in the dorsal striatum (Volkow et al., 2006). Cocaine cues have also been shown to elicit dorsal striatal dopamine release in animal studies (Ito et al., 2002). Therefore, we speculate that after dorsal striatum injury, dopamine release from the dorsal striatum induced by smoking cues is decreased, thus, blocking this addiction mechanism.

Second, the dorsal striatum is extensively linked to the orbitofrontal cortex (Fornito et al., 2013), and studies have confirmed that the orbitofrontal cortex is closely related to drug abuse and drug addiction (Kasanetz et al., 2013). The striatum

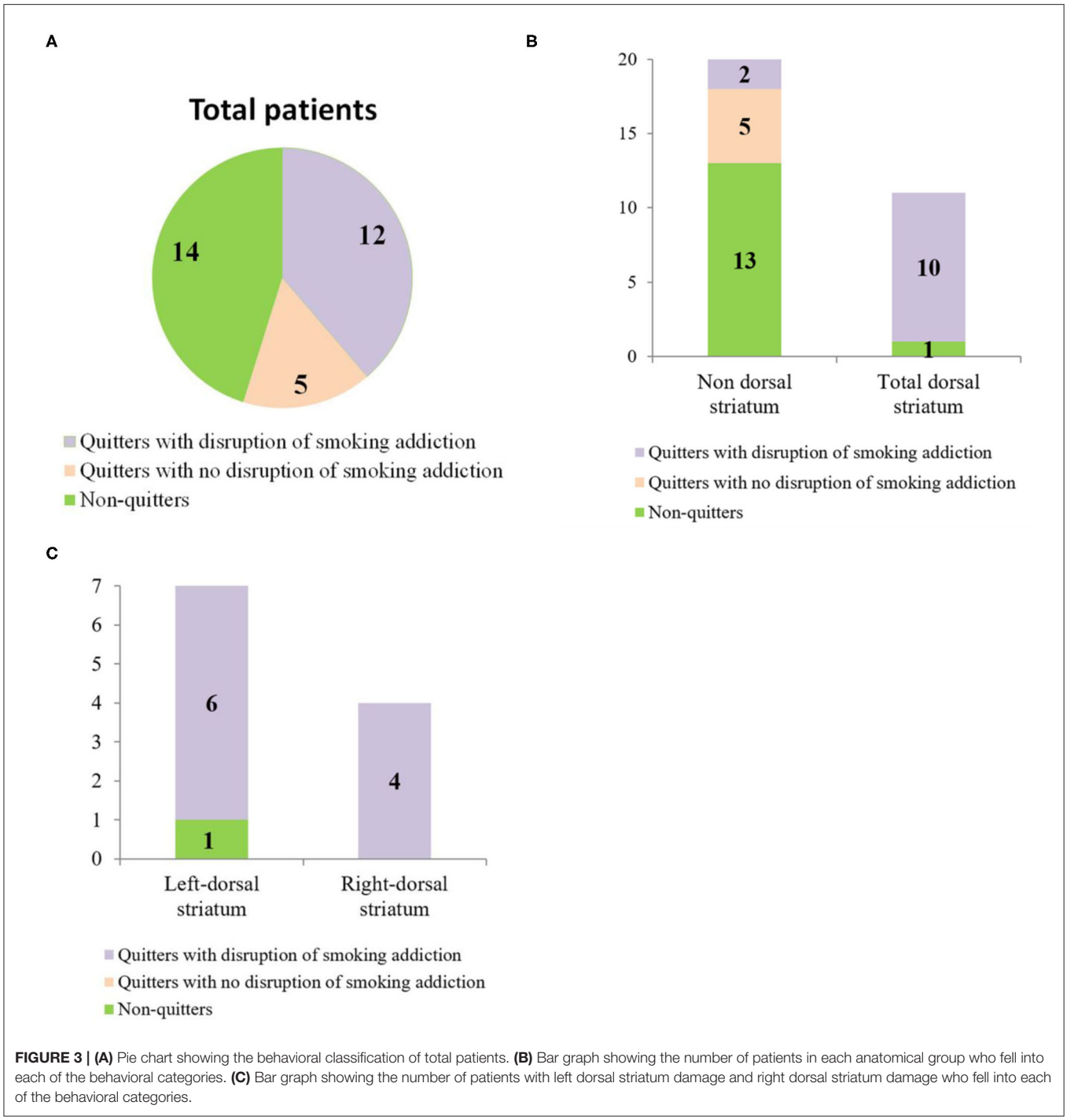

FIGURE 3 | (A) Pie chart showing the behavioral classification of total patients. **(B)** Bar graph showing the number of patients in each anatomical group who fell into each of the behavioral categories. **(C)** Bar graph showing the number of patients with left dorsal striatum damage and right dorsal striatum damage who fell into each of the behavioral categories.

TABLE 3 | Number of participants in different groups.

		NQ/n(%)	NDSA/n(%)	DSA/n(%)	Phi	*P*-value
Dorsal striatum	left	1 (7.1%)	0 (0.0%)	6 (50.0%)	0.726641	0.000608
	right	0 (0.0%)	0 (0.0%)	4 (33.3%)	0.774597	0.001412
	total	1 (7.1%)	0 (0.0%)	10 (83.3%)	0.794770	0.000015
Non-dorsal striatum	total	13 (92.9%)	5 (100.0%)	2 (16.7%)	–	–

NQ, Non-quitters; NDSA, Quitters with no disruption of smoking addiction; DSA, Quitters with disruption of smoking addiction.

TABLE 4 | Some studies on the disruption of addiction after injury to different brain regions.

Author	Participants	Etiologies	Lesion region	Addictive substances	Addiction time	Outcome
Miller et al. (2006)	1	Methadone overdose	Globus pallidus	Alcohol, LSD, marijuana, cocaine, opiates, and Ecstasy	>10 years	No longer experienced pleasure from drugs and four serial urine toxicology screens were negative for 6 months.
Béchir et al. (2010)	1	Acute hemorrhagic stroke	Posterior cingulate	Cigarettes	18 years	Continued to be abstinent after 12 months.
Muskens et al. (2012)	1	Ischemic stroke	Dorsal striatum	Cigarettes	>20 years	Continued to be abstinent after 3 months.
Moussawi et al. (2016)	1	Methadone overdose	Globus pallidus	Alcohol and opiates	Not mentioned	Continued to be abstinent after 10 years.
Gaznick et al. (2014)	63	Acute stroke	Basal ganglia and insula	Cigarettes	Not mentioned	Had significantly higher and more sustained rates of smoking cessation
Naqvi et al. (2007)	69	–	Insula	Cigarettes	>2 years	Likely to quit smoking easily.
Abdolahi et al. (2015)	156	Ischemic stroke	Insula	Cigarettes	Smoked at least one cigarette per day during the month prior to their stroke and at least 100 in their lifetime	Had a lower WSWS score and MNWS score, appeared to be less likely to use NRT during admission.

directly receives the projection of glutamate neurons from the orbitofrontal cortex, and addictive behavior may enhance the projection of the glutamate system from the orbitofrontal cortex to the dorsal striatum through repeated stimulation. Glutamate activates the neurons in the dorsal striatum, thus, increasing the release of dopamine in the striatum, and the increase of dopamine in the striatum activates GABA projection neurons, which express D1 receptors (Surmeier et al., 2007). Therefore, the inhibitory GABA neurons projecting to the substantia nigra pars reticulata (SNr) increase, which can inhibit the function of the SNr. Then, the inhibitory GABA neurons projecting from the SNr to the thalamus are reduced. Furthermore, the release of glutamate from the thalamus to the cortex is increased, and the high level of glutamate in the cortex is projected to the dorsal striatum through the cortex-striatum pathway; thus, forming a positive feedback mechanism. Damage to the dorsal striatum may hinder this feedback mechanism, thereby blocking the addiction process.

Moreover, numerous studies have found that cortical and subcortical structures play an important role in automatic behavior and motor planning (Johnson-Frey, 2004; Johnson-Frey et al., 2005; Lewis, 2006). Subjects with damage in these brain regions usually exhibit different types of apraxia or deficits in general action planning and execution (Lewis, 2006). Dorsal striatum circuits are known to interact with thalamic-cortical circuits that are involved in the planning and execution of motor responses. Smoking behavior becomes highly automatic after repeated practice in people with smoking addiction. We speculate that damage to the dorsal striatum may block the connection between the dorsal striatum and the thalamus, thus, impairing the planning and execution of smoking.

Our findings suggest that the dorsal striatum may play an important role in the process of smoking addiction. Therapies that modulate the function of the dorsal striatum may be useful to help smokers quit. However, we acknowledge that our sample sizes were too small. Thus, we look forward to more clinical studies in the future to provide more detailed explanations of the processes involved in addiction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of The First Affiliated Hospital of Xiamen University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ChuJ performed the statistical analysis. ChangJ drafted the manuscript. LZ, GH, JZ, LY, NS, and TZ carried out the acquisition of data. QM helped to draft the manuscript. JF conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

FUNDING

The project was funded by the grants from the Science and Technology Major Project of Xiamen (Grants nos. 3502Z20171005-20170801), the Medical Health Science and Technology Program of Xiamen (Grants nos. 3502Z20194002), the School Management Project of Fujian University of Traditional Chinese Medicine (Grants nos. XB2020160), and the Medical and Health Key Project of Xiamen (Grants nos. 3502Z20204006).

ACKNOWLEDGMENTS

We thank all the subjects who took part in this study for their collaboration. In addition, we thank Dr. Fengyang Cao for her assistance with the statistical analysis and Zhimin Wei for his assistance with MRI acquisition.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

OPINION
published: 13 April 2021
doi: 10.3389/fnbeh.2021.660667

Neuromodulation of Foraging Decisions: The Role of Dopamine

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Keywords: decision making, dopamine, foraging, neuromodulation, patch-leaving

When searching for food, animals need to decide whether they can maximize rewards by harvesting at a current resource, or whether they should instead leave for another foraging site. Humans face similar types of problems when deciding whether to stay with their current job, or to move to a new one with a prospect of better career opportunities. Such decisions to leave, often referred to as patch-leaving decisions, require dynamically weighing the time and energy costs of leaving, as well as the benefits of encountering more rewarding resources at new locations. How neuromodulators are involved in patch-leaving decisions, especially in humans, is, at present, scarcely researched. In their recent study, Le Heron et al. (2020) fill this gap by investigating how these decisions are causally affected by dopaminergic state in an ecologically valid foraging scenario. In their study, participants could choose between collecting reward (milk filling a bucket) at one location (patch) or leaving for another patch which incurred a cost in the form of a fixed travel time. As soon as participants started harvesting (collecting milk) from one patch, the reward per time in that patch decreased exponentially, emulating a depleting resource. To maximize their reward rate, participants were thus faced with the task of continuously comparing the rewards at current location against potential rewards at other locations, whilst taking into account the time cost for leaving.

The optimum solution to this foraging problem is given by the Marginal Value Theorem (MVT, Charnov, 1976; Stephens and Krebs, 1986), which has been shown to predict foraging behavior in many species (Cassini et al., 1993; Hayden et al., 2011). MVT states that the optimal time to leave the current patch is when its marginal reward rate ("foreground") drops below the average reward rate in the environment ("background"). To separately manipulate background and foreground reward rates, the authors created patches that differed in their (initial) reward rates (low, medium, and high yield). These could be encountered in either a rich or poor environment. In the rich environment, participants were most likely to transition to a high yield patch upon leaving the current patch, whereas in the poor environment, encountering a low yield patch was most likely. The reward obtained in the current patch thus constituted the foreground, whereas the proportion of the different patch types determined the background reward rate. MVT predicts that optimally behaving agents will.

H1: leave patches within an environment (i.e., equal background) at the same reward rate for all patch types; therefore leave patches with lower initial foreground reward earlier than patches with higher foreground reward.

H2: leave earlier in general when in rich compared to poor environments (high vs. low background reward rate).

A main effect of background reward rate on patch leaving times was observed, supporting H2. In contrast, pertaining to H1, participants left patches with lower foreground reward rate earlier, but they seemed to exhibit a tendency to stay longer in high yield patches, in contrast with the prediction that at leaving, the foreground rate is the same for all patch types. Additionally, participants stayed in patches longer than optimal ("overharvested") across all patch types, leading to less reward obtained than predicted by MVT. Overharvesting is a phenomenon reported

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *01 February 2021*
Accepted: *15 March 2021*
Published: *13 April 2021*

Citation:
Marzecová A, Kaiser LF and Maddah A (2021) Neuromodulation of Foraging Decisions: The Role of Dopamine. Front. Behav. Neurosci. 15:660667. doi: 10.3389/fnbeh.2021.660667

ubiquitously in the foraging literature (see e.g., Hayden et al., 2011; Kane et al., 2019) and has been related to different factors, including time preferences (Kane et al., 2019), and behavioral variability (Cash-Padgett and Hayden, 2020).

Evidence on neuromodulatory mechanisms underlying value comparisons in foraging environments remains scant. Tonic dopamine (DA) levels have been previously suggested to scale with the average background reward rate (average of prediction errors) in the environment (Niv et al., 2007; Beierholm et al., 2013) and could therefore be considered a key element in signaling decisions to leave a patch (Constantino et al., 2017). Le Heron et al. (2020) thus hypothesized that tonic DA levels would modulate the impact of the background, but not the foreground reward rate on patch-leaving decisions. To test this hypothesis, a group of elderly participants was tested twice on the foraging task under the influence of either placebo or the D2 receptor agonist cabergoline. When "on" cabergoline, participants left patches in the poor environment earlier. In contrast, cabergoline did not modulate the effect of the foreground reward rate on patch leaving. This pattern resonates well with the hypothesized role of tonic DA in encoding the average background reward rate. Since participants generally overharvested, this may also imply a shift toward more optimal behavior.

A 1 mg dose of cabergoline was hypothesized to specifically influence the perceived background reward by increasing tonic DA levels, acting via postsynaptic mechanisms (Brooks et al., 1998). However, there have been discussions of whether similar doses of D2 agonists would instead impact phasic rather than tonic DA signaling (Santesso et al., 2009; Norbury et al., 2013) through a modulation of presynaptic autoreceptors (Frank and O'Reilly, 2006). Given that there has been no possibility to assess pre- vs. post-synaptic medication effects in the current study, one may not exclude the possibility that the cabergoline dose resulted in a reduction of the phasic tone (Frank and O'Reilly, 2006). A recent study has shown that a reduction of phasic DA may lead to an increase in (random) exploration (Cinotti et al., 2019), and could thus promote patch-leaving behavior. Contributions of both the phasic and the tonic mode in modulating perceived background reward rate may be considered, bearing in mind it has recently been suggested that the distinction between tonic and phasic DA release and its relation to behavior may not be as clear-cut as previously thought (Berke, 2018).

In another recent study, DA depletion associated with Parkinson's disease (PD), has been linked to a lower estimate of background reward rates in a previous study. PD patients overharvested to a larger extent than control participants when "off" DA medication, while their performance was comparable to controls when "on" medication (Constantino et al., 2017). In that study, the richness of the environment varied due to long and short travel costs. Notably, the difference in leaving time between control and PD participants was more pronounced in the richer (short travel) environment. This may imply multiplicative effects on the perceived richness of the environment, but contrasts with Le Heron et al. (2020) finding of effects in poorer environments only. Since participants in both studies discussed above can be assumed to differ with respect to their baseline DA levels, and potential compensatory changes to DA systems, different ceiling effects may have brought about differing patterns of results. Noteworthy, Le Heron et al. (2020) increased DA levels by targeting D2 receptors, while the depletion of DA in PD is likely to affect both D1 and D2 type receptors (Seeman and Niznik, 1990). However, D2 receptors, owing to their higher affinity for DA, may be still sensitive to (subtle) variations in DA concentration in PD patients. Additionally, whether the effects of DA manipulation extend to younger healthy populations (with likely higher baseline DA levels) is an open question. Future work should seek to delineate under which specific circumstances DA modulates the influence of perceived environmental richness on behavior.

Importantly, the specific drug effects might potentially be considered in relation to different manipulations of environmental richness in the two studies. According to MVT, the background reward rate is determined by the value of potential alternatives as well as by costs of accessing these options. During traveling, the net reward intake is zero, therefore the agent needs to consider whether the potential benefits in alternative patches are worth the invested cost of time (i.e., the foregone reward while traveling). As in Constantino et al. (2017) study, decreasing travel time costs should lead to earlier patch leaving, since it translates into an increased background reward rate. Travel times have been previously found to influence the leaving threshold in patch leaving tasks (Hayden et al., 2011; Wolfe, 2013; Ramakrishnan et al., 2019). In the study by Le Heron et al. (2020), travel costs were kept constant in both environments. However, since the average expected reward rate is different in both environments, the opportunity costs of time differ. The equal travel times therefore potentially have a distinct effect in poor and rich environments. While the relationship between DA modulations and subjective travel cost estimates has been scarcely addressed so far (Constantino et al., 2017), there is a rich literature about the effects of DA on cost-benefit decisions (Salamone et al., 1994; Beeler and Mourra, 2018). In these paradigms, subjects usually decide whether a potential outcome is worth a certain effort, which is a conceptually similar question as in the reported foraging scenario: "Is my investment worth the expected payoff?". A potential route to an increase in the subjective estimate of environmental richness may be a decrease in the subjective estimate of the opportunity costs of time. It would be interesting for further research to explicitly vary travel time costs to assess the contribution of costs to estimates of environmental richness. Combining the experimental manipulations of travel time costs (Constantino et al., 2017) and patch reward yield proportions determining environmental richness (Le Heron et al., 2020) could thus prove useful to further a comprehensive framework on how DA modulates patch-leaving. To build a full picture of dopaminergic control of patch-leaving behavior, future research should systematically consider pharmacological effects of particular drug manipulations, behavioral consequences of experimental manipulations, and the extent to which learning takes place in the task.

Research on the role of other neuromodulators implicated in patch-leaving decisions has started to emerge. The locus-coeruleus (LC) noradrenaline system may be involved in patch-leaving, as it promotes behavioral flexibility (Aston-Jones and Cohen, 2005). A recent study reported that tonic LC stimulation in rats led to an earlier patch leaving, which was related to an increased decision noise (Kane et al., 2017). Conversely, optogenetic stimulation of serotonergic cells in the dorsal raphe nucleus led to later leaving times in a patch leaving task (Lottem et al., 2018). Additionally, a recent whole-brain imaging evidence showing that persistent serotonergic activity correlates with a state of exploitation (Marques et al., 2020). Furthermore, GABA and glutamate concentrations in the anterior cingulate cortex have been shown to predict patch-leaving behavior in healthy participants (Kaiser et al., 2021).

Understanding patch leaving decisions and their underlying neurochemical mechanisms is of fundamental relevance to understanding many neuropsychiatric disorders (Addicott et al., 2015). Le Heron et al. (2020) and Constantino et al. (2017) therefore provide new evidence of high practical importance by exploring a modulatory role of DA in the encoding of background reward rates in patch leaving decisions.

AUTHOR CONTRIBUTIONS

AMar: conceptualization (equal), investigation (equal), writing-original draft (lead), writing-review, and editing (equal). LK: conceptualization (equal), investigation (equal), writing-original draft (supporting), writing-review, and editing (equal). AMad: conceptualization (equal), investigation (supporting), writing-review, and editing (equal). All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Research Foundation Flanders (FWO) postdoctoral fellowship (12V5620N) and a grant from the Deutsche Forschungsgemeinschaft (JO-787/6-1).

ACKNOWLEDGMENTS

We thank Monja Froböse, Tom Verguts, Bahador Bahrami, Gerhard Jocham, and two reviewers for helpful discussions on the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 28 May 2021
doi: 10.3389/fnbeh.2021.663761

Anhedonic Type Behavior and Anxiety Profile of Wistar-UIS Rats Subjected to Chronic Social Isolation

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: 03 February 2021
Accepted: 26 April 2021
Published: 28 May 2021

Citation:
Acero-Castillo MC, Ardila-Figueroa MC and Botelho de Oliveira S (2021) Anhedonic Type Behavior and Anxiety Profile of Wistar-UIS Rats Subjected to Chronic Social Isolation. Front. Behav. Neurosci. 15:663761. doi: 10.3389/fnbeh.2021.663761

Chronic Social Isolation (CSI) is a model of prolonged stress employed in a variety of studies to induce depression and anxious behavior in rats. The present study aims to evaluate the effect of CSI on male Wistar rats in terms of "anhedonic-type" behavior in the Sucrose Preference Test (SPT) and anxiogenic profile in the elevated-plus-maze (EPM) test, as well as evaluating the effect of resocialization upon sucrose consumption. A total of 24 adolescent male Wistar rats were evaluated. The animals were housed either together (communally) or socially isolated for 21 days, and then exposed for four consecutive days to the SPT test [water vs. a 32% sucrose solution (SS)]. Four days later, they were again subjected to the SPT test (32% vs. 0.7% SS), and then tested on the EPM apparatus 3 days later. Following the completion of the anxiogenic profile of the model, the animals were resocialized for 72 h and then re-tested once again using the SPT (32% vs. 0.7% SS). Twenty-four hours after this final consumption, the animals were euthanized to record the weight of their adrenal glands (AG). It was found that exposure to CSI produces anhedonic-type behavior and an anxiogenic profile in adolescent male rats, as evidenced in both the SPT and EPM tests, as well as in the animals' physiological stress response. It was also demonstrated that resocialization does not reverse the anhedonic-type behavior, nor the physiological response to stress.

Keywords: anhedonia, anxiety, depression, chronic social isolation, resocialization

INTRODUCTION

Exposure to stress, especially at early ages, has been shown to be a determining factor in the appearance of depression and anxiety in humans (Starr et al., 2016; Bavley et al., 2017; Gawali et al., 2017; Sangenstedt et al., 2017; Smith and Pollak, 2020). Chronic Social Isolation (CSI) in rodents and non-human primates has been used to model neurobiological alterations linked to

such behaviors as depression, anhedonia, and, anxiety (Botelho et al., 2007; Fone and Porkess, 2008; Starr et al., 2016; Shetty and Sadananda, 2017; Mumtaz et al., 2018; Brenes et al., 2020); as well as the development of post-traumatic stress (Berardi et al., 2014). In particular, the Sucrose Preference Test (SPT) has been employed to understand the mechanisms underlying anhedonia. This animal experimentation model is based on the appetitive nature of sweet solutions (Cantora and López Ramírez, 2005; Díaz et al., 2010; Alvarez, 2015), wherein the preference pattern of sucrose solutions (SS) at varying concentrations (0.7%, 1%, 2%, 4%, 5%, 8%, 12%, 16%, 32%, and 35%) is used as an indicator of anhedonic-type behavior (Muscat et al., 1991; Grippo et al., 2003; Cortés et al., 2005; Martínez et al., 2008; Rodríguez et al., 2012; Páez-Ardila and Botelho, 2014; He et al., 2020; Wang et al., 2020; Wright et al., 2020).

In this context, an array of studies have found that animals subjected to CSI consume a greater quantity of high-concentration SS (24%, 32%, and 34%; Muscat et al., 1991; Hall et al., 1997; Brenes Sáenz et al., 2006; Brenes and Fornaguera, 2008, 2009; Wright et al., 2020), even when given the option to choose solutions of various concentrations (Sammut et al., 2002; Martínez et al., 2008). Such alternations in reward–sensitivity have been widely found to be related to the mesolimbic dopaminergic system (Fone and Porkess, 2008; Rodrigues et al., 2011; Noschang et al., 2021). It has also been found that this pattern of consumption can be reversed using antidepressant drugs such as fluoxetine (Brenes and Fornaguera, 2009). The majority of studies have interpreted increased consumption of high-concentration SS as enhanced incentive motivation and reward–sensitivity. In addition, when other models of chronic stress are used, reduced consumption of low–concentration SS has been reported (0.1%, 1%, and 2%; Willner et al., 1987; Papp et al., 2016; Qin et al., 2017; He et al., 2020; Wang et al., 2020). In this context, anhedonia can be defined as a low reward–sensitivity (Rygula et al., 2005; Willner, 2005).

Social isolation also produces anxiety-type behaviors in rodents, which are demonstrated by their performance in tests such as the Open Field and Elevated-Plus-Maze (EPM; Botelho et al., 2007; Pritchard et al., 2013; Kumari et al., 2016; Shetty and Sadananda, 2017; Sequeira-Cordero et al., 2019). The EPM is one of the most widely used models for the evaluation of anxiety-related behavior (Botelho et al., 2007; Amancio-Belmont et al., 2020). It is based upon the natural preference of these animals for dark and protected places, and their anxiety is thus measured by the proportion of test time that they remain in the closed arms of the maze, without coming into contact with the open areas (Pellow et al., 1985; Botelho et al., 2007; Casarrubea et al., 2016). Research indicates that exposure to social isolation in adolescence results in an increased level of locomotor activity, as well as a reduced number of entries into—and time spent in—the open arms of the maze (Kumari et al., 2016; Shetty and Sadananda, 2017; Amancio-Belmont et al., 2020); a pattern which has shown itself to be reversible through the use of various types of anxiolytic drugs such as Diazepam, Alprazolam y Pentylenetetrazol (Pellow et al., 1985; Sorregotti et al., 2013; Sprowles et al., 2021).

In general, the behavioral, cognitive, and neural effects resulting from social isolation can be reversed by means of resocialization (Wright et al., 1991; Chen et al., 2016; An et al., 2017), however, this method cannot reverse the effects of social isolation imposed immediately after weaning (Einon and Morgan, 1977). Nevertheless, when social isolation is applied during adolescence or adulthood, a variety of the effects caused thereby can indeed be reversed by means of resocialization (Rivera et al., 2021).

This study takes into account the potential of the social isolation to model the neurobiology of depression and anxiety; and operationalizes an increase in the consumption of high–concentration sucrose solutions as an indicator of anhedonic-type behavior potentially associated with an elevated pleasure threshold. The objective of the present study is therefore to evaluate the effect of CSI in male Wistar rats upon their anhedonic-type behavior in the SPT and their anxiogenic profile in the EPM. Additionally, the study aims to evaluate the effect of resocialization upon sucrose consumption.

MATERIALS AND METHODS

Subjects

The present study employed 24 male adolescent Wistar rats, each weighing between 135 and 170 g (Nistiar et al., 2012), and sourced from the vivarium of the Industrial University of Santander (UIS). The test animals were given ad libitum access to food and water, and were handled only for the provision of food and daily hygiene under a controlled lighting regimen of 12 h light and darkness (with the lit period beginning at 07:00) and maintained at an ambient temperature of 22°C. The test subjects were housed either individually (in the case of the experimental group), or communally (the control group). In the former group (n = 12), each rat was housed in an acrylic box which allowed for visual, olfactory, and auditory contact with the other animals, but without providing an opportunity for physical contact. The remaining rats (n = 12) were housed in groups of six per box; these being considered as the control group. All of the experiments were performed in accordance with the approved ethical standards for animal experimentation (Congreso Nacional de la República de Colombia, 1989).

Materials

Acrylic boxes measuring 23 × 23 × 34 cm were provided for individual housing, with stainless steel boxes of 40 × 33 × 16 cm used for communal housing. SPT: the reward response of the animals was measured by evaluating their preference for appetizing sucrose solutions over pure water (Willner et al., 1987). EPM: this maze was constructed using a four-armed wooden cross raised 50 cm from the ground. Two of the arms measured 50 × 10 cm and were open (unwalled), while the other two arms measured the same, but included walls 40 cm in height, and were open-roofed. The two open and two closed arms were arranged perpendicular to each other (Pellow et al., 1985). The

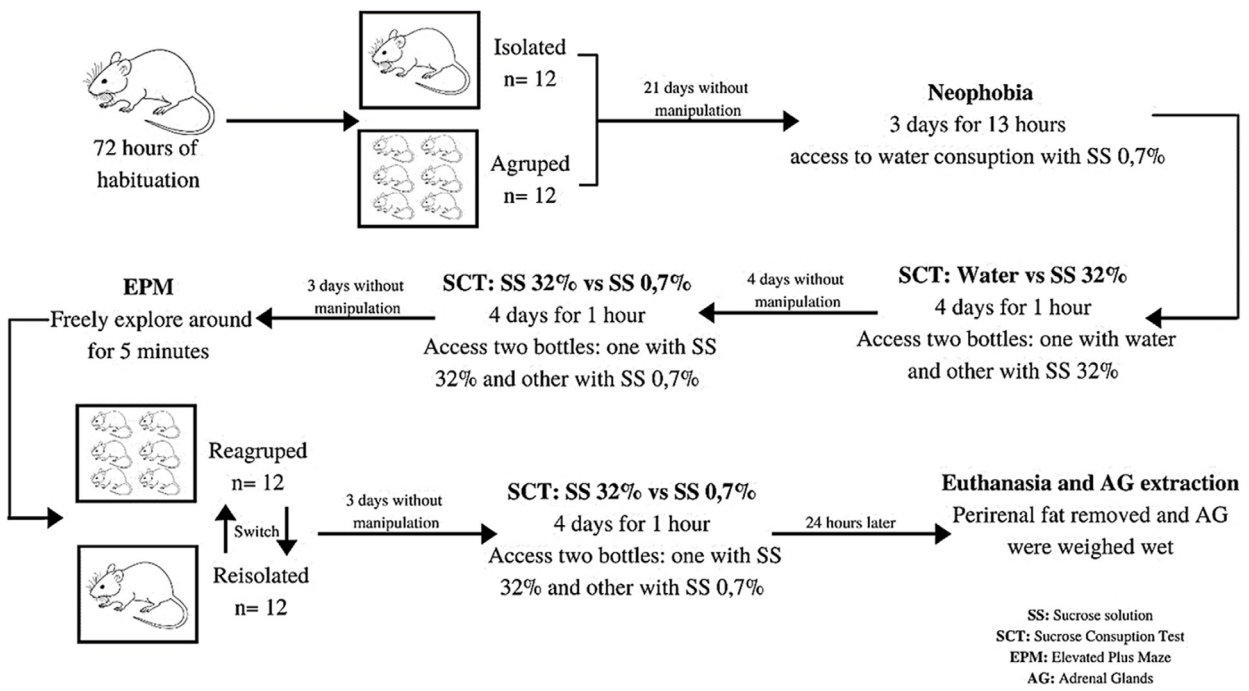
frequency with which the animals entered and the time they remained, in the open arms of the maze were used as operational indicators for anxiety-type behavior in this model (Pellow et al., 1985; File and Zangrossi, 1993). The percentage of instances in which the animals entered the open arms were calculated in relation to the total number of times they entered any arm. The total number of entries into both the open and closed arms, as well as the number of times the rats crossed between arms, were taken as an operational indicator of their level of locomotor activity (Pellow et al., 1985; Botelho et al., 2007). Ethogram: a Microsoft Excel file was created to record the frequency of entries and time of stay in each of the arms of the maze (Conde, unpublished data; Ethogram is a file programmed in Excel by one of the researchers of the research group for recording behavior of rats in the Elevated Cross Maze).

Procedure

Upon their arrival in the laboratory, the rats were given 72 h to adapt to their environment, after which they were randomly assigned into two housing regimens—individual and communal—for 21 days (Brenes and Fornaguera, 2009; Weintraub et al., 2010). Thereafter, they were given access to a drinking spout which provided a 0.7% SS. This spout was made available for 13 h each day (18:00–07:00) over a period of 3 days for the purpose of habituating the animals to the consumption of sucrose and avoiding possible neophobia (Hall et al., 1997). Upon the completion of these 3 days of habituation, the animals were evaluated with respect to their sucrose consumption preferences over the following four consecutive days. On each of those days, the rats were moved to the testing area and kept for 1 h in an acrylic box (with the same characteristics as those used for individual housing) where they were given access to two drinking spouts: one which provided pure water and the other a 32% sucrose solution. The consumption of the rats was measured for six animals simultaneously; three of the individually housed rats, and three of those housed communally. The schedule for these consumption evaluations was assigned randomly (**Scheme 1**).

Following the evaluation of their consumption preferences for the 32% sucrose solution, the animals were allowed to rest for 4 consecutive days in the same housing conditions as they had previously experienced (i.e., individual or communal), and provided only pure water to drink. Thereafter, they were once again evaluated for their preference for SS at 32% and 0.7% for a further four consecutive days, using the same evaluation procedure as described previously (**Scheme 1**).

Seventy-two hours after the completion of this latest round of SPT evaluation, the animals were tested *via* the EPM procedure. This test consisted of placing the animal in the center of the maze, facing towards one of the closed arms, and allowing it to explore freely for 5 min (**Scheme 1**). During this time, its number of entries into the open and closed arms, and the duration of its stay therein, was recorded. One entry was defined to occur when the animal positioned itself with all four of its paws within the arm of the maze (Pellow et al., 1985). Each of these sessions was monitored and recorded *via* closed-circuit television. The



SCHEME 1 | Experiment model.

sessions were recorded "online" by a video camera positioned over the maze, with the videos analyzed "offline" by the same researcher, with the results recorded using the ethogram (Conde, unpublished data).

Following the completion of the latter evaluation, the animals were resocialized, with the housing regimen reversed. The animals previously housed individually were now housed communally, and those previously housed communally were placed into individual boxes. Seventy-two hours after this resocialization (Páez-Ardila and Botelho, 2014), the rats were exposed to one final round of SPT (32% and 0.7% SS) using the same protocol as previously described (**Scheme 1**). It was considered that the effect of re-exposing the rats to the EPM could generate a degree of acquisition and retention, with potential changes to the animals' behavior due to their experience in the first exposure (Rodgers et al., 1996; Lamprea et al., 2000; Belviranli et al., 2012). This, in turn, could result in an intensification of fear and generate a state of phobia (File and Zangrossi, 1993), thus, the effect of the resocialization upon the rats' anxiogenic profile was not evaluated. Finally, 24 h after the last day of evaluation using the SPT (at 32% vs. 0.7%), the rats were euthanized, and their adrenal glands (AG) were extracted. The periadrenal fat was removed, and the wet weights of the glands were measured as a physiological indicator of stress (Selye, 1936; Grippo et al., 2003; Rygula et al., 2005; **Scheme 1**).

ANALYSIS OF THE RESULTS

To evaluate the effects of CSI upon sucrose consumption for days 1 (D1), 2 (D2), 3 (D3), and 4 (D4) of the first and second exposures, a 3-way analysis of variance (ANOVA) was carried out, followed by a *t*-test for multiple comparisons (using the Holm–Sidak method).

Factor 1 refers to the GROUP (type of housing: individual or communal); factor 2, to the CONCENTRATION of the sucrose solutions (pure water, 0.7%, and 32%); and factor 3, to the DAY of exposure. To evaluate the effects of CSI on the anxiety-type behavior in the EPM and the weight of the AG, analysis was performed using *t*-tests for independent samples; parametric or non-parametric, depending on the distribution of the data and the homogeneity of the sample (student's *t*-test or Mann–Whitney, respectively). These tests compared the two groups: isolated (AISL) and communal (AGRUP).

Finally, to evaluate the effects of the 72 h of resocialization upon the consumption of sucrose for days 1, 2, 3, and 4; a three-way ANOVA test was used, followed by a *t*-test for multiple comparisons (Holm–Sidak method). Factor 1 refers to the GROUP [type of housing: REAIS (Animals initially communal and later isolated) vs. REGRUP (Animals initially isolated and later communal)]; factor 2, to the CONCENTRATION of the sucrose solutions (0.7% and 32%); and finally, factor 3 to the DAY of exposure. In all statistical analyses, significance was defined as $p < 0.05$. All of the statistical analyses were performed using SigmaStat software (Systar Software Inc., 2016).

RESULTS

Consumption of Pure Water vs. 32% Sucrose Solution

For the first exposure (pure water vs. 32% sucrose solution), it was found that the factors of group ($F_{(1,176)} = 29.667$, $p < 0.001$), concentration ($F_{(1,176)} = 494.048$, $p < 0.001$), and day ($F_{(3,176)} = 9.583$, $p < 0.001$), as well as the interaction between group and concentration ($F_{(1,176)} = 56.397$, $p < 0.001$) and the interaction between concentration and day ($F_{(3,176)} = 19.412$, $p < 0.001$) were sources of variation. The Holm–Sidak *t*-test for multiple comparisons showed that the two groups, both isolated ($t = 21.027$) as well as communal ($t = 10.407$), consumed more of the 32% sucrose solution than pure water. Nevertheless, the isolated rats consumed more of the sucrose solution ($t = 9.162$) than did the other group.

With respect to the per day consumption of SS, the analyses demonstrated that the isolated animals consumed more on D4 as compared to D1 ($t = 4.873$; $p = 0.009$) and D2 ($t = 2.896$; $p = 0.01$), however, no statistically significant differences were found in the consumption of SS between D4 and D3 ($t = 2.193$; $p = 0.017$). Similarly, they consumed more on D3 as compared to D1 ($t = 2.68$; $p = 0.013$). For their own part, the communally housed animals consumed more SS on D3 than on D1 ($t = 3.077$; $p = 0.009$). Also, specifically on D2 ($t = 3.138$; $p = 0.05$) and D4 ($t = 4.691$; $p = 0.05$), it was the isolated animals who consumed more.

In accordance with the previous results, it can be seen that both the communally housed and isolated animals consumed more of the SS on D4 ($t = 8.446$; $p = 0.009$), D3 ($t = 6.923$; $p = 0.01$), and D2 ($t = 3.725$; $p = 0.017$), as compared with D1. It is also evident that on D1 ($t = 5.339$; $p = 0.05$), D2 ($t = 10.242$; $p = 0.05$), D3 ($t = 13.427$; $p = 0.05$), and D4 ($t = 15.447$; $p = 0.05$), the animals consumed more of the 32% SS than of pure water (**Figure 1**).

Consumption of 32% vs. 0.7% Sucrose Solution

The 3-way ANOVA carried out for the analysis of the second exposure (consumption of 0.7% vs. 32% sucrose solution), revealed statistically significant differences related to the group ($F_{(1,176)} = 64.413$, $p < 0.001$) and concentration ($F_{(1,176)} = 1051.101$, $p < 0.001$) variables. It also indicated that the interaction between these two factors ($F_{(1,176)} = 52.138$ $p < 0.001$) was a source of variation.

According to the Holm–Sidak *t*-test, both groups, the isolated ($t = 28.031$; $p < 0.05$) as well as the communally housed rats ($t = 17.819$; $p < 0.05$), consumed more of the 32% SS than the 0.7% solution. Nevertheless, the group of isolated rats exhibited a higher consumption of the 32% solution ($t = 10.781$; $p < 0.05$) than that of the communally housed animals.

Similarly, socially isolated animals consumed more than the grouped animals on days D1 ($t = 2.957$; $p = 0.05$), D2 ($t = 5.152$; $p = 0.05$), D3 ($t = 2.762$; $p = 0.05$), and D4 ($t = 5.181$; $p = 0.05$). On D4, a higher consumption of SS 32% was recorded as compared to D2 ($t = 3.246$; $p = 0.009$) and D3 ($t = 2.643$; $p = 0.01$); and

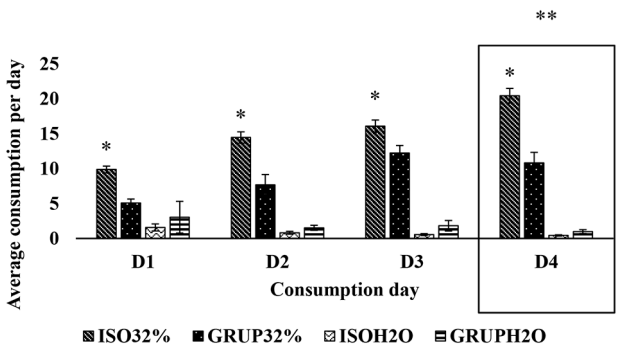


FIGURE 1 | Consumption of pure water and 32% sucrose solution by groups on D1, D2, D3, and D4. Average consumption (PROM ± EE) of pure water vs. 32% SS on D1, D2, D3, and D4, of the groups ISO32% (isolated 32%); GRUP32% (grouped 32%); ISOH2O (isolated water); and GRUPH2O (grouped water). *Higher consumption of 32% SS tan water in isolated D1, D2, D3 y D4. **Higher consumption of 32% SS in isolated and grouped on D4 than D1, D2, and D3 (Holm–Sidak Test *t*, $p < 0.05$).

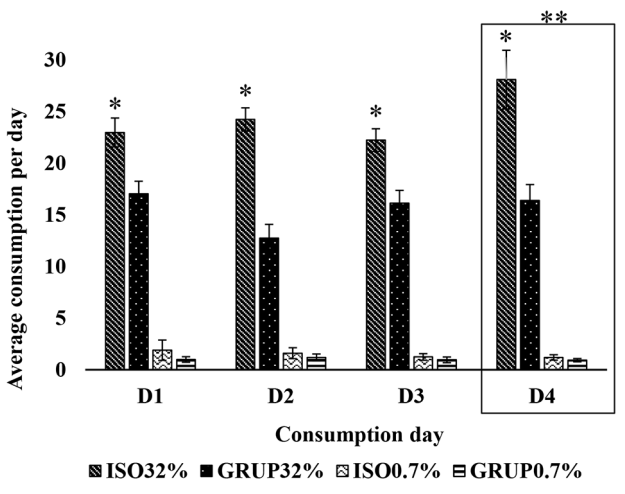


FIGURE 2 | Consumption of 32% sucrose solution vs. 0.7% sucrose solution by groups on D1, D2, D3, and D4. Average consumption (PROM ± EE) of 32% SS vs. 0.7% SS on D1, D2, D3 and D4, in the groups ISO32% (isolated 32%); GRUP32% (grouped 32%); ISO0.7% (isolated 0.7%); GRUP0.7% (grouped 0.7%). *Higher consumption of SS in ISO32% than ISO0 7%. **Higher consumption of 32% SS in isolated and grouped on D4 than D1, D2 and D3 (Holm–Sidak Test *t*, $p < 0.05$).

on days D1 ($t = 16.07$; $p < 0.05$), D2 ($t = 14.791$; $p < 0.05$), D3 ($t = 15.651$; $p < 0.05$), and D4 ($t = 18.33$; $p < 0.05$), the animals of both groups consumed a greater quantity of the 32% sucrose solution (**Figure 2**) than of the 0.7% solution.

Taken together, the results obtained from the SS consumption tests demonstrate that both in conditions of CSI or communal housing, the subject animals prefer to consume more of the sweeter solution (32%) than of the less sweet (0.7% or water); although in the socially isolated rats, this preference was seen to be more pronounced than in the communally housed ones.

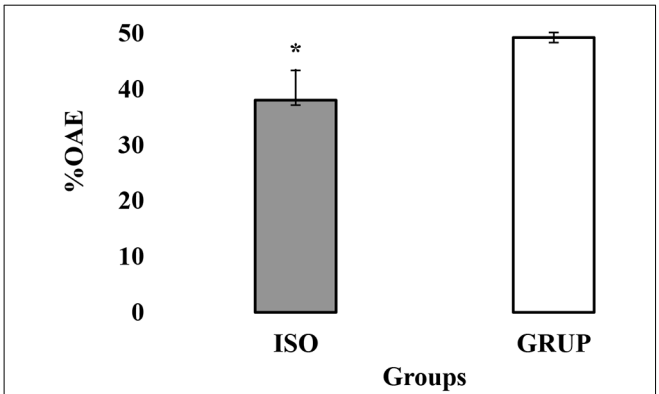


FIGURE 3 | Exploration of the open arms of the elevated-plus-maze (EPM). Exploration of the open arms of the EPM. OAE y %OAE (PROM ± EE) in EPM, in the groups ISO (isolated) y GRUP (grouped). *Less %OAE in ISO than GRUP; Mann–Whitney Test *t*, $p < 0.05$.

Exploration in the Elevated-Plus-Maze (EPM)

In general terms, no statistically significant differences were found between the time and percentage of stay in the open arms (TBA) and closed arms (TBC) of the EPM between the rats assigned to the AISL and AGRUP groups ($p > 0.05$). Nonetheless, the student's *t*-test indicated that the animals from the AISL group entered the open arms with lower frequency (EBA; $t = -2.314$, $p = 0.030$) and %EBA ($U = 110.500$, $p = 0.0024$) than those assigned to AGRUP (**Figure 3**). Logically, then, the AGRUP rats entered the closed arms of the maze (%EBC) less often than those of the AISL group ($t = -2.231$, $p = 0.036$).

The student's *t*-test revealed a statistically significant difference between the AISL and AGRUP groups with respect to locomotor activity. The AISL animals exhibited a lower number of total entries (EBA+EBC; $t = -2.283$, $p = 0.032$) and number of crossings of the EPM ($t = -2.378$, $p = 0.026$) than the AGRUP animals.

Consumption of 32% vs. 0.7% Sucrose Solution After 72 h of Resocialization

To analyze the second exposure to 32% vs. 0.7% SS after 72 h of resocialization, a 3-way ANOVA was carried out, which indicated that the factors of group ($F_{(1,176)} = 13.815$, $p < 0.001$), concentration ($F_{(1,176)} = 2490.816$, $p < 0.001$), and day ($F_{(3,176)} = 22.563$, $p < 0.001$) were all sources of variation. Statistically significant differences were also found for the interactions between group and concentration ($F_{(1,176)} = 13.623$, $p < 0.001$), group and day ($F_{(3,176)} = 2.954$, $p = 0.034$), and concentration and day ($F_{(3,176)} = 25.654$, $p < 0.001$).

The Holm–Sidak *t*-test indicated that the two groups, REAIS ($t = 32.68$; $p < 0.05$) and REGRUP ($t = 37.9$; $p < 0.05$), consumed more of the 32% solution than of the 0.7%. Nevertheless, it was the REGRUP group that consumed a greater quantity of the 32% solution ($t = 5.238$; $p < 0.05$). Thus, the animals originally subjected to CSI for 21 days consumed a greater quantity of the sweeter solution, suggesting that their subsequent resocialization

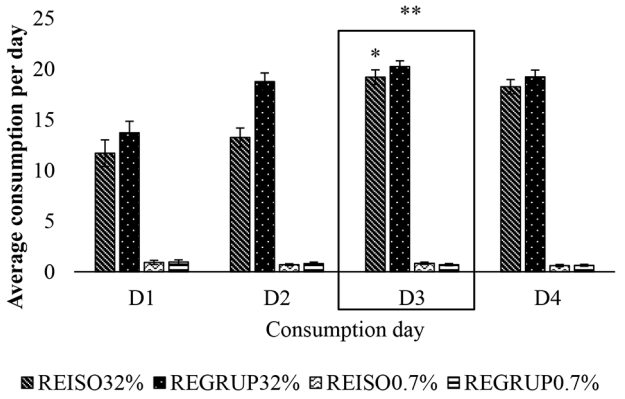


FIGURE 4 | Average consumption of 32% sucrose solution vs. 0.7% sucrose solution on D1, D2, D3, and D4 after exposure to 72 h of resocialization. Average consumption (PROM ± EE) of 32% SS vs. 0.7% SS on D1, D2, D3, and D4, in the groups REISO32% (Grouped animals that became isolated 32%); REGRUP32% (Isolated animals that became grouped 32%); REISO0.7% (Grouped animals that became isolated 0.7%); and REGRUP0.7% (Isolated animals that became grouped 0.7%) after 72 h of resocialization. *Higher consumption of SS in ISO32% than ISO0.7% on D4, D2, D1 and D3. **Higher consumption of SS in ISO32% than ISO0.7% on D3 than D4, D2 and D1 (Holm–Sidak Test *t*, $p < 0.05$).

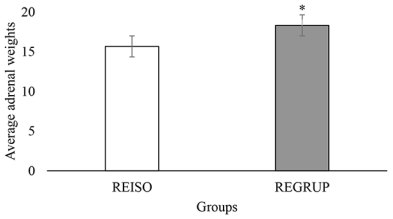


FIGURE 5 | Average weight of adrenal glands (AG) by group. Average weight (PROM ± EE) of the AG in the REISO (grouped animals that became isolated) and REGRUP (isolated animals that became grouped) groups. *Greater weight of the AG in REGRUP than REISO (Student Test *t*, $p < 0.05$).

did not reverse the effect of chronic stress upon their preference for 32% SS.

The animals were found to consume more of the sucrose solution on days D3 ($t = 10.943$; $p = 0.009$), D4 ($t = 9.406$; $p = 0.01$), and D2 ($t = 3.304$; $p = 0.017$), as compared to D1; and more on D3 ($t = 5.799$; $p = 0.013$) and D4 ($t = 4.262$; $p = 0.025$) than on D2. As well, it can be seen that on days D1 ($t = 18.325$; $p < 0.05$), D2 ($t = 23.748$; $p < 0.05$), D3 ($t = 29.527$; $p < 0.05$), and D4 ($t = 28.217$; $p < 0.05$) the animals consumed more of the 32% sucrose solution than the 0.7%, most notably so on D3 (**Figure 4**).

Considering the importance of having objective measurements of emotion in rats, the present study used the weight of the AG (measured as a function of the animal's total weight 24 h after the final SPT) as a physiological indicator of its response to stress. Per the student's *t*-test, the animals from the REGRUP group exhibited heavier glands ($t = -2.651$, $p = 0.002$) than those of the animals from the REAIS group (**Figure 5**). This is to say that the animals which were initially isolated for 21 days exhibited heavier glands than those of the animals which were originally housed communally. Once again, and in alignment with the results from the SPT consumption tests, it appears that resocialization did not reverse the effect of CSI upon the rats' physiological response to stress.

DISCUSSION

The idea of the current study is to contribute to the knowledge about the consequences of CSI in male Wistar rats on their anhedonic-type behavior in the SPT and their anxiogenic profile in the EPM. Moreover, the study looks forward to evaluating the effect of resocialization upon sucrose consumption. In general terms, it was seen that both the communally housed and isolated animals demonstrated a preference for the consumption of 32% sucrose solution, however, the animals subjected to CSI consumed more of the sweeter solution and exhibited heavier AG than the animals from the AGRUP control group. Furthermore, they demonstrated an anxiogenic profile in the EPM test. Resocialization reversed neither the increased preference for 32% SS nor the weight of the AG.

These results align well with other studies which show that exposure to chronic stressors (Gawali et al., 2017; Nelemans et al., 2017; Steudte-Schmiedgen et al., 2017), especially social deprivation, leads to a series of behavioral changes, including some related to the reward system (Pritchard et al., 2013). The results of the present study are thus in agreement with those of Brenes Sáenz et al. (2006) and Brenes and Fornaguera (2008) who showed that rats subjected to CSI immediately after weaning consume more 32% sucrose solution than pure water. These authors affirm that this increased consumption of 32% SS was found to be associated with sensitivity to reinforcement, and can be understood as a sign of behavioral desperation (Brenes Sáenz et al., 2006; Brenes and Fornaguera, 2008). In support of the above, Hall et al. (1997) utilized CSI as a stressor followed by evaluation of varied concentrations of sucrose solutions (0.7%, 2.1%, 7%, 21%, and 34%). These authors used two schemes to present the test subjects with the solutions; the first in ascending order of concentration, and the latter in a descending order. It was discovered that rats subjected to CSI consumed more 34% SS in both schemes. These results were discussed in light of the effects of CSI upon the animals' reactiveness to highly appetizing concentrations of SS and emphasized the involvement of the dopaminergic system in the nucleus accumbens as a modulator of the incentivizing effects of sucrose (Wright et al., 2020).

Furthermore, in a study by Cortés et al. (2005), it was shown that female rats subjected to CSI consumed more 32% SS than 0.7% solution or pure water; as well as finding that animals housed individually exhibited a heightened preference for 32% SS as compared to animals housed communally. The authors demonstrated that CSI resulted in depression and anxiety-type behaviors, which appeared in both the SPT and EPM tests, respectively (Cortés et al., 2005).

Notably, the consumption of 32% SS in the present study was incremented in an ascending fashion over the

full course of the experiment; that is, at both the first and second exposures, with consumption on D4 being higher than consumption during the preceding days. This result aligns with those of related studies, which found the consumption of sweet (32%) sucrose solutions to follow a gradually increasing path (Hall et al., 1997; Sammut et al., 2002; Cortés et al., 2005) while that of water or less sweet SS to diminish over the course of several days (Hall et al., 1997; Ayuso-Mateos et al., 2012). Martínez et al. (2008) hypothesize that the preference for sweeter solutions could demonstrate the importance of pleasure thresholds along the course of "anhedonic-type behavior."

Páez-Ardila and Botelho (2014) evaluated the effects of CSI upon SS consumption in young orchiectomized adult rats and found that independently of treatment (i.e., castrated or uncastrated), those animals which were subjected to CSI exhibited a greater consumption of 32% SS than those housed communally, although uncastrated rats exhibited the highest overall consumption. These results demonstrate the importance of the hormonal factor upon the course of Major Depressive Disorder (MDD), although various human studies on the subject have found that reduced levels of testosterone appear to be associated with the appearance of this disorder (Ayuso-Mateos et al., 2012; Rice and Sher, 2017). According to Rice and Sher (Rice and Sher, 2017), the tendency towards MDD associated with a hormonal component could be related to the age of the subjects, since young rats with the highest testosterone levels have been found to be most closely associated with "depressive-type behavior" (Torres-González et al., 2009). Furthermore, in a study carried out by Rodríguez et al. (2012), male rats were subjected to a CSI model, with those animals thus treated tending to consume a higher quantity of 32% SS. Additionally, the consumption of sweet SS was greater in animals subjected to the forced-swim test before the SPT, a finding which highlights the stressor effect of the forced-swim model.

Contrary to the results of the present study, Papp et al. (2016) found that chronic stress led to reduced ingestion of 1% sucrose solution. This behavior was normalized by means of treatment with imipramine, ketamine, rivastigmine, and donepezil; which was interpreted to indicate their antidepressant effects. Similarly, Qin et al. (2017) and Sun et al. (2017) stated that treatment with melatonin was able to reverse this reduced consumption of low–concentration SS, as a consequence of deficiencies in reward systems produced by chronic unpredictable mild stress (CUMS), and which have traditionally been associated with "anhedonic-type behavior" (Willner et al., 1987; Qin et al., 2017; Sun et al., 2017; He et al., 2020). Also, Muscat et al. (1991) demonstrated that Raclopride exhibits an effect upon the behavior of male rats, creating anhedonic-type behavior, as shown by their preference for the consumption of high-concentration (34%) SS over 0.7% and 7% concentrations. These results indicate that the mesolimbic dopaminergic system plays a fundamental role in the response to reward stimuli since Raclopride is a dopamine receptor antagonist. Similarly, Wang et al. (2020) reported that CUMS also led to a reduction in body mass and depression-type behavior, as evidenced by a reduction in the consumption of 1% sucrose solution; a pattern reversible using fluoxetine.

Various methodological factors could explain the reduction of low–concentration SS consumption in the studies mentioned above; among them, the type of stressor utilized, the consumption measurement scheme, and the age of the test subjects. Additionally, the effect could occur due to the withholding of water and food before exposure to the SS, as this could affect the animals' ingestion.

Furthermore, it is noteworthy that CSI which occurs in young animals or immediately after weaning exhibits a distinct form of impact upon anhedonic-type behavior, which could, in turn, result in elevated pleasure thresholds (Martínez et al., 2008; Díaz et al., 2010; Páez-Ardila and Botelho, 2014). This observation finds basis in studies which show that the resocialization of animals subjected to SCI immediately after weaning, as adolescents, or as young animals is not capable of reversing the emotional behavior produced by this model, and results in behavioral deficits in the long term (Einon and Morgan, 1977; Whitaker et al., 2003).

From a pharmacological point of view, various studies have indicated that chronic treatment with antidepressants only reverses the pattern of consumption of 32% sucrose solution (Sammut et al., 2002). Some, such as Brenes and Fornaguera (2009), demonstrate that the increase in consumption of sweet (32%) SS produced by CSI can be reversed by fluoxetine; a finding which is consistent with the involvement of the serotonergic system in the regulation of motivated behavior towards a palatable incentive.

Taking into account the incongruencies found in the operational definition of anhedonia, it is possible that these can be attributed to the stress model used to induce anhedonic-type behavior (Brenes Sáenz et al., 2006), as the majority of studies that have linked anhedonic-type behavior to reduced SS consumption have utilized the CUMS model, while those that have linked it to increased consumption have used the CSI, as in the present study.

Another possible cause may arise from the sex of the animals, due to the differential susceptibility to stress responses caused thereby (Liang et al., 2008; Burke et al., 2016). Page et al. (2016) reported that among the female and male rats exposed to a model of social defeat, the female animals exhibited anhedonic-type behavior, this being taken as a reduction in their consumption of low–concentration SS. In an opposite finding, however, Burke et al. (2016) observed that when animals of both sexes were subjected to stress inducers, the males exhibited a reduction in their consumption of a low–concentration SS, which was taken as an operational definition of anhedonic-type behavior.

Additionally, Liang et al. (2008) found that, upon subjecting male and female rats to a chronic mild stress model, females in heat consumed a greater amount of SS compared to the males and the females not in heat. This result suggests that the females exhibited increased susceptibility to stressors relative to their stage of the estrous cycle (Liang et al., 2008). Supporting these results, Cortés et al. (2005) showed that females subjected to CSI exhibited an increased consumption of high–concentration sucrose solutions.

These findings could be explained by the action of gonadal hormones, which play a fundamental role in regulating mood (Martínez-Mota et al., 2012).

In the same manner, in studies carried out with human test subjects, even though women exhibit higher indices of MDD prevalence than men, they also demonstrate a more effective recovery by means of antidepressants and other treatments; while men respond more slowly to therapy (Liang et al., 2008; Díaz Sotelo, 2016) and exhibit a higher incidence of suicidal ideation and behavior; especially when this occurs simultaneously with low testosterone levels experienced by the senior population (Díaz Sotelo, 2016; Nelemans et al., 2017), as well as the heightened levels found in adolescents and young adults (Rice and Sher, 2017).

In line with the marked comorbidity which exists between depression and anxiety (Nelemans et al., 2017; Sangenstedt et al., 2017; Steudte-Schmiedgen et al., 2017), this study sought to evaluate the effect of CSI upon the animals' exploration of the EPM. Taken together, the results of the present study align with those of previous studies which show CSI to produce an anxiogenic profile on the EPM (Pellow et al., 1985; Papp et al., 2016; Bavley et al., 2017; Biala et al., 2017; Sun et al., 2017; Viana Borges et al., 2019). According to some authors, male adolescent rats exhibited less exploration of the open arms and lower levels of locomotor activity on the EPM, thus demonstrating a reaction to stress. These authors suggest that this anxiogenic profile may be modulated by hormonal states (Liang et al., 2008; Viana Borges et al., 2019). This proposal is supported by similar results in female rats by Kumari et al. (2016), who suggested that in light of the differences attributable to sex, although greater presence of anxious behaviors are found in females, it is, in fact, males who, when subjected to chronic stressors such as CSI, increase and maintain high levels of these behaviors. Weintraub et al. (2010) add that females exhibited higher percentages of entries into the open arms of the EPM (vs. the closed arms) when compared to males; indicating that despite being subjected to the same stressor, the males demonstrated higher anxiety levels than the females.

Finally, the present study sought to determine the effect of resocialization upon SS consumption preferences. The results showed that the anhedonic-type behavior of the male rats produced *via* CSI was not reversed by 72 h of resocialization. These results align with the literature, which indicates that resocialization is not able to reverse the adverse effects caused by CSI such as sensorimotor deficits, difficulty with the fear response, and circadian rhythm irregularities (Einon and Morgan, 1977; Whitaker et al., 2003; Weintraub et al., 2010). Specifically, resocialization did not reverse aggressivity and acute anxiety produced by CSI applied immediately following weaning in male rats (Walker et al., 2008). In a similar fashion, it has been demonstrated that the behavioral effects of acute social deprivation can be reversed by resocialization, but not those produced by chronic exposure to the same (Einon and Morgan, 1977).

On the other hand, it has been demonstrated that resocialization can reverse some of the effects produced by CSI (Tulogdi et al., 2014). For example, in a study conducted by Tulogdi et al. (2014), despite the fact that resocialization did not reverse the aggressivity produced by CSI applied immediately after weaning, adult animals were able to overcome the resulting deficiencies in pro-social behavior. This discrepancy among the results in the aforementioned studies may be explained by methodological differences, which include the sex and exact breed of animal, the duration of social isolation and the age at which it was applied, the physical conditions of housing, as well as the number of rats that are housed communally (Einon and Morgan, 1977; Hellemans et al., 2004).

Additionally, pioneering researchers in this field have suggested that resocialization is only effective when it is implemented for twice as long as the preceding isolation; this is to say, in the present study, the animals should have been resocialized for approximately 80 days, considering that they underwent an experimental period of 40 days between their initial isolation and the end of the protocol. Nevertheless, in a study conducted by Martínez et al. (2008), it was reported that resocialization for a period of 72 h did successfully reverse the effects of CSI upon anhedonic-type behavior but not of anxiety, as the latter may respond to the particularities of an array of disorders. As well, many studies report that the effects of CSI applied after weaning or during adolescence are not reversible *via* resocialization (Selye, 1936; Einon and Morgan, 1977), in contrast to when the animal is subjected to CSI in adulthood (Hellemans et al., 2004).

Finally, the animals that were originally isolated for 21 days exhibited heavier AG than those that were initially housed together. This is to say; 72 h of resocialization did not reverse the effect of CSI upon their physiological stress response. In a study conducted by Rygula et al. (2005), it was found that rats exposed to a model of social defeat produced AG heavier than those of the control group. Similarly, Grippo et al. (2003) demonstrated that rats subjected to mild chronic stress over the course of 4 weeks exhibited heavier AG than those of the control group. These results align with various studies which demonstrate that different stressors lead to heavier rodent AG as a consequence of the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Selye, 1936; Rygula et al., 2005; Pariante and Lightman, 2008; Walker et al., 2008; Díaz et al., 2010; Gawali et al., 2017). These findings highlight the importance of stress in the etiology of anxiety and depression (Pariante and Lightman, 2008; Gawali et al., 2017; Sangenstedt et al., 2017; Steudte-Schmiedgen et al., 2017).

Overall, the results of the present study reinforce the hypothesis that anhedonic-type behavior can be associated with an elevated pleasure threshold. Future studies may explore this hypothesis from a neurochemical point of view, using the same experimental protocol. Additionally, it would be desirable to study the difference which arises with respect to the effect of CSI upon anhedonia and anxiety profiles as relates to sex, and evaluate whether resocialization, for a period of time longer than that of isolation, is able to reverse the behavioral and physiological effects produced by social deprivation.

CONCLUSION

In the present study, it was demonstrated that exposure to CSI produces changes in the natural reward sense, as exhibited by a greater consumption of high–concentration sucrose solutions (which was not reversible through resocialization) and an anxiogenic profile in male adolescent rats.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://repository.upb.edu.co/handle/20.500.11912/7769.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Universidad Pontificia Bolivariana.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

This research was carried out with the financial support of the Universidad Pontificia Bolivariana Seccional Bucaramanga, under the heading of support for the investigative activities of the Laboratory of Neurosciences and Behavior.

ACKNOWLEDGMENTS

We wish to thank the Universidad Pontificia Bolivariana Seccional Bucaramanga, and the Laboratory of Neuroscience and Behavior of the same, for providing the space and equipment necessary to carry out the present study. Also, we extend our thanks to the Laboratory of Neuroscience and Behavior of the Universidad Industrial de Santander for the provision of the animals used in this research.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 20 August 2021
doi: 10.3389/fnbeh.2021.722456

Anticipatory 50-kHz Precontact Ultrasonic Vocalizations and Sexual Motivation: Characteristic Pattern of Ultrasound Subtypes in an Individual Analyzed Profile

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We verified the hypothesis of the existence of forms of individual-specific differences in the emission of anticipatory precontact vocalization (PVs) indicating individualization related to sexual experience and motivation in male rats. Long-Evans males were individually placed in a chamber and 50-kHz ultrasounds were recorded during 5-min periods. In experiment 1, PVs were recorded before the introduction of a female in four consecutive sessions during the acquisition of sexual experience. In experiment 2, PVs were analyzed in three groups of sexually experienced males: with the highest, moderate, and the lowest sexual motivation based on previous copulatory activity. In both experiments, the total number of ultrasounds, as well as 14 different specific subtypes, was measured. The ultrasound profiles for each male were created by analyzing the proportions of specific dominant subtypes of so-called 50-kHz calls. We decided that the dominant ultrasounds were those that represented more than 10% of the total recorded signals in a particular session. The number of PVs was positively correlated with the acquisition of sexual experience and previous copulatory efficiency (measured as the number of sessions with ejaculation). Furthermore, PVs showed domination of the frequency modulated signals (complex and composite) as well as flat and short with upward ramp ultrasounds with some individual differences, regardless of the level of sexual motivation. The results show a characteristic pattern of PVs and confirm the hypothesis that the number of PVs is a parameter reflecting the level of sexual motivation.

Keywords: 50-kHz ultrasonic vocalizations, anticipatory behavior, male rats, sexual experience, sexual motivation, ultrasound subtypes

OPEN ACCESS

Edited by:
Fabrizio Sanna, University of Cagliari, Italy

Reviewed by:
Nicola Simola, University of Cagliari, Italy
Wendy Portillo, National Autonomous University of Mexico, Mexico

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *08 June 2021*
Accepted: *03 August 2021*
Published: *20 August 2021*

Citation:
Bogacki-Rychlik W, Rolf M and Bialy M (2021) Anticipatory 50-kHz Precontact Ultrasonic Vocalizations and Sexual Motivation: Characteristic Pattern of Ultrasound Subtypes in an Individual Analyzed Profile. Front. Behav. Neurosci. 15:722456. doi: 10.3389/fnbeh.2021.722456

INTRODUCTION

Ultrasonic vocalization is one of the most intensively studied components of social behavior in rodents. In rats, two main types of ultrasonic vocalization (USV) can be distinguished based on the dominant frequency of the signal: 22-kHz and 50-kHz (Sales and Pye, 1974; Barfield et al., 1979; Brudzynski, 2015, 2021). The first is characterized by long-lasting flat calls (up to 3 s in duration) with a relatively narrow frequency range around 22-kHz (Barfield and Geyer, 1972). In the frustration state (situation of the absence of expected appetitive reinforcement), these calls can

be frequency modulated (20–35 kHz) with the preceding element at a frequency of about 45-kHz (Geyer et al., 1978; Bialy et al., 2019b). The so-called 50-kHz USV includes shorter ultrasounds (typically lasting up to several tens of milliseconds) with frequencies from 30–35 to 80-kHz. Signals of such calls, after undergoing Fourier transformations, display various shapes with 14 different subtypes (Wright et al., 2010). The rats emit 50-kHz USV during different elements of behavior related to their high arousal states (Bell, 1974; Berz et al., 2021) such as: socio-sexual interactions including copulation (Barfield et al., 1979; Bialy et al., 2000; Burgdorf et al., 2008), fighting (Sales, 1972; Burke et al., 2017), playing (Reinhold et al., 2019), and even tickling by the experimenter (Burgdorf et al., 2005; Panksepp, 2005). Additionally, pharmacologically induced high levels of general arousal [related to movement activity and sensory sensitization associated with increased level of wakefulness during activation of the gigantocellular reticular nucleus and associated structures (Pfaff, 2017)] result in the expression of 50-kHz vocalizations with a strong positive correlation between the number of 50-kHz ultrasounds emitted and the level of activation of the dopaminergic and noradrenergic pathways (Brudzynski, 2007, 2015; Wright et al., 2012; Simola, 2015; Hamed et al., 2016; Simola and Costa, 2018; Kuchniak et al., 2019).

Contrary to 50-kHz USV and according to the arousal hypothesis, 22-kHz USV occurs during abrupt decreases of arousal (Bell, 1974) and in this case can reflect a relaxation state after ejaculation (Bialy et al., 2016) or a safety signal during aversive conditioning (Jelen et al., 2003).

Although USV is accompanied by certain behavioral situations, the question about the function of 50-kHz vocalizations is still open. One hypothesis assuming a non-semiotic communicative character of vocalization was proposed by Sales and Pye in 1974. An alternative hypothesis postulated that USV can simply be artifacts associated with breathing patterns in rodents (Blumberg and Alberts, 1991). Some researchers have also suggested that it is possible to accurately assess a rat's emotional state based on the profile of the signals emitted (Brudzynski, 2015; Simola and Granon, 2019).

In this context, the phenomenon of USV can be considered as a complex central response containing an easily measurable autonomic reflex component (objective component) along with a co-occurring mental constituent (subjective component) that is difficult to measure. While the precise division between subjective and objective emotional components is not easy to establish, an accurate behavioral analysis may assess the proportion between them. To categorize some aspects of behavior as a reflection of subjective mental processes, it is necessary to demonstrate individual differences in USV expression. This form of individualization in the case of USV emission was shown in mice (Holy and Guo, 2005; Arriaga and Jarvis, 2013; Asaba et al., 2014; Chabout et al., 2015; Zala et al., 2020).

Moreover, Matsumoto et al. (2016) revealed that rats have the potential ability to discriminate calls emitted by their own vs. conspecific. Based on electrophysiological measurements, authors showed an independent reaction of different groups of neurons in the dorsal amygdala (area of the lateral amygdaloid nucleus) responded specifically to own vs. conspecifics ultrasounds calls.

Furthermore, rats seem to be able to diminish the emission of USV while playing hide-and-seek with humans when hiding themselves which requires being quiet even when having an elevated level of arousal (Reinhold et al., 2019). Such results provide a possible approach for the conscious use of USV by rats.

In sexual behavior, 50-kHz vocalization can be observed during anticipation, during the initiation of copulation, during the copulatory performance, and during the late phase of the postejaculatory interval (Barfield et al., 1979; Bialy et al., 1996, 2019b). Furthermore, the medial preoptic area and the nucleus accumbens—the neuronal circuits important in sexual activity (Hull et al., 2002) are involved in 50-kHz emissions (Fu and Brudzynski, 1994), also during sexual activity (Gao et al., 2019; Karigo et al., 2021), and anticipatory sexual behavior (Bialy et al., 2010). The anticipatory precontact 50-kHz vocalization (PVs) is convenient for the analysis of individual male vocalizations related to general arousal along with sexual motivation (Bialy et al., 2019a). The usefulness of this model derives, from the possibility, to separately analyze the ultrasounds emitted by the male in a conveniently short period (absence of the female provides a single source of USV). It allows describing the factors that trigger and modify individual vocalization in an appetitive state. Furthermore, it can also be a convenient parameter in the experimental models based on the acquisition of socio-sexual experience and extinction reactions, facilitate recognition of crucial cues regulating acquisition/extinction reactions. It is due to the direct relationship of PVs with the memory of emotional state, copulatory efficiency, and reward value of preceding socio-sexual interactions (Bialy et al., 2000).

However, this relationship concerns the total number of emitted ultrasounds, while the question of the relationship between the level of sexual motivation and emitted subtypes remains unknown. In the present experiments, we recorded PVs emitted during the acquisition of sexual experience as well as those emitted by sexually experienced males with different levels of copulatory activity. We have classified 50-kHz calls into one of 14 subtypes (Wright et al., 2010) and created individual USV profiles for each male. We compared the profiles thus obtained with the copulatory history of each rat. We would answer whether PVs subtypes profiles have the traits suggesting the uniqueness for each animal. If they have, is there any relation between this emerging inter-individual differentiation and the level of sexual motivation for each animal?

The purpose of this study was to answer the question: are there individual-specific distinctions of 50-kHz vocalization associated with the level of sexual experience and sexual motivation in the ultrasound recordings? If not, what is the general physiological pattern of anticipatory precontact vocalizations in the sexual behavior of the male rat?

MATERIALS AND METHODS

Animals

Long-Evans rats, 6–7 months old, were the subjects in this study.

The choice of animals' age was dictated by the stabilized profile of sexual parameters occurring between 150 and 500 days of their life (allows to omit the first life period with significant fluctuation in levels of sex hormones and corresponding tissue sensitivity) (Larsson, 1956, 1967).

Males and females were housed in separate rooms with a reversed 12-h light-dark cycle (lights switched off at 09:00 h) and at a temperature maintained at 22 ± 1°C. All of the animals had food and water freely available. The food consisted of standard laboratory chow (experiment 1 and 2) with some enrichment in experiment 2 (cereals, fresh vegetables, and fruits) due to the effort to achieve more natural-like conditions conducive to the emergence of social hierarchies. The males arrived from the Department of Experimental Medicine, Medical University of Silesia, Katowice, from different cohorts for experiments 1 and 2. The males were sexually naïve at the beginning of the experiments and have been described separately in the corresponding experiments.

The females ($N = 20$, 10 in the first and 10 in the second experiment), were housed 2–3 animals per standard laboratory cage (55.6 cm × 33.4 cm × 19.5 cm).

The housing conditions of males are described in corresponding "experiment 1 and 2" subsections.

All of the cages were provided with wood shavings and dedicated plastic tubes as enrichment. The ovariectomized females were brought into estrus with a single injection of estradiol benzoate (50 μg/rat s.c., Sigma-Aldrich) and progesterone (500 μg/rat s.c., Sigma-Aldrich). Hormones were dissolved in sesame oil and administered at a dose of 0.05 ml per individual. Hormonal injections were given 48–72 h before the test for estradiol and 4–8 h before the test for progesterone. Estrus was induced not more often than once a week and not less often than once every 2 weeks. The females were sexually experienced at the beginning of experiments. During an experimental day, a female copulated with up to two males.

Behavioral Procedures

All of the behavioral tests were conducted between 13:00 and 17:00 h during the dark phase of the light-dark cycle. We maintained at least a 1-week interval between tests to counteract the influence of the sexual exhaustion phenomenon on the sexual parameters, which is particularly important in less active groups of males (Larsson, 1956).

The test chamber was a transparent Plexiglas container (50 cm × 25 cm × 30 cm) in experiment 1 and a container made of transparent reinforced polyethylene (39 cm × 59 cm × 37 cm) in experiment 2.

Before an experiment, all of the males were acclimated 3–4 times to the experimental chamber for 10 min the first time and then for 5 min in consecutive acclimating sessions.

Acquisition of Sexual Experience

A male was introduced into the experimental chamber and a female was introduced 5 min later. Ultrasounds were recorded during the 5-min period between the introduction of the male and the female to the experimental chamber (precontact anticipatory ultrasonic vocalizations—PVs). The session was conducted until the first ejaculation and ended just after the male resumed copulatory activity. The maximum duration of a single session was 30 min and the session was terminated after this time.

Ultrasound Analysis and Behavior Recording

Visual recording of behavior was made using the Noldus EthoVision system.

Simultaneous to visual recording, ultrasounds were recorded on the same computer using the Metris Sonotrack system.

The microphone was placed 50 cm above the floor during ultrasonic recording. Spectral analysis of ultrasounds was performed using the Sonotrack software. Each ultrasound was analyzed manually using the Sonotrack cursor and was assigned to one of the 14 subtypes according to the classification proposed by Wright et al. (2010) based on its characteristic shape, complexity, and average frequency.

Each session was independently analyzed by two experienced observers to minimize bias in classification. The most inconclusive results were found between the complex and composite categories. For this reason, we decided to modify the classification and distinguish only 13 subtypes. Therefore, the complex and the composite subtypes were combined into one complex/composite category.

In this classification, subtypes of ultrasounds are defined as: complex/composite, trill, flat-trill, trill with jumps, split, step up, step down, multi-step, flat, short, upward ramp, downward ramp, and inverted U. In ambiguous cases, the ultrasounds were listened to at slow speed to classify them correctly.

Subsequently, we counted the total number of ultrasounds and the proportion of their subtypes emitted by each male. The identification of dominant subtypes in the recording was used to create a simplified code characteristic for each individual. Subtypes accounting for at least 10% of the total number of signals were understood to be dominant. Additionally, a low proportion (less than 10%) of the flat vocalization, which is usually frequently emitted by male rats, was considered as a distinguishing feature. In experiment 1, a characteristic vocalization profile for each male was created based on the dominant subtypes visible in all sessions with vocalizations above 9 USV.

Experiment 1

Males ($N = 17$) were housed 2–3 animals per standard laboratory cage (55.6 cm × 33.4 cm × 19.5 cm). All were sexually naïve at the beginning of the experiment. They acquired sexual experience during four consecutive copulatory sessions. Our previous results showed that during four sessions changes in sexual parameters related to the acquisition of sexual experience are most relevant and stabilized around the fifth session (Bialy et al., 2000).

All of the precontact 50-kHz vocalizations (PVs), quantification of every subtype and their percentage share in the total spectrum were analyzed. The sum of PVs was additionally correlated with copulatory efficiency measured as the sum of sessions when the male achieved ejaculation (during sessions 2–4 after the male had his first sexual experience).

One male was excluded from the experiment due to extremely aggressive behavior.

Experiment 2

Males ($N = 20$) were housed in the enriched environment as the group contained up to five animals which supported hierarchization within the group. These special larger cages had a vertical structure with wire sidewalls and a solid base (base dimensions 48 cm × 80 cm and 142 cm high) and were equipped with three levels of wooden platforms. The males before the experimental test achieved socio-sexual experience during five copulatory training sessions.

After such training, rats achieved a relatively stable level of sexual performance related to sexual experience (Bialy et al., 2000). During each session, the male was placed individually with a receptive female in one cage. Training sessions were terminated after 30 min. As the indicator of copulatory efficiency, we used the number of sessions when males achieved ejaculation(s) (or lack of them) from all five training sessions. Then we divided each male into one of three groups based on their copulatory history.

Alpha males ($N = 8$) ejaculated at least during two sessions, beta males ($N = 7$) ejaculated only once and gamma males ($N = 5$) never ejaculated during the five copulatory sessions.

One week after the last training session, precontact anticipatory ultrasonic vocalizations were recorded. A male was introduced for 5 min into the familiar experimental/training chamber where odor cues from receptive females were present. The scent stimuli were provided by the earlier placement of three randomly selected receptive females for 5 min into the experimental cage. Prior to the introduction of the male, the females were taken out.

Males that emitted less than 10 ultrasounds were excluded from statistical analysis. This criterion was adopted because, in the case of few ultrasounds, a single vocalization showed a disproportionately high percentage of a given subtype of vocalization, which led to a significant error in the interpretation of the results. This criterion was met by a total of three males—one male from each examined group.

Statistics

Data from the acquisition phase (the number of ultrasounds, mount, intromission, and ejaculation latencies) were analyzed by non-parametric Friedman repeated measure ANOVA and Dunn's *post hoc* tests (Experiment 1). The number of ultrasounds in Experiment 2 was analyzed by non-parametric Kruskal–Wallis ANOVA and Mann–Whitney for independent groups. Additionally, in experiment 1, the Spearman test was used to correlate the number of the session with ejaculation(s) with the total number of PVs.

RESULTS

Experiment 1

Number of PVs

The number of PVs (**Figure 1**) increased significantly in males ($N = 16$) during four consecutive sessions of the acquisition of sexual experience (Fr = 12.816, $P < 0.01$ with statistically significant differences between sessions 1 and 3 ($P < 0.05$), and sessions 1 and 4 ($P < 0.01$). Mount latency decreased significantly (Fr = 17.591, $P < 0.001$) with statistically significant differences between sessions 1 and 2 ($P < 0.05$), sessions 1 and 3 ($P < 0.01$) and 1 and 4 ($P < 0.01$. Intromission and ejaculation latencies display no significant differences ($P = 0.0815$ and $P = 0.077$ respectively).

A significant correlation (Spearman $r = 0.5608$, $P < 0.05$, $N = 16$) was found between the number of sessions with ejaculation (sessions 2–4) and the total number of PVs emitted by a male during these sessions (**Figure 2**).

PVs Subtypes

We counted the percentage of different subtypes when a male vocalized 10 times or more in a session. An analysis of USV subtypes was performed and more than 10 vocalizations occurred the most often in three sessions for the same male (six males) and less frequently: in four sessions (five males), in two sessions (four males), and one male vocalized less than 10 times in all four sessions. Those subtypes that appeared at least 10% in the total pool of male vocalizations were marked as the dominant calls. **Table 1** shows those subtypes that predominated in all sessions (100%) and those that occurred in at least two sessions out of three or four (50–75%) sessions and in only one session (25%). The complex/composite and flat (or short or upward ramp) subtypes occurred most frequently. The complex/composite and flat (or short or upward ramp) profile was typical to anticipatory precontact vocalizations regardless of the male's copulatory efficiency as measured by the number of ejaculations achieved during sessions 2–4.

Subtypes of PVs did not differ as a function of the acquisition of sexual experience. The percentage of different subtypes showed a similar level during all four copulatory sessions (**Figure 3**).

Experiment 2

Number and Subtypes of PVs

The number of PVs differed significantly between the alpha, beta, and gamma groups [H(2), $N = 17$] = 6.0396, $P < 0.05$. The alpha group vocalized more frequently compared with the gamma group ($P < 0.05$). The beta group did not differ significantly compared with the alpha and gamma groups (**Figure 4**).

The three most frequent subtypes of ultrasounds detected were: complex/composite (alpha 39.9%, beta 42.4%, and gamma 38% of total ultrasounds), flat (alpha 23.1%, beta 23.8%, gamma 24% of total ultrasounds) and upward ramp (alpha 10.2%, beta 11.5%, gamma 9.2% of total ultrasounds).

There were no statistically significant differences in the percentage of selected subtypes in the total pool of ultrasounds emitted by males when these three groups were compared (**Figure 5**). **Table 2** shows the main ultrasonic subtypes emitted by each male.

DISCUSSION

The results indicate a significant positive relationship between the total number of anticipatory precontact vocalizations and the number of sessions with achieved ejaculation(s). It reveals

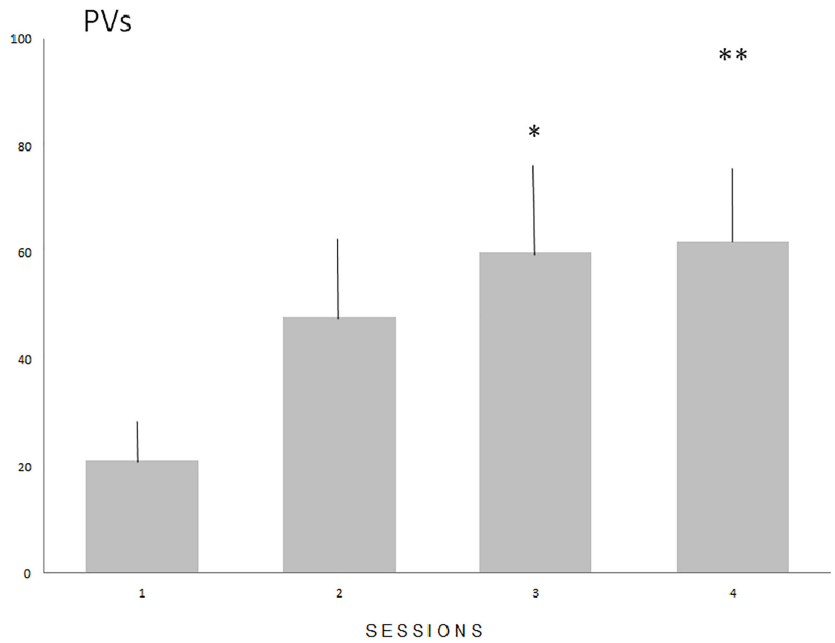

FIGURE 1 | The means number (SE) of PVs during the acquisition of sexual experience. Numbers on the Y axis represent the means value of PVs emitted during subsequent sessions. Statistically significant differences compared with session 1 (*$P < 0.05$ and **$P < 0.01$).

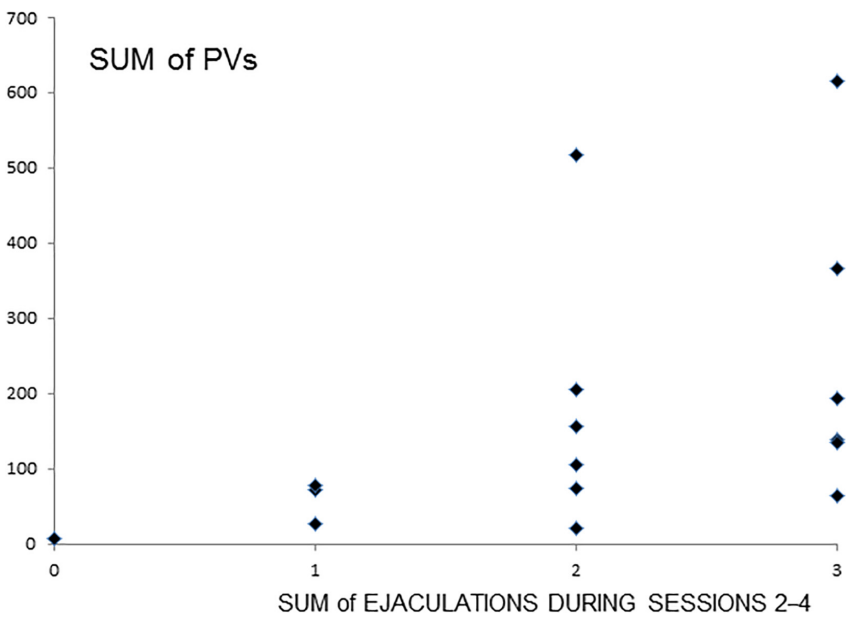

FIGURE 2 | Total number of PVs with relation to the sum of ejaculations during sessions 2–4. Numbers on the X-axis represent the sum of ejaculation from 0 to 3 achieved by the male during all three sessions 2–4. 0- means that male did not achieve any ejaculation during three sessions, respectively, 1—only one ejaculation during three sessions, 2—two ejaculations, and 3—ejaculated in each session. Numbers on the Y-axis represent the sum of PVs emitted by the male during sessions 2–4. The diamond represents an individual male. $r = 0.5608$, $P < 0.05$.

an association between the level of socio-sexual motivation and the expression of PVs. Shortening of mount latency—parameter related to sexual motivation (Hull et al., 2002) during the acquisition of sexual experience additionally supports the relation between PVs and sexual motivation.

In two independent groups of rats (experiments 1 and 2), similar patterns of emitted ultrasounds were observed. In all of the males, regardless of their level of sexual motivation or sexual experience, the dominant signals were the frequency modulated complex/composite (CC) signals and the unmodulated flat (F)

TABLE 1 | PVs and ultrasonic subtypes during consecutive sessions.

#	Copulatory status	100%	50–75%	25%	Note
1	3	Sh, UR	F, SU, CC	DR, IU	
2	3	CC, F, Sh	UR	Trills	
3	3	CC, Sh	UR	SU, Trills	Rare flat
4	3	UR, F, CC	Sh, SU	/	
5	3	F, Sh, SD, CC	/	/	
6	3	CC	Trills, UR, Sh	F	
7	2	UR, Sh, F	SD, CC, SU	/	
8	2	CC	Trills, SU, MS	SD, UR, Sh, IU	Rare flat
9	2	CC, Sh, UR	Trills, SU	/	Rare flat
10	2	F, CC	SD, Sh	SU,UR, Trills	
11	2	Trills, CC	/	F, Sh	
12	2	CC, F	/	Trills, SD, Sh	
13	2	F, CC	/	Trills, Sh, UR	
14	1	CC	Sh, UR, Trills, F	MS, SU	
15	1	CC, UR, Trills	/	/	Rare flat
16	0	/	/	/	

Subtypes of ultrasounds are defined as: complex and composite (CC), trill, flat-trill, and trill with jumps (Trills), split (S), step up (SU), step down (SD), multi-step (MS), flat (F), short (Sh), upward ramp (UR), downward ramp (DR), and inverted U (IU). The columns show subtypes emitted always (100%), frequently (50–75%), and sporadically (25%). Within the columns, the order from left to right indicates a decreasing percentage of emitted subtypes.

and, less frequently, the slightly modulated short (Sh) and upward ramp (UR) ultrasounds appeared as co-occurring subtypes. Trills were emitted on an elevated level in experiment 1 only. Other signals were seen sporadically and rarely exceeded 10% of the total PVs.

PVs and Sexual Activity

Anticipatory precontact vocalizations depend on the level of sexual activity and reward value of the contacts. In experiment 1, the number of PVs increased during the acquisition of sexual experience.

Moreover, the total number of PVs relates to the number of ejaculations. Ejaculation has the highest reward value compared with other elements of copulatory behavior (Tenk et al., 2009) and, in this context, PVs are related to the reward value of socio-sexual contacts. In experiment 2, the active males vocalized on a significantly higher level compared with much less active or sexually inactive males.

A more detailed explanation of the processes related to anticipatory ultrasonic precontact vocalizations follows. The number of PVs depends on the acquisition of sexual experience, conditioning to odor and background cues, the reward value of contact, NMDA (Bialy et al., 2000), and D1 receptor activity (Bialy et al., 2010).

Blocking of the NMDA receptor (receptor important in neuronal plasticity processes) inhibited changes in PVs during the acquisition of sexual experience but have no significant effect on PVs in sexually experienced males (Bialy et al., 2000). Acquisition of sexual experience and PVs are also related to D1 receptor activity. Surprisingly, peripheral repeated administration of both: antagonist and agonist of D1 receptor inhibited changes in PVs during the acquisition

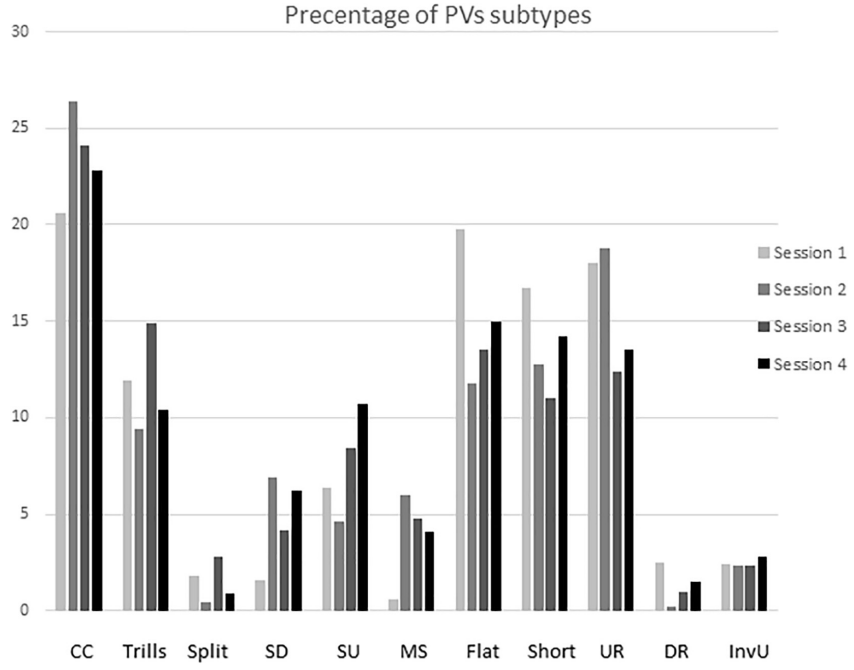

FIGURE 3 | Means percentage of different ultrasonic subtypes during the acquisition of sexual experience. Subtypes are represent as: complex and composite (CC), trill, flat-trill and trill with jumps (Trills), split, step up (SU), step down (SD), multi-step (MS), flat, short, upward ramp (UR), downward ramp (DR), and inverted U (IU). Subtypes on the X-axis are shown from left to right of each column as sessions 1, 2, 3, and 4.

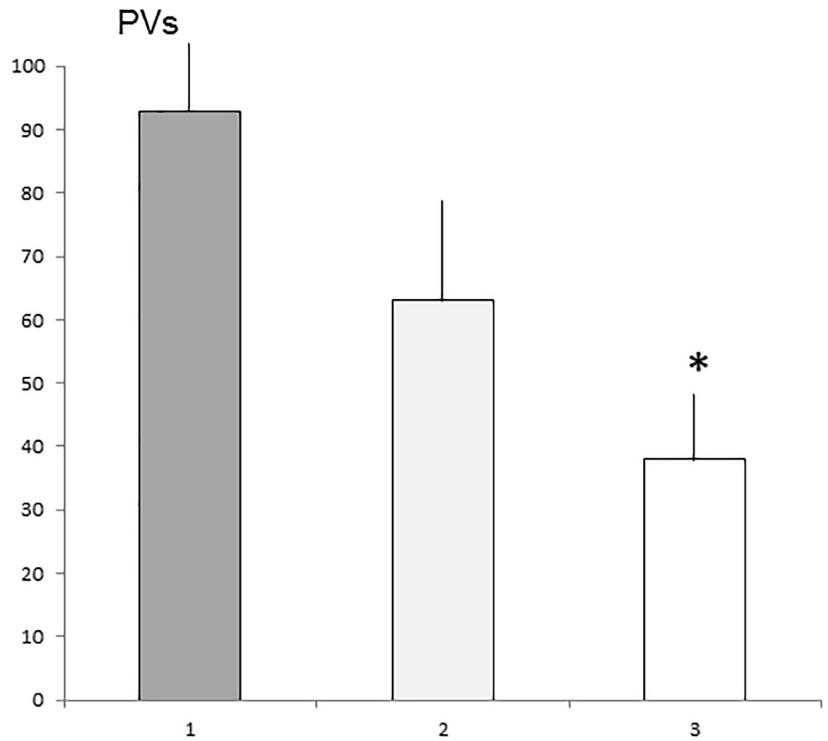

FIGURE 4 | The means number (SE) of PVs emitted by alpha, beta, and gamma groups. 1—Alpha group, 2—Beta group, and 3—Gamma group. *Statistically significant differences ($P < 0.05$) compared with the alpha group.

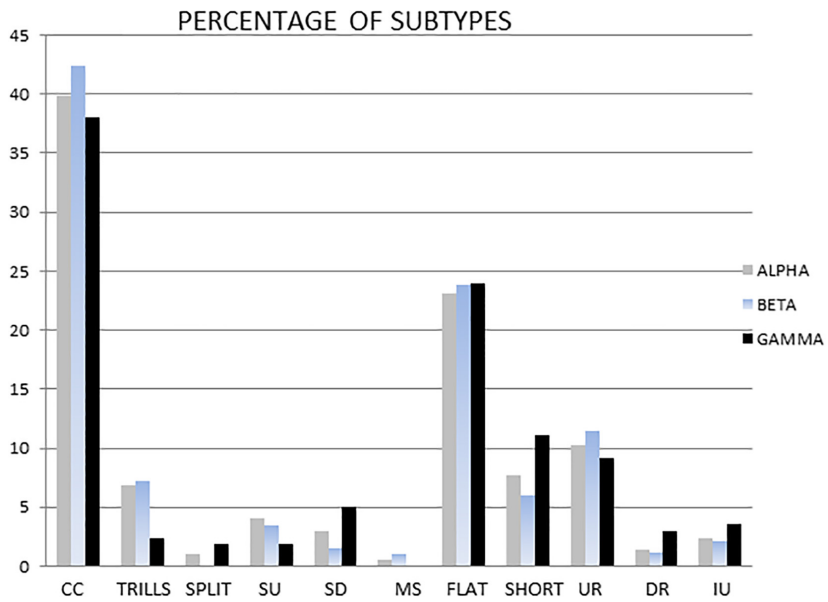

FIGURE 5 | The dominant means percentage of different subtypes in the alpha, beta and gamma groups. Subtypes are shown from left to right as: complex and composite (CC), trill, flat-trill and trill with jumps (Trills), split, step up (SU), step down (SD), multi-step (MS), flat, short, upward ramp (UR), downward ramp (DR), and inverted U (IU).

phase (Bialy et al., 2010) but, D1 receptor agonist had a minor effect on PVs in sexually experienced males (Beck et al., 2002). Repeated D1 receptor agonist injections into the nucleus accumbens similarly diminished PVs during the acquisition phase and this effect prolonged at least 4 weeks.

TABLE 2 | The individual profiles of emitted ultrasounds.

Male number	Group	Pattern
1	Alpha	CC, Sh, -F
2	Alpha	CC, F
3	Alpha	CC, F
4	Alpha	F, CC
5	Alpha	CC, F, UR
6	Alpha	CC, F, Sh, UR
7	Alpha	CC, F, UR
8	Beta	F,CC
9	Beta	CC, UR, F
10	Beta	CC, T -F
11	Beta	CC -F
12	Beta	CC, UR, F
13	Beta	F, CC
14	Gamma	F, CC, UR, SD
15	Gamma	F, CC
16	Gamma	CC, UR, -F
17	Gamma	CC, Sh, F

In columns, the order from left to right indicates decreasing percentage.Individual vocalization patterns based on the dominant subtypes of individual males appear in the last column of the table.The rare occurrence of the flat subtype is also considered as a minus F.Subtypes of ultrasounds are defined as: complex and composite (CC), trills (T), step down (SD), multi-step (MS), flat (F), short (Sh), upward ramp (UR), downward ramp (DR), and inverted U (IU).

Previously, were identified the specific, dopaminergic neuronal group in the nucleus accumbens responding either to appetitive unconditioned and conditioned stimuli that regulating, reward related behavior (Fiorillo et al., 2003). Thus, probably any significant disturbance of their activity: inhibition or overstimulation can be responsible for such attenuation of the acquisition phase and diminishing PVs.

In this context, PVs reflect learning and emotional memory processes. The number of PVs seems to be related to the level of general arousal and sexual motivation rather than to sexual arousal (Bialy et al., 2019a). In addition, social motivation should also be taken into account, as contacts with an anestrus female also has a triggering effect on PVs although at a significantly lower level (Bialy et al., 2000). We can assume that in the present experiments, PVs in low sexually active males were related to social motivation and general arousal rather than sexual motivation. All of these data seem to be consistent and repeatable, enabling the changing number of PVs to be used as a parameter for the functioning of the memory circuits related to the reward system.

PVs and Individualization

We observed individual differences in the percentage of non-dominant (less than 10% of the total spectrum) subtypes as well as in the occasionally very low level of the flat subtype. The composite signals contained within the CC group consisted of a modulated sound of the complex subtype with a directly accompanying sound of the short subtype. The distinction between the two ultrasounds was often only possible after listening to the signal. In some studies, this type of signal is listed as a "dual-type." However, due to the high subjectivity in the evaluation between different observers and the inability to listen on different recording systems other than our system, we decided on a common category. The composite subtype calls, consisting of calls other than complex and short components, occurred very rarely. Furthermore, the individual specificity of vocalizations was manifested in different proportions in the short, upward ramp, and flat subtypes. In general, the presented form of individualization seems to be subtle and not easy to use as a behavioral parameter.

This limitation is also related to the fact that a vast majority of inter-individual differentiation can occur within the so-called frequency-modulated non-trill group (split, step down, step up, multistep, downward, and inverted U) Despite their lack of visibility in the profiles after applying our quantitative criterion, variation within these types exists. Each of them is characterized by its specific shape, although their acoustic parameters, such as duration, mean frequencies, or modulation ranges, are heterogeneous. In summary, the observed individualization additionally occurs within the types of ultrasound that occur sporadically, in their exact parameters, and their potential combinations (like syllables).

Nevertheless, given the existence of the presented pattern, we can assume that it reflects a relatively constant emotional state. In this context, large, comparatively easy to visualize deviations from it may indicate significant central changes. The question of a behaviorally relevant reason for the existence of certain forms of individualization remains open.

USV Profiles

The main question, and aim of our experiments, was to answer the question: do changes in the level of PVs related to elevated general arousal and sexual motivation correspond to changes in the profile of the ultrasound subtypes. Qualitative analysis of ultrasound subtypes revealed an existing characteristic vocalization pattern with complex/composite subtypes combined with flat, short, and upward ramp subtypes dominating in the recordings. This model of vocalization appears similar in both sexually inactive and active males. Moreover, it seems constant during consecutive sessions of sexual experience acquisition and sexual motivation influenced the number of PVs but not the subtype profile. In experiment 2, more escalated social hierarchization was evident in the form of an increase in the non-copulatory number of rats (the gamma group). Regardless of this, the pattern of the profiles remained similar.

In our experiments, trills were visible in some sexually moderately active males. They did not change as a function of sexual experience. Probably they are related to the general arousal level but not sexual motivation *per se*. This hypothesis can be supported by the results of experiments with the repeated administration of psychostimulant drug agents (amphetamine and derivatives, apomorphine, cocaine) which evokes the expression of the trills subtype (Wright et al., 2012, 2013; Mulvihill and Brudzynski, 2018). However, the biological context of the expression of this type of vocalization is not entirely

clear. For example, Mahler et al. (2013) observed no difference in the number of trill emissions during the methamphetamine self-administration period and the subsequent extinction period, obtaining an increase only after re-exposure of the animals to the drug.

Furthermore, many authors, due to the simplicity of ultrasonic classification, often cluster all frequency modulated subtypes (Taracha et al., 2012; Brenes and Schwarting, 2015; Mulvihill and Brudzynski, 2018). The lack of correlation between sexual motivation and the profile of PVs could indicate that the specific type of frequency modulated calls presented in our experiments is an innate pattern of general anticipatory activity. In this context, the degree of modulation could represent a continuous behavioral spectrum, with complex/composite subtype domination associated with more physiological situations and the trills subtype domination reflecting highly aroused states. Further research is needed to confirm this hypothesis. Also, the neurobehavioral context of flat, unmodulated subtype is not clear. In these experiments, we observed flat call as one of the dominant types in most rats. However, in few animals, we observed a noticeable decrease of flats calls in profiles (**Tables 1**, **2**). This reduction wasn't related to copulatory status (activity), indicating the involvement of a factor other than socio-sexual motivation. Recently, some authors proposed that this type can be associated with social-coordinating function and alimentary appetitive reactions, including the food approach (Brudzynski, 2021). According to our results, additional data are needed to clarify this issue.

Further Implications

We have shown that the pattern of physiological anticipatory vocalization was relatively constant and persisted from one experimental session to the next. It is worth noting, however, that vocalization can change significantly in some situations. We observed rapid changes in vocalization occurring during a single session with characteristic transitions between the 50-kHz and 22-kHz frequencies during the frustrated states (Bialy et al., 2019b). Using an analogous non-contact model, changes in the vocalization profile across consecutive sessions have also been demonstrated in mice (Zala et al., 2020). Similarly, significant changes during consecutive experimental sessions have been shown repeatedly with addiction models, e.g., morphine (Covington and Miczek, 2003; Hamed and Kursa, 2018), cocaine (Ma et al., 2010), and methamphetamine (Mahler et al., 2013). Another example of such an application is the change in vocalization profiles that have been observed in induced Parkinson's disease in rats where ultrasound flattening occurs as the disease progresses (Ciucci et al., 2009). Individual vocalization and own vs. foreign recognition can be disturbed in schizophrenic-like symptoms in the rat model (Matsumoto et al., 2016). Furthermore, changes in the vocalization profile occur in the rat model of affective disorders and psychotic states (Nikiforuk et al., 2013; Wendler et al., 2019; Wöhr, 2021) as well as in suggested models of autism (Caruso et al., 2020).

CONCLUSION

We proposed a relatively simple method to discriminate individual characters of ultrasonic vocalization in rats based on dominant subtype ultrasonic vocalizations. Our results indicate a persistent similar vocalization pattern during anticipatory behavior, regardless of the level of socio-sexual motivation or experience. The number of ultrasonic vocalizations but not the different subtypes seems to be the most related to sexual motivation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by First Local Ethical Committee in Warsaw.

AUTHOR CONTRIBUTIONS

WB-R and MB designed and performed experiments, analyzed data, and prepared the manuscript. MR analyzed USV data in experiments 2. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Medical University of Warsaw and the MUW Young Researcher Grant: 1MA/5/M/MB/N/20/20. The study was carried out with the use of the CePT infrastructure financed by the European Union—the European Regional Development Fund within the Operational Program "Innovative Economy" for 2007–2013.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 20 October 2021
doi: 10.3389/fnbeh.2021.725856

Patterned Feeding of a Hyper-Palatable Food (Oreo Cookies) Reduces Alcohol Drinking in Rats

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While a bidirectional positive link between palatable food intake and alcohol drinking has been suggested, several rodents studies report reduced alcohol drinking following palatable diets exposure. These studies utilized purified rodents' diets high in sugar/fat; however, the effects of hyper-palatable food (HPF) rich in fat and sugar on alcohol drinking remain unclear. Furthermore, neural substrates involved in HPF-mediated changes in alcohol consumption are poorly understood. Therefore, the present study evaluated the effects of patterned feeding of a hyper-palatable food (Oreo cookies) on alcohol drinking as well as dopamine (DA) and serotonin (5-HT) content in rat's mesocorticolimbic (medial-prefrontal cortex, orbitofrontal cortex, amygdala, and nucleus accumbens) circuitry. Male Long Evans rats received 8-weeks of intermittent (Mon, Tue, Wed) Oreo cookies access, which induced a patterned feeding, in which rats in the Oreo group overconsumed calories on HPF days whereas underconsumption was observed on chow only (Thu, Fri) days. Following HPF exposure, alcohol consumption was evaluated while patterned feeding continued. Alcohol intake in the Oreo group was significantly lower as compared to the chow controls. However, alcohol intake in the Oreo group increased to the levels seen in the group receiving chow following the suspension of patterned HPF feeding. Finally, DA levels in the nucleus accumbens were significantly greater, whereas its metabolite (DOPAC) levels were lower in the Oreo group compared to the chow controls. Surprisingly, 5-HT levels remained unaltered in all tested brain areas. Together, these data suggest that HPF-associated increased DA availability and reduced DA turnover within mesocorticolimbic circuitry may regulate alcohol drinking following patterned HPF feeding.

Keywords: alcohol use disorder, high-sugar/fat diet, palatable diet, alcohol drinking, dopamine, nucleus accumbens

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *15 June 2021*
Accepted: *27 September 2021*
Published: *20 October 2021*

Citation:
Leon Z, Shah K, Bailey LS, Karkhanis AN and Sirohi S (2021) Patterned Feeding of a Hyper-Palatable Food (Oreo Cookies) Reduces Alcohol Drinking in Rats. Front. Behav. Neurosci. 15:725856. doi: 10.3389/fnbeh.2021.725856

INTRODUCTION

Problematic caloric intake is not only a core component of some eating disorders but also is linked to several public health concerns. For example, binge eating disorder, characterized by consuming a large amount of food in a short period with a behavioral loss of control over eating, can significantly impact overall health, quality of life, and healthcare costs (Ágh et al., 2016).

Hyper-palatable foods (HPF), rich in sugar and fat, are the typically preferred foods consumed during these episodes (Leigh et al., 2018) and data suggest that individuals who engage in such problematic eating behavior are at higher risk for developing substance abuse, overweight/obesity, and worsening depressive symptoms (Ross and Ivis, 1999; Swanson et al., 2011; Skinner et al., 2012; Mehlig et al., 2018). Furthermore, substance use disorder frequently co-occurs with eating disorders (Bulik et al., 2004; Harrop and Marlatt, 2010), and a significant proportion of the college-aged population has been reported to engage in episodes of dysregulated drinking/eating, an experience that could trigger alcohol/drug abuse and numerous health concerns (Callas et al., 2004; Ferriter and Ray, 2011; Kelly-Weeder, 2011).

Converging research evidence suggests common neurochemical, behavioral, and physiological determinants of maladaptive eating and alcohol drinking (Fortuna, 2010; Morganstern et al., 2011; Nogueiras et al., 2012). For instance, feeding peptides, traditionally known for controlling appetite and energy metabolism, also regulate the intake and reinforcing properties of alcohol (Vadnie et al., 2014). It is also becoming apparent that hyper-palatable foods can interact with brain reward circuitry (Volkow et al., 2012), and changes in several of these neurochemical systems have been reported following HPF and alcohol intake (Barson et al., 2011; Volkow et al., 2012, 2017). For example, both food and drug reward stimulate DA release in the nucleus accumbens (NAc; Hernandez and Hoebel, 1988; Yoshimoto et al., 1992; Rada et al., 2005; Liang et al., 2006), and alterations in DA turnover and DA receptors gene expression have been reported following prolonged alcohol and HPF exposure (Hajnal and Norgren, 2002; Vasconcelos et al., 2003; Davis et al., 2008; Villavasso et al., 2019). Similarly, serotonergic (5-HT) neurotransmission is also implicated in mediating behavioral and emotional impairments following chronic exposure to alcohol and high-calorie diets (Kurhe and Mahesh, 2015; Zemdegs et al., 2016; Li et al., 2020; Popova et al., 2020). Together, these studies suggest that both hypercaloric foods and alcohol may affect central monoaminergic systems, and neuroadaptations in the mesocorticolimbic circuitry could mediate the effects of chronic HPF and alcohol exposure.

Impaired emotional status resulting from chronic alcohol exposure has been shown to promote escalated alcohol intake (Kissler et al., 2014). Considering that prolonged dysregulated eating of HPFs is capable of triggering negative emotional states (Cottone et al., 2009; Iemolo et al., 2012), excessive consumption of hyper-palatable food and resultant changes in neuroendocrine signaling along with negative affective states could trigger alcohol use disorder (AUD). Interestingly, some studies have suggested a bidirectional positive link between HPF intake and alcohol drinking (Pekkanen et al., 1978; Mitchell et al., 1985; Krahn and Gosnell, 1991; Avena et al., 2004; Carrillo et al., 2004). However, studies have also demonstrated reduced alcohol drinking following both sugar- and fat-rich palatable diets exposure (Yung et al., 1983; DiBattista and Joachim, 1999; Stickel et al., 2016; Takase et al., 2016; Cook et al., 2017; Gelineau et al., 2017; Sirohi et al., 2017b; Villavasso et al., 2019; Shah et al., 2020). It is important to note that several procedural/experimental differences among these studies could explain the differential impact of palatable diets on alcohol drinking and have been reviewed recently (Brutman et al., 2020). Briefly, some studies reported increased alcohol intake provided palatable diets in the acute or chronic manner and assessed alcohol drinking following palatable diets suspension (Pekkanen et al., 1978; Carrillo et al., 2004). On the other hand, studies from our lab provided intermittent access and evaluated alcohol drinking while rats were still maintained on intermittent palatable diet cycling (Sirohi et al., 2017b; Villavasso et al., 2019). Furthermore, hedonic fat or sugar consumption has been suggested to produce fundamentally different behavioral states (Avena et al., 2009). In this regard, a decrease in anxiety-like behavior has been reported by studies providing intermittent access to the high-fat diet in a non-consecutive manner (Mon, Wed, Fri; Sirohi et al., 2017b), whereas when a high-sugar diet was provided in an intermittent but on two consecutive days in a week, increase in anxiogenic behavior was reported (Cottone et al., 2009). In short, how hyper-palatable food overconsumption impact behavioral processes that regulate alcohol drinking is poorly understood.

While most of these studies provided rodent's purified diets high in sugar or fat, typical dysregulated feeding episodes in the real-world involve hyper-palatable food rich in both fat and sweet. Therefore, the present study evaluated patterned feeding of real-world hyper-palatable food (Oreo double stuffed cookies) on alcohol drinking and monoamines (DA and 5-HT) levels in rat's corticolimbic areas. Previously, we have shown that a 2-week of patterned high-fat diet pre-exposure is sufficient to reduce alcohol drinking in rats (Villavasso et al., 2019). Considering that the length and exposure history of a calorie-rich food can produce fundamentally different behavioral outcomes (Tracy et al., 2015; Krishna et al., 2016), alcohol drinking in the present study was evaluated following extended (three times a week for 10-weeks), patterned HPF administration. It was hypothesized that patterned feeding of hyper-palatable food would increase alcohol drinking by associated changes in the monoamines content in the brain reward circuitry.

MATERIALS AND METHODS

Animals

Male Long Evans rats (Envigo RMS, Inc, Indianapolis, IN) initially weighing ∼300 g were used. Animals were individually housed in a temperature (∼70°F) and humidity (∼60%) controlled vivarium on a standard 12 h reverse light-dark cycle (lights on at 1:00 AM and off at 1:00 PM). Upon arrival, animals were gently handled before any experimental manipulation, and baseline body weight, food intake, and water intakes were recorded. Cage cleaning and changes occurred every Monday ∼10:30 AM. All procedures were approved by the Institutional Animal Care and Use Committee guidelines at the Xavier University of Louisiana.

Diet

All animals received *ad libitum* access to standard rodent chow (Tekland-Envigo Diets #2020X, 3.1 kcal/g with 16% Calories from fat and 60% Calories from carbohydrates) and water. Rats in the experimental group also received intermittent access to

Double Stuff Oreo cookies (Walmart, 4.83 kcal/g with 24% Calories from fat and 72% Calories from carbohydrates, of which 45% Calories were derived from sugar). Other test diets used in the study were high-fat diet (HFD; Research Diets #D03082706, 4.5 kcal/g with 40% Calories from fat and 46% Calories from carbohydrates, of which 7.9% Calories were derived from sugar) and high-sugar diet (HSD; Research Diets #D10001, 3.9 kcal/g with 11.5% Calories from fat and 67.7% Calories from carbohydrates, of which 51% Calories were derived from sugar).

General Experimental Procedure

Male Long Evans rats (n = 5/group) with no significant between-group difference in body weight, food intake, and water intake were randomly divided into control (Chow) and hyper-palatable food (HPF/Oreo) groups. All rats had *ad libitum* access to standard rodent chow and water for the entire duration of the experiment. Rats in the HPF group also received intermittent (Mon, Tues, and Wed) access to Double Stuff Oreos cookies (3.0 cookies/session) ∼2.0 h into the dark cycle. The control group received chow during this period. Standard chow was available to all rats for the rest of the week, as shown in **Figure 1A**. Food intake was manually recorded every 24 h, and body weight was measured every Monday and Thursday. Any leftover cookie crumbs were recovered from the cage at the end of HPF access. Following an initial 8 weeks of patterned HPF feeding, a series of tests (**Figure 1B**), as described below, were conducted while the animals still maintained on a patterned feeding schedule, unless noted otherwise. Rats were weighed simultaneously just before respective food/fluid presentation.

We first examined the impact of withdrawal from patterned feeding of an HPF exposure on high fat or high-sugar diet preference. In the 9th week, ∼24 h following termination of HPF patterned feeding cycle, both control and the experimental groups received eight pellets of HFD and HSD (11:00 AM), and hourly food intake was recorded for 4 h. HFD and HSD testing occurred over a period around light dark switch so that food intake could be captured at least 2 h before and 2 h after this switch. These time points were chosen as rodents typically consume their biggest meals around this switch. Also, since the goal of the manuscript was to assess the effects of withdrawal from hyper-palatable food, 2 h post switch testing time point was chosen as it was ∼24 time period following Oreo cookies access was suspended. All rats resumed patterned HPF cycling following this testing.

In the 10th week, alcohol drinking was evaluated on chow-only days (Thu, Fri, Sat) in a two-bottle choice paradigm, as explained below. Alcohol (20% v/v) and water bottles were provided in the rat's home cages (∼2.0 h into the dark cycle), and alcohol intake was manually recorded 2 or 24 h following administration. Alcohol testing was conducted for 5 weeks on chow-only days (Thu, Fri, and Sat) while patterned HPF cycling continued. Next, we examined alcohol drinking once dietary manipulations were released. For this, patterned HPF cycling was suspended (Mon, Tue, and Wed), and all animals received alcohol in a similar manner on regular chow only days. Following the conclusion of alcohol drinking studies, intermittent HPF cycling was reinitiated until animals were euthanized.

Finally, all animals were euthanized on chow access day (∼24 h after the last patterned HPF cycle), and their brains were immediately snap-frozen. The medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), nucleus accumbens (NAc), and amygdala (Amyg) were micro- dissected and prepped for dopamine (DA), serotonin (5-HT), and their metabolites content analysis.

Alcohol Drinking Procedure

Alcohol testing occurred using a two-bottle choice paradigm, as done previously (Sirohi et al., 2017b; Villavasso et al., 2019). On alcohol testing days, rats were given 20% v/v unsweetened alcohol and water bottles. The position of alcohol and water bottles was switched in each session to reduce and minimize conditioning effects on alcohol intake. Body weight and weight of alcohol and water bottles were manually recorded on testing days. Data represents 15 separate alcohol drinking sessions where 2 or 24 h alcohol intake was measured. Alcohol preference was calculated as alcohol intake/water intake.

Brain Neurotransmitters Analysis

Brain tissue samples (n = 3–5/brain region) from the mPFC, OFC, NAc, and Amyg were collected using tissue punches and were weighed for wet weight. Tissue samples were then homogenized in 0.2 M Perchloric acid (1 ml EDTA, 10 ml 60% $HClO_4$, filled to 500 ml with ultrapure water) using sonication. Homogenized samples were centrifuged for 15 min at 12,000 rpm and 4°C (Sorvall Legend Micro 21R Centrifuge; Thermo Scientific; Waltham MA). The supernatant was collected for analysis and the pellet was discarded. Samples were analyzed using high-performance liquid chromatography (HPLC). The mobile phase consisted of the following composition: 8% acetonitrile, 100 mM phosphoric acid, 100 mM citric acid, 0.1 mM EDTA.Na2, 600 mg/L octanesulfonic acid sodium salt, pH = 3.0. All chemicals used were HPLC grade and dissolved in 18.0 MΩ purity water. A sample volume of 5 μl sample was injected for analyses using an autosampler (AS 110, Antec, Zoeterwoude, Netherlands). Standards of known concentrations of dopamine and, 3,4-Dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin, and 5-HIAA were used to quantify concentrations of the respective monoamines and their metabolites in the brain samples. An ALEXYS Neurotransmitter Analyzer (Antec, Zoeterwoude, the Netherlands) consisting of a stationary phase C18 column (Acquity UPLC BEH, 1.7 μm diameter, and 1 × 100 mm length) and a DECADE Elite electrochemical detector (senCell 2 mm GC sb, Antec, Zoeterwoude, Netherlands). Clarity software (Prague, The Czech Republic) was used for quantifying neurotransmitter concentrations. Data are expressed as nM/mg of tissue weight.

Statistical Analysis

A mixed-model two-way ANOVA analyzed body weight, food intake, and alcohol drinking data, with appropriate *post hoc* (Sidak) analysis. The within subject variable was time intervals, and the between-group variable was diet exposure. HFD/HSD testing data were analyzed by the two-way repeated measure of

ANOVA, followed by *post hoc* analysis (Turkey). A Student's *t*-test was also used wherever applicable, particularly for brain monoamine data analysis. Statistical comparisons were conducted at 0.05 α level using GraphPad Prism 7.05 (GraphPad Software Inc).

RESULTS

HPF Patterned Feeding: Body Weight

A mixed-model two-way ANOVA identified a main effect of time ($F_{15,120}$ = 575.1, $p < 0.0001$) and a significant HPF exposure × time interaction ($F_{15,120}$ = 5.79, $p < 0.0001$) during 8 weeks initial intermittent HPF exposure compared to the chow controls. However, no significant ($p > 0.05$) between-group body weight differences existed during this period (**Figure 1C**). Similarly, no significant ($p > 0.05$) between group body weight differences existed during any of the testing time points (**Figure 1D**).

HPF Patterned Feeding: Food Intake

Intermittent HPF exposure induced a patterned feeding cycle, in which rats in the Oreo group overconsumed on HPF access days, whereas underconsumption was observed on chow only days (**Figures 2A–C**). A mixed-model two-way ANOVA identified a main effect of time ($F_{38,304}$ = 25.11, $p < 0.0001$), a significant HPF exposure × time interaction ($F_{38,304}$ = 24.01, $p < 0.0001$) and a significant between-group effect ($F_{1,8}$ = 42.49, $p < 0.001$). On the other hand, the caloric intake of rats in the chow group was consistently similar during this period. Food intake was also measured on two separate weekends only and no significant between-group difference existed these days. Furthermore, significant ($p < 0.0001$) caloric overconsumption (∼93% of kcal from Oreo cookies) occurred within 0–2 h of the presentation, whereas a significant ($p < 0.0001$) caloric underconsumption occurred 2–18 h following the suspension of HPF (**Figures 2D,E**). The caloric intake at other time points was identical in both groups.

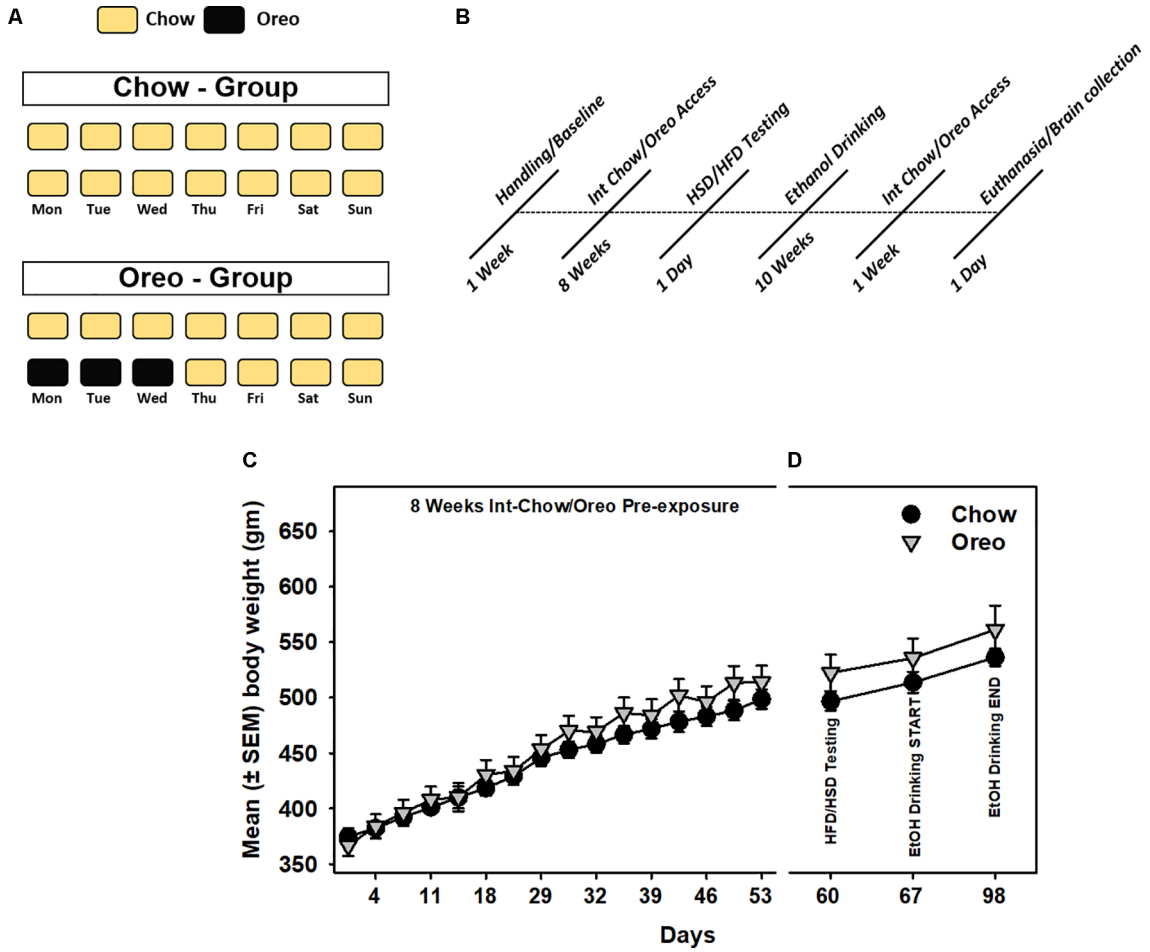

FIGURE 1 | Schematic representation of patterned feeding paradigm and associated body-weight changes. **(A)** During the feeding paradigm, experimental group of rats (n = 5/group) received 24 h intermittent access (Mon, Tue, and Wed) to Double Stuff Oreo cookies whereas control group of rats received chow. **(B)** A timeline showing a series of behavioral and biochemical testing following initial 8 weeks of patterned feeding. All rats had *ad libitum* access to chow and water. Mean (± SEM) body weight readings during **(C)** initial 8 weeks of pre-exposure and **(D)** subsequent testing sessions. There was no significant difference between body weights of the control and the experimental group at any time point.

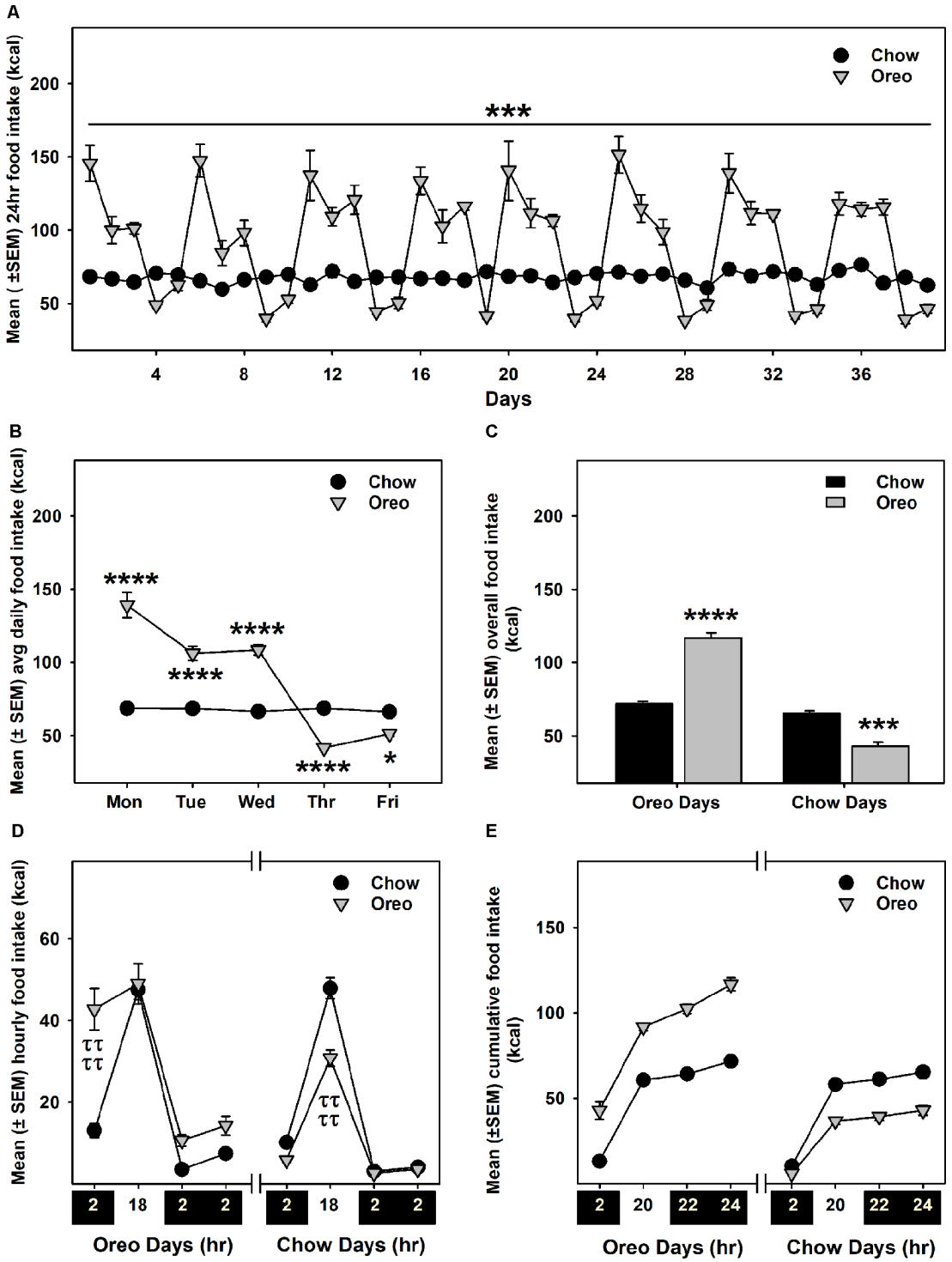

FIGURE 2 | Energy intake during the 8-weeks of patterned HPF feeding. Data compare mean (± SEM) 24 h food intake (kcal) between chow and HPF group. **(A)** Rats in the HPF group followed a pattern where they significantly overconsumed on HPF access days (i.e., Mon, Tue, and Wed) and under-consumed on chow-only access days. ***$p < 0.001$ main effect of diet exposure. HPF group of rats significantly overconsumed and under-consumed on Oreo-access days and chow-access days respectively when data were compared as indicated by the average of daily food intake **(B)** and overall food intake during the exposure period **(C)**. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ compared to chow controls. Food intake (kcal) was also measured at different timepoints after initiation and suspension of HPF respectively. Data represents (± SEM) hourly **(D)** and cumulative **(E)** food intake at these time-points. HPF rats significantly overconsumed during the first 2 h (0–2 h) on the Oreo days, and significantly under-consumed during the 2–18 h timeframe on Chow days. ττττ$p < 0.0001$ compared to chow controls. ***$p < 0.001$, ****$p < 0.0001$ main effect of diet exposure. HPF, hyper-palatable food.

HPF Patterned Feeding: HFD/HSD Preference

HFD/HSD preference data were analyzed by a two-way repeated measure ANOVA. In the rats receiving chow only, there was a main effect of time ($F_{3,12}$ = 20.98, $p < 0.0001$), a significant diet × time interaction ($F_{6,24}$ = 20.51, $p < 0.0001$) and a significant between-diets effect ($F_{2,8}$ = 73.9, $p < 0.0001$; **Figure 3A**). Similar results were obtained in the rats receiving intermittent access to Oreo, where there was a main effect of time ($F_{3,12}$ = 15.07, $p < 0.001$), a significant diet × time interaction ($F_{6,24}$ = 7.679, $p < 0.001$) and a significant between-diets effect ($F_{2,8}$ = 98.2, $p < 0.0001$; **Figure 3B**). While the HFD intake in both Chow ($p < 0.0001$) and Oreo ($p < 0.01$) group of rats was significantly elevated compared to the chow and HSD intake, HSD intake was not significantly different compared to chow intake in either group (**Figures 3C,D**). Furthermore, HFD/HSD intake was not significantly ($p > 0.05$) different between the Chow and Oreo group of rats. These data suggest that while both the Chow and the Oreo group of rats preferred HFD over chow and HSD, this preference was not impacted by 8 weeks patterned HPF feeding.

HPF Patterned Feeding: Alcohol Drinking

Alcohol drinking data were analyzed by a mixed-model two-way ANOVA, which identified a main effect of time ($F_{14,112}$ = 2.74, $p < 0.01$), a significant diet × time interaction ($F_{14,112}$ = 2.628, $p < 0.01$) and a non-significant trend between-group effect ($F_{1,8}$ = 4.774, p = 0.06; **Figure 4A**). While alcohol drinking was not significantly different between groups in the first week, further analysis revealed a gradual reduction in alcohol drinking behavior over 5 weeks of testing. A significant between-group difference in alcohol drinking was observed on fourth ($F_{1,8}$= 10.01, p = 0.01, power = 0.79) and fifth ($F_{1,8}$= 13.12, p = 0.0068, power = 0.89) alcohol testing week. Interestingly, rats in the chow group repeatedly displayed escalated alcohol intake on renewed alcohol access (on Thursday), an effect reduced over the next testing days each week. To examine if alcohol drinking differed on testing days, we compared each alcohol drinking day (Thu, Fri, and Sat) across 5 weeks of alcohol testing in both chow and Oreo groups. In the chow group, a repeated measure two-way ANOVA identified a main effect of testing days ($F_{2,8}$ = 40.36, $p < 0.05$) but no significant week's effect (**Figure 4B**). Post-hoc analysis further revealed that alcohol intake on Thursday was significantly elevated compared to Friday ($p < 0.01$) and Saturday ($p < 0.0001$). In addition, Friday alcohol drinking was also elevated compared to Saturday ($p < 0.05$). On the other hand, in the case of Oreo group, a repeated measure two-way ANOVA identified a main effect of the week ($F_{4,16}$ = 3.671, $p < 0.05$) but no significant testing day effect (**Figure 4C**). *Post hoc* analysis further revealed that alcohol intake in the 4th ($p < 0.05$) and the 5th ($p < 0.01$) week was significantly reduced compared to the 1st week in the Oreo group. Two-hour alcohol intake was also evaluated on Thu and Fri and a mixed-model two-way ANOVA identified the main effect of time ($F_{9,72}$ = 5.458, $p < 0.0001$), a significant food × time interaction ($F_{9,72}$ = 2.163, $p < 0.05$), and a significant between-group effect ($F_{1,8}$ = 6.414, $p < 0.05$; **Figure 4D**). Similar to 24 h alcohol intake data, alcohol drinking in the chow group significantly escalated on renewed alcohol access, an effect absent in the Oreo group of rats. Total fluid intake was also not significantly different between groups.

A mixed-model ANOVA also evaluated water (**Figure 5A**) and total fluid (**Figure 5B**) intake data over 5 weeks of alcohol testing period and found no significant between-group differences. Since alcohol drinking in the Oreo group gradually reduced over 5 weeks and it became only significant towards the last weeks, we evaluated changes in water and total fluid intake during the last 3 weeks. A repeated measure one-way ANOVA revealed that from week 3–5, average weekly alcohol intake remained unchanged in the chow group, whereas alcohol drinking in the Oreo group of rats gradually reduced significantly ($F_{1.415,5.659}$ = 6.202, $p < 0.05$; **Figure 5C**). While water intake in the chow group remained unchanged, it significantly ($F_{1.729,6.915}$ = 14.91, $p < 0.01$) escalated in the Oreo group of rats (**Figure 5D**). Total fluid intake did not change under these conditions in either group (**Figure 5E**). These data suggest that the Oreo group of rats gradually increased their water intake during the same time when their alcohol intake was reducing and together, their fluid intake remained unchanged during the last alcohol testing weeks. These data also suggest reduced alcohol preference in the Oreo group of rats, which was supported by the repeated measure one-way ANOVA (**Figure 5F**) to be significant ($F_{1.369,5.477}$ = 8.224, $p < 0.05$), whereas alcohol preference remained unchanged during this testing period for chow group of rats.

Next, we evaluated alcohol drinking following the suspension of intermittent HPF cycling. Compared to the alcohol drinking (week 5 in **Figure 4**) before suspension (**Figure 6A**), alcohol intake in the Oreo group was not significantly different and returned to the chow controls drinking level (**Figure 6B**).

HPF Patterned Feeding Selectively Affected Dopamine in the NAc

The mesolimbic and mesocortical dopamine system is highly implicated in reward processing and affect. Given our behavioral data showing decreased ethanol intake in rats exposed to HPF patterned feeding, we quantified dopamine tissue content in regions receiving dopamine inputs from the VTA, namely, the NAc, mPFC, OFC, and amygdala. Dopamine content in the NAc (**Figure 7A**) was significantly greater in Oreo group of rats compared to the chow control rats ($t_{(8)}$ = 3.146; p = 0.0137). We also evaluated DA metabolites in the NAc and found that DOPAC was significantly ($t_{(8)}$ = 4.275; p = 0.0027) reduced in the Oreo group of rats compared to the chow controls (**Figure 7B**). NAc HVA content was not significantly different between groups (**Figure 7C**). No significant ($p > 0.05$) between-group differences in dopamine or its metabolites content were observed in other brain regions (**Table 1**). Since the above-mentioned regions also receive serotonin inputs from the dorsal raphe, we assessed the impact of ethanol and HPF patterned feeding on serotonin levels in these regions. Serotonin or its metabolite content was not significantly ($p > 0.05$) different in any of the brain regions examined (**Table 2**).

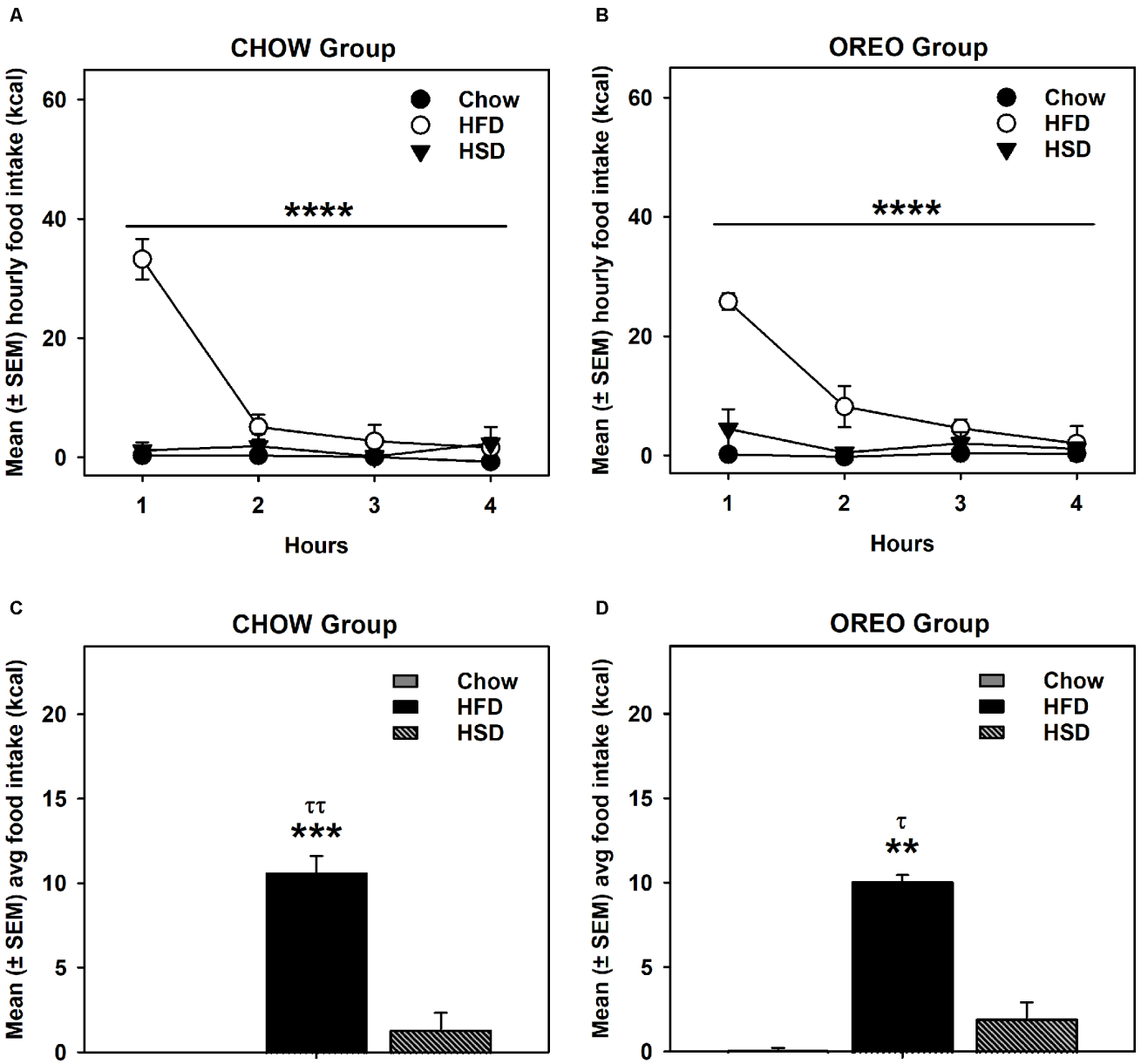

FIGURE 3 | HFD/HSD preference during acute withdrawal from patterned HPF feeding. In the 9th week, 24 h after the termination of intermittent HPF access, rats were tested for their HFD/HSD preference. Data represent mean (± SEM) hourly and average food intake from each diet (chow, HFD, and HSD). A significant between-diet effect was noted in the hourly food intake in both Chow **(A)** and Oreo **(B)** groups. $^{****}p < 0.0001$ between diet effect. A significant preference of HFD over both HSD and chow was noted in both Chow **(C)** and Oreo **(D)** groups. However, no significant between diet type differences in HFD or HSD intake were evident. There was no significant preference for HSD compared to chow in either group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to chow. $^{\tau}p < 0.05$, $^{\tau\tau}p < 0.01$ compared to HSD. HFD, high-fat diet; HSD, high-sugar diet.

DISCUSSION

The present study's goal was to evaluate the impact of prolonged patterned feeding of hyper-palatable real-world food (Oreo double stuffed cookies) on alcohol drinking and alterations in the central monoamine levels. We found that HPF intermittent access induced patterned feeding and reduced alcohol drinking in rats. In addition, dopamine concentration was significantly elevated in the NAc of rats receiving patterned feeding of HPF compared to the chow controls. These data collectively suggest that patterned feeding of HPF reduces alcohol drinking, potentially by modulating dopaminergic neurotransmission in the brain reward circuitry in rats.

We and others have utilized an intermittent palatable diet access model to induce sustained bouts of caloric overconsumption and underconsumption, a hallmark of

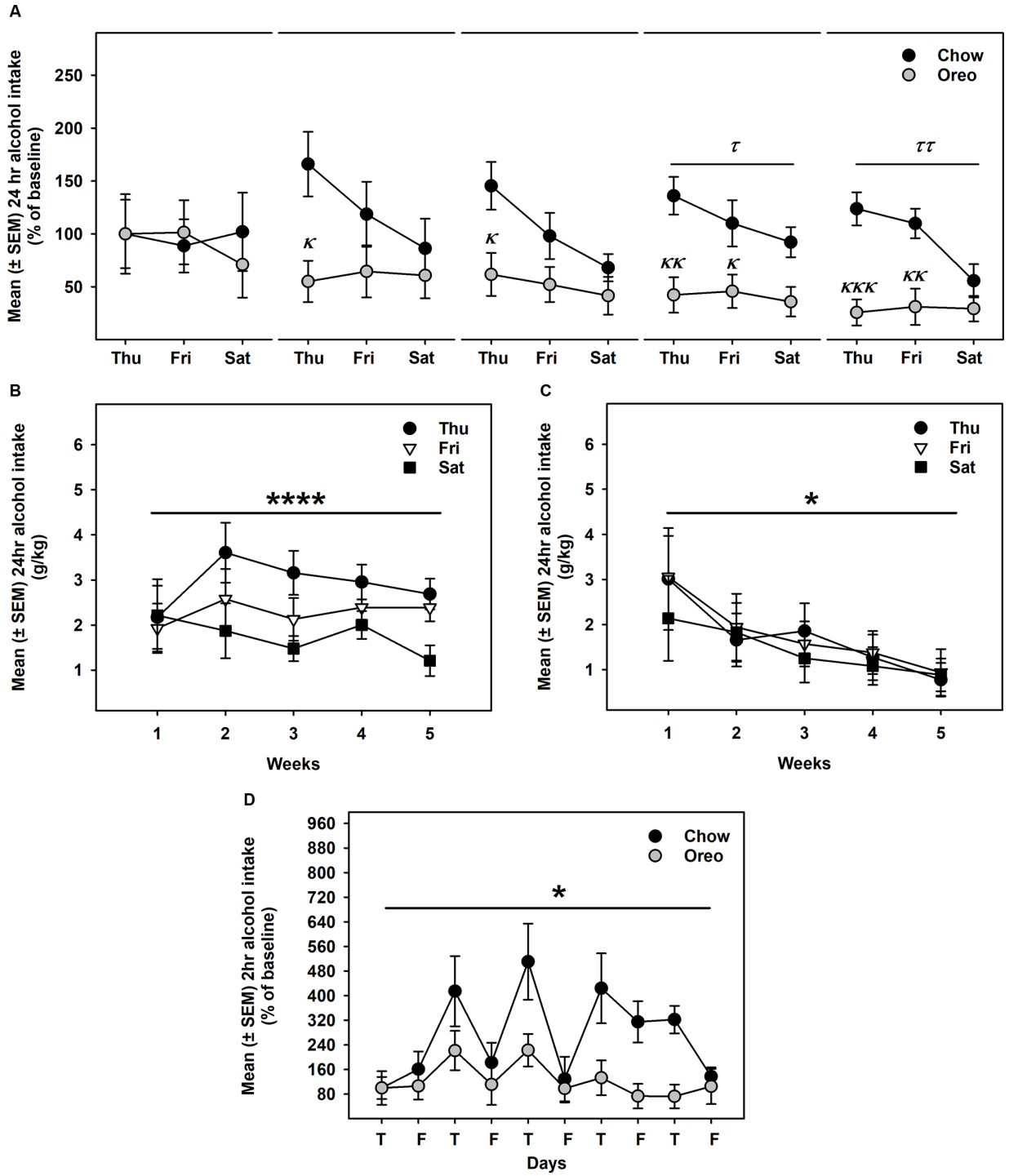

FIGURE 4 | Alcohol drinking following patterned feeding of HPF. Alcohol (20% v/v) was provided, and intake was recorded on chow-only days (Thr, Fri, Sat) for 5 weeks. **(A)** Mean (±SEM) % of baseline (day 1) alcohol intake is plotted. While there was no significant difference in alcohol intake between groups in the first week, alcohol drinking was gradually reduced in the case of the Oreo group. During the 4th and the 5th week/session, the difference markedly increased with HPF rats significantly consuming less alcohol than the chow controls. $^{\tau}p = 0.01$, $^{\tau\tau}p = 0.0068$ main effect of diet. $^{\kappa}p < 0.05$, $^{\kappa\kappa}p < 0.01$, $^{\kappa\kappa\kappa}p < 0.001$ compared to chow controls. Furthermore, alcohol consumption on each testing day was compared across the 5 weeks. **(B)** In chow group, a significant difference in alcohol consumption was noted among testing days, a decreasing order from Thursday to Saturday. No significant week's effect was noted. $^{****}p < 0.0001$ main effects of testing days. **(C)** In the Oreo group, there was no difference in alcohol consumption over different testing days; however, there was a significant decrease in alcohol intake across the weekly testing sessions. $^{*}p < 0.05$ effect of the week. **(D)** Similar to 24-h alcohol intake, 2-h alcohol intake in the Oreo group was significantly lower as compared to the chow controls. $^{*}p < 0.05$ main effect of diet.

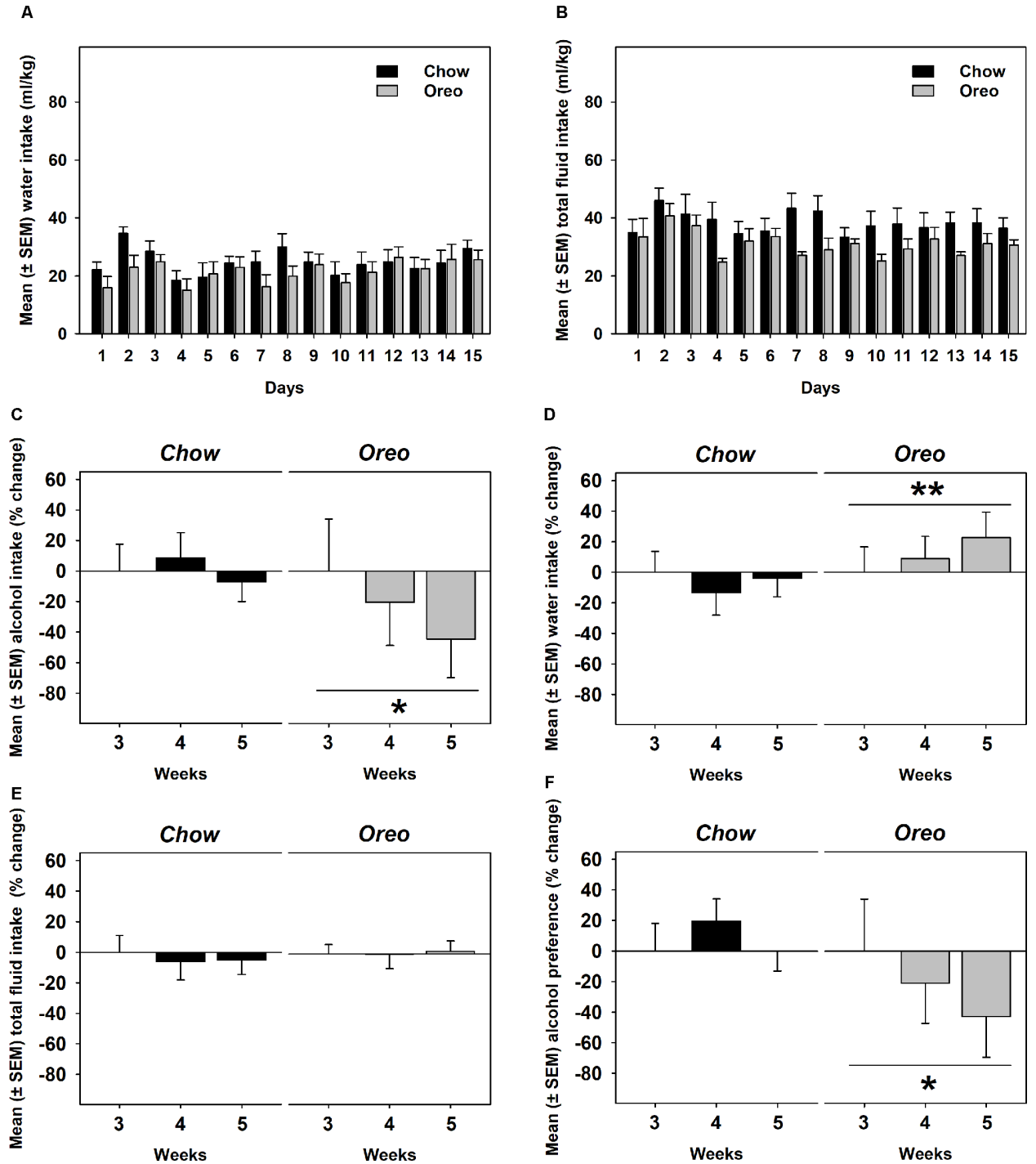

FIGURE 5 | Water, total fluid intake, and alcohol preference following suspension of patterned HPF cycling. Data compares the mean (± SEM) **(A)** water and **(B)** total fluid intake (ml/kg) between chow and HPF groups and no significant between group effects were observed. As noted earlier that significantly reduced alcohol drinking behavior only emerged towards last weeks of the alcohol testing period, we evaluated average **(C)** alcohol, **(D)** water, and **(E)** total fluid intakes during the last 3 weeks along with **(F)** alcohol preference. In the Oreo group, alcohol drinking and alcohol preference was reduced, water intake was increased but the total fluid intake did not change. On the other hand, no such changes were observed in the chow group of rats. $^{**}p < 0.01$, $^{*}p < 0.05$ main effect of time.

dysregulated/binge-like feeding behavior (Davis et al., 2007; Corwin and Babbs, 2012; Sirohi et al., 2017b; Villavasso et al., 2019). It is important to note that our intermittent feeding paradigm is distinct from *ad libitum* protocols that induce acute bouts of caloric overconsumption, which are not sustained and lead to an increase in body weight. In our paradigm, rats

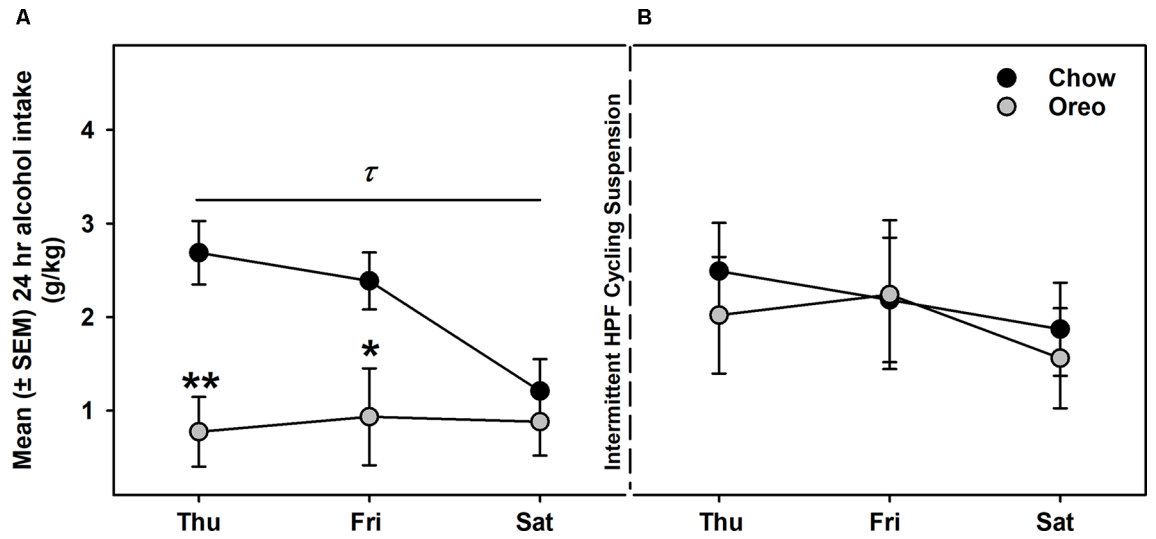

FIGURE 6 | Alcohol intake following suspension of patterned HPF cycling. Data compares the mean (± SEM) alcohol intake (g/kg) between **(A)** the 5th week of regular alcohol testing and **(B)** after a week of patterned HPF cycling suspension. While the Oreo group had a significantly reduced alcohol consumption as compared to chow in the 5th week, they returned to the chow controls drinking level when the patterned HPF cycling was suspended. $^{\tau}p < 0.05$ main effect of diet. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to chow controls.

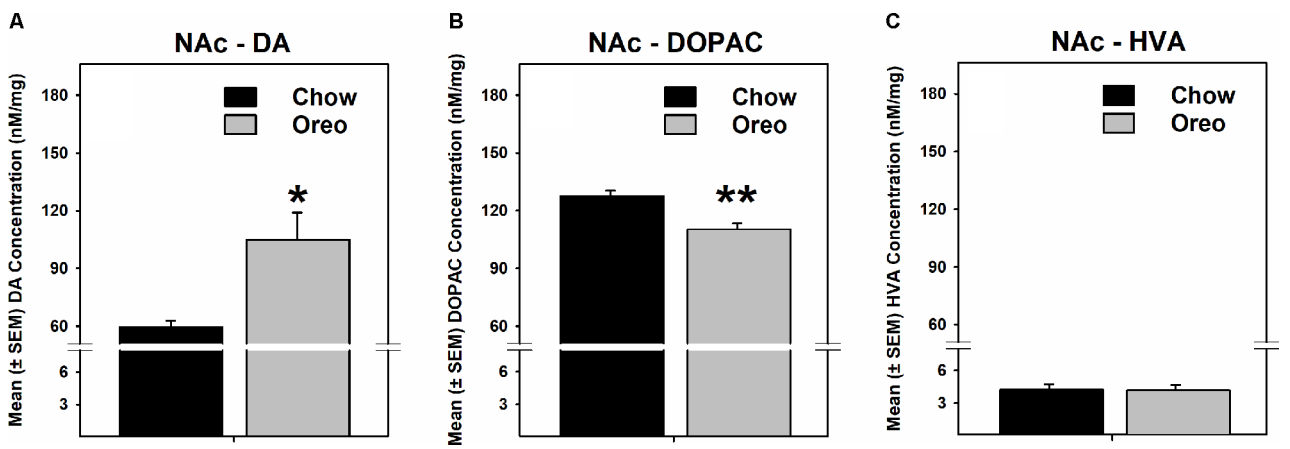

FIGURE 7 | Effect of patterned feeding of HPF on dopamine levels in the NAc. Data represent mean (± SEM) concentrations (nM/mg of tissue wt) of **(A)** DA, **(B)** DOPAC and **(C)** HVA in the nucleus accumbens (NAc). DA levels were significantly elevated, whereas DOPAC levels were significantly reduced in the NAc of the Oreo group of rats compared to the chow controls. HVA levels were not significantly different between groups. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to chow. DA, dopamine; DOPAC, 3,4-Dihydroxyphenylacetic acid.

display sustained bouts of hyperphagia but no body weight gain; therefore, our results are not confounded by the presence of an obese phenotype. In the present study also, rats receiving intermittent access to Oreo cookies developed a feeding pattern of overconsumption on HPF access days (Mon, Tue, and Wed) and underconsumption on chow only access days (Thu and Fri; **Figures 2A–E**). It is possible that restricting access to palatable food could facilitate overconsumption of palatable food intake (Fisher and Birch, 1999); in fact, a previous study utilizing a similar approach reported an escalation in overconsumption following renewed access to a palatable diet over a 5 weeks period (Cottone et al., 2009). While the caloric intake in the present study was significantly higher on the first day of renewed HPF access compared to the second and third days, no escalation in this feeding behavior was seen over a period of 8 weeks (**Figures 2A,B**). We also evaluated average weekly caloric intake on Oreo access days across 8 weeks and found no evidence of escalated feeding behavior.

Preference for a high-sugar or high-fat diet under acute abstinence conditions (24 h following termination of HPF cycling) was also examined. While HFD was preferred over HSD by both chow and Oreo groups, there was no between-group difference in caloric intake (**Figures 3A–D**), suggesting that HPF's patterned feeding did not induce a compulsive

TABLE 1 | Effect of patterned feeding of HPF on dopamine, DOPAC, and HVA levels in the brain reward circuitry.

Brain region	Chow	Oreo	*p*-value
DOPAMINE			
mPFC	2.13 (± 0.29)	2.18 (± 0.06)	0.87
OFC	3.14 (± 0.10)	4.85 (± 0.96)	0.15
NAc	59.71 (± 3.06)	104.93 (± 14.04)	0.03*
Amyg	3.06 (± 0.85)	4.32 (± 1.62)	0.57
DOPAC			
mPFC	2.94 (± 1.11)	2.33 (± 0.33)	0.62
OFC	6.09 (± 0.62)	25.19 (± 12.52)	0.20
NAc	127.64 (± 2.70)	110.32 (± 3.02)	0.003**
Amyg	27.29 (± 13.79)	5.03 (± 0.88)	0.21
HVA			
mPFC	9.05 (± 2.01)	8.26 (± 1.52)	0.76
OFC	5.05 (± 1.16)	5.96 (± 0.99)	0.58
NAc	4.23 (± 0.48)	4.18 (± 0.47)	0.94
Amyg	14.11 (± 0.69)	20.03 (± 3.00)	0.14

*Data represent mean (± SEM) dopamine and its metabolites (DOPAC and HVA) concentrations (nM/mg of tissue wt) in the medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), nucleus accumbens (NAc), and amygdala (Amyg) of Chow and Oreo group of rats. *$p < 0.05$, **$p < 0.01$ compared to chow. HPF, hyper-palatable food; DOPAC, 3,4-Dihydroxyphenylacetic acid; HVA, homovanillic acid.*

feeding phenotype. In the present study, rats in the experimental group had a choice between chow and Oreo cookies, which was not the case in Cottone et al. (2009) where animals in the intermittent access group had access to a palatable diet only during binge episodes and displayed compulsive feeding behavior. It is important to note that free choice palatable diet access mimics real-world situations to model neurobiological and behavioral consequences of overconsumption of palatable diets, which could differ if the palatable diet is provided as the only choice (Slomp et al., 2019).

Both animals and human studies have shown that disordered eating behavior shares many similarities and neurobiological characteristics of alcohol and substance use disorders (Cottone et al., 2009; Gearhardt et al., 2009; Volkow et al., 2012) and individuals who engage in such problematic eating behavior are at higher risk for developing alcohol use disorder, overweight/obesity, and worsening depressive symptoms (Ross and Ivis, 1999; Swanson et al., 2011; Skinner et al., 2012; Mehlig et al., 2018). Considering that hyper-palatable food is typically consumed during dysregulated eating episodes and activate similar brain reward circuitry as of drugs of abuse, including alcohol, several studies have examined the impact of overconsumption of palatable diets on alcohol (Pekkanen et al., 1978; Yung et al., 1983; Mitchell et al., 1985; Krahn and Gosnell, 1991; DiBattista and Joachim, 1999; Avena et al., 2004; Carrillo et al., 2004; Stickel et al., 2016; Takase et al., 2016; Cook et al., 2017; Gelineau et al., 2017; Sirohi et al., 2017b; Villavasso et al., 2019). While still unclear, many studies have observed a reduction in alcohol drinking (Pekkanen et al., 1978; Forsander and Sinclair, 1988; DiBattista and Joachim, 1999; Takase et al., 2016; Cook et al., 2017; Gelineau et al., 2017; Sirohi et al., 2017a,b; Constant et al., 2018; Villavasso et al., 2019; Shah et al., 2020). Paradoxical effects of HPF feeding on alcohol drinking could be attributed to palatable diets with different macronutrient compositions (high-fat or high-sugar), palatable diets exposure duration (acute, intermittent, or chronic), and alcohol testing conditions (Brutman et al., 2020). It is important to note that majority of these studies utilized commercially available purified diets high in sugar or fat. Therefore, the impact of binge-like intake of a real-world hyper-palatable food on alcohol drinking remained to be investigated and was the primary goal of the present manuscript.

TABLE 2 | Effect of patterned feeding of HPF on 5-HT and 5-HIAA levels in the brain reward circuitry.

Brain region	Chow	Oreo	*p*-value
5-HT			
mPFC	1.19 (± 0.19)	1.22 (± 0.09)	0.89
OFC	10.98 (± 0.83)	10.70 (± 0.52)	0.78
NAc	4.69 (± 0.70)	4.64 (± 0.31)	0.96
Amyg	0.88 (± 0.17)	1.04 (± 0.03)	0.44
5-HIAA			
mPFC	60.41 (± 8.81)	59.58 (± 3.16)	0.93
OFC	64.21 (± 2.88)	61.34 (± 2.27)	0.47
NAc	102.21 (± 6.22)	89.76 (± 7.98)	0.25
Amyg	70.10 (± 9.21)	65.19 (± 4.38)	0.65

Data represent mean (± SEM) serotonin (5-HT) and its metabolite (5-HIAA) concentrations (nM/mg of tissue wt) in the medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), nucleus accumbens (NAc), and amygdala (Amyg) of Chow and Oreo group of rats.

In order to examine the impact of withdrawal from an extended intermittent palatable diet exposure on alcohol drinking, alcohol intake was evaluated on chow access days following 10 weeks of patterned HPF feeding. Interestingly, rats in the chow group displayed acute deprivation-induced escalated alcohol drinking (24 h) on renewed access to alcohol (Thursday), which gradually reduced to baseline levels over 3 days (**Figures 4A,B**). On the other hand, rats in the Oreo group were not only protected from this effect but also their 24 h alcohol intake gradually decreased, with significantly decreased alcohol drinking observed on the 4th and 5th week of alcohol testing (**Figures 4A,C**). Furthermore, 2 h alcohol intake (**Figures 4D**) was also significantly reduced in the Oreo group of rats accompanied by gradually reduced alcohol preference (**Figure 5F**). While we predicted that such prolonged cycling of HPF would increase alcohol drinking, surprisingly, alcohol drinking was significantly gradually reduced in the Oreo group of rats. These data are consistent with previous studies from our lab in which reduced alcohol drinking was observed following patterned feeding of a high-fat diet (Sirohi et al., 2017b; Villavasso et al., 2019). While blood alcohol levels were not assessed in the present study, a previously published study (Sirohi et al., 2017b) from our lab using Long Evans rats reported pharmacologically relevant blood alcohol levels (∼25 mg/dl) following similar alcohol intake.

It has been suggested that withdrawal from fat and sugar produce fundamentally different behavioral states (Avena et al., 2009). On macronutrient levels, Oreo cookies are composed of 24% Calories from fat and 72% Calories from carbohydrates, of which 45% Calories were derived from sugar. On the other hand, high-fat diet previously used in our paradigm consisted of 40% Calories from fat and 46% Calories from carbohydrates, of which 7.9% Calories are derived from

sugar (Sirohi et al., 2017a,b; Villavasso et al., 2019; Shah et al., 2020). Surprisingly reduced alcohol drinking behavior following patterned feeding to both Oreo cookies and high-fat diet suggests a role of palatability rather than macronutrient composition in reduced alcohol drinking following patterned feeding of HPF as reported by a recent study from our lab (Shah et al., 2020).

While reduced alcohol drinking was observed at least until 72 h following the suspension of the HPF, it was unclear how long this effect would last. To address this question, we suspended patterned HPF cycling and carried out alcohol testing exactly as done earlier on chow only access days (Thu, Fri, and Sat). Interestingly, alcohol drinking in the Oreo group of rats increased compared to the last alcohol drinking session and was not significantly different from chow controls (**Figure 6B**). These data are similar to what we had reported recently when alcohol drinking gradually increased to the level of chow controls within a week following intermittent high-fat diet suspension, suggesting that acute availability of a palatable diet is critical to observe reduced alcohol drinking behavior (Villavasso et al., 2019). In this context, an alternative explanation of reduced alcohol drinking following patterned feeding of a palatable diet could be the caloric overload before alcohol testing as a contributing factor in reduced alcohol drinking. However, previous studies from our lab have repeatedly shown that reduced alcohol drinking following patterned high-fat diet feeding is seen on days when rats did not restrict calories voluntarily (Sirohi et al., 2017b; Villavasso et al., 2019). On a similar note, alcohol drinking was gradually reduced over 5 weeks on a patterned HPF feeding cycle, whereas this behavior disappeared within a week following HPF suspension. Importantly, when tested under similar conditions, feeding peptides remained unchanged following patterned feeding of a high-fat diet (Villavasso et al., 2019). These data collectively suggest that energy homeostasis mechanisms are less likely to drive reduced alcohol drinking following patterned HPF feeding.

This notion also aligns with the data from a recent study from our lab in which selective alterations in the neurotransmitter receptors gene expression was observed in the brain reward circuity compared to the brain region involved in energy homeostasis following patterned feeding of a palatable diet (Villavasso et al., 2019). Mesocorticolimbic dopamine neuronal connectivity from VTA to NAc is highly implicated in food and drug reward processing (Wise, 2006), and palatable foods rich in fat and sugar increase extracellular dopamine in the NAc similar to drugs of abuse (Hernandez and Hoebel, 1988; Rada et al., 2005; Liang et al., 2006). While studies have reported blunted DA release following repeated/chronic palatable food/solution exposure, limited/intermittent access to sugar and fats repeatedly triggers increased DA release in the NAc (Bassareo and Di Chiara, 1997; Rada et al., 2005; Liang et al., 2006). Similarly, increased DA turnover and enhanced behavioral responses to psychostimulants have been registered following intermittent/restricted access to palatable diet (Hajnal and Norgren, 2002, but see Moore et al. 2020). However, prolonged intermittent or chronic access to palatable diets have been shown to reduce extracellular DA levels in the NAc, referred to as deficits in the mesolimbic dopamine neurotransmission (Geiger et al., 2009; Fordahl et al., 2016). While their extracellular DA levels were low, rats in the intermittent palatable diet group display increased behavioral sensitization and a greater increase in extracellular levels following the psychostimulants challenge (Fordahl et al., 2016). These effects are hallmarks of a hypodopaminergic state at baseline with an overactive dopamine system in response to external stimuli—largely due to greater dopamine availability as a result of enhanced synthesis (Mathews et al., 2009; Karkhanis et al., 2016, 2019).

Although it is unclear if extracellular levels were different, the present study found greater DA and attenuated DOPAC levels selectively in the NAc homogenates of the Oreo group compared to the chow controls rats (**Figure 7A**). It is possible that increased NAc DA availability in the Oreo group of rats promotes heightened sensitivity to the intoxicating effects of alcohol in the Oreo group of rats, thereby reducing the amount of alcohol needed to achieve a state similar to the chow controls, a hypothesis that needs further investigation. Since rats in the Oreo group were voluntarily restricting calories on alcohol testing days, reduced alcohol intake could reflect enhanced reward sensitivity. It is important to note that brain sections were collected on chow only access days ∼24 h following patterned palatable food intake ended and >100 h following termination of alcohol drinking. While animals had no access to alcohol on that day, previous alcohol testing occurred under identical conditions on the chow only access days. Since both alcohol anticipation and ingestion increase extracellular DA levels in the NAc (Weiss et al., 1993), increased DA content in the NAc could reflect anticipation of alcohol reward. Future studies are needed to precisely understand the role of mesolimbic DA neurotransmission in regulating alcohol intake following this paradigm. Future studies are also needed to identify any sex differences in the impact of patterned feeding of HPF on alcohol drinking.

In conclusion, the present study identifies that patterned feeding of hyper-palatable food reduces alcohol drinking in rats, and alterations in the dopaminergic neurotransmission within mesolimbic circuitry could mediate reduced alcohol drinking behavior following patterned feeding. These data are consistent with previous studies from the lab and systematically replicates reduced alcohol drinking following patterned feeding a palatable high-fat diet and extend these observations to real-world food.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee guidelines at the Xavier University of Louisiana.

AUTHOR CONTRIBUTIONS

SS was responsible for conceptualization, funding acquisition, methodology, investigation, and formal analysis. SS, ZL, and KS conducted feeding and alcohol drinking experiments, wrote original draft and final version of the manuscript. LB and AK conducted HPLC analysis and wrote corresponding sections. All authors contributed to the article and approved the submitted version.

FUNDING

This publication was made possible by funding, in part, by NIGMS-SCORE # 5 SC3 GM127173-03 (SS), NIMHD-RCMI # G12MD007595-10 (SS) and NIGMS-BUILD # UL1GM118967 (SS), RL5GM118966, T32 AA025606 (LB), R01 AA028228 (AK) and NIAAA K01-AA023874 (AK). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

We thank Ms. Starr Villavasso for technical assistance. SS thanks Dr. Nicholas Gilpin at Louisiana State University Health Sciences Center, New Orleans, and Dr. Jon F. Davis at Washington State University, Pullman, WA, for several helpful discussions during this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 21 October 2021
doi: 10.3389/fnbeh.2021.758252

Cotinine: Pharmacologically Active Metabolite of Nicotine and Neural Mechanisms for Its Actions

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Tobacco use disorder continues to be a leading public health issue and cause of premature death in the United States. Nicotine is considered as the major tobacco alkaloid causing addiction through its actions on nicotinic acetylcholine receptors (nAChRs). Current pharmacotherapies targeting nicotine's effects produce only modest effectiveness in promoting cessation, highlighting the critical need for a better understanding of mechanisms of nicotine addiction to inform future treatments. There is growing interest in identifying potential contributions of non-nicotine components to tobacco reinforcement. Cotinine is a minor alkaloid, but the major metabolite of nicotine that can act as a weak agonist of nAChRs. Accumulating evidence indicates that cotinine produces diverse effects and may contribute to effects of nicotine. In this review, we summarize findings implicating cotinine as a neuroactive metabolite of nicotine and discuss available evidence regarding potential mechanisms underlying its effects. Preclinical findings reveal that cotinine crosses the blood brain barrier and interacts with both nAChRs and non-nAChRs in the nervous system, and produces neuropharmacological and behavioral effects. Clinical studies suggest that cotinine is psychoactive in humans. However, reviewing evidence regarding mechanisms underlying effects of cotinine provides a mixed picture with a lack of consensus. Therefore, more research is warranted in order to provide better insight into the actions of cotinine and its contribution to tobacco addiction.

Keywords: behavior, cotinine, neuropharmacology, nicotine, nicotinic acetylcholine receptor

OPEN ACCESS

Edited by:
Satoshi Ikemoto, National Institute on Drug Abuse (NIDA), United States

Reviewed by:
Valentina Echeverria Moran, Bay Pines VA Healthcare System, United States
Alvin V. Terry, Augusta University, United States

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *13 August 2021*
Accepted: *27 September 2021*
Published: *21 October 2021*

Citation:
Tan X, Vrana K and Ding Z-M (2021) Cotinine: Pharmacologically Active Metabolite of Nicotine and Neural Mechanisms for Its Actions. Front. Behav. Neurosci. 15:758252. doi: 10.3389/fnbeh.2021.758252

INTRODUCTION

Cigarette smoking remains to be a leading public health issue. Despite a steady decline over the past decades, smoking rate remained at 17.2 percent in people aged 12 or older in 2018 in the United States (Substance Abuse and Mental Health Services Administration [SAMHSA], 2019). In addition, an estimated 3.6 million middle and high school students were current users of electronic cigarettes in 2020 in the United States, posing additional risk to youth (Wang et al., 2020a). Relapse rates are high in smokers; approximately 55% adult smokers made quit attempts, but only 7.5% successfully quit smoking in 2018 (Creamer et al., 2019). Nicotine is widely accepted as the major addictive component in cigarette, and it mainly activates nicotinic acetylcholine receptors (nAChRs) to produce its reinforcing and rewarding effects (Prochaska and Benowitz, 2016).

Pharmacotherapies targeting effects of nicotine (e.g., nicotine replacement therapy and varenicline) have been approved to aid in smoking cessation, but only produced modest effectiveness in promoting abstinence (Rosen et al., 2018). Therefore, there is a remaining need for better understanding of mechanisms underlying nicotine addiction and tobacco smoking.

There are growing efforts investigating the potential involvement of minor tobacco alkaloids and nicotine metabolites in nicotine's effects and tobacco use (Crooks and Dwoskin, 1997; Hoffman and Evans, 2013). Cotinine is a minor tobacco alkaloid and the major metabolite of nicotine. It is most commonly used as a biomarker for nicotine exposure (Benowitz and Jacob, 1994; Zhu et al., 2013). It is safe and well tolerated in humans with short-term exposure (Bowman and McKennis, 1962; Hatsukami et al., 1997), and much less toxic in rodents than nicotine (Borzelleca et al., 1962; Riah et al., 1999). Since an early study revealed cotinine's behavioral and physiological effects (Yamamoto and Domino, 1965), accumulating evidence indicates that cotinine produces diverse effects across multiple systems, including the nervous system (Fuxe et al., 1979; Dwoskin et al., 1999), cardiovascular system (Dominiak et al., 1985; Chahine et al., 1996), endocrine system (Barbieri et al., 1989; Sofikitis et al., 2000), immune system (Rehani et al., 2008; Li et al., 2021), as well as neurobehavioral systems (Risner et al., 1985; Buccafusco and Terry, 2003). In addition, there is recognition that cotinine may contribute to some effects of nicotine (Crooks and Dwoskin, 1997; Majdi et al., 2019), raising the possibility that cotinine may play a role in tobacco use, abuse, and dependence. Herein, we review findings supporting cotinine as a neuroactive metabolite of nicotine, and discuss potential mechanisms underlying its effects. The focus is on cotinine interactions with the nervous system, and on neuropharmacological and behavioral effects of cotinine (**Figure 1**).

ORIGIN OF COTININE

Cotinine is one of the minor tobacco alkaloids which include nornicotine, anabasine, anatabine and others in tobacco products. These minor alkaloids account for approximately 5% of total tobacco alkaloids, and nicotine makes up the remainder (Benowitz et al., 1983a; Leete, 1983). Cotinine was shown to form in small quantities in fermented tobacco leaves during the tobacco curing and aging processes after harvesting, potentially through chemical oxidation of and/or bacterial actions of nicotine (Frankenburg and Vaitekunas, 1957; Wada et al., 1959). Minimal biosynthesis of cotinine was found in the living *Nicotiana Glauca* plant with negligible conversion of nicotine to cotinine (Leete and Chedekel, 1974). Cotinine is also found in other plant specifies, e.g., Carica papaya and Cestrum nocturnum (Leete, 1983). For each cigarette smoked, cotinine was absorbed in the range of 9-57 μg, far less than that of nicotine at 0.8-3 mg (Schmeltz and Hoffmann, 1977; Benowitz and JacobIII, 1984, Benowitz and Jacob, 1994; Gori and Lynch, 1985).

Cotinine, however, is the predominant metabolite of nicotine in humans and animals through enzyme-mediated oxidation of nicotine (Bowman et al., 1959; Hucker et al., 1959). Early work indicated that this enzymatic process mainly occurred in the liver involving a two-step reaction. Nicotine was first converted to 5′-hydroxynicotine by an enzyme system requiring triphosphopyridine nucleotide and O_2, and then 5′-hydroxynicotine was oxidized to cotinine by an aldehyde oxidase (Hucker et al., 1959, 1960). An important discrete intermediate during this process was later identified as nicotine-$\Delta^{1'(5')}$-iminium ion, which was catalyzed from nicotine by a cytochrome P450 (CYP)-linked oxidase, and was in rapid equilibrium with 5′-hydroxynicotine (Murphy, 1973; Brandänge and Lindblom, 1979b; Peterson et al., 1987). The oxidation of the intermediate to cotinine was demonstrated in mouse liver microsomes to also be mediated by aldehyde oxidase (Hill et al., 1972; Gorrod and Hibberd, 1982). The aldehyde oxidase was also referred to as "iminium oxidase" and was shown to exhibit high affinity for nicotine-$\Delta^{1'(5')}$-iminium ion (Brandänge and Lindblom, 1979a). Subsequent research determined CYP2A6 to be the major enzyme responsible for nicotine conversion to nicotine-$\Delta^{1'(5')}$-iminium ion (Cashman et al., 1992; Nakajima et al., 1996). In addition to the liver, there is evidence suggesting the metabolism of nicotine and formation of cotinine in the brain, mechanisms of which remain less clear (Jacob et al., 1997).

PHARMACOKINETICS OF COTININE

In humans, an average of 70-80% of absorbed nicotine was converted to cotinine (Benowitz and Jacob, 1994; Zhu et al., 2013). Blood cotinine levels in regular smokers typically range between 250 and 350 ng/ml (1.4–2.0 μM), but can reach 800–900 ng/ml (4.5–5.0 μM) in some heavy smokers, greatly exceeding typical blood nicotine levels in the range of 10–50 ng/ml (0.06–0.3 μM) (Benowitz et al., 1983a; Benowitz and Jacob, 1994; Geng et al., 1995; Schneider et al., 2001). Oral administration of cotinine resulted in rapid absorption leading to peak systemic cotinine levels within 45 min. Bioavailability exceeded 95% following oral administration, suggesting minimal first-pass metabolism of cotinine (De Schepper et al., 1987). This is in contrast to approximate 70% of first-pass metabolism of nicotine (Matta et al., 2007). The steady-state volume of distribution was 0.7–1.0 L/kg for cotinine and 2.6–2.8 L/kg for nicotine. Plasma clearance was 0.4–1.0 ml/min/kg for cotinine and 16–17 ml/min/kg for nicotine. The elimination half-life of cotinine ranged from 12 to 16 h, in contrast to 2–2.5 h for nicotine (Benowitz et al., 1983b; De Schepper et al., 1987; Curvall et al., 1990; Benowitz and Jacob, 1994; Zevin et al., 2000; Zhu et al., 2013). The half-life of cotinine, derived from nicotine, can be up to 19–20 h, longer than that of cotinine administered as cotinine, possibly due to slow release of nicotine from tissue to blood (Benowitz et al., 1983b; Benowitz and Jacob, 1994; Zevin et al., 1997). Chronic smoking appeared to reduce the clearance half-life of cotinine (Kyerematen et al., 1982). Approximately 10–12% of administered cotinine was excreted unchanged in the urine (De Schepper et al., 1987; Curvall et al., 1990). Neither nicotine conversion to cotinine nor cotinine elimination appeared to be different between men and women (Benowitz and Jacob, 1994; Zhu et al., 2013). Plasma protein

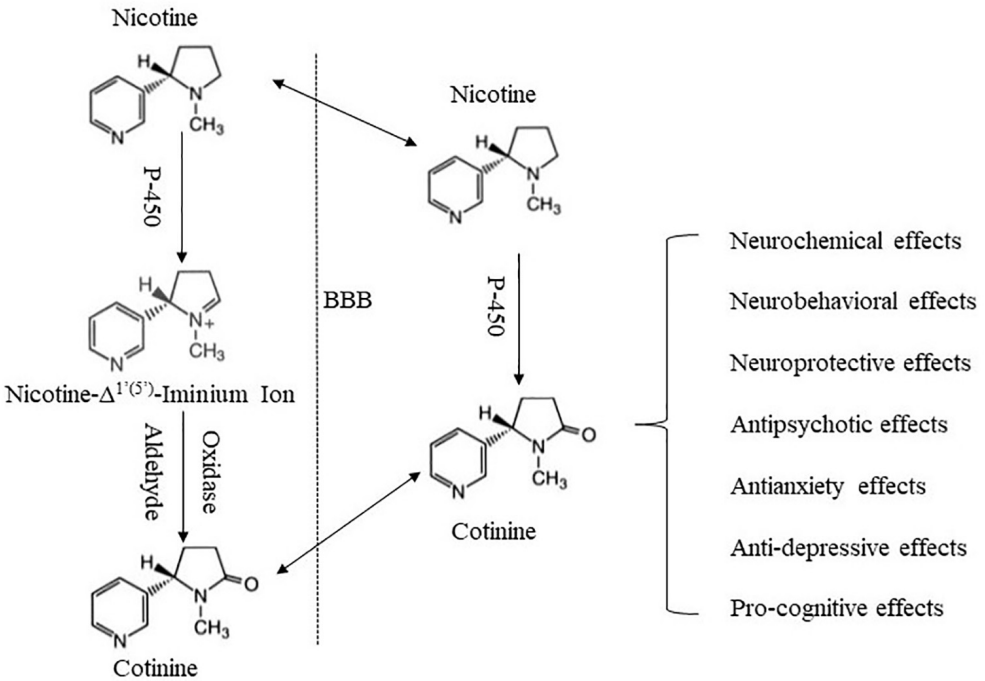

FIGURE 1 | Schematic summary of cotinine formation and its major effects. Cotinine is formed mainly in the periphery via an enzyme-mediated process. Following its formation, cotinine penetrates the BBB and enters the brain. Cotinine in the brain may also be derived from *in situ* metabolism of nicotine. Cotinine then interacts with the brain to produce a variety of effects.

binding of cotinine was concentration-independent and averaged at 2–3%. Blood and plasma cotinine ratio averaged at 0.88, and the ratio between red blood cell and unbound plasma cotinine concentration averaged at 0.74 (Benowitz et al., 1983b). Cotinine did not seem to alter nicotine disposition or metabolism, nor was it converted back to nicotine (Keenan et al., 1994; Zevin et al., 1997; Hatsukami et al., 1998b).

In rats, approximately 60% of absorbed nicotine was converted to cotinine (Hucker et al., 1960). The half-life of cotinine formation ranged from 0.33 to 0.46 h, and maximal plasma cotinine concentrations were reached about 1.5 h after intravenous bolus administration of nicotine (Adir et al., 1976; Miller et al., 1977). Steady-state volume of distribution was 0.7–1.5 L/kg for cotinine and 2.0–5.0 L/kg for nicotine. Plasma clearance was 2.5–4.4 L/h/kg for nicotine and 0.12–0.21 L/h/kg for cotinine (Adir et al., 1976; Miller et al., 1977; Li et al., 2015). The clearance half-lives were about 5.0–9.0 h for cotinine and 20–70 min for nicotine, both of which were slightly longer in adult than early adolescent rats (Miller et al., 1977; Kyerematen et al., 1988; Sastry et al., 1995; Craig et al., 2014). Approximately 17–18% cotinine and 10–11% nicotine were excreted in urine in its unchanged form (Miller et al., 1977).

In mice, blood cotinine peaked within 10 min after intraperitoneal injection of nicotine (Petersen et al., 1984), and clearance half-life was in the range of 20–40 min, longer than that of nicotine at 6–7 min, respectively (Thompson et al., 1982; Petersen et al., 1984). Exposure to smoke from commercial cigarettes showed slowed cotinine peak time at ∼120 min and cotinine half-life could be up to ∼80 min (El Mubarak et al., 2020). Cotinine formation and elimination in mice appeared to be strain-dependent and to be influenced by mouse genotypes. For example, DBA/2Ibg mice attained 1.5 fold higher blood cotinine levels and 60-80% longer half-life than C57BL/6Ibg and C3H/2Ibg mice (Petersen et al., 1984). In combination, these disparate facts combine to demonstrate that nicotine is rapidly converted to cotinine that then is slowly removed. As a result, the body is exposed to high concentrations of cotinine for a prolonged period of time.

BLOOD BRAIN BARRIER PENETRATION OF COTININE

A wealth of evidence indicates that cotinine can penetrate BLOOD BRAIN BARRIER (BBB) and enter the brain. Early studies, using whole-body radiography, reported that intravenous injection of radiolabeled nicotine resulted in uniform and diffuse cotinine-related radioactivity in the brain of mice and cats, and that cotinine could be isolated from brain tissue in mice (Appelgren et al., 1962; Schmiterlöw et al., 1967). A later study indicated that brain uptake of cotinine in mice was brain region dependent with greater cotinine levels detected in cerebral cortex and basal ganglia than in hippocampus or cerebellar cortex following systemic injection of nicotine (Essman, 1973). In addition, the time-course of cotinine penetration of BBB was influenced by routes of nicotine administration. Intravenous

injection of nicotine led to rapid detection of cotinine in the brain within 2-5 min, with peak levels detected 10-20 min post-injection (Stålhandske, 1970; Petersen et al., 1984; Sastry et al., 1995). Subcutaneous administration of nicotine resulted in detection of cotinine as the major metabolite in the brain at 15-30 min post-injection, which peaked around 4 h, and remained detectable 18 h after nicotine administration (Crooks et al., 1995, 1997, Katner et al., 2015). A microdialysis study showed that cotinine was detected approximately 45 min following intragastric administration of nicotine, and continued to increase during the 125-min collection period in the nucleus accumbens, a central reward zone (Katner et al., 2015). Cotinine accumulated in the brain following chronic administration of nicotine via an osmotic minipump in rats, with brain levels lower than serum levels (Oliver et al., 2007). Chronic nicotine exposure did not alter cotinine penetration of BBB in rats (Lockman et al., 2005).

Studies with direct cotinine administration confirmed cotinine penetration of BBB. An early autoradiography study performed in mice observed highest radioactivity in the dense cell area of the cerebellum following intravenous administration of cotinine (Bowman et al., 1964). Another study suggested that cotinine uptake into the brain was relatively homogenous with minimal regional differences (Lockman et al., 2005). In rats, subcutaneous administration of cotinine induced time- and dose-dependent accumulation of cotinine in the brain. Cotinine started to accumulate in the brain within 5 min, reached maximal levels at 20-60 min, and then gradually decreased over time with significant levels of cotinine still detected in the brain at 18 h post-administration (Crooks et al., 1997; Riah et al., 1998). In addition, subcutaneous and intravenous administration resulted in more efficient cotinine penetration than intraperitoneal administration (Riah et al., 1998). No cotinine metabolite was detected following cotinine administration, suggesting little or no biotransformation of cotinine in the brain (Crooks et al., 1997). These studies indicate that cotinine readily crosses the BBB. However, it can't be excluded that nicotine may undergo *in situ* metabolism in the brain, thus contributing to cotinine accumulation in the brain following peripheral nicotine administration (Jacob et al., 1997).

Cotinine appears to be less efficient than nicotine in crossing BBB. In contrast to the widespread distribution of nicotine in the mouse brain, cotinine did not concentrate in the brain nearly as well (Hansson and Schmiterlow, 1962; Schmiterlöw and Hansson, 1962; Bowman et al., 1964). The brain uptake of nicotine was approximately 10 times greater than that of cotinine (Lockman et al., 2005), and an approximately 10 times higher dose of cotinine than nicotine was required to produce comparable concentrations in rats (Riah et al., 1998). In addition, the peak brain/plasma ratio for cotinine was 0.26, much lower than the 0.65 ratio for nicotine (Reavill et al., 1990; Riah et al., 1998). A human positron emission tomography study reported much lower uptake of cotinine than nicotine, in an approximately 1:6 ratio, into the frontal cortex of healthy non-smokers (Halldin et al., 1992). Nicotine is a tertiary amine, and its un-ionized form is highly lipophilic, whereas cotinine is more polar and less lipophilic (Halldin et al., 1992; Crooks and Dwoskin, 1997; Herzig et al., 1998). In addition, nicotine is transported as a mono-protonated cation across the BBB by organic cationic transport systems, whereas no active transport system has been reported for cotinine (Majdi et al., 2019). These differences in passive diffusion and active transport may contribute to the lower penetration of BBB by cotinine as compared to that of nicotine.

Brain half-lives of cotinine were 20–30 min in mice, and ∼350 min in rats, significantly longer than those of nicotine in mice at 6–7 min and in rats at ∼50–90 min (Petersen et al., 1984; Sastry et al., 1995; Ghosheh et al., 1999; Craig et al., 2014). It was estimated that, for average plasma cotinine levels at 250–350 ng/ml, the influx rate of cotinine through BBB was 0.5–0.7 ng per second per gram brain tissue, which was ∼40% of the nicotine influx estimated with average nicotine levels at 40–50 ng/ml, suggesting that cotinine may penetrate the BBB to a significant degree that would allow central actions (Lockman et al., 2005). This is consistent with evidence demonstrating cotinine's neuropharmacological and behavioral effects in animals (Goldberg et al., 1989; Crooks and Dwoskin, 1997; Terry et al., 2005), and psychoactive effects in humans (Keenan et al., 1994; Hatsukami et al., 1998b).

PHARMACODYNAMICS OF COTININE

Cotinine appears to be a weak agonist of nAChRs, but there are substantial discrepancies in the literature regarding its potency (**Table 1**). In rat brain membrane preparations, two studies reported that Ki values of cotinine for displacing [^{3}H]nicotine or [^{3}H]epibatidine binding were ∼1–4 μM, and Ki values for nicotine were ∼5–15 nM, with cotinine being ∼200–250 fold less potent than nicotine (Abood et al., 1981; Vainio and Tuominen, 2001). These Ki values of cotinine are within the range of blood cotinine concentrations attained in human smokers (Hukkanen et al., 2005). Other studies reported that the potency of cotinine for displacing [^{3}H]nicotine binding was 1-3 mM, and the potency of nicotine was 0.6–200 nM for nicotine, with cotinine being ∼10,000 to ∼1.5 million fold less potent than nicotine (Sloan et al., 1984; Anderson and Arneric, 1994; Riah et al., 1999). The potency of cotinine is greatly higher than physiological levels of cotinine in smokers (Hukkanen et al., 2005). Ki values for displacing [^{3}H]cytisine binding (presumably high-affinity α4β2* subtype; the * denotes other nAChR subunits) were over 200 μM for cotinine and 0.6 nM for nicotine (Anderson and Arneric, 1994). Consistently, cotinine up to 1 μM produced minimal effect on [^{3}H]cytisine binding, whereas nicotine induced over 70% inhibition of [^{3}H]cytisine binding in rat cerebral cortex preparations (Sziraki et al., 1999). Cotinine and nicotine were reported to display equal efficacy in displacing [^{125}I]α-bungarotoxin binding (presumably low-affinity α7 nAChRs), but cotinine was ∼100 fold less potent than nicotine, with IC_{50} values at 1 mM and 10 μM, respectively (Riah et al., 1999).

In squirrel monkey preparations, cotinine inhibited ^{125}I-α-conotoxinMII (a ligand for α3/α6β2* nAChRs) binding in the caudate with an IC_{50} value of ∼3.5 μM, which was ∼600-fold less potent than nicotine at 5.7 nM. Cotinine also inhibited [^{125}I]A-85380 (a ligand for both α3/α6β2* and α4β2* nAChRs) binding

TABLE 1 | Summary of receptor binding and agonistic potency of cotinine for nAChRs and specific subtypes.

Receptor	Test system	Potency (μM)		References
		(–)-Cotinine	(–)-Nicotine	
nAChRs	Rat brain membrane	1–2	0.006–0.01	Abood et al., 1981
		3–4	0.011–0.016	Vainio and Tuominen, 2001
		> 1,000	0.0006	Anderson and Arneric, 1994
		2,000	0.2	Riah et al., 1999
		2,800	0.03	Sloan et al., 1984
	Torpedo membrane	520	0.3	Abood et al., 1981
		200,000	500	Riah et al., 1999
	Bovine chromaffin cells	130–310	0.3–1.6	Vainio and Tuominen, 2001
α4β2*	Rat brain membrane	> 200	0.0006	Anderson and Arneric, 1994
	Monkey striatal synaptosomes	65–79	0.008	O'Leary et al., 2008
	Chinese hamster ovary cells	85	0.8	Alijevic et al., 2020
α7	Rat brain membrane	1,000	10	Riah et al., 1999
	Torpedo membrane	50	25	Riah et al., 1999
	Xenopus oocytes (α7V274T mutant)	70	0.94	Briggs et al., 1999
α3/α6β2*	Monkey striatal synaptosomes	3.1–3.5	0.006	O'Leary et al., 2008

Nicotine data, when available in the same studies, are included for comparisons.

with an IC_{50} value of 65–80 μM, ∼10,000-fold less potent than nicotine at 7.53 nM. Complete inhibition of ^{125}I-a-conotoxinMII or [^{125}I]A-85380 binding by cotinine occurred at ∼ 1 mM. This study suggested that cotinine might be more potent at α3/α6β2* than α4β2* receptors (O'Leary et al., 2008).

In cultured bovine chromaffin cells, EC_{50} values were 130 μM for cotinine and 0.3 μM for nicotine for displacing high-affinity [^{3}H]epibatadine binding, and were 310 μM for cotinine and 1.6 μM for nicotine for displacing low-affinity [^{3}H]epibatidine binding (Vainio and Tuominen, 2001). In Torpedo membrane, Ki values for displacing [^{3}H]nicotine were 520 μM for cotinine and 310 nM for nicotine (Abood et al., 1981). Another study reported that IC_{50} values for inhibiting [^{3}H]nicotine binding were 200 mM for cotinine and 0.5 mM for nicotine. IC_{50} values for displacing [^{125}I]α-bungarotoxin binding were 50 μM for cotinine and 25 μM for nicotine. In these assays, cotinine appeared to be only 50% efficacious compared to nicotine (Riah et al., 1999). These studies suggested that cotinine might have greater potency at low-affinity α7 nAChRs in Torpedo membrane.

In cultured *Xenopus* oocytes or Chinese hamster ovary (CHO) cells expressing human α7 nAChRs, cotinine at concentrations up to 1 mM did not elicit appreciable activation of these receptors (Briggs and McKenna, 1998; Terry et al., 2015a; Alijevic et al., 2020). However, cotinine functioned as a full agonist of a mutant human α7 nAChR (α7V274T) with its EC_{50} value at 70 μM (Briggs et al., 1999). In cultured *Xenopus* oocytes, cotinine up to 100 μM didn't activate human α4β2 nAChRs (Terry et al., 2015a). On the other hand, cotinine activated human α4β2 nAChRs with EC_{50} value at ∼90 μM in CHO cells, and cotinine was ∼115 fold less potent and 40% less efficacious than nicotine, suggesting a weak partial agonist activity of cotinine on α4β2 nAChRs (Alijevic et al., 2020). Pretreatment with cotinine up to 100 μM did not alter acetylcholine-induced currents in either α7 or α4β2 nAChRs, whereas short term cotinine incubation increased acetylcholine-induced currents in α7, but not α4β2 receptors, suggesting that short-term exposure to cotinine upregulated acetylcholine activation of α7 receptors (Terry et al., 2015a). On the other hand, cotinine was shown to inhibit acetylcholine-elicited response in human α7 nAChRs with IC_{50} values at 175 μM; cotinine was ∼250 fold less potent than nicotine, but similarly efficacious to nicotine (Briggs and McKenna, 1998).

These studies revealed a complex landscape of the interaction between cotinine and nAChRs, with cotinine functioning mainly as a weak agonist of α3/α6β2*, α4β2*, and α7 nAChRs. In addition, the potency and efficacy of cotinine appear to be influenced by subunit compositions of nAChRs. Interestingly, α7 and β2 subunits can form functional α7β2 heteromeric nAChRs in the brain (Wu et al., 2016). Whether the α7β2 nAChRs would interact with cotinine remains to be determined. More importantly, most of these studies reveal that the potency values of cotinine greatly exceed the physiological levels of cotinine obtained in smokers, suggesting that nAChRs may not be the main target of cotinine in smokers. However, more research will be needed to identify receptors that cotinine can interact with at physiological levels.

Cotinine did not show significant binding to serotonin receptors (Fuxe et al., 1979), muscarinic receptors (Anderson and Arneric, 1994), or NMDA receptors (Aizenman et al., 1991). A recent study was in line with these findings (Terry et al., 2015a). In addition, cotinine at 10 μM was found to lack significant binding to or action on more than 70 molecular targets, including major neurotransmitter receptors and transporters (adenosine, adrenergic, dopamine, GABA, glutamate, glycine, histamine, muscarinic, opioid, serotonin, sigma 1 and 2), ion channels (Ca^{2+}, K^+, Na^+), second messengers (e.g., nitric oxide), prostaglandins (e.g., leukotriene and thromboxane), brain/gut peptides (e.g., angiotensin II, bradykinin, endothelin, neurokinin, neuropeptide), and enzymes (acetylcholine esterase, phosphodiesterase, protein kinase A and C) (Terry et al., 2015a).

Interestingly, one study reported the isolation of a putative cotinine receptor from rat brain. This 40-kDa protein had greater affinity for cotinine than for α-bungarotoxin, nicotine and acetylcholine, with IC_{50} values at 0.19 μM, 1.7 μM, 110 μM, and 160 mM, respectively. Amino acid sequence analysis of this protein showed no identity to then known proteins except for the homology to the human p205 synovial fluid protein (Riah et al., 2000). A recent study demonstrated that both cotinine and nicotine bound with similar affinity (∼10–20 μM) to the myeloid differentiation protein 2, an accessory protein of Toll-like receptor 4, to regulate glia-mediated neuroinflammation

in a nAChRs-independent manner (Li et al., 2021). These studies suggest that cotinine may function through non-nAChRs-mediated mechanisms. However, whether these mechanisms may underlie cotinine's physiological effects remains to be determined.

NEUROPHARMACOLOGICAL EFFECTS OF COTININE

Several studies indicated that cotinine altered serotonin turnover in the brain. Chronic exposure of rats to cotinine in drinking water increased daily urinary excretion of 5-hydroxyindoleacetic acid, the major metabolite of serotonin, suggesting that cotinine might alter serotonin turnover (De Clercqm and Truhaut, 1963). Systemic administration of cotinine in mice significantly increased tissue content of serotonin and 5-hydroxyindoleacetic acid in mesencephalon and diencephalon, but not in the cerebral cortex. The effect of cotinine on serotonin levels was similarly robust to nicotine in mesencephalon, but less robust in diencephalon. In contrast, elevation of 5-hydroxyindoleacetic acid was more pronounced following cotinine treatment in both regions (Essman, 1973). Repeated intraperitoneal injections of cotinine attenuated α-propyldopacetamide-induced cortical serotonin depletion to a similar degree as nicotine treatment, which was not altered by mecamylamine pretreatment (Fuxe et al., 1979). In addition, low concentrations of cotinine, but not nicotine, reduced serotonin uptake, and increased spontaneous serotonin release *in vitro* in neocortical slices (Fuxe et al., 1979).

Cotinine can increase brain dopamine transmission. In rat striatal slices or minces, cotinine increased [^{3}H]dopamine overflow in concentration-, Ca^{2+}-, and nAChRs-dependent manners, with EC_{50} values ranging from 30 to 350 μM (Dwoskin et al., 1999; Oliver et al., 2007). This increase appears to be due mainly to facilitated synaptic dopamine release, but not dopamine uptake (Dwoskin et al., 1999). Cotinine was ∼1000 fold less potent than nicotine, but was as fully efficacious as nicotine (Oliver et al., 2007). In squirrel monkeys, cotinine stimulated [^{3}H]dopamine release from striatal synaptosomes through both α3/α6β2* and α4β2* nAChRs-dependent mechanisms. EC_{50} values were 270 and 500–750 μM for α3/α6β2*- and α4β2*-mediated release, respectively, which were 200–750 fold less potent than nicotine. Cotinine was equally efficacious to nicotine in producing α4β2*-mediated release, but was only 50% efficacious in inducing α3/α6β2*-mediated release in the medial caudate (O'Leary et al., 2008).

Cotinine has also been shown to alter peripheral catecholamine activity. Cotinine induced concentration-dependent depolarization of mouse sympathetic superior cervical ganglion, which may lead to altered catecholamine release. Cotinine was ∼80 fold less potent than nicotine (Schroff et al., 2000). In isolated rabbit heart, cotinine inhibited [^{3}H]norepinephrine release evoked by sympathetic nerve stimulation, whereas nicotine increased stimulated release of [^{3}H]norepinephrine (Chahine et al., 1993). In cultured bovine adrenal chromaffin cells, cotinine increased the release of [^{3}H]noradrenaline, which was accompanied by increased protein kinase C expression and phorbol dibutyrate binding. Cotinine was less potent than nicotine in inducing these effects (Vainio et al., 1998b). In isolated rat adrenal gland, cotinine inhibited catecholamine release evoked by high calcium and acetylcholine, but not by high K^+, whereas nicotine produced biphasic effect on catecholamine release induced by acetylcholine and high K^+. Both cotinine and nicotine depressed catecholamine release evoked by activation of nicotinic or M1 muscarinic receptors (Koh et al., 2003).

Cotinine affected extracellular amino acid levels in the brain. Perfusion of striatum with cotinine via reversed microdialysis in rats decreased the levels of aspartic acid, serine, and glutamine, but did not change the levels of glutamic acid, glycine, taurine, or threonine (Toth et al., 1993).

These studies suggest that cotinine can alter neurochemistry in the nervous system, especially monoamine neurotransmission. However, it remains unknown how these cotinine-induced neurochemical changes may contribute to the effects of cotinine on behavior. Given the important role of monoamine neurotransmitters, especially dopamine, in nicotine reinforcement and the development of nicotine addiction (De Biasi and Dani, 2011), it will be interesting to determine potential roles of cotinine in nicotine reinforcement and use.

NEUROPROTECTIVE EFFECTS OF COTININE

In cultured PC12 cells or rat primary cortical neurons, cotinine attenuated loss in cell viability induced by growth factor withdrawal, $Aβ_{1-42}$ incubation, and excessive glutamate, with the (−) isomers more effective than the (+) isomers. In these effects, cotinine showed similar potency and efficacy to nicotine (Buccafusco and Terry, 2003; Terry et al., 2005; Burgess et al., 2012; Gao et al., 2014). Cotinine was shown in *in vitro* studies to bind to $Aβ_{1-40}$ peptides, and to inhibit $Aβ_{1-42}$ peptide precipitation and aggregation with similar affinity (Ka ∼10 nM) and efficacy to nicotine (Salomon et al., 1996; Szymańska et al., 2007; Echeverria et al., 2011). Cotinine increased neurotrophic factors level, and activated pro-survival signaling markers (Sadigh-Eteghad et al., 2020). In addition, cotinine attenuated 6-hydroxydopamine-induced cytotoxicity (a Parkinson's disease model) in cultured human neuroblastoma cells. Cotinine was equally effective to nicotine at a lower concentration of 6-hydroxydopamine, but less effective than nicotine at a higher concentration of 6-hydroxydopamine (Riveles et al., 2008). Cotinine was shown to increase total antioxidant capacity and reduce oxidative stress. Cotinine reduced O_2 consumption, H_2O_2 accumulation, and the production of oxygen free radicals to the similar degree as nicotine (Srivastava et al., 1989; Soto-Otero et al., 2002; Sadigh-Eteghad et al., 2020). Cotinine also attenuated production of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokines (Rehani et al., 2008; Bagaitkar et al., 2012; Sadigh-Eteghad et al., 2020). These anti-oxidative stress, anti-inflammatory, and pro-survival effects of cotinine may contribute to the neuroprotective effects of cotinine, suggesting potential beneficial effects of cotinine in neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease.

BEHAVIORAL EFFECTS OF COTININE

Effects of Cotinine on Locomotor Activity

Low doses of cotinine were shown to alter locomotor activity, with one study reporting reduced (Wiley et al., 2015), and another demonstrating increased locomotor activity (Wang et al., 2020b). Interestingly, low doses of nicotine produced biphasic effects with initial decrease followed by subsequent increase of locomotor activity (Wiley et al., 2015). These findings suggest differential effects of low doses of cotinine and nicotine on locomotor activity. Repeated daily treatment with cotinine decreased locomotor activity overtime in adult, but not adolescent rats, suggesting age-dependent effects (Marusich et al., 2017). Chronic cotinine treatment decreased locomotor activity in mice receiving chronic restraint stress, but not in non-stressed mice, suggesting an interaction between cotinine and stress on locomotor activity (Grizzell et al., 2014a).

Cotinine was shown to alter motor function induced by either nicotine or ethanol. Adding a low dose of cotinine to nicotine solution enhanced the locomotor-stimulating effect of nicotine (Clemens et al., 2009). Intra-ventricular or intra-cerebellar administration of cotinine or nicotine attenuated ethanol-induced motor incoordination in mice, with cotinine producing less robust effects than nicotine. The nAChR antagonists, hexamethonium and trimethaphan, blocked these effects of cotinine. Cotinine and nicotine also antagonized effects of adenosine agonists on ethanol-induced motor incoordination. These data suggest an interaction between nicotinic cholinergic and adenosinergic systems within the cerebellum, and its involvement in modulating ethanol-induced motor incoordination (Dar et al., 1993, 1994).

Effects of Cotinine on Conditioning-Related Behaviors

A series of studies by Goldberg and colleagues indicated that cotinine altered schedule-controlled, food-conditioned responding in a species-, schedule-, and dose-dependent manner. In dogs, cotinine decreased response rates during a fixed-ratio (FR) and a fixed-interval (FI) schedule, whereas nicotine decreased response rates during the FR schedule, but produced biphasic effects during the FI schedule (Risner et al., 1985). In squirrel monkeys, cotinine reduced overall responses during the FR schedule, and produced biphasic effects during the FI schedule. Nicotine produced biphasic effects during both schedules (Risner et al., 1985). In rats, cotinine dose-dependently increased response rate during a FI, but not a FR schedule. Nicotine produced biphasic effects during the FI schedule, but only decreased response rates during the FR schedule (Goldberg et al., 1989). Interestingly, the effects of nicotine, but not cotinine, were antagonized by the pretreatment with the non-selective nAChRs antagonist mecamylamine in rats (Goldberg et al., 1989). These studies suggest that cotinine may alter reinforcement-related behavior.

Several studies indicated that cotinine could be substituted for nicotine in producing nicotine-like discriminative stimulus effects. Intra-ventricular administration of cotinine fully generalized to nicotine in inducing discriminative stimulus effects in rats trained on nicotine under a variable-interval schedule in a training dose-dependent manner (Rosecrans and Chance, 1977; Rosecrans et al., 1978). Later studies by Goldberg and colleagues demonstrated that systemic cotinine was nearly completely substituted for nicotine in both rats and squirrel monkeys; EC_{50} value of cotinine was approximately 30 mg/kg and cotinine was 1000-2000 fold less potent than nicotine (Goldberg et al., 1989; Takada et al., 1989). These authors noted that there was up to 0.1% of nicotine as impurity in continine, which was speculated to contribute to effects of cotinine. However, no pharmacological or blood nicotine data were provided to support this speculation. These authors also found that cotinine's effects on food-reinforced behaviors were insensitive to nAChR blockade with mecamylamine (Goldberg et al., 1989). Therefore, the potential confound with nicotine impurity remains unproven.

In a recent study, we demonstrated that cotinine was self-administered intravenously in rats in a dose-dependent manner (Ding et al., 2021). Rats acquired self-administration of cotinine over time and responded more on an active lever than an inactive lever. Cotinine induced more infusions and greater breakpoints than vehicle under both fixed-ratio and progressive-ratio schedules. The comparison between cotinine and nicotine revealed similarities and differences in self-administration which were dependent on reinforcement schedule and dose. In general, cotinine self-administration was less robust than that of nicotine. In addition, this study found that pharmacological manipulation of nAChRs with mecamylamine and varenicline only reduced nicotine, but not cotinine, self-administration, suggesting differential involvement of nAChRs in cotinine and nicotine self-administration. It remains unknown how cotinine may contribute to nicotine self-administration.

EFFECTS OF COTININE ON NEUROPSYCHIATRIC SYMPTOMS

The neuropsychiatric disease schizophrenia is characterized by impairment in sensorimotor gating. Schizophrenia is comorbid with high rates of tobacco smoking and it has been proposed that nicotine can provide protective effects against neuropsychiatric symptoms in schizophrenia (Lucatch et al., 2018). Pre-pulse inhibition (PPI) of the acoustic startle reflex is a widely used experimental model for schizophrenia. The PPI paradigm for measuring sensorimotor gating measures suppression of the amplitude of a startle reflex to a startling stimulus when it is preceded by a weaker pre-pulse stimulus (Geyer et al., 2001). In a series of studies, deficits in PPI was induced in rats by the non-selective dopamine receptor agonist apomorphine, the non-competitive NMDA receptor antagonist MK-801, and non-specific muscarinic receptor antagonist scopolamine. Pretreatment with cotinine reversed deficits in PPI induced by these compounds (Buccafusco and Terry, 2003; Terry et al., 2005). In addition, in DBA/2 mice exhibiting spontaneous deficits in hippocampal sensory inhibition, both acute and chronic cotinine increased conditioning amplitude in a hippocampal

sensory inhibition test, suggesting that cotinine may attenuate deficits in sensory inhibition (Wildeboer-Andrud et al., 2014). These results suggest that cotinine may be beneficial for schizophrenia and other sensory gating disorders.

Post-traumatic stress disorder (PTSD) is an anxiety disorder triggered by exposure to life-threatening traumatic events. Epidemiological studies indicate a high prevalence of tobacco use in PTSD patients, and tobacco use has been proposed as a form of self-medication to improve neuropsychiatric symptoms in PTSD (Leonard et al., 2001). In rodent fear conditioning models of PTSD, cotinine administration, either systemically or locally into the hippocampus and medial prefrontal cortex, reduced the retention of fear memory and facilitated extinction of fear memory (Zeitlin et al., 2012; Aguiar et al., 2013; Alvarez-Ricartes et al., 2018; Oliveros-Matus et al., 2020). Interestingly, the effects of cotinine in the hippocampus were shared by nicotinic antagonists, such as mecamylamine, dihydro-β-erythroidine, and methyllycaconitine, suggesting that inhibition of nAChRs may underlie cotinine's effects in hippocampus (Aguiar et al., 2013). Co-administration of methyllycaconitine but not dihydro-β-erythroidine, with cotinine into the medial prefrontal cortex abolished the effect of cotinine on extinction of fear conditioning, suggesting an involvement of α7 nAChRs-, but not α4β2* nAChRs-mediated mechanisms within the medial prefrontal cortex (Oliveros-Matus et al., 2020). In addition, systemic cotinine reduced anxiety-like behaviors in the elevated plus maze test and the open field test following fear conditioning. These effects of cotinine were accompanied by an increase of phospho-ERK1/2 (Zeitlin et al., 2012; Aguiar et al., 2013), calcineurin (Alvarez-Ricartes et al., 2018), and GFAP + immunoreactivity (Oliveros-Matus et al., 2020) in hippocampus and PFC. These findings suggest that cotinine may have therapeutic potential for PTSD-like symptoms.

Cotinine reduced depressive-like behaviors induced by chronic stress (Grizzell et al., 2014a,b; Perez-Urrutia et al., 2017), fear conditioning (Alvarez-Ricartes et al., 2018), chemotherapy (Iarkov et al., 2016), and the development of Alzheimer's disease (Patel et al., 2014). These effects of cotinine were associated with increase of vascular endothelial growth factor, pAKT-GSK3β phosphorylation, synaptic density and PSD95 expression, and calcineurin in the hippocampus and/or prefrontal cortex (Grizzell et al., 2014a,b; Patel et al., 2014; Alvarez-Ricartes et al., 2018). These results suggest that cotinine may provide beneficial effects for depression.

EFFECTS OF COTININE ON COGNITIVE FUNCTIONS

It is well known that nicotine can enhance cognitive functions (Valentine and Sofuoglu, 2018), and recent preclinical findings indicate that cotinine can also provide cognitive benefits. In a series of studies using the delayed matching-to-sample task to measure working memory and attention in Macaques, cotinine was shown to increase the overall task accuracy by itself, and produced persistent attenuation of ketamine- and distractor-induced impairment in task accuracy (Buccafusco and Terry, 2003, 2009; Terry et al., 2005). Cotinine improved sustained attention in rats tested in the five choice serial reaction time task. The non-competitive NMDA receptor antagonist MK-801 reduced overall accuracy rate, increased impulsive- and compulsive-like behaviors, and caused cognitive inflexibility. Both acute and chronic cotinine significantly attenuated MK-801-induced impairments in task accuracy, and reduced impulsive- and compulsive-like behaviors (Terry et al., 2012). In Swiss mice, cotinine suppressed the scopolamine-induced deficit in short-term spatial memory in Y-maze test, and its effects were less efficacious than those of nicotine (Callahan et al., 2021). These studies suggest that cotinine may have therapeutic potential for neuropsychiatric disorders by improving attention and memory, especially those characterized by alterations in glutamate and cholinergic neurotransmission.

Repeated cotinine improved spatial recognition memory in a novel location recognition test in rats receiving chemotherapy (Iarkov et al., 2016). Chronic cotinine treatment improved working memory performance in the radial arm water maze test (Grizzell et al., 2014a), and reversed the deficit in visual recognition memory in the novel object recognition test after prolonged restraint stress in mice (Grizzell et al., 2014a; Perez-Urrutia et al., 2017; Mendoza et al., 2018b). These behavioral changes were accompanied by normalization of the number and arborization of GFAP + cells (Perez-Urrutia et al., 2017; Mendoza et al., 2018b), increases in GSK3β phosphorylation, and enhancement of synaptic density in prefrontal cortex and hippocampus (Grizzell et al., 2014a).

Cotinine attenuated age- and neurodegeneration-related cognitive impairments. In senescent mice, chronic cotinine treatment reversed impairments in spatial and recognition learning and memories in the Morris water maze and novel object recognition tasks in a α7 nAChR-dependent manner (Sadigh-Eteghad et al., 2020). In both transgenic and Aβ-induced models of Alzheimer's disease, chronic cotinine administration prevented working and reference memory impairments, and improved cognitive performance in several learning and memory tasks, including circular platform, radial arm water maze, Y-maze, and cognitive interference task. Cotinine also restored short-term visual recognition memory performance in a novel object recognition test. Such a protective effect was not observed in the Morris water maze or platform recognition task, suggesting a task-dependent effect (Echeverria et al., 2011; Grizzell et al., 2017; Boiangiu et al., 2020). These beneficial effects were associated with reduction in Aβ, p-Tau, neuroinflammation, and acetylcholinesterase activity, as well as increase in neurotrophic factors, total antioxidant capacity, pro-survival signaling, and synaptic plasticity in hippocampus and/or prefrontal cortex (Echeverria et al., 2011; Patel et al., 2014; Grizzell et al., 2017; Boiangiu et al., 2020, 2021). All these factors may converge to promote neuronal synaptic plasticity and long-term potentiation, inhibit neuronal cell death, and improve memory and attention.

Cotinine also improved cognitive performance in $Fmr1^{-/-}$ mice, a murine model of Fragile X syndrome. Cotinine rescued deficits in spatial memory in the coordinate and categorical spatial processing tests, increased the performance toward a novel object in the novel object recognition test, and reversed memory

impairment in the temporal order memory test. This study also established the causal role of the AKT-GSK3β signaling pathway in mediating cotinine's effects by demonstrating that cotinine failed to enhance cognition in GSK3β knockin mice that exhibited impaired phosphorylation of GSK3β (Pardo et al., 2017).

EFFECTS OF COTININE IN HUMANS

Cotinine at doses producing blood levels up to 3000 ng/ml (∼17 μM) appeared to be safe and well-tolerated in humans, with no major side effects other than dizziness and headache (Bowman and McKennis, 1962; Hatsukami et al., 1997, 1998a). Cotinine had no appreciable cardiovascular effects, did not alter heart rate, systolic or diastolic blood pressure, or electrocardiogram in healthy non-smokers (Zevin et al., 1997, 2000; Herzig et al., 1998) or abstinent smokers (Benowitz et al., 1983b; Keenan et al., 1994). Cotinine didn't change skin temperature, weight, or caloric intake (Benowitz et al., 1983b; Hatsukami et al., 1997, 1998b). Cotinine didn't appear to alter mood state in healthy non-smokers (Herzig et al., 1998; Zevin et al., 2000). Discontinuation from repeated cotinine administration did not induce drug-like effects or withdrawal-like symptoms (Hatsukami et al., 1997). Therefore, cotinine appears to have a more favorable toxicology profile compared to nicotine.

There is evidence that cotinine alters withdrawal-related psychological and physiological signs and symptoms. Acute cotinine infusion in abstinent smokers reduced self-reported desire to smoke, irritability, low energy, anxiety and tension. A tendency to think less about smoking was also observed. These effects were small and subtle, and there was no placebo control group included for a comparison (Benowitz et al., 1983b). One randomized, double-blind, placebo-controlled, counterbalanced study examined the effects of cotinine on symptoms related to acute smoking cessation. Compared to placebo, cotinine slightly increased the self-ratings of "pleasant" and "sedated," but reduced the self-ratings of "restless," "anxious/tense," "insomnia," suggesting cotinine alterations of subjective ratings during acute withdrawal (Keenan et al., 1994). On the other hand, a sebsequent study reported that cotinine increased "restless" and "impatience", and as dose increased, tended to increase then decrease "depressed mood" and "difficulty concentrating" during acute withdrawal (Schuh et al., 1996). Another study demonstrated that cotinine caused a greater severity of "difficulty concentrating," an increase in fatigue, and potentially less abstinence in abstinent smokers compared to nicotine patch and nicotine plus cotinine treatments. In addition, cotinine completely eliminated nicotine patch's effects on reducing withdrawal symptoms (Hatsukami et al., 1998b). Cotinine did not alter the self-reported number of cigarettes smoked, the average weights of the collected cigarette butts, or alveolar carbon monoxide levels in current smokers (Hatsukami et al., 1998a).

Cotinine was shown to cause cognitive deficit in healthy non-smokers. Cotinine significantly impaired memory on the long list of a verbal recall task, and slowed serial information processing in a visual choice reaction time task (Herzig et al., 1998). On the other hand, cotinine did not alter cognitive function in several attention-related tasks, including the Symbol Digit Modalities test, the Stroop test, and Letter Cancellation test (Hatsukami et al., 1998b). These different results suggest that effects of cotinine on cognitive function may be task-dependent. Given the cognition-enhancing effects of cotinine in animal models of various neuropsychiatric and neurodegenerative diseases (Terry et al., 2005; Echeverria and Zeitlin, 2012; Mendoza et al., 2018a), it will be interesting to examine the potential effects of cotinine on cognitive function in these disease states.

In these studies, cotinine was administered either acutely or repeatedly over a short period of time, usually fewer than 14 days. Given the chronic relapsing nature of habitual smoking, it would be worth examining effects of cotinine over a longer-term administration period to better understand the chronic effects of cotinine in humans.

POTENTIAL MECHANISMS UNDERLYING COTININE'S EFFECTS

Potential Involvement of Nicotinic Acetylcholine Receptors?

Since cotinine has been shown to be a weak agonist of nAChRs, most studies have focused on determining whether nAChRs could mediate the effects of cotinine. There are a number of studies indicating that certain effects of cotinine are dependent on activation of nAChRs. Cotinine blunted pain perception and this was blocked by mecamylamine (Erenmemisoglu and Tekol, 1994). Cotinine increased phorbol binding and intracellular Ca^{2+} concentrations in cultured bovine adrenal chromaffin cells, and these effects were antagonized by nAChR antagonists, hexamethonium, chlorisondamine, and dihydro-β-erythroidine (Vainio et al., 1998b, 2000). Cotinine-increased striatal dopamine overflow was attenuated by mecamylamine and dihydro-β-erythroidine (Dwoskin et al., 1999; Oliver et al., 2007). Cotinine-attenuated production of pro-inflammatory cytokines was reversed by α-bungarotoxin (Rehani et al., 2008; Bagaitkar et al., 2012). Dihydro-β-erythroidine and α-bungarotoxin prevented cotinine-induced reversal of sensory inhibition deficits in mice (Wildeboer-Andrud et al., 2014). Methyllycaconitine abolished the effect of cotinine on extinction of fear conditioning (Oliveros-Matus et al., 2020) and age-related cognitive impairments (Sadigh-Eteghad et al., 2020). Dihydro-β-erythroidine, but not methyllycaconitine, co-infusion abolished cotinine's effect on GFAP (Oliveros-Matus et al., 2020). The preponderance of evidence, therefore, suggests that the effects of cotinine in these physiological domains may be mediated through nAChRs.

There is also evidence, however, suggesting that certain effects of cotinine are not mediated by nAChRs. Effects of cotinine on cortical serotonin depletion and on food-reinforced operant responding were not antagonized by mecamylamine (Fuxe et al., 1979; Goldberg et al., 1989). The nAChR antagonist hexamethonium reduced nicotine toxicity, but enhanced cotinine toxicity (Riah et al., 1999). Cotinine alters BBB permeability of saquinavir and sucrose, and these effects

are not altered by α-bungarotoxin, methyllycaconitine, or mecamylamine (Abbruscato et al., 2002; Manda et al., 2010). The effects of cotinine on Aβ-induced cell death was not affected by mecamylamine (Burgess et al., 2012). Cotinine self-administration in rats were not altered by mecamylamine or varenicline (Ding et al., 2021). Furthermore, the inhibitory effects of cotinine on lipopolysaccharide-induced pro-inflammatory factors were not affected by either mecamylamine or RNAi-mediated down-regulation of α7 nAChRs (Li et al., 2021).

These differences may be driven by the varying experimental systems implemented in these studies. In addition, they suggest that cotinine may act through both nAChRs- and non-nAChR-mediated mechanisms, which echoes findings implicating cotinine's binding and interactions with both nAChRs and other protein targets reviewed above. However, these non-nAChRs mechanisms remain to be further characterized.

Cotinine as a Nicotinic Acetylcholine Receptors Desensitizing Agent?

Cotinine has been proposed as a desensitization agent for nAChRs (Buccafusco et al., 2009). There are several lines of evidence supporting this hypothesis. First, cotinine increased dopamine overflow from rat striatal slices; the increase peaked shortly after cotinine superfusion, but gradually diminished during continued cotinine incubation, suggesting the development of receptor desensitization overtime (Dwoskin et al., 1999). Second, cotinine pretreatment diminished several effects mediated by the activation of nAChRs, including nicotine-induced increase of intracellular Ca^{2+} concentrations and norepinephrine release in cultured bovine chromaffin cells (Vainio et al., 1998a, 2000), nicotine-induced dopamine release from striatal minces (Oliver et al., 2007) and nucleus accumbens (Sziraki et al., 1999), nicotine-stimulation of mouse sympathetic superior cervical ganglion neurons (Schroff et al., 2000), acetylcholine-stimulated catecholamine release from adrenal gland (Koh et al., 2003), and ganglionic stimulant-mediated increase of arterial blood (Buccafusco et al., 2007). Third, cotinine treatment upregulated protein expression of α4β2 nAChRs, and favored the assembly of high sensitivity $(\alpha4)_2(\beta2)_3$ stoichiometry on plasma membrane of cultured undifferentiated mouse neuroblastoma 2a cells (Fox et al., 2015), consistent with desensitization-induced upregulation of nAChRs (Henderson and Lester, 2015).

On the other hand, there is also evidence which doesn't support cotinine as a nAChR desensitizer. First, several studies indicate that cotinine pretreatment does not alter effects mediated by activation of nAChRs; these effects include the inhibitory effects of nicotine on high voltage spindles in electroencephalographic recording in rats (Radek, 1993), acetylcholine-induced currents in human α7 or α4β2 nAChRs expressed in oocytes (Terry et al., 2015a), or nicotine's effects on locomotor activity or ultrasonic vocalization (Wang et al., 2020b). Second, cotinine pretreatment enhanced acetylcholine-induced currents in human α7 nAChRs expressed in oocytes, implicating cotinine as a α7 nAChR sensitizer (Terry et al., 2015a,b). Third, chronic cotinine treatment reduced both high-affinity [^{3}H]epibatidine and low-affinity [^{125}I]α-bungarotoxin binding in various rat brain regions (Buccafusco and Terry, 2003; Terry et al., 2005), and α6β2β3 receptor density in mouse neuroblastoma 2a cells (Fox et al., 2015), which is inconsistent with the up-regulation of nAChRs induced by desensitization (Henderson and Lester, 2015). Interestingly, chronic cotinine treatment also reduced M_2 muscarinic acetylcholine receptor binding in several rat brain regions, although the importance of these changes remains unknown (Terry et al., 2005).

Cotinine as a Potential Positive Allosteric Modulator of α7 Nicotinic Acetylcholine Receptors?

Cotinine has also been hypothesized to be a potential Positive Allosteric Modulator (PAM) of α7 nAChRs to explain its behavioral effects in various animal models involving cognitive impairments (Grizzell and Echeverria, 2015; Echeverria et al., 2016; Oliveros-Matus et al., 2020). Currently, there is no direct evidence supporting this hypothesis. One study reported that sustained exposure to cotinine at 1 μM, but not 0.1 or 10 μM potentiated acetylcholine-induced currents in human α7 nAChRs expressed in *Xenopus* oocytes, which may partially support this proposal (Terry et al., 2015a). It is noted that the potentiation effects of cotinine occurred only after 8 min of exposure, but not after shorter exposure, raising the possibility of cotinine as a sensitizer of α7 nAChRs (Terry et al., 2015b). An earlier study found that cotinine inhibited acetylcholine-mediated currents in α7 nAChRs expressed in *Xenopus* oocytes with the IC_{50} value at ∼175 μM (Briggs and McKenna, 1998). In addition, cotinine at 3.7-33.3 μM inhibited acetylcholine-mediated currents in α7 nAChRs expressed in Chinese hamster ovary cells, but not in *Xenopus* oocytes (Alijevic et al., 2020). Therefore, more evidence will be needed for this hypothesis.

FUTURE DIRECTIONS

Most studies have focused on the interactions between cotinine and α4β2* and α7 nAChRs, and few studies have examined potential involvement of other nAChR subtypes. For example, cotinine was shown to be more potent toward α3/α6β2* than α4β2* nAChRs in monkey striatum (O'Leary et al., 2008). Moreover, cotinine treatment down-regulated α6β2β3* receptor density in mouse neuroblastoma 2a cells (Fox et al., 2015). Therefore, it will be important to study binding and interaction profiles of cotinine for other nAChR subtypes to provide more insights into the mechanisms involved in cotinine's effects.

Since cotinine is a weak agonist at nAChRs, many studies employed relatively high concentrations of cotinine to study its effects (Goldberg et al., 1989; Takada et al., 1989; Vainio et al., 1998a, 2000; Oliver et al., 2007). Although these results provided valuable information, cotinine concentrations used in these studies were greatly higher than blood cotinine levels attained in habitual smokers. In addition, it was noted that potential contamination of cotinine samples with small amount of nicotine as an impurity confounded the interpretation of

some studies (Goldberg et al., 1989; Schroff et al., 2000). Some recent studies applied cotinine at doses yielding blood cotinine levels close to physiological levels, and reported various effects of cotinine (Terry et al., 2005, 2012; Echeverria et al., 2011; Grizzell et al., 2014a; Ding et al., 2021). Therefore, it will be imperative to study cotinine's effects with cotinine concentrations at or close to the physiological levels in smokers to increase translational value of the findings. In addition, given the chronic nature of smoking, it is highly valuable to investigate long term adaptive changes within the brain at molecular, cellular, and circuit levels following chronic cotinine exposure at these physiological levels. Such information has potential translational significance and may shed light on the development of therapeutic strategy targeting cotinine and its effects.

Another remaining question is whether cotinine can contribute to the development of nicotine use, abuse and addiction. Our recent study (Ding et al., 2021) indicates that cotinine supported intravenous self-administration in rats, suggesting that cotinine may be reinforcing by itself. These reinforcing effects of cotinine may play a role in nicotine reinforcement. Therefore, it will be interesting to determine how cotinine may alter nicotine reinforcement. Such studies will provide valuable evidence supporting potential therapeutic value of targeting cotinine and its effects for treating nicotine addiction.

AUTHOR CONTRIBUTIONS

Z-MD conceptualized and drafted the manuscript. KV and XT contributed to the draft and review of the manuscript. All authors approved the final manuscript.

FUNDING

This study was supported in part by a grant from the National Institutes of Health [DA044242 (Z-MD)]. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of National Institutes of Health.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 29 October 2021
doi: 10.3389/fnbeh.2021.771114

Social Contact Reinforces Cocaine Self-Administration in Young Adult Male Rats: The Role of Social Reinforcement in Vulnerability to Drug Use

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *06 September 2021*
Accepted: *20 October 2021*
Published: *29 October 2021*

Citation:
Smith MA, Cha HS, Griffith AK and Sharp JL (2021) Social Contact Reinforces Cocaine Self-Administration in Young Adult Male Rats: The Role of Social Reinforcement in Vulnerability to Drug Use. Front. Behav. Neurosci. 15:771114. doi: 10.3389/fnbeh.2021.771114

Drug-using peers are recognized as a leading factor influencing drug use among adolescents and young adults. One mechanism by which peers influence drug use is by providing social reinforcement for using drugs. Social reinforcement may be provided in multiple ways, including by making social contact contingent on drug use (i.e., an individual must use drugs to gain/maintain access to a peer). The purpose of this study was to develop a preclinical model in which intravenous cocaine self-administration was positively reinforced by access to a social partner. Young adult male rats were trained to self-administer cocaine in operant conditioning chambers with a guillotine door that could be opened to an adjacent compartment housing either a social partner or a non-social stimulus. Once cocaine self-administration was established, the guillotine door was activated, and cocaine intake was reinforced by brief access to either a social (age- and sex-matched peer) or non-social (black-and-white athletic sock) stimulus. Contingent access to a social partner rapidly increased cocaine self-administration. Total cocaine intake was 2- to 3-fold greater in rats assigned to the social versus non-social condition across a 100-fold dose range. Cocaine intake rapidly increased when rats in the original non-social group were later provided with social partners, whereas cocaine intake resisted change and remained elevated when rats in the original social group had their partners removed. These data indicate that contingent access to a social partner increases drug intake and suggest that social reinforcement may represent a vulnerability factor that is particularly resistant to psychosocial interventions.

Keywords: addiction, preclinical model, social influence, social learning, substance use disorder

INTRODUCTION

Epidemiological studies reveal that one of the most reliable predictors of whether an adolescent or young adult will use drugs is whether his or her friends use drugs (Wills and Cleary, 1999; Bahr et al., 2005; Simons-Morton and Chen, 2006; Tompsett et al., 2013; Barnett et al., 2014; Schuler et al., 2019). Consequently, drug use among an individual's peers represents a major vulnerability factor determining whether an individual will use drugs and develop a substance use disorder. Theoretical

approaches to explain the high concordance rate of drug use among peers have focused on the roles of (1) selection, in which an individual self-selects peers based on shared interests (e.g., drug use), and (2) social learning, in which peers establish and maintain drug use amongst one another *via* associative learning mechanisms (see reviews by Kandel, 1986; Andrews and Hops, 2010; Pandina et al., 2010 further discussion of selection and socialization theories). Although these theoretical approaches are not mutually exclusive, only the latter lends itself to behavioral interventions that may reduce drug use among vulnerable populations.

Social learning models of drug use posit that drug use is established and maintained by contingencies operating in an individual's social environment (Akers, 1977; Strickland and Smith, 2014). For instance, drug use is established by observing and modeling the behavior of a peer using drugs, and drug use is maintained by social reinforcement provided by the peer. This reinforcement could be in the form of verbal encouragement or simply by continued access to the peer. Unfortunately, empirical support for the role of social learning in drug use is limited. Ethical constraints limit the degree to which drug use, particularly illicit drug use, can be modeled and reinforced in humans, and animal models, particularly those that use intravenous drug self-administration, have traditionally been limited by the necessity of testing animals in isolation.

Recently, several studies have described the use of modified operant conditioning chambers that permit one or more animals to intravenously self-administer drugs in proximity to a social partner, which has rapidly advanced our understanding of how social contact can increase or decrease drug intake. For instance, these studies have shown that drug intake is increased in the presence of a partner self-administering drugs (Smith, 2012; Smith et al., 2014; Robinson et al., 2016, 2017), drug intake is decreased in the presence of a partner without access to drugs (Smith, 2012; Smith et al., 2014; Peitz et al., 2013; Robinson et al., 2016, 2017), patterns of drug intake between partners become more similar over time (Lacy et al., 2014), and subjects will maintain voluntary abstinence when given a choice between drugs and access to a social partner (Venniro et al., 2018, 2019, 2021). Importantly, social contact serves as a positive reinforcer in laboratory animals, and contingent access to a social partner can establish responding in experimentally naïve rats (Angermeier, 1960) and maintain rates of responding similar to those of consummatory reinforcers (e.g., food; Evans et al., 1994). The extent to which social reinforcement increases drug intake in these models has not been examined.

The aim of this study was to establish an animal model in which drug intake is positively reinforced by access to a social partner. To this end, male rats were implanted with intravenous catheters and trained to self-administer cocaine in modified operant conditioning chambers in which a solid guillotine door could be opened to an adjacent compartment housing either a social partner or a non-social (i.e., negative control) stimulus behind a metal screen. Once cocaine self-administration was established, the guillotine door was activated, and cocaine intake was reinforced by brief access to either the social or non-social stimulus. We predicted that cocaine intake would be greater in the social than non-social condition, thus supporting a role for social reinforcement in the facilitation of drug use.

MATERIALS AND METHODS

Subjects

Male, Long-Evan rats were obtained on postnatal day 49 and housed individually in polycarbonate cages in a temperature- and humidity-controlled colony room maintained on a 12:12 h light-dark cycle. All rats had access to bedding, enrichment materials (e.g., gnaw sticks, plastic enclosures), and water throughout the study. Food was available *ad libitum*, except during the brief period of lever press training described below. All subjects were maintained in accordance with the requirements of the Davidson College Animal Care and Use Committee and the guidelines described in the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research).

Materials

All experimental events took place in operant conditioning chambers purchased from Med Associates, Inc. (St. Albans, VT, United States). Each chamber contained a houselight, a retractable response lever, a stimulus light located above the response lever, and a guillotine door leading to a smaller, adjacent compartment containing either a social or non-social stimulus. The two compartments were further separated by a metal screen that allowed rats in adjacent compartments full visual, auditory, and olfactory contact, as well as limited tactile contact with one another, but prevented each subject from traversing to the opposite compartment. Each chamber was housed within a larger, sound-attenuating cabinet, and white noise was continuously present during training and testing. Subjects self-administered cocaine intravenously through a Tygon tube surrounded by a stainless-steel spring connected to a swivel at the top of the cage and an infusion pump located outside of the cage.

Cocaine HCl was generously supplied by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC, United States) and dissolved in sterile saline for intravenous administration.

Lever-Press Training

One week after arrival, rats were restricted to 90% of their free-feeding body weight and trained to lever press using food reinforcement. These training sessions were conducted in operant conditioning chambers that were different from those that would later be used for drug self-administration, with a different configurational arrangement and located in a different testing room. During training, lever presses were reinforced with a single, 45-mg grain pellet on a fixed ratio (FR1) schedule of reinforcement. Sessions terminated once 40 reinforcers had been earned or 2 h elapsed, whichever occurred first. Training ended when a rat earned 40 reinforcers during any four training sessions, and all rats met this criterion within one week.

Surgery

After lever-press training, rats were implanted with intravenous catheters into the right jugular vein under isoflurane anesthesia. Rats were treated with ketoprofen immediately after surgery and at 12-h intervals for 2 days. Wounds were treated with topical antibiotic immediately after surgery and daily for 2 days. Catheters were flushed daily with heparinized saline to maintain patency and ticarcillin to prevent infection.

Social Partnering

One day before self-administration training commenced, all rats were paired with a stimulus rat of the same sex and age but had not undergone surgery. A single partnering session occurred in which the two rats were placed together in a clean, neutral cage and allowed unlimited social interaction for 15 min. One stimulus rat was typically partnered with at least 2 rats that would subsequently be trained for drug self-administration.

Cocaine Self-Administration Training

Self-administration training began approximately one week after surgery and one day following social partnering. Training sessions were conducted in the operant conditioning chambers containing the guillotine door with adjacent side compartment; however, the guillotine door was not operational during these sessions, and no stimulus was placed in the adjacent compartment. Each session began with illumination of the house light, insertion of the lever inside the chamber, activation of the stimulus light above the response lever, and a non-contingent infusion of cocaine (0.5 mg/kg). For the remainder of the session, cocaine was available on an FR1 schedule of reinforcement. Each lever press produced an infusion of 0.5 mg/kg cocaine, retracted the response lever, and turned off the stimulus light above the lever. After 30 s, the lever was inserted back into the chamber, and the stimulus light above the lever was illuminated again. All sessions terminated automatically after 60 min. Training continued in this manner for five consecutive days.

Introduction of the Social/Non-social Stimulus

After 5 days of training, contingencies were changed, and each lever press produced 0.5 mg/kg cocaine, retracted the response lever, turned off the stimulus light above the lever, and opened the guillotine door to the adjacent side compartment. Consequently, each cocaine infusion was simultaneously reinforced by 30-s exposure to either the social or non-social stimulus located in the adjacent compartment. After 30 s, the guillotine door closed, the stimulus light turned off, and the lever was reinserted into the chamber. Rats assigned to the social and non-social conditions were matched based on the number of reinforcers obtained during the 5 days of testing. Rats that were used for partnering (see above) were used as the social stimuli, such that each partner was assigned to a subject with which it had previous contact during the partnering session. A clean, black-and-white athletic sock of similar size and coloring as a Long-Evans rat served as the non-social stimulus. These conditions remained in effect for 10 consecutive days. Similar to training, all sessions were 60 min in duration.

Dose-Effect Curve Determinations

After 10 sessions of exposure to the social/non-social stimuli, a cocaine dose-effect curve was determined in both the social and non-social groups. In these sessions, the dose of cocaine available in each infusion changed each day, such that a 100-fold dose range was examined (0.01–1.0 mg/kg). Five doses were tested in a pseudorandom order with the stipulation that no more than two ascending or descending doses could be tested in a row. In addition, a saline substitution test was conducted in which each infusion contained the vehicle.

Following an initial determination of the cocaine dose-effect curve, we tested whether reducing the magnitude of the social/non-social stimuli would reduce their reinforcing effects on cocaine intake. To this end, the duration in which the guillotine door was open was reduced from 30 to 5 s, thus decreasing the duration of access to the social and non-social stimuli. After 5-s exposure to the social/non-social stimulus, the guillotine door closed, but the response lever remained retracted for an additional 25 s to keep all other temporal parameters consistent across conditions. The cocaine-dose effect curve was then redetermined (and a saline substitution tested was reconducted) under the conditions described above over six consecutive sessions.

Social/Non-social Stimulus Switch

Following redetermination of the cocaine dose-effect curve, the two groups functionally switched conditions, such that each rat originally assigned a social partner was now assigned a non-social stimulus (i.e., black-and-white athletic sock), and each rat originally assigned a non-social stimulus was assigned a social partner. Test sessions began on the following day with rats in their new group assignments, and cocaine self-administration was reinforced with 30-s access to the social or non-social stimulus. All other conditions were identical to those described above. The dose of cocaine (0.5 mg/kg/infusion) was held constant, and data were collected across 10 consecutive sessions.

Data Analysis

A total of 21 rats (social: $n = 12$; non-social: $n = 9$) contributed to the acquisition and dose-response analyses. Three rats (original social: $n = 2$; original non-social = 1) lost catheter patency following the stimulus switch and were not included in the data analysis from the last 10 days of testing.

Data obtained during acquisition were analyzed *via* two-way, mixed-factor ANOVA, with group (social vs. non-social) serving as the between-subjects factor and session serving as the repeated-measure. Data from the dose-response analyses were analyzed *via* two-way, mixed-factor ANOVA, with group serving as the between-subjects factor and dose serving as the repeated-measure. Data from saline substitution tests were analyzed *via* independent-samples *t*-tests. Data obtained during the stimulus switch were analyzed *via* two-way, mixed-factor ANOVA, with group serving as the between-subjects factor and session serving as the repeated-measure. *Post hoc* tests

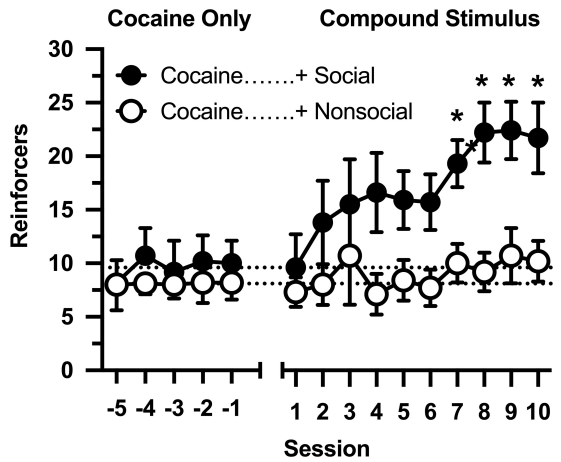

FIGURE 1 | Number of reinforcers obtained during daily, 60-min test sessions. Data reflect the mean (SEM) number of cocaine infusions from rats assigned to the social ("+ Social," filled symbols; n = 12) and non-social ("+ Non-social," open symbols; n = 9) groups. For the first five sessions (Sessions –5 to –1), responding was maintained only by cocaine (0.5 mg/kg). For the following 10 sessions (Sessions 1 to 10), responding was maintained by cocaine and 30-s access to either a social (sex- and age-matched partner) or non-social (black-and-white athletic sock) stimulus. Dashed lines originating at the X-intercept reflect the average number of cocaine infusions during the first five sessions (social: 9.6 infusions; non-social: 8.1 infusions). Asterisks indicated significant differences between social and non-social groups.

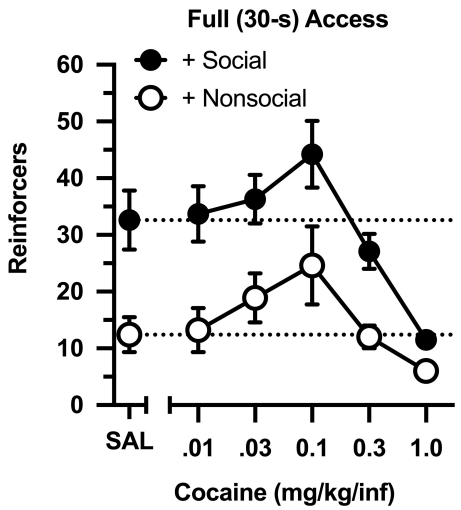

FIGURE 2 | Number of reinforcers obtained during daily, 60-min test sessions under full (30-s) access conditions. Data reflect the mean (SEM) number of infusions from rats assigned to the social ("+ Social," filled symbols; n = 12) and non-social ("+ Non-social," open symbols; n = 9) groups. Responding was maintained by various doses of cocaine (0.01–1.0 mg/kg/inf) and either a social (sex- and age-matched partner) or non-social (black-and-white athletic sock) stimulus. Dashed lines originating at the X-intercept reflect the average number of infusions during a saline substitution test (SAL).

were conducted *via* independent-samples or paired-samples t-tests where appropriate, following by the Holm's-Bonferroni correction for multiple comparisons. All statistical tests were two-tailed, and the alpha value was set to 0.05.

RESULTS

All rats responded on the first day of cocaine availability, receiving approximately 9 infusions per session (**Figure 1**: Sessions –5 to –1). The number of reinforcers was consistent over 5 consecutive days in which responding was maintained by cocaine, and no differences were observed between rats that would later by assigned to the social and non-social conditions (no main effect of group, main effect of session, or group × session interaction was observed).

Cocaine intake was selectively impacted by the social stimulus when operant contingencies changed and responding resulted in opening the guillotine door (**Figure 1**: Sessions 1 to 10). Cocaine intake progressively increased in rats in the social partner condition [main effect of session: $F(9,171) = 4.691$, $p < 0.001$], resulting in significantly greater cocaine intake in this group relative to rats in the non-social condition [main effect of group: $F(1,19) = 5.530$, $p = 0.030$]. In contrast, cocaine intake did not increase in the non-social condition [group × session interaction: $F(9,171) = 1.984$, $p = 0.044$], resulting in significant between-group differences in cocaine intake during sessions 6 through 10.

Across a 100-fold dose range, cocaine intake was characterized by an inverted, U-shaped dose-effect curve in both groups (**Figure 2**), with ascending and descending limbs converging at 0.1 mg/kg [main effect of dose: $F(4,76) = 15.851$, $p < 0.001$]. Across all doses, cocaine intake ranged from 2- to 3-fold higher in the social group than in the non-social group [main effect of group: $F(1,19) = 13.421$, $p = 0.002$]. Importantly, responding was greater in the social than non-social group during a saline substitution test [$t(19) = 9.661$, $p = 006$], reflecting the differential reinforcing effects of the social/non-social stimuli in the absence of cocaine.

The dose-effect curve was redetermined after reducing the magnitude of the social and non-social stimuli (**Figure 3**). Reducing the duration of door opening from 30 to 5 s did not impact the dose-effect curve in either group (compare **Figures 2** and **3**). Similar to the original determination, cocaine intake was characterized by an inverted, U-shaped dose-effect curve [main effect of dose: $F(4,76) = 13.793$, $p < 0.001$], and intake ranged from 2- to 3-fold greater in the social than non-social group [main effect of group: $F(1,19) = 11.647$, $p = 0.003$]. Responding was also greater in the social than non-social group during a saline substitution test [$t(19) = 5.191$, $p = 034$].

Following determinations of the cocaine dose-effect curve, conditions were switched (**Figure 4**), such that rats in the original social condition were switched to the non-social condition (i.e., partners were switched for socks), and rats in the original non-social condition were switched to the social condition (i.e., socks were switched for partners). Cocaine intake increased across 10 consecutive sessions [main effect of session: $F(9,144) = 4.326$, $p < 0.001$], and this was driven by selective increases in intake in the new social group [group × session interaction: $F(9,144) = 2.645$, $p = 0.007$]. Cocaine intake was significantly

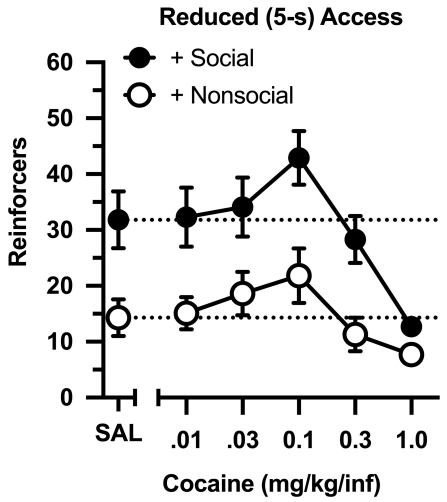

FIGURE 3 | Number of reinforcers obtained during daily, 60-min test sessions under reduced (5-s) access conditions. Data reflect the mean (SEM) number of infusions from rats assigned to the social ("+ Social," filled symbols; $n = 12$) and non-social ("+ Non-social," open symbols; $n = 9$) groups. Responding was maintained by various doses of cocaine (0.01–1.0 mg/kg/inf) and either a social (sex- and age-matched partner) or non-social (black-and-white athletic sock) stimulus. Dashed lines originating at the X-intercept reflect the average number of infusions during a saline substitution test (SAL).

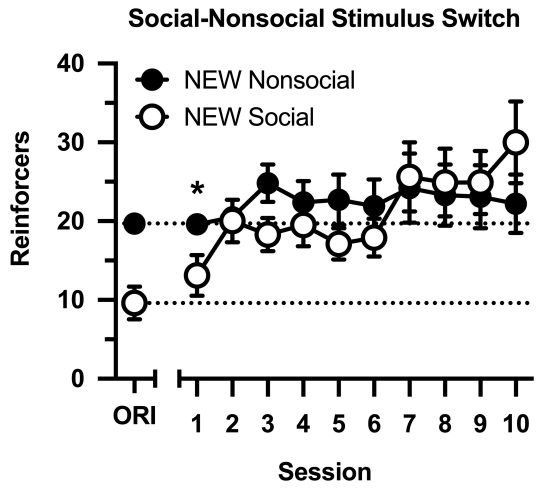

FIGURE 4 | Number of reinforcers obtained during daily, 60-min test sessions. Data reflect the mean (SEM) number of cocaine infusions from rats newly switched to the non-social (filled symbols; $n = 10$) and social (open symbols; $n = 8$) groups. During all sessions, responding was maintained by cocaine (0.5 mg/kg) and 30-s access to either a social (sex- and age-matched partner) or non-social (black-and-white athletic sock) stimulus. Dashed lines originating at the X-intercept reflect the average number of cocaine infusions during Session 10 of the groups' original assignments (ORI). Asterisk indicates significant difference between social and non-social groups.

less in the new social group during the first session, but the groups did not differ during the final nine sessions. Notably, cocaine intake remained high and stable in the new non-social group, reflecting the persisting effects of exposure to the social stimulus.

DISCUSSION

This study used a novel preclinical model to determine whether access to a social partner positively reinforces cocaine self-administration and thus facilitates cocaine intake. We found that contingent access to a social partner, but not a non-social stimulus, rapidly increases cocaine intake resulting in a 2- to 3-fold escalation of cocaine intake relative to both baseline and control conditions. These data emphasize the importance of social peers in the escalation of drug intake and demonstrate the role of social reinforcement in vulnerability to drug abuse.

Responding was under control of both reinforcers. Lever pressing was readily established with cocaine, and cocaine intake in both groups was characterized by an inverted, U-shaped dose-effect curve, which is typical of responding maintained by cocaine on simple FR schedules of reinforcement (Lynch and Carroll, 2001). Lever pressing increased rapidly when the social stimulus was introduced, both at the beginning of the study in the original social group, and toward the end of the study in the non-social group after the stimulus switch. Moreover, twice as many reinforcers were obtained during a saline substitution test in the social group than the non-social group, revealing the effects of the social contingency in the absence of cocaine. Finally, it is notable that high doses of cocaine markedly reduced responding relative to vehicle (i.e., saline) control values, thus functioning as a positive punisher to reduce responding otherwise maintained by social contact.

Recent studies have used several experimental designs to examine how social contact interacts with drug reward. For instance, studies using the conditioned place preference procedure report that social contact increases the conditioned rewarding effects of a drug if the stimuli are conditioned in the same compartment (Thiel et al., 2008; Reyna et al., 2021). In contrast, social contact can block the conditioned rewarding effects of a drug if social contact is provided exclusively in the opposite compartment (Fritz et al., 2011; Zernig and Pinheiro, 2015). Drug self-administration studies demonstrate the presence of a partner that is also self-administering drugs enhances drug intake, whereas the presence of a partner that is not self-administering drugs inhibits drug intake (Smith, 2012; Robinson et al., 2016). Unlike the present study, the presence of the partner was not contingent on drug intake in those previous studies. Finally, a social partner can inhibit drug intake in a discrete-choice procedure when selection of that partner specifically excludes drug delivery (Venniro et al., 2018, 2019).

Several behavioral mechanisms have been used to explain how drug use may be established and maintained among peers, and they include classic learning phenomena such as imitation/modeling, social reinforcement, emulation, social facilitation, local enhancement, stimulus enhancement, and reinforcement enhancement (Strickland and Smith, 2014). Although this study was not designed to systematically test all possible mechanisms, the current data may be used

in conjunction with data collected previously to rule out several possibilities. Explanations based on imitation/modeling, emulation, local enhancement, and stimulus enhancement require a model to be engaged in behavior that increases a subject's attention to (or engagement with) a discriminative stimulus, behavioral action, or reinforcing stimulus. Social partners did not have access to either response levers or intravenous cocaine, thus limiting their ability to increase the salience of any component of the lever-press/cocaine infusion contingency. Social facilitation or reinforcement enhancement represent two potential explanations for these findings, but multiple studies report the presence of a non-intoxicated social partner reliably *decreases* drug intake across a range of experimental conditions (Smith, 2012; Peitz et al., 2013; Smith et al., 2014; Robinson et al., 2016, 2017; Venniro et al., 2018, 2019, 2021). The experimental design of the present study specified that social contact was contingent on pressing a response lever and self-administering cocaine; consequently, social reinforcement reflects the most parsimonious explanation for the observed findings, especially after other explanations are ruled out. Importantly, the non-social (i.e., negative control) condition ruled out the possibility that extraneous components of the reinforcing stimulus (e.g., guillotine door opening, presence of novel stimulus) was responsible for the escalation of cocaine intake.

One observation that questions the role of social reinforcement in the maintenance of elevated responding is that cocaine intake was not responsive to either a reduction in the magnitude of the social stimulus (i.e., a decrease in the duration of social access) or the removal of the social stimulus (i.e., replacing a social partner for an athletic sock). Previous studies examining social reinforcement reveal that greater social contact (visual plus partial physical contact > only visual contact; Angermeier, 1960) and greater social deprivation (longer deprivation > shorter deprivation; Achterberg et al., 2016) increase the reinforcing effects of social contact. We do not know of any studies that manipulated the duration of social contact when measuring social reinforcement; however, studies using conditioned place preference report that longer durations of social contact do not lead to greater reward (10 min contact = 30 min contact; Thiel et al., 2008). In the present study, the elevated levels of cocaine intake in the social group were remarkably stable and not responsive to further manipulations, including the removal of the social stimulus. These data suggest that other behavioral mechanisms were also recruited to produce long lasting increases in drug intake that were resilient to further behavioral manipulations. This observation is of translational concern because it indicates that once drug use increases in response to social reinforcement from others, it will persist even in the absence of those individuals and may be less responsive to some first-line psychosocial interventions.

Collecting additional measures of behavior would help identify the mechanisms contributing to the differences in responding between social and non-social conditions. For instance, responding on an inactive lever would reveal the degree to which a social partner increased general levels of activity. Alternatively, the presence of a second active lever that resulted in only cocaine delivery (or only social access) would determine the preference for each stimulus individually relative to the compound stimulus. Furthermore, the presence of a second active lever that resulted in a different stimulus (e.g., sucrose, electric shock) would determine the extent to which social contact could differentially reinforce behaviors maintained at high rates (e.g., food-maintained responding) versus low rates (e.g., shock-maintained responding). Our hypothesis would be that the effects of social reinforcement would generalize broadly to other behaviors, particularly those maintained at low rates (Herrnstein, 1961).

We did not collect within-session patterns of responding or videotape the rats during test sessions. In the former case, we made a programming error that prevented the creation of cumulative records; in the latter case, video cameras were not available to us at the time of data collection. Consequently, we do not know the extent to which the subjects interacted with the social and non-social stimuli, nor do we know the nature of the interaction (e.g., active exploratory, passive avoidance). Relating the temporal pattern of lever-pressing to behaviors emitted during the 30-s stimulus presentation would provide further insight into the mechanisms responsible for the stimulus control of cocaine self-administration.

Females should be tested in future iterations of this model, especially when considering that male and female adolescents respond differently to social pressure on measures of drug use (Dick et al., 2007; Schulte et al., 2009; Mason et al., 2014; McMillan et al., 2018). An additional area ripe for investigation involves the effects of male-female interactions on measures of drug intake. There is nothing about the present model that would prevent the use of sexually mature male and female subjects as social partners, including female subjects during various phases of behavioral estrous. Although sex is frequently included as a biological variable in behavioral assays, interactions with the opposite sex are frequently ignored outside of studies specifically examining courtship/mating behavior.

The experimental design intentionally limited cocaine intake by restricting cocaine availability. Levers were retracted during periods of social/non-social stimulus presentation to encourage engagement with the reinforcing stimuli, and sessions were limited to 60 min to prevent satiation of the social stimulus. Limited access procedures do not model problematic patterns of drug intake that characterize addictive behavior (Smith, 2020), so data from this study are more translationally relevant to social drug use than drug abuse and addiction. This study also only examined the effects of social reinforcement on intravenous cocaine self-administration. Future studies must examine other drugs and routes of administration, particularly drugs and routes of administration that are more relevant to adolescent and young adult populations (e.g., oral alcohol consumption, inhaled nicotine/cannabinoid consumption).

The most obvious translational implication of this study is that social reinforcement escalates cocaine use, identifying it as a vulnerability factor for drug abuse and addiction. Prevention programs for at-risk groups (e.g., younger adolescents involved in predelinquent behavior) should target group norms that are permissive or even encouraging of drug use. Alternatively, it is

equally likely that abstinence-related behaviors (e.g., attending school, involvement in religious and community activities, showing up for work) could be differentially reinforced by non-drug rewards, including social activities with abstinent individuals. Community reinforcement programs often make use of social reinforcement (e.g., integrating the person in a social network that engages in non-drug recreational activities) to encourage abstinence, and these programs have higher success rates than standard-care control groups (see review by Meyers et al., 2011). This could easily be modeled in future studies by using a differential reinforcement of other behavior (DRO) schedule in which social reinforcement is contingent on behavior that excludes drug self-administration. Indeed, previous studies have successfully modeled "voluntary abstinence" by providing rats with a discrete choice between drug delivery and social contact after a history of drug self-administration. Those studies reveal that social contact decrease drug intake, prevents the incubation of craving, and decreases measures of relapse in both males and females (Venniro et al., 2017, 2019).

CONCLUSION

This study describes a preclinical model in which cocaine self-administration is positively reinforced by contingent access to either a social (age- and sex-matched rat) or non-social (black-and-white athletic sock) stimulus. Contingent access to a social partner rapidly increased cocaine intake 2- to 3-fold across an extensive dose range. These increases in cocaine intake were persistent over time and resistant to later reductions in and removal of the social stimulus. Data collected in this model suggest that social reinforcement may contribute to the escalation of drug intake among peers and represents a vulnerability factor that may be particularly resistance to psychosocial interventions once established.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Davidson College Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MS conceived the study and wrote the manuscript. HC, AG, and JS collected the data. MS and JS analyzed the data. All authors approved the final draft and are accountable for the work.

FUNDING

This work was supported by the National Institutes of Health (Grant Numbers DA045364 and DA031725).

ACKNOWLEDGMENTS

The authors thank the National Institute on Drug Abuse for generously supplying the study drug.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 29 October 2021
doi: 10.3389/fnbeh.2021.744715

A Novel Assay Allowing Drug Self-Administration, Extinction, and Reinstatement Testing in Head-Restrained Mice

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Edited by:
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Reviewed by:
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David James Barker, Rutgers, The State University of New Jersey, United States

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *20 July 2021*
Accepted: *30 September 2021*
Published: *29 October 2021*

Citation:
Vollmer KM, Doncheck EM, Grant RI, Winston KT, Romanova EV, Bowen CW, Siegler PN, Green LM, Bobadilla AC, Trujillo-Pisanty I, Kalivas PW and Otis JM (2021) A Novel Assay Allowing Drug Self-Administration, Extinction, and Reinstatement Testing in Head-Restrained Mice. Front. Behav. Neurosci. 15:744715. doi: 10.3389/fnbeh.2021.744715

Multiphoton microscopy is one of several new technologies providing unprecedented insight into the activity dynamics and function of neural circuits. Unfortunately, some of these technologies require experimentation in head-restrained animals, limiting the behavioral repertoire that can be integrated and studied. This issue is especially evident in drug addiction research, as no laboratories have coupled multiphoton microscopy with simultaneous intravenous drug self-administration, a behavioral paradigm that has predictive validity for treatment outcomes and abuse liability. Here, we describe a new experimental assay wherein head-restrained mice will press an active lever, but not inactive lever, for intravenous delivery of heroin or cocaine. Similar to freely moving animals, we find that lever pressing is suppressed through daily extinction training and subsequently reinstated through the presentation of relapse-provoking triggers (drug-associative cues, the drug itself, and stressors). Finally, we show that head-restrained mice will show similar patterns of behavior for oral delivery of a sucrose reward, a common control used for drug self-administration experiments. Overall, these data demonstrate the feasibility of combining drug self-administration experiments with technologies that require head-restraint, such as multiphoton imaging. The assay described could be replicated by interested labs with readily available materials to aid in identifying the neural underpinnings of substance use disorder.

Keywords: two-photon (2P), calcium imaging, addiction, ensembles, longitudinal tracking of individual cells

INTRODUCTION

Multiphoton microscopy is a powerful tool used to help dissect the neurobiological substrates that coordinate behavior. By harnessing fluorescent imaging strategies based on genetically encoded constructs, its never-before-seen spatiotemporal resolution in living tissues enables observation and tracking of single neurons (e.g., calcium ion concentration or voltage changes; Tian et al., 2009; Chen et al., 2013; Lin and Schnitzer, 2016; Villette et al., 2019), neurotransmitter release (e.g., receptor-activation-based neurotransmitter sensors; Sun et al., 2018; Feng et al., 2019) and

cell morphology (e.g., dendritic spine plasticity; Muñoz-Cuevas et al., 2013; Moda-Sava et al., 2019) in awake, behaving animals. Such technology could be transformative for studying substance use disorder (SUD), which is rooted in plasticity in the neural circuits that govern motivated behavior (Russo and Nestler, 2013). Unfortunately, experiments involving multiphoton imaging generally require head immobilization, which has prevented its integration with preclinical models of SUD.

Perhaps the most powerful preclinical model for SUD involves training animals to voluntarily self-administer drugs of abuse, an approach that has both predictive and construct validity for treatment outcomes and abuse liability (Shalev et al., 2002; Epstein et al., 2006; Haney and Spealman, 2008). In drug self-administration, animals are reinforced for performing an operant task (e.g., lever pressing) by drug delivery (e.g., intravenous), after which seeking behavior is extinguished through reward omission. Animals can then be tested for reinstatement of drug seeking in response to stimuli known to produce craving and relapse in humans, such as drug-associated cues, a single dose of the drug, or stressors. Distinct phases of self-administration can be used to study the cycle of intoxication, withdrawal, and drug seeking, which characterizes SUD (Koob and Le Moal, 1997) and is paralleled by neural plasticity in the brain's reward circuits (Gardner, 2000). By pairing self-administration with bench approaches, critical factors underlying drug-seeking behavior have been identified at the cellular, molecular, and circuit levels (Koob and Le Moal, 1997; Kalivas and Volkow, 2005; Deadwyler, 2010; Russo et al., 2010). Despite these advances, our ability to precisely observe, longitudinally track, and manipulate these implicated factors in live, behaving animals has been limited.

Here we design and develop a new approach for drug self-administration in head-restrained mice, an assay that would allow simultaneous multiphoton imaging. Overall, we find that head-restrained mice will reliably learn to press an active, but not inactive, lever that results in the delivery of a tone cue followed by intravenous heroin or cocaine, but not saline. We find that omission of the cue and drug results in extinction learning, wherein active lever pressing is suppressed across days. Following extinction training, presentation of the drug-associated cue, the drug itself, or stressors cause reinstatement of drug seeking, as is typically observed in freely moving studies. Finally, we show that mice will similarly self-administer a sucrose reward, delivered orally, which is a common control used in drug self-administration experiments. Interestingly, cue presentation, but not drug or stress exposure, results in reinstatement of sucrose seeking after extinction. These results demonstrate the feasibility of head-restrained drug self-administration and control sucrose self-administration experiments in mice, enabling the integration of novel technologies that require head immobilization (e.g., two-photon microscopy) to study the neuronal mechanisms of SUD.

MATERIALS AND METHODS

Subjects

Male and female C57BL/6J mice (8 weeks old/20 g minimum at study onset; Jackson Labs) were group-housed pre-operatively and single-housed post-operatively under a reversed 12:12-hour light cycle (lights off at 8:00am) with access to standard chow and water *ad libitum*. Experiments were performed in the dark phase and in accordance with the NIH Guide for the Care and Use of Laboratory Animals with approval from the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Surgeries

Mice were anesthetized with isoflurane (0.8–1.5% in oxygen; 1 L/min) and placed within a stereotactic frame (Kopf Instruments) for head ring implantation surgeries. Ophthalmic ointment (Akorn), topical anesthetic (2% Lidocaine; Akorn), analgesic (Ketorlac, 2 mg/kg, ip), and subcutaneous sterile saline (0.9% NaCl in water) treatments were given pre- and intra-operatively for health and pain management. A custom-made ring (stainless steel; 5 mm ID, 11 mm OD) was adhered to the skull using dental cement and skull screws. Head rings were scored on the base using a drill for improved adherence. Following surgeries, mice received antibiotics (Cefazolin, 200 mg/kg, sc).

Drug self-administration mice were allowed at least 7 days of recovery from head ring implantation before catheterization occurred. Once recovered, mice were anesthetized as described above and implanted with indwelling intravenous catheters for drug self-administration. Catheters (Access Technologies #071709H) were custom-designed for mice, with 4.5 cm of polyurethane tubing (0.012″ ID; 0.025″ OD, rounded tip) between the sub-pedestal/indwelling end of the back-mounted catheter port (Plastics One #8I313000BM01) and silicone vessel suture retention bead, from which 1.0 cm extended intravenously via the right external jugular vein toward the right atrium of the heart. Larger tubing (1.5 cm of 0.025″ ID; 0.047″ OD) encased the smaller attached to the pedestal, and components were adhered by ultraviolet curation [catheter construction was based on Doncheck et al. (2018)]. Catheters were implanted subcutaneously using a dorsal approach, with back mounts externalized through $\leq$ 5 mm midsagittal incisions posterior to the scapulae and tubing running from the sub-pedestal base over the right scapula into the <1 mm incision in the right external jugular vein. Silk sutures adhered the catheter tubing retention bead to the external jugular at the site of incision and negative pressure-induced blood backflow was assured prior to closing skin incisions with non-absorbable monofilament sutures. All animals received analgesic, ophthalmic, and antibiotic treatments as described above in addition to topical antibiotic ointment and lidocaine (2%) jelly application to incisions. Mice were allowed to recover for a minimum of 1 week before behavioral experiments. Following implantation, catheters were flushed daily with heparinized saline (60 units/mL, 0.02 mL) to maintain patency. Animals with non-patent catheters were to be excluded from the study, however, all animals remained patent throughout acquisition; catheter patency was no longer monitored once the animals entered extinction. When necessary, patency was determined by giving mice an i.v. infusion of brevital (2 mg/mL, 0.02 mL).

Self-Administration Chambers

Self-administration chambers were custom designed and created using readily available and cost-effective components. Each chamber was a two-door cabinet (24″ × 16″ × 30″; NewAge Products; #50002) equipped with (1) soundproofing, (2) infusion pump, (3) restraint tube, (4) laptop with MATLAB and Arduino software, (5) breadboard, (6) head-fixation station, (7) operant levers, (8) speaker, and (9) an Arduino (see **Supplementary Figure 1**).

1. Soundproofing: Acoustic foam (2.5″ × 24″ × 18″ UL 94, Professional Acoustics; 12″ × 12″ × 1.5″ egg crate acoustic foam tiles, OBCO) was glued to the inner chamber.
2. Infusion pump: Arduino-controlled infusion pumps (Med Associates #PHM-100VS-2) were used for drug delivery. For drug delivery, a 3 mL syringe filled with drug (cocaine, heroin, or saline) was connected to tubing (Tygon, 0.02″ ID; 0.06″ OD) which could be fitted over backmounted i.v. catheters. For sucrose delivery, a 3 mL syringe filled with sucrose (12.5%) was connected to tubing (Tygon, 0.05″ ID; 0.09″ OD) that attached to a lick spout positioned in front of the mouse.
3. Restraint tube: Partial body restraint was used to avoid self-injury. Rotary grinders were used to make slits in 50 mL conical tubes (Fisher; 01-812-55) to allow for catheter port externalization while mice were partially restrained within head-fixation stations. The open end of the conical faced the lever box and was positioned to allow free range of movement for the front limbs.
4. Laptop: A laptop (Lenovo Ideapad 330S; 81F5006GUS) interfaced with other electronics via Arduino. Arduino- (Arduino 1.8.12) and MATLAB- (MathWorks) software were used to control equipment and record behavioral events.
5. Breadboards: An aluminum breadboard (12″ × 18′ × 1/2″) was used as the base to allow for chamber components to be screwed in place (ThorLabs; MB1218). A smaller aluminum breadboard (4″ × 6″ × 1/2″) was used to secure the head-fixation station into place and to provide a behavioral platform (ThorLabs; MB4).
6. Head-fixation station: Head-fixation stations were custom-made (Clemson University Engineering Department) and attached to the breadboard. Stainless steel inverted square-edged U-frames with slits in the central crossbar allowed for mouse insertion by head rings. A second crossbar clamped the head rings down to prevent head movement.
7. Operant levers: Two operant levers (Honeywell; 311SM703-T) were cemented into a hollowed-out 12-well plate (Fisher; FB012928) and wired for active/inactive response functionality through the Arduino board. The levers extended outward by 5 cm from this "lever box" and the ends aligned centrally 3.5 cm from the edge of the restraint conical. Forelimb extension was required for mice to reach the levers.
8. Speaker: Arduino-controlled piezo buzzers (Adafruit; #1739) were positioned above the head-fixation frames for the delivery of auditory, reward-conditioned cues. The cue was initiated immediately following an active lever press during a non-timeout period.
9. Arduino board: Arduino (Arduino Uno Rev 3; A000066) interfaced with two electronic breadboards (Debaser Electronics; DE400BB1-1) for control of self-administration equipment.

Behavioral Procedure

Mice were given at least 7 days to recover from their previous surgery (head ring implantation or catheterization) before beginning behavior. Once recovered, mice underwent 3 days of habituation, during which they were head-restrained for 30 min in the operant chambers without access to the levers. During acquisition, the operant levers were placed in front of the mice. Mice self-administering heroin, cocaine, or saline received intravenous infusions via tubing connecting the syringe pump and their i.v. catheters. Mice self-administering sucrose received liquid droplets via a lick spout placed in front of their mouth. Pressing the active lever resulted in immediate cue presentation (8 kHz, 2 s), followed by a gap in time (trace interval, 1 s), and finally intravenous drug or saline infusion (2 s; 12.5 μl) or sucrose droplet (2 s; 12.5 μl). The trace interval is included for isolation of sensory cue- and reward-related neural activity patterns during multiphoton imaging, similar to previous appetitive learning experiments (Otis et al., 2017; Grant et al., 2021). Reinforced active lever presses also resulted in a timeout period wherein further active lever presses were recorded but not reinforced with the cue or reward. Inactive lever presses resulted in neither cue nor reward delivery.

Heroin Self-Administration

Mice underwent 14 days of heroin self-administration on a fixed ratio 1 (FR1) schedule of reinforcement using a decreasing dose design (Day 1–2: 0.1 mg/kg/12.5 μl heroin, 10 infusion maximum; Day 3–4: 0.05 mg/kg/12.5 μl heroin, 20 infusion maximum; Day 5–14: 0.025 mg/kg/12.5 μl heroin, 40 infusion maximum), for a maximum of 1 mg/kg of heroin per session (similar to previously described experiments in freely moving mice; Corre et al., 2018). Due to quick responding on the active lever, mice were capped to receiving 1 mg/kg per session to prevent overdose. Concurrently, a separate group of mice underwent 14 days of saline (12.5 μl infusions; 40 infusion maximum) self-administration sessions on an FR1 schedule of reinforcement. Session durations for both heroin and saline self-administering mice were a maximum of 2 h. Following acquisition, heroin and saline self-administering mice underwent 2-h extinction training sessions, wherein active lever presses resulted in neither cue nor drug delivery until extinction criteria were reached. Extinction criteria were determined *a priori*, defined as (1) ≥ 10 days of extinction training and (2) 2 of the last 3 days resulting in ≤20% of the average active lever pressing observed during the last 2 days of acquisition. Heroin self-administering mice that did not reach extinction criteria were excluded from analyses and subsequent reinstatement testing (n = 4/28). Saline self-administering mice were immediately tested after the 10th day of extinction due to low responding during acquisition, and thus an inability

to "extinguish" lever pressing. Due to initial piloting, only a subset of saline self-administering mice underwent extinction and reinstatement testing (n = 4). Following establishment of extinction criteria, heroin and saline self-administering mice underwent reinstatement testing in a pseudorandom order, for cue-, drug-, yohimbine-, predator odor-, or saline-induced reinstatement tests. Animals were tested first for cue-induced reinstatement, but the subsequent stimuli were tested using a counterbalanced design. For cue-induced reinstatement testing, active lever presses resulted in cue presentation in a manner identical to acquisition, but drug infusions were excluded. For drug-primed reinstatement, heroin (1 mg/kg, i.p.) was delivered immediately prior to the reinstatement session. A heroin-primed reinstatement dose-response pilot study revealed this dose produced responding most comparable to that observed during both acquisition and other reinstatement tests (data not shown). For reinstatement testing in response to a pharmacological stressor, yohimbine (0.625 mg/kg, i.p.; Sigma Chemical; King and Becker, 2019) was given 30 min before the session. Yohimbine, the alpha-2-noradrenergic receptor antagonist, may influence behavior in a manner that is not purely stress-related (e.g., Chen et al., 2015). Thus, we included an additional stress-induced reinstatement test provoked by the predator odor 2,3,5-trimethyl-3-thiazoline (TMT), a synthetically derived component of fox feces chosen due to its ethological relevance and validity as a stressor (Janitzky et al., 2015). For predator odor-induced reinstatement, TMT (30 μL; 1% v/v ddH2O) was placed in front of each head-restrained mouse on a gauze pad inside a container connected to a vacuum (to restrict odor spread) for 15 min and removed immediately before behavioral testing (King and Becker, 2019). For saline-primed reinstatement, 0.9% NaCl (10 mL/kg, i.p.) was delivered immediately before behavioral testing. Heroin and saline self-administering mice were re-extinguished to criteria between reinstatement tests. Due to initial piloting, not all heroin self-administering mice that completed acquisition and extinction underwent all 5 reinstatement tests.

Cocaine Self-Administration

Mice underwent 14 days of 2-hour cocaine (0.75 mg/kg/12.5 μl cocaine infusion; 40 infusion maximum) self-administration sessions on an FR1 schedule of reinforcement similar to that described in freely moving mice (Heinsbroek et al., 2017). The criteria for extinction were defined in the same manner as for heroin (see above), and no mice were excluded for failure to reach criteria. Mice underwent reinstatement testing using a counterbalanced design similar to the methods described above for heroin self-administration reinstatement testing, with the exception of drug-primed reinstatement involving cocaine (5 mg/kg, i.p.) rather than heroin injections immediately before the session. A subset of cocaine self-administering mice (n = 4/15) did not undergo extinction or reinstatement testing due to initial piloting. The saline self-administering mice detailed above (see section "Heroin Self-administration") underwent acquisition concurrently with cocaine self-administration mice and were thus included as a control for both experiments.

Sucrose Self-Administration

Mice underwent 14 days of 2-hour sucrose self-administration sessions on an FR1 schedule of reinforcement. As sucrose self-administration is often used as a control for drug self-administration, sucrose self-administration mice followed an intake design similar to the methods described above for heroin self-administration animals. While sucrose droplets were capped (Day 1–2: 12.5 μl of 12.5% sucrose, 10 droplets maximum; Day 3–4: 12.5 μl of 12.5% sucrose, 20 droplets maximum; Day 5–14: 12.5 μl of 12.5% sucrose, 40 droplets maximum), the volume of sucrose delivered per reward did not change. Following the last day of acquisition, sucrose self-administering mice underwent extinction and reinstatement testing using the same criteria and pseudorandom design, respectively, as described for heroin self-administration (see above). No mice were excluded for failure to reach extinction criteria.

Data Collection and Statistics

Parameters for behavioral sessions were set using a custom MATLAB graphical user interface that controlled an Arduino and associated electronics. Data were recorded using MATLAB, extracted using Python, and analyzed and graphed using GraphPad PRISM (version 8), and illustrated using Adobe Illustrator. Analysis of variance (ANOVA; 2-way or 3-way repeated measurers) or t-tests (paired) were used to analyze data collected for the experiments. Independent variables included lever (active vs. inactive), day (e.g., extinction vs. reinstatement), and sex (male vs. female). Sidak's *post hoc* tests were used following significant main effects and interactions within the ANOVA analyses. Due to initial electrical issues with the Arduino circuit board, data showed that some mice exhibited lever responding exceeding what is feasible (∼60 Hz). This electrical issue was due to improper grounding of the electrical components within the behavioral chamber, and the subsequent static resulted in unachievable lever press counts. We promptly corrected the drifting ground by ensuring the electrical connection between levers and the Arduino circuit board was secure. To remove the variance caused by this electrical issue, we excluded outliers greater than two standard-deviations away from the mean for each session (14 out of 1022 total acquisition sessions from all drug and sucrose self-administration animals). Additionally, during initial troubleshooting phases of the experiments we lost three files that were not saved to the computer, and thus that data is not included in acquisition (3 out of 392 heroin self-administration acquisition sessions).

RESULTS

Head-Restrained Heroin Self-Administration

Acquisition

Following recovery from surgery and habituation (see **Figure 1A** for timeline), mice began head-restrained heroin (n = 28; 13 males and 15 females) or saline self-administration (n = 8; 5 males and 3 females; **Figures 1B,C**). Heroin self-administering

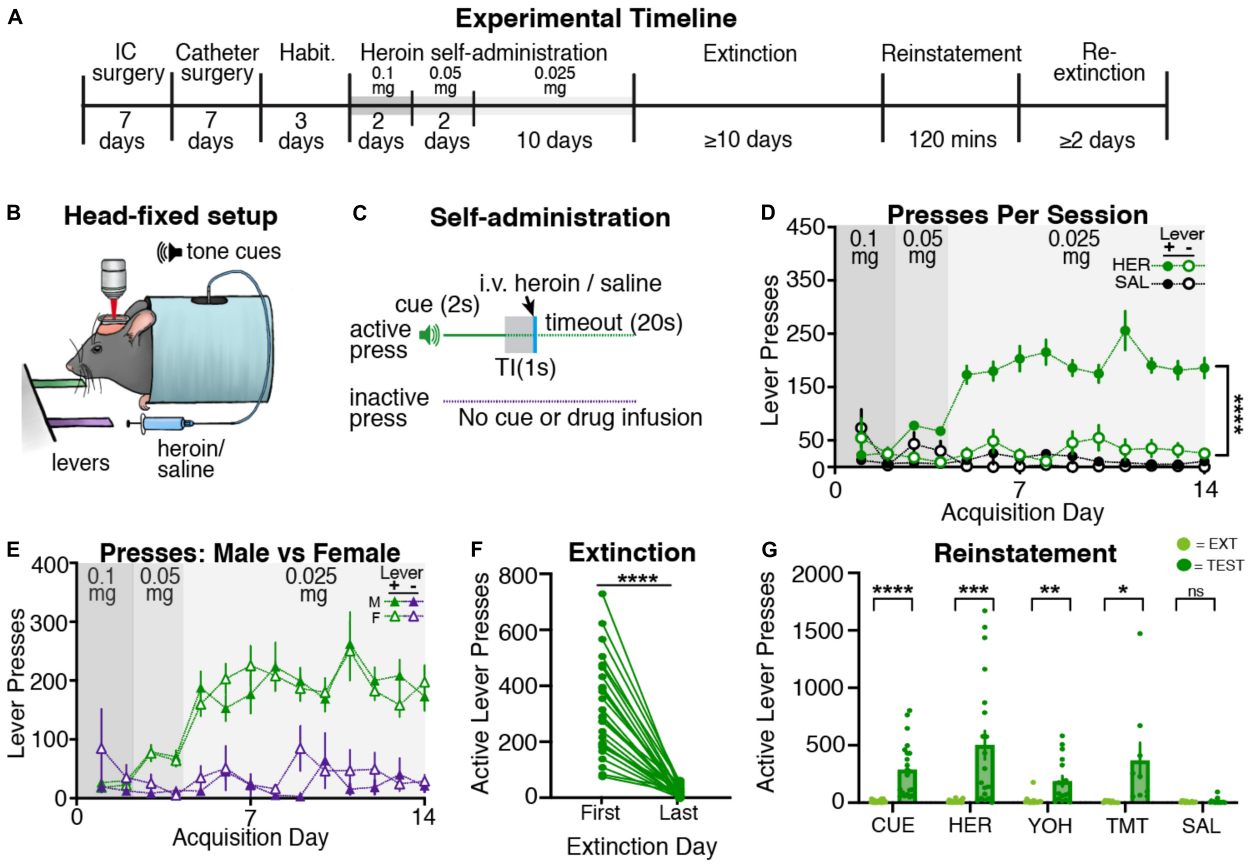

FIGURE 1 | Head-restrained mice self-administer heroin and display extinction and reinstatement of heroin seeking. **(A)** An experimental timeline demonstrating the behavioral procedure used for head-restrained heroin self-administration. **(B)** Illustration of head fixation for heroin self-administration experiments. Active lever presses resulted in a tone cue that predicted intravenous heroin [at doses of 0.1 mg/kg (day 1–2), 0.05 mg/kg (day 3–4), or 0.025 mg/kg (day 5–14)] or intravenous saline. **(C)** Schematic for heroin/saline self-administration experiments, where active but not inactive lever presses resulted in a tone cue (2s), a gap in time (TI, trace interval; 1s), an infusion of heroin or saline, and a timeout period (20s). **(D)** Data showing that heroin self-administering mice pressed the active lever more than the inactive lever (****$p < 0.001$), whereas saline self-administering mice did not. **(E)** A comparison of active and inactive lever pressing across sexes. Male and female heroin self-administering mice showed no differences in lever pressing across acquisition. **(F)** During extinction, heroin self-administering mice significantly decreased active lever pressing from the first day of extinction to the last (****$p < 0.001$). **(G)** Data showing that mice displayed cue- (****$p < 0.001$), heroin- (***$p = 0.001$), yohimbine- (**$p = 0.002$), and predator odor (TMT; *$p = 0.048$)-induced reinstatement of active lever pressing as compared with the previous extinction test. Mice did not reinstate following an injection of saline.

mice learned to reliably press the active lever more than the inactive lever across acquisition, but saline animals did not exhibit a preference for pressing either lever (**Figure 1D**). A three-way ANOVA revealed a significant effect of drug [$F(1,68) = 62.0$, $p < 0.001$] and a drug by lever interaction [$F(1,68) = 35.5$, $p < 0.001$], but no drug by lever by day interaction [$F(13,862) = 1.67$, $p = 0.06$]. Following two-way ANOVAs (day × lever separated for each group) revealed an effect of lever for heroin self-administering mice [$F(1,54) = 133.5$, $p < 0.001$], but not for saline self-administering mice [$F(1,14) = 0.01$, $p = 0.92$]. The heroin self-administering mice also received more infusions as compared with the saline self-administering mice (**Supplementary Figure 2A**), as a two-way ANOVA revealed an effect of drug [$F(1,34) = 2655$; $p < 0.001$] and day by drug interaction [$F(13,434) = 147.7$, $p < 0.001$]. *Post hoc* tests revealed that heroin self-administering mice began receiving significantly more infusions starting on day 2 of acquisition (day 1, $p = 0.68$; days 2–14, $ps < 0.05$). Thus, mice rapidly and readily acquired heroin self-administration while head restrained.

Extinction and Reinstatement

Following acquisition, heroin self-administering mice underwent extinction training, wherein active lever presses no longer resulted in heroin or cue delivery. We find that head-restrained, heroin self-administering mice significantly decrease active lever pressing [**Figure 1F**; $t(27) = 8.95$; $p < 0.001$], but not inactive lever pressing [**Supplementary Figure 3A**; $t(27) = 0.92$; $p = 0.37$] from the first to last day of extinction. Next, heroin self-administering mice underwent cue- ($n = 22$; male = 10, female = 12), heroin- ($n = 20$; male = 8, female = 12), yohimbine- ($n = 17$; male = 8, female = 9), predator odor- ($n = 9$; male = 4, female = 5), and/or saline- ($n = 14$; male = 7, female = 7) induced reinstatement tests (**Figure 1G**). Paired *t*-tests revealed that heroin self-administering mice significantly increased active

lever pressing during cue- [t(21) = 5.41; $p < 0.001$], heroin- [t(19) = 3.97; $p = 0.001$], yohimbine- [t(16) = 3.87; $p = 0.002$], and predator odor-induced [t(8) = 2.33; $p = 0.048$] reinstatement tests, but not following an injection of saline [t(13) = 0.89; $p = 0.39$], relative to responding during the previous extinction session. Heroin self-administering mice did not increase inactive lever pressing during cue- [t(21) = 1.61; $p = 0.12$], heroin- [t(19) = 0.77; $p = 0.45$], yohimbine- [t(16) = 1.48; $p = 0.15$], predator odor- [t(8) = 0.69; $p = 0.50$], or saline- [t(13) = 1.58; $p = 0.13$] induced reinstatement tests relative to the previous extinction session (**Supplementary Figure 3B**). Overall, we find that head-restrained animals readily self-administer heroin, extinguish lever pressing, and display reinstatement of heroin seeking similar to freely moving mice (Corre et al., 2018).

A subset of saline self-administering mice ($n = 4$) underwent extinction training and reinstatement testing despite displaying low levels of lever responding throughout acquisition. As a result of this low responding, saline self-administering mice did not show a significant decrease of active lever pressing [t(3) = 0.0; $p > 0.99$] from the first day of extinction to the last (**Supplementary Figure 4A**). Additionally, saline self-administering mice did not increase active lever pressing to any testing conditions [**Supplementary Figure 4B**; *t*-tests, cue: t(3) = 0.86, $p = 0.45$; heroin: t(3) = 1.89, $p = 0.15$; yohimbine: t(3) = 2.29, $p = 0.11$; saline: t(3) = 1.13, $p = 0.34$]. These data are consistent with previous findings showing that saline self-administering rodents will not extinguish or reinstate active lever pressing (Kruyer et al., 2019; Chioma et al., 2021).

Sex Differences

During acquisition, we did not observe sex differences in lever press rates for heroin self-administering mice (**Figure 1E**), as a three-way ANOVA revealed no effect of sex, sex by lever interaction, or sex by lever by day interaction (*F*-values < 0.83; *p*s > 0.50). Male and female heroin self-administering mice also received a similar number of infusions (**Supplementary Figure 2A**) throughout acquisition, as confirmed by a two-way ANOVA revealing no effect of sex or sex by drug interaction (*F*-values < 1.0, *p*s > 0.5). During extinction, we find male and female mice display equivalent active lever presses from the first to last day (**Supplementary Figure 2B**; two-way ANOVA, effects of sex: *F*-values < 0.3, *p*s > 0.5). Lastly, we did not observe sex differences in reinstatement responding for heroin self-administering mice (**Supplementary Figure 2C**; two-way ANOVAs, effects of sex: *F*-values < 2.4, *p*s > 0.1; sex by day interactions: F-values < 1.45, *p*s > 0.25).

Head-Restrained Cocaine Self-Administration

Acquisition

Following surgery and habituation (see **Figure 2A** for timeline), mice ($n = 15$; 8 males and 7 females) underwent head-restrained cocaine self-administration (**Figures 2B,C**). Cocaine self-administering mice learned to press the active lever more than the inactive lever across acquisition, and when compared to saline self-administering animals, showed significantly more active lever pressing (**Figure 2D**). A three-way ANOVA revealed a significant effect of drug [F(1,42) = 48.51, $p < 0.001$] and a drug by lever interaction [F(1,42) = 6.15, $p < 0.02$], but no drug by lever by day interaction [F(13,512) = 0.19, $p = 0.99$]. Following two-way ANOVAs (day × lever separated for each group) revealed an effect of lever for cocaine self-administering mice [F(1,28) = 11.91, $p = 0.002$], but not for saline self-administering mice [F(1,14) = 0.01, $p = 0.92$]. Cocaine self-administering mice also received significantly more infusions than saline self-administering mice throughout acquisition (**Supplementary Figure 2D**), with a mixed-model two-way ANOVA showing an effect of drug [F(1,21) = 1564, $p < 0.001$], but no interaction between day and drug [F(13,266) = 0.70, $p = 0.67$]. Thus, mice reliably acquire cocaine self-administration behavior while head restrained.

Extinction and Reinstatement

Following cocaine self-administration, mice underwent extinction until criteria were met ($n = 11$; 6 males and 5 females). A paired *t*-test confirmed that cocaine self-administering mice significantly decreased both active [t(10) = 3.48; $p = 0.006$; **Figure 2F**] and inactive [t(10) = 2.58; $p = 0.027$; **Supplementary Figure 3C**] lever pressing from the first day of extinction to the last day. Next, mice underwent cue- ($n = 11$; 6 males, 5 females), cocaine- ($n = 10$; 6 males, 4 females), yohimbine- ($n = 10$; 6 males, 4 females), predator odor- ($n = 11$; 6 males, 5 females), and saline- ($n = 10$; 6 males, 4 females) induced reinstatement tests (**Figure 2G**). Paired *t*-tests revealed that, compared to the respective prior day's extinction responding, mice significantly increased active lever pressing during cue- [t(10) = 3.34; $p = 0.007$], cocaine- [t(9) = 4.38; $p = 0.002$], yohimbine- [t(9) = 2.93; $p = 0.017$], and predator odor-induced [t(10) = 2.79; $p = 0.019$] reinstatement tests, but not but not following an injection of saline [t(9) = 0.77; $p = 0.45$]. Cocaine mice did not increase inactive lever pressing during cue- [t(10) = 1.88; $p = 0.08$], cocaine- [t(9) = 0.98; $p = 0.34$], yohimbine- [t(9) = 1.09; $p = 0.29$], predator odor- [t(10) = 1.85; $p = 0.08$], or saline-induced [t(9) = 0.31; $p = 0.76$] reinstatement tests relative to inactive lever responding during the most recent extinction session [**Supplementary Figure 3D**]. Overall, we find that head-restrained animals self-administer cocaine, extinguish lever pressing, and display reinstatement of cocaine seeking similar to freely moving mice (Heinsbroek et al., 2017).

Sex Differences

During acquisition, we did not observe sex differences in lever press rates for cocaine self-administering mice (**Figure 2E**), as a three-way ANOVA revealed no effect of sex, sex by lever interaction, or sex by lever by day interaction (*F*-values < 1.17; *p*s > 0.3). Male and female cocaine self-administering mice also received a similar number of infusions (**Supplementary Figure 2D**) during acquisition, as confirmed by a two-way ANOVA showing no effect of sex or sex by drug interaction (*F*-values < 1.3, *p*s > 0.6). During extinction, males and females display a similar number of active lever presses from the first to last day (**Supplementary Figure 2E**; two-way ANOVA, effects of sex: *F*-values < 0.1, *p*s > 0.5).

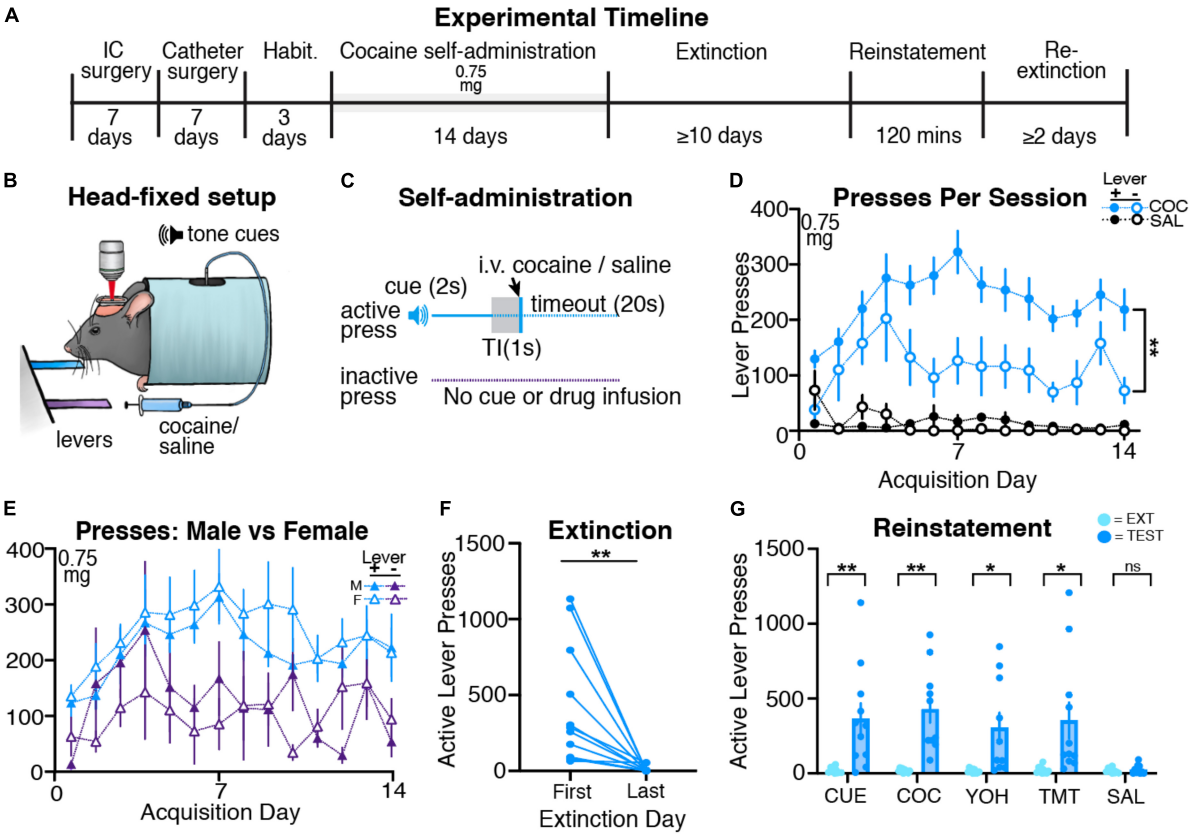

FIGURE 2 | Head-restrained mice self-administer cocaine and display extinction and reinstatement of cocaine seeking. **(A)** An experimental timeline demonstrating the behavioral procedure used for head-restrained cocaine self-administration. **(B)** Illustration of head fixation for cocaine self-administration experiments. Active lever presses resulted in a tone cue that predicted intravenous cocaine (0.75 mg/kg on all days) or saline. **(C)** Schematic for cocaine self-administration experiments, where active but not inactive lever presses resulted in a tone cue (2s), a gap in time (TI, trace interval; 1s), an infusion of cocaine or saline, and a timeout period (20s). **(D)** Data showing that cocaine self-administering mice pressed the active lever more than the inactive lever (**p = 0.002). **(E)** A comparison of active and inactive lever pressing across sexes. Male and female cocaine self-administering mice showed no differences in lever pressing across acquisition. **(F)** All cocaine self-administering mice significantly decreased active lever pressing from the first day of extinction to the last (**p = 0.006). **(G)** Mice displayed cue- (**p = 0.007), cocaine- (**p = 0.002), yohimbine- (*p = 0.017), and predator odor (TMT; *p = 0.019)- induced reinstatement of active lever pressing as compared with the previous extinction test. Mice did not reinstate following an injection of saline.

We did not observe sex differences during the cue-, cocaine-, yohimbine-, and predator odor-induced reinstatement tests (two-way ANOVAs: main effect and interactions including sex: F-values < 0.82, $ps > 0.05$), but did see a significant effect of sex for the control injection of saline [$F(1,8) = 7.92$, $p = 0.023$; **Supplementary Figure 2F**]. *Post hoc* tests revealed that males pressed the active lever significantly more than females on the test day ($p = 0.014$), but not the previous extinction day ($p = 0.974$).

Head-Restrained Sucrose Self-Administration

Acquisition

Since drug self-administration protocols often include natural reward (sucrose) self-administration as a control, we determined whether mice would self-administer liquid sucrose droplets within our head-restrained model. Following recovery from surgery and habituation (see **Figure 3A** for timeline), mice ($n = 32$; 15 males, 17 females) began head-restrained sucrose self-administration (**Figures 3B,C**), using a similar behavioral protocol as the previous heroin self-administration experiment. Sucrose self-administering mice learned to reliably press the active lever more than the inactive lever across acquisition (**Figure 3D**). A mixed-model two-way ANOVA showed a day by lever interaction [$F(13,800) = 3.93$, $p < 0.001$], and *post hoc* tests revealed that mice pressed the active lever more than the inactive lever during each day of acquisition (days 1–14, $ps < 0.05$). Sucrose self-administering mice often reached the maximum number of sucrose droplets across all sessions (**Supplementary Figure 2G**). Thus, sucrose self-administering mice were able to reliably acquire lever pressing for sucrose rewards while being head-restrained.

Extinction and Reinstatement

Following sucrose self-administration, mice underwent at least 10 days of extinction until criteria were met ($n = 26$, 14 males, 12 females). Sucrose self-administering mice significantly decreased

FIGURE 3 | Head-restrained mice self-administer sucrose and display reinstatement of sucrose seeking to the reward-associated cue. **(A)** An experimental timeline demonstrating the behavioral procedure used for head-restrained sucrose self-administration. **(B)** Illustration of head fixation for sucrose self-administration experiments. Active lever presses resulted in a tone cue that predicted a sucrose droplet (12.5 μl of 12.5% sucrose). **(C)** Schematic for sucrose self-administration experiments, where active but not inactive lever presses resulted in a tone cue (2s), a gap in time (TI, trace interval; 1s), a palatable sucrose droplet, and a timeout period (20s). **(D)** Sucrose self-administering mice pressed the active lever significantly more than the inactive lever on each day of acquisition (*ps < 0.05). **(E)** A comparison of active and inactive lever pressing across sexes. Male and female sucrose self-administering mice showed no differences in lever pressing across acquisition. **(F)** All sucrose self-administering mice significantly decreased active lever pressing from the first day of extinction to the last (****p < 0.001). **(G)** Sucrose self-administering mice displayed an increase in active lever pressing to cue- (****p < 0.001), but not heroin-, yohimbine-, predator odor (TMT)-, or saline-induced reinstatement tests.

active lever pressing [**Figure 3F**; t(25) = 5.63, p < 0.001], but not inactive lever pressing [**Supplementary Figure 3E**; t(25) = 1.50, p = 0.15], from the first day of extinction to the last. Following extinction, sucrose self-administering mice underwent cue- (n = 8; 4 males, 4 females), heroin- (n = 8; 3 males, 5 females), yohimbine- (n = 8; 3 males, 5 females), predator odor- (n = 8; 4 males, 4 females), and saline- (n = 8; 4 males, 4 females) induced reinstatement tests (**Figure 3G**). Paired t-tests revealed that, compared to the previous extinction session, mice significantly increased active lever pressing during cue-induced reinstatement [t(7) = 5.93, p < 0.001], but not during heroin- [t(7) = 0.55, p = 0.60], yohimbine- [t(7) = 0.76, p = 0.48], predator odor- [t(7) = 1.18, p = 0.28], or saline-induced [t(7) = 0.21, p = 0.84] reinstatement tests. Sucrose mice did not increase their inactive lever pressing during any reinstatement tests (**Supplementary Figure 3F**; t-values < 1.5, ps > 0.15). Overall, we find that head-restrained animals will self-administer sucrose, extinguish lever pressing, and display reinstatement of sucrose seeking to the sucrose-associated cue similar to freely moving mice (Bobadilla et al., 2020).

Sex Differences

Male and female mice did not differ in lever pressing during acquisition (**Figure 3E**), as a three-way ANOVA showed that, while there was an effect of sex [F(1,60) = 4.29, p = 0.04], there was no sex and lever interaction [F(1,60) = 0.12, p = 0.73] or day by sex by lever interaction [F(13,774) = 1.11, p = 0.34]. Furthermore, *post hoc* tests revealed that there were no sex differences for active (days 1–14, ps > 0.66) or inactive (days 1–14, ps > 0.35) lever pressing across acquisition. We find that male and female mice also receive a similar number of sucrose droplets (**Supplementary Figure 2G**) throughout acquisition (two-way ANOVA, effects of sex: F-values < 0.3, ps > 0.6). During extinction, males and females displayed a similar number of active presses on each day (**Supplementary Figure 2H**; effects of sex: F-values < 2.3, ps > 0.12). Additionally, we did not observe

sex differences during any reinstatement tests (**Supplementary Figure 2I**; effects of sex: F-values < 2.1, $ps > 0.12$).

DISCUSSION

Here we demonstrate that mice will readily self-administer heroin and cocaine, but not saline, while head restrained through active lever pressing behavior. Mice will extinguish active lever pressing when the drug and drug-associated cues are omitted. After extinction, mice with drug experience will resume drug seeking upon re-exposure to stimuli known to provoke relapse in humans (i.e., drug-associated cues, the drug itself, and stressors; Kalivas and Volkow, 2005). However, we find that sucrose self-administering mice will only resume seeking following presentation of the reward-associated cue, whereas heroin and stressors do not provoke sucrose seeking. These findings indicate that our head-restrained procedure may share similar construct and predictive validity as drug self-administration assays in freely moving rodents (Epstein et al., 2006; Sanchis-Segura and Spanagel, 2006). Furthermore, this study demonstrates the feasibility of combining preclinical drug self-administration experiments with novel technologies that require head immobilization.

Head-Restrained vs. Freely Moving Drug Self-Administration

Similar to freely moving drug self-administration studies, our head-restrained drug self-administration procedure allows mice to acquire, extinguish, and reinstate drug seeking behaviors. Despite these similarities, we find that head-restrained mice display some differences that are important to consider. Most notably, the number of lever presses is generally higher when comparing our results with experiments in freely moving mice (Heinsbroek et al., 2017). This increase is likely a result of immobilization, which isolates mice in front of the levers. Furthermore, the stress involved in head restraint could be a factor, as stress is known to facilitate and provoke drug seeking and taking (Piazza and Le Moal, 1998). An additional difference that we observe is a relatively high inactive lever press rate during acquisition for cocaine self-administering mice. This could be due to the behavioral sensitization caused by repeated cocaine exposure, an effect that substantially increases movement in mice (for review, see Steketee and Kalivas, 2011). It is possible that head restraint limits increased motor activity primarily to the forelimbs, resulting in stereotyped inactive lever pressing. Despite the increase in inactive pressing, it is important to note that head-restrained cocaine self-administering mice discriminate between active and inactive levers, suggesting goal-directed behavioral responding.

Sex Differences During Head-Restrained Drug Self-Administration

Sex differences are often described in disorders associated with dysfunctional reward seeking (Fattore et al., 2008; Galmiche et al., 2019), with females displaying elevated cravings, relapse susceptibility, and loss of control over intake as compared with male counterparts (Rubonis et al., 1994; Robbins et al., 1999; Kippin et al., 2005; Striegel-Moore et al., 2009; Moran-Santa Maria et al., 2014). Sex differences have accordingly been found in rodent drug and sucrose self-administration experiments, wherein females reportedly show an enhanced rate of acquisition of drug taking (Lynch et al., 2001; Roth et al., 2002; Hu et al., 2004; Jackson et al., 2006), and greater reinstatement of drug (Fuchs et al., 2005; Anker and Carroll, 2010; Feltenstein et al., 2011) and sucrose seeking (Wei et al., 2021). However, a lack of sex differences in these behaviors have also been reported (Roth and Carroll, 2004; Doncheck et al., 2020). Within our head-restrained model, we find that males and females acquire cocaine, heroin, and sucrose self-administration at equivalent rates and display comparable levels of reinstatement. While head restraint limits the behavioral repertoire exhibited in freely moving animals, which may influence complex factors to differentially promote seeking in both sexes, our head-restrained procedure could be adapted for sex-specific studies. For example, allowing head-restrained mice to have extended drug access could be advantageous for revealing sex differences, as others have shown that females will significantly elevate drug intake during long-access cocaine or heroin self-administration (Roth and Carroll, 2004; Towers et al., 2019). To study propensity to relapse, our procedure could be modified to include abstinence, as it has been reported that females will exhibit greater sucrose (Wei et al., 2021) and drug-seeking reinstatement following a period of forced abstinence (Anker and Carroll, 2010; Feltenstein et al., 2011). Regardless of behavioral sex differences, when coupled with *in vivo* imaging techniques, our head-restrained procedure could provide insight into the sex-specific neurophysiology that underlies acquisition and reinstatement to drug seeking.

Applications of Head-Restrained Drug Self-Administration

The field of preclinical *in vivo* neuroimaging has seen rapid innovation within recent years, facilitated by the development and improvement of imaging technologies and *in vivo* sensors. For example, through the application of head-restraint, scientists have been able to perform functional MRI (Chang et al., 2016; Stenroos et al., 2018; Desjardins et al., 2019; Liu et al., 2020), functional ultrasound (Brunner et al., 2021; Wang et al., 2021), wide-field (Kim et al., 2016; Silasi et al., 2016; Yoshida et al., 2018) and multiphoton fluorescence (Otis et al., 2017, 2019; Namboodiri et al., 2019; Rossi et al., 2019; Grant et al., 2021) imaging experiments in awake rodents. Coupling innovative preclinical imaging techniques with behavioral tasks can provide an extraordinary view into how complex neural systems mediate relevant behavioral states. Here, we combine head-restraint with an operant drug self-administration task so that we may investigate the precise neuroadaptations associated with the development of SUD. Pairing operant drug self-administration with the described *in vivo* imaging techniques could aid in elucidation of the neuronal underpinnings of SUD.

Using Multiphoton Imaging to Track Activity and Morphological Plasticity in Cell-Type Specific Neurons From the Onset of Drug Use to Relapse

The development of SUD involves complex and long-lasting changes to activity (Koob and Volkow, 2016) and structural plasticity (for review, see; Russo et al., 2010; Spiga et al., 2014; Wolf, 2016; Kruyer et al., 2020) in brain reward circuitry, but how these adaptations develop in cell-type specific neurons and predict relapse vulnerability is unknown. With the advent of multiphoton microscopy (Denk et al., 1990) along with genetically encoded calcium indicators (Chen et al., 2013), we can now longitudinally measure activity (Otis et al., 2017, 2019; Namboodiri et al., 2019; Rossi et al., 2019; Grant et al., 2021) and visualize cell morphology (Muñoz-Cuevas et al., 2013; Moda-Sava et al., 2019) in deep brain, cell-type specific neurons for weeks to months in awake, behaving animals. This combinatorial approach can be exploited to monitor activity not only in hundreds to millions of neuronal cell bodies simultaneously (Dombeck et al., 2007; Kim et al., 2016), but also in dendrites (Lavzin et al., 2012), dendritic spines (Chen et al., 2011), and axons (Lovett-Barron et al., 2014; Otis et al., 2019). Although calcium indicators are by far the most commonly used for visualizing activity, other sensors are available and under continued development for visualization of ground-truth voltage (Gong et al., 2015), neurotransmitter release and binding (Nguyen et al., 2010; Jing et al., 2018; Marvin et al., 2018, 2019); molecular signaling (Greenwald et al., 2018; Muntean et al., 2018), and more (Marvin et al., 2011; Aper et al., 2016; Díaz-García et al., 2019). These powerful technologies could be combined with drug self-administration studies in head-restrained mice to provide an unparalleled view into the abnormal neural circuit activity patterns and the morphological adaptations that arise from the onset of drug use to relapse.

Evaluating the Function of Neuronal Ensemble Activity Patterns and Morphological Plasticity in Drug Use and Seeking

Studies using multiunit recordings and calcium imaging technologies often reveal complex activity patterns within single brain regions during natural reward (Otis et al., 2017, 2019; Grant et al., 2021) and drug reward-seeking (Drouin and Waterhouse, 2004; Ahmad et al., 2017; Siciliano et al., 2019). These heterogeneous activity patterns are not just common at the population level, but also at the level of neuronal subpopulations, as recordings from genetically defined or projection-specific neurons also reveal profound cell-to-cell variability (Seong and Carter, 2012; Dembrow and Johnston, 2014; Al-Hasani et al., 2015; Yang et al., 2018). The inherent complexity of neuronal circuit activity patterns has made it difficult to selectively manipulate unique neuronal ensembles to determine their function for behavior. However, existing and emerging technologies that combine multiphoton microscopy with optogenetics now allow for manipulation of activity in experimenter-defined neurons in 3-dimensional space (Yang et al., 2018; Marshel et al., 2019). Furthermore, multiphoton laser-directed lesion experiments have been employed at the level of cell bodies, axons, dendrites, and dendritic spines *in vivo* (Allegra Mascaro et al., 2010; Canty et al., 2013; Hill et al., 2017; Park et al., 2019). These technologies could now be combined with drug self-administration studies to identify the function of unique neuronal ensembles and morphological plasticity for drug use and seeking.

Future Directions and Limitations

A potential limitation of our protocol is that it is unknown whether head restraint produces greater basal stress levels compared to freely moving, albeit catheter-tethered mice. This caveat is important given that chronic stress can lead to escalation of drug intake (Mantsch and Katz, 2007), which may produce behavioral phenotypes akin to those observed in animals with a greater history of intake (i.e., "long-access" or "extended-access," rather than "short-access," animals; Mantsch et al., 2016). Future modifications to our approach could be beneficial for reducing the possible effects of stress on drug seeking and allow for improved behavioral resolution within the task itself. For example, inclusion of a running wheel, treadmill, or trackball would provide an avenue for mice to move while head restrained and would also provide a behavioral readout of locomotor activity – a behavioral variable that is often studied due to its robust modulation by repeated drug use and correlation with addiction vulnerability (Piazza et al., 1989; Zhou et al., 2019). Notably, as our mice still reinstate in response to other stressors, head restraint does not prohibit investigation of stress effects on reward seeking. Altogether, the influence of restraint-related stress should be acknowledged and considered when designing drug self-administration experiments in head-restrained animals. Moreover, future adaptations of the assay could improve its strength for studying the neural circuit underpinnings of SUD.

CONCLUSION

By coupling multiphoton imaging with simultaneous intravenous drug self-administration, we can characterize and manipulate adaptations in neuronal circuits that evolve from the onset of drug use to relapse. The replication of effects observed in freely moving animals indicates that our head-restrained approach could build upon, rather than diverge from, decades of foundational research provided by freely moving drug self-administration studies. Most importantly, this could accelerate discovery of novel therapeutic interventions for SUD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

KV, ED, and JO designed the experiments and wrote the manuscript. KV, ED, RG, KW, ER, CB, PS, LG, and IT-P performed the experiments. AB and PK provided intellectual support and training for self-administration studies. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by NIDA (R01-DA051650) and a pilot award from the MUSC Cocaine and Opioid Center on Addiction (COCA Pilot Core C; P50-DA046373) to JO, the NIDA T32 (DA00728829) grant awarded to ED, KV, and RG, the NIH/NIDA F31 to KV (DA052186), the NIH/NIDA F32 to ED (DA053830), the NIH/NIDA Specialized Center of Research Excellence (U54-DA016511) pilot award to ED, an NIH IMSD grant awarded to RG (R25-GM072643), the NIH Post-Baccalaureate Research Education Program (R25-GM113278) award to PS and KW, and the NIH/NIDA K99 (DA046522) and P20GM121310 to AB and the VA Merit Award BX004727 to PK.

ACKNOWLEDGMENTS

We would like to thank Dr. Madalyn Hafenbreidel, Dr. Carmela Reichel, Dr. Jakie McGinty, Dr. Howard Becker, Eric Dereschewitz, and Maribel Vasquez-Silva for technical advice and support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.744715/full#supplementary-material

Supplementary Figure 1 | Head-restrained drug self-administration behavioral chamber. *Left:* each two-door cabinet has soundproofing foam **(1)** lining the doors and walls. An infusion pump **(2)** sits atop each behavioral chamber and has tubing extending to the restrained mouse (mouse not shown) **(3)**, allowing i.v. infusions. Each behavioral chamber is equipped with a laptop **(4)**, which interfaces with the Arduino circuit board to control equipment and record behavior. *Right:* Electronics, levers, and the head-fixation station are secured to an aluminum breadboard **(5)** within each behavioral chamber. The head-fixation station **(6)** holds the head-ring which is surgically implanted onto the mouse's skull. Once the mouse is secured within the head-fixation station, its body is placed within the restraint tube. The mouse can use its forelimbs to press the operant levers **(7)**. When the active lever is pressed, the speaker **(8)** can deliver an auditory cue during acquisition and cue-induced reinstatement sessions. The Arduino circuit board **(9)** and associated electronic breadboards interface with the computer to record behavioral events, as well as control other aspects of the session (e.g., auditory cues, infusions, and session length).

Supplementary Figure 2 | Sex comparison across head-fixed drug and sucrose self-administration experiments. *Heroin self-administration.* **(A)** Male and female mice did not differ in the number of i.v. heroin infusions received during acquisition. However, grouped data shows heroin self-administering mice received more infusions as compared with the saline self-administering mice ($p < 0.001$). **(B)** Male and female mice did not differ in active lever pressing rates during extinction of heroin seeking. **(C)** Male and female mice did not display differences during cue-, heroin-, yohimbine-, predator odor (TMT)-, or saline-induced reinstatement tests. *Cocaine self-administration.* **(D)** Male and female mice did not differ in the number of i.v. cocaine infusions received during acquisition. However, grouped data shows cocaine self-administering mice received more infusions as compared with the saline self-administering mice ($p < 0.001$). **(E)** Male and female mice did not differ in active lever pressing rates during extinction of cocaine seeking. **(F)** Male and female mice did not display differences during cue-, cocaine-, yohimbine-, or predator odor (TMT)-induced reinstatement tests. However, males pressed the active lever significantly more than females during the saline control test ($p = 0.014$). *Sucrose self-administration.* **(G)** Male and female mice did not differ in the number of sucrose droplets received during acquisition. **(H)** Male and female mice did not differ in active lever pressing rates during extinction of sucrose seeking. **(I)** Male and female mice did not display differences during cue-, heroin-, yohimbine-, predator odor (TMT)-, or saline-induced reinstatement tests.

Supplementary Figure 3 | Inactive lever pressing across head-fixed drug and sucrose self-administration experiments. *Heroin self-administration.* **(A)** Mice showed no difference in inactive lever pressing across extinction. **(B)** Mice showed no change in inactive lever pressing from extinction to cue-, heroin-, yohimbine-, predator odor (TMT)-, or saline-induced reinstatement. *Cocaine self-administration.* **(C)** Mice significantly decreased inactive lever pressing from the first day of extinction to the last. **(D)** Mice showed no change in inactive lever pressing from extinction to cue-, cocaine-, yohimbine-, predator odor (TMT)-, or saline-induced reinstatement. *Sucrose self-administration.* **(E)** Mice showed no difference in inactive lever pressing across extinction. **(F)** Mice showed no change in inactive lever pressing from extinction to cue-, heroin-, yohimbine-, predator odor (TMT)-, or saline-induced reinstatement.

Supplementary Figure 4 | Active lever pressing across extinction and reinstatement testing for saline self-administration experiments. **(A)** Due to low lever responding across acquisition, saline self-administering mice did not decrease active lever pressing from the first day of extinction to the last. **(B)** Saline self-administering mice did not increase active lever pressing to cue-, heroin-, yohimbine-, or saline-induced reinstatement tests.

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