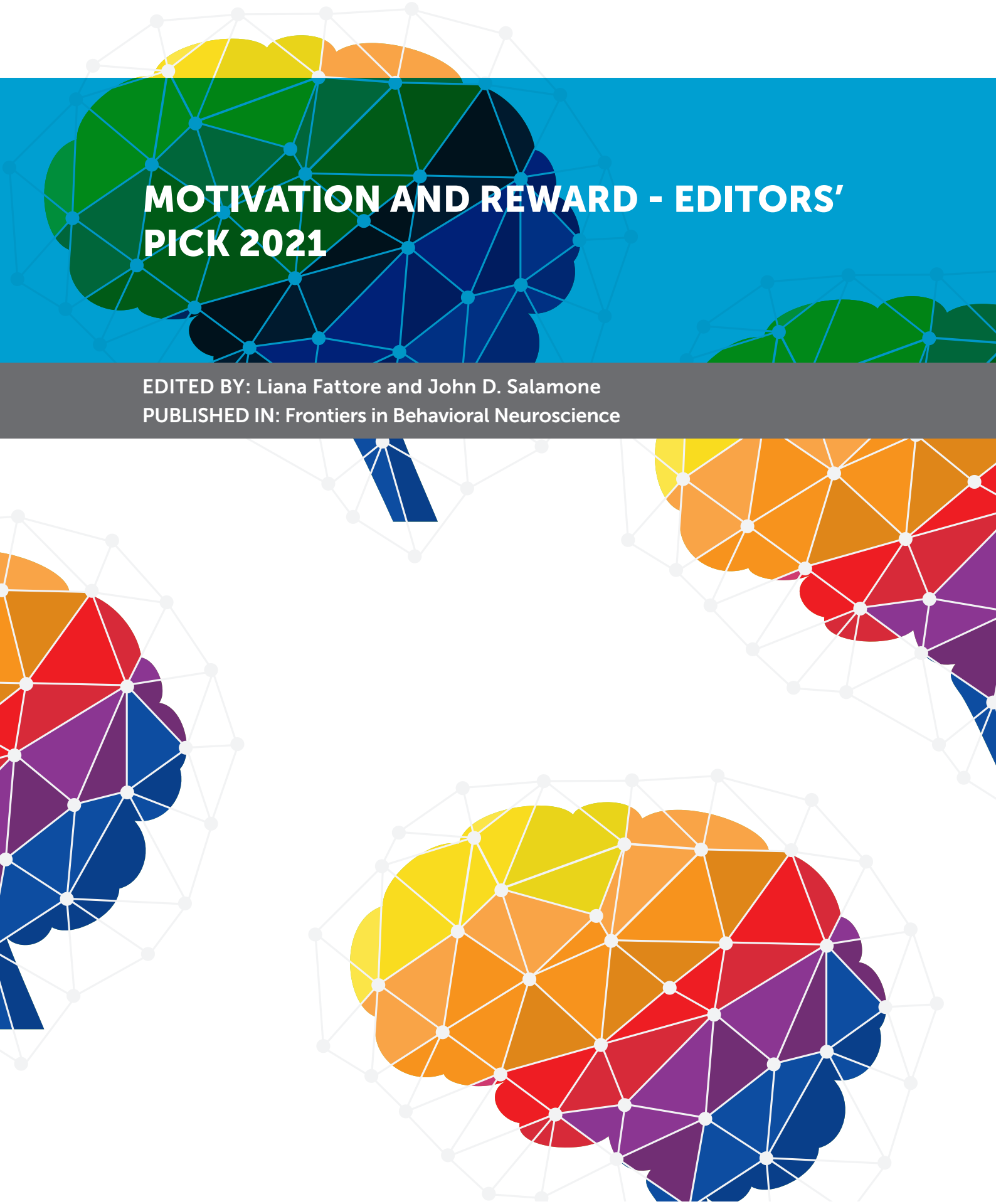
MOTIVATION AND REWARD - EDITORS' PICK 2021

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



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-197-0
DOI 10.3389/978-2-88971-197-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

MOTIVATION AND REWARD - EDITORS' PICK 2021

Topic Editors:
Liana Fattore, National Research Council (CNR), Italy
John D. Salamone, University of Connecticut, United States

Citation: Fattore, L., Salamone, J. D., eds. (2021). Motivation and Reward - Editors' Pick 2021. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-197-0

Table of Contents

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 15 January 2019
doi: 10.3389/fnbeh.2018.00331

Ethanol Preference and Drinking Behavior Are Controlled by RNA Editing in the Nucleus Accumbens

Takahira Shirahase[1,2,3], Yoshihisa Watanabe[1], Atsushi Tsujimura[1], Shin Kwak[4], Toshiro Yamamoto[3], Narisato Kanamura[3] and Masaki Tanaka[2]**

[1]Department of Basic Geriatrics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Department of Anatomy and Neurobiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, [3]Department of Dental Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, [4]Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan

RNA editing plays critical roles in normal brain function, and alteration of its activity causes various disorders. We previously found that chronic consumption of ethanol was associated with increased levels of RNA editing of serotonin 2C receptor in the nucleus accumbens (NAc). However, it remains unknown whether RNA editing in the NAc modulates alcohol addiction through the brain reward system. To investigate the involvement of NAc RNA editing in alcohol addiction, we generated NAc-specific knockout mice of the double-stranded RNA-specific adenosine deaminase *ADAR2* using AAV-GFP/Cre and conducted a battery of behavioral tests including anxiety- and depression-like behaviors. In addition, NAc-specific *ADAR2* knockout mice were exposed to ethanol vapor for 20 days, followed by ethanol-drinking and conditioned place preference (CPP) tests. NAc-specific *ADAR2* knockout mice showed a significant decrease in locomotor activity in the open field test although they did not develop anxiety- and depression-like behaviors. In addition, the enhancements of ethanol intake and ethanol preference that are usually observed after chronic ethanol vapor exposure were significantly reduced in these mice. These results suggest that ADAR2-mediated RNA editing in the NAc is involved in determination of alcohol preference after chronic alcohol consumption.

Keywords: RNA editing, ethanol preference, ethanol drinking behavior, ADAR2, nucleus accumbens

OPEN ACCESS

Edited by:
Rainer Spanagel, Central Institute for Mental Health, Germany

Reviewed by:
Luigia Trabace, University of Foggia, Italy
Amy Lasek, University of Illinois at Chicago, United States

***Correspondence:**
Yoshihisa Watanabe
y-watana@koto.kpu-m.ac.jp
Masaki Tanaka
mtanaka@koto.kpu-m.ac.jp

Received: *07 September 2018*
Accepted: *17 December 2018*
Published: *15 January 2019*

Citation:
Shirahase T, Watanabe Y, Tsujimura A, Kwak S, Yamamoto T, Kanamura N and Tanaka M (2019) Ethanol Preference and Drinking Behavior Are Controlled by RNA Editing in the Nucleus Accumbens. Front. Behav. Neurosci. 12:331. doi: 10.3389/fnbeh.2018.00331

INTRODUCTION

Adenosine deaminases acting on RNA (ADAR1 and 2) modify adenosine to inosine in double-stranded RNA, a process called RNA editing (Nishikura, 2016). RNA editing is involved in neuronal functions including synaptic plasticity and neuronal development (Behm and Öhman, 2016). The precursor mRNAs of several neuroreceptors such as serotonin 2C receptor (5-HT_{2C}R), glutamate receptor A2 (GluA2), and γ-aminobutyric acid type A receptor ($GABA_A$) undergo RNA editing, which modulates synaptic transmission (Behm and Öhman, 2016). 5-HT_{2C}R mRNA is edited at five positions by ADAR1 and ADAR2, substituting three amino acids in the second intracellular loop region (Burns et al., 1997; Niswender et al., 1998). Edited $5HT_{2C}$R isoforms show reductions in 5-HT potency, agonist binding affinity, constitutive activity and G-protein coupling activity compared with non-edited isoforms (Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000; Gurevich et al., 2002). In mice, alteration in expression of $5HT_{2C}$R

isoforms causes abnormal emotional, feeding and alcohol-drinking behaviors (Kawahara et al., 2008; Mombereau et al., 2010; Martin et al., 2013; Watanabe et al., 2014; Aoki et al., 2016). AMPA receptor subunit GluA2 is also edited at the Q/R site by ADAR2, resulting in reduced Ca^{2+} permeability (Sommer et al., 1991). Impairment of RNA editing in motor neurons of the anterior horn causes neurodegeneration and leads to development of amyotrophic lateral sclerosis (Kawahara et al., 2004; Yamashita and Kwak, 2014).

Accumbal RNA editing is involved in alcohol-drinking, cocaine-seeking and despair behaviors (Watanabe et al., 2014; Schmidt et al., 2015; Aoki et al., 2016). Chronic alcohol exposure causes enhanced 5-HT_{2C}R RNA editing following an increase in ADAR1 and ADAR2 expression in the nucleus accumbens (NAc) and dorsal raphe nucleus (DRN) of C57BL/6J mice (Watanabe et al., 2014). As a result of alteration in expression of 5-HT_{2C}R isoforms, voluntary alcohol intake is enhanced (Watanabe et al., 2014). Moreover, mice that exclusively express the unedited (INI) isoform of 5-HT_{2C}R mRNA on a C57BL/6J background exhibit a reduction in neuropeptide Y (NPY) expression in accumbal neurons and elevated despair behavior compared with wild-type mice (Aoki et al., 2016). In addition, GluA2 RNA editing at the Q/R site in the NAc shell is reduced by forced cocaine abstinence, and ADAR2 overexpression in the NAc shell attenuates cocaine-seeking behavior (Schmidt et al., 2015).

To investigate the roles of accumbal RNA editing in brain function, we produced NAc-specific conditional *ADAR2* knockout mice (*ADAR2*$^{flox/flox}$; Hideyama et al., 2010), and performed anxiety- and depression-like behavioral tests, home-cage activity test, and ethanol drinking and ethanol conditioned place preference (CPP) tests.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with international guidelines for the care and use of experimental animals. *ADAR2*$^{flox/flox}$ mice were maintained under a 12-h light-dark cycle (lights on from 08:00 to 20:00 h) with free access to water and food (Hideyama et al., 2010). Behavioral testing was performed between 9:00 and 18:00 h. After testing, the apparatus was cleaned with 70% ethanol to prevent bias caused by olfactory cues. All animal experiments, including production, maintenance protocols and behavioral studies, were reviewed and approved by the Animal Care and Use Committee of the Kyoto Prefectural University of Medicine (M26-250). Mice were anesthetized with a midazolam/medetomidine/butorphanol cocktail (4,0.3 and 5 mg/kg, respectively) during surgery. Prior to brain removal, all mice were euthanized with an excess of pentobarbital (50 mg/kg).

Generation of a Conditional ADAR2 Knockout Mouse Using AAV-GFP/Cre

Male *ADAR2*$^{flox/flox}$ mice with a C57BL/6J genetic background (10–13 weeks old) were anesthetized and mounted in a stereotaxic apparatus (Narishige, Tokyo, Japan). AAV-GFP/Cre (Addgene plasmid # 49056) was a gift from Dr. Fred Gage (Kaspar et al., 2002). pAAV-GFP control vector was purchased from Cell Biolabs (San Diego, CA, USA). For recombinant AAV (rAAV) production, the vectors were cotransfected into HEK293 cells with pAAV-DJ and pHelper (Cell Biolabs), according to the manufacturer's instructions. rAAV particles were purified by discontinuous step gradient using iodixanol (Opti-Prep; Nycomed Pharma, Oslo, Norway), as described previously (Aoki et al., 2016). Iodixanol fractions were concentrated and dialyzed by centrifugation through a Biomax 100K filter (Millipore, Billerica, MA, USA) with phosphate-buffered saline (PBS) containing 5% sorbitol. rAAV titers were determined by quantitative PCR and aliquoted rAAVs were stored at −80°C. AAV-GFP/Cre (1.63×10^{13} viral genome particles/ml) or AAV-GFP (2.2×10^{12} viral genome particles/mL) containing Fast Green dye (Nacalai Tesque, Kyoto, Japan) was bilaterally microinjected (1 μl/site) into the NAc (AP, +2 mm from Bregma; ML, ±0.8 mm from midline; DV, +4.3 mm below the skull surface) using a 30-gauge Hamilton syringe needle (Hamilton, Reno, NV, USA) at a rate of 0.2 μl/min. The needle was left in place for 12 min after each injection before slow retraction. GFP/Cre or GFP expression was allowed to develop for 4 weeks. After behavioral analysis, GFP/Cre and GFP gene expression were confirmed by immunohistochemical analysis using anti-GFP antibodies (MBL, Nagoya, Japan). Genomic DNA from the NAc was prepared using the NucleoSpin TriPrep kit (MACHEREY-NAGEL, Düren, Germany). Primers (5′-CTGGTTCATAACAGATCCTCAGGG-3′ and 5′-GTCTCCCTTGTCCTTCCAGGTAGC-3′) were used for genomic ADAR2 PCR (Hideyama et al., 2010).

Immunohistochemical Analysis

Under deep anesthesia, AAV-GFP/Cre- or AAV-GFP- injected mice (n = 5) were perfused *via* the left cardiac ventricle with 20 ml chilled 0.1 M PBS followed by 40 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed in the same fixative at 4°C overnight. After cryoprotection in 25% sucrose in 0.1 M PB for 48 h at 4°C, coronal sections (10-μm thick) were cut using a cryostat and mounted on coated glass slides (Fisherbrand Superfrost Plus; Fisher Scientific, Hampton, NH, USA). Sections were exposed to an antigen retrieval solution (Histo VT One; Nacalai Tesque, Kyoto, Japan) for 20 min at 70°C. After washing in PBS and incubating in blocking solution (Blocking One Histo; Nacalai Tesque, Kyoto, Japan), sections were incubated with mouse monoclonal anti-ADAR2 antibody (1:200; sc-73409, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and rabbit monoclonal anti-GFP antibody (1:2,000; MBL, Nagoya, Japan) for 24 h at 4°C. For detection of primary antibodies, Alexa 594-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit antibodies (1:500; Life Technologies, Carlsbad, CA, USA) were used. For NeuN detection, monoclonal anti-NeuN (1:200; MAB377, Merck, Burlington, MA, USA) and Alexa 594-conjugated goat anti-mouse (1:500; Life Technologies, Carlsbad, CA, USA) were used as primary and secondary

TABLE 1 | Primer sequences.

5-HT_{2C}R Fw	5′-ATTGCTGATATGCTGGTGGGACTAC-3′
5-HT_{2C}R Rv	5′-GCTTTCGTCCCTCAGTCCAATCACA-3′
CYFIP2 Fw	5′-AACTGGATGCCAAGAAGAGAATCAAC-3′
CYFIP2 Rv	5′-CAGCCCTGAGCCTGTCACCACCTCG-3′
GluR2 Fw	5′-AGCAGATTTAGCCCCTACGAG-3′
GluR2 Rv	5′-CAGCACTTTCGATGGGAGACAC-3′

antibodies, respectively. Sections were coverslipped and observed using an inverted fluorescence microscope (IX83 cellSense Dimension; Olympus, Tokyo, Japan) or inverted laser-scanning confocal microscope (LSM510 META; Carl Zeiss, Oberkochen, Germany). Images were captured as Z-stacks (8 z-sections, 0.5 μm apart).

Measurement of RNA Editing Efficiency

ADAR2$^{flox/flox}$ mice were injected with AAV-GFP (n = 10) or AAV-GFP/Cre (n = 10) into the NAc. Mouse brains were obtained 1 month later, and quickly frozen in liquid nitrogen. Coronal sections (15 μm thick) were cut using a cryostat and mounted onto membrane slides (PEN-membrane 2.0 μm; Leica Microsystems, Wetzlar, Germany). NAc samples from mounted coronal sections were obtained by 1-mm punch biopsy, followed by RT-PCR amplification using VolcanoCell2G 2 × RT-PCR Master Mix (myPLOS Biotec, Konstanz, Germany). PCR products were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and subjected to sequencing reaction (BigDye Terminator v1.1 Cycle Sequencing Kit, ThermoFisher, Waltham, MA, USA). Primer sequences are described in **Table 1**. Sequencing analysis was performed with an Applied Biosystems 3130 Genetic Analyzer (ThermoFisher, Waltham, MA, USA). DNA sequencing data were converted to waveform data, and the peaks of adenosine (A) and guanosine (G) were quantified using ImageJ software[1]. RNA editing frequency was calculated as A/(A + G) × 100 (%).

Behavioral Testing Experimental Design

ADAR2$^{flox/flox}$ mice were injected with AAV-GFP (n = 55) or AAV-GFP/Cre (n = 66) into the NAc. Mice were subjected to anxiety- and depression-like behavior tests and ethanol consumption tests. The order of each testing session was: (1) anhedonia test; (2) light/dark transition test; (3) open field test; (4) elevated plus maze (EPM); (5) forced swimming test; (6) tail suspension test (TST). After chronic ethanol exposure; (7) ethanol-drinking behavior test; and (8) CPP test were performed. A home-cage activity test was performed using an independent cohort of mice. The test battery was performed at 1–3 day inter-test intervals. We checked correct microinjection of AAV into the bilateral NAc after behavioral tests.

Anhedonia Test

Mice were allowed free access to water and food prior to the anhedonia test. During testing, mice were given free choice between two bottles, one with water and another with 1% sucrose solution, for 24 h (Shirahase et al., 2016). Mice were allowed free access to food during the test period. After 12 h, the positions of the bottles were switched to exclude possible effects of side preference in drinking behavior. The consumption (ml) of sucrose solution and water were estimated after 24 h. Sucrose consumption was calculated as sucrose intake (g) per sucrose volume (ml) per body weight (kg). Water intake and total intake were calculated as the volume of fluid intake (ml) per body weight (kg).

Light/Dark Transition Test

The light/dark transition test was performed as previously described (Aoki et al., 2016). The apparatus used for the light/dark transition test comprised a cage (14 × 14 cm) divided into two sections of equal size by a partition with a door (Melquest Co., Ltd, Toyama, Japan). One chamber was brightly illuminated (400 lux), whereas the other chamber was dark (2 lux). Mice were placed into the dark side and allowed to move freely between the two chambers with the door open for 10 min. The total number of transitions, time spent in each compartment, and latency of first movement to the light side were automatically recorded using an animal movement analysis system (SCANET-40; Melquest Co., Ltd).

Open Field Test

Each subject was allowed to move freely in the open field box (60 × 60 cm) for 30 min. Time spent in the center area of the open field (30 × 30 cm) was measured using SMART v3.0 software (Panlab SL, Barcelona, Spain).

Elevated Plus Maze (EPM)

The EPM test was performed as described previously (Aoki et al., 2016). The EPM consisted of two open arms (30 × 5 cm) and two enclosed arms of the same size, with 13-cm-high transparent walls. The arms and central square were constructed from gray plastic sheets elevated to a height of 50 cm above the floor. To minimize the likelihood of animals falling from the apparatus, 5-mm-high Plexiglas sides were used for the open arms. Arms of the same type were arranged at opposite sides to each other. The device was set up under low illumination (center square, 300 lux). Each mouse was placed in a closed arm of the maze. Mouse behavior was recorded during a 10-min test period. The number of entries into and the time spent on open arms were recorded. For data analysis, the following two measures were used: percentage of entries into open arms and duration of stay on open arms. Data acquisition and analysis were performed using SMART v3.0 software.

Forced Swim Test (FST)

The FST apparatus consisted of four Plexiglas cylinders. The cylinders were filled with water at a temperature of 23°C up to a height of 10 cm. Mice were placed into the cylinders and images recorded over a 10-min period. Data acquisition and analysis were performed using SMART v3.0 software. Immobility time was measured during 2–6 min of the test period (Aoki et al., 2016).

[1]http://rsb.info.nih.gov/ij

Tail Suspension Test (TST)

The TST was performed according to a previously described procedure (Watanabe et al., 2011). Mice were suspended 30 cm above the floor in a visually isolated area using adhesive tape placed 1 cm from the tip of the tail. Immobility duration was recorded over a 10-min test period. Images were captured using a USB camera (Webcam C270; Logicool, Tokyo, Japan). Data acquisition and analysis were performed using SMART v3.0 software.

Home-Cage Activity Test

Mice were placed in a home cage (22 × 22 × 18.5 cm) for 7 days prior to the home-cage activity test. Home-cage activity was recoded for 3 days using an animal movement analysis system (SCANET-40). Mice were maintained under a 12-h light/dark cycle (lights on from 08:00 to 20:00 h) with free access to water and food.

Chronic Ethanol Vapor Exposure

Chronically ethanol-exposed mice were produced as previously described (Yoshimoto et al., 2012). Briefly, mice were exposed to 22–27 mg/l of ethanol vapor during dark phase for 20 days using an intermittent 3–6 h/day schedule that mimics cyclical patterns of ethanol consumption. Blood ethanol concentration of mice was not measured. Mice were maintained in the ethanol vapor chamber under a 12-h light/dark cycle (lights on from 08:00 to 20:00 h) with free access to water and food.

Ethanol-Drinking Behavior Test

After chronic ethanol exposure, mice were withdrawn from ethanol and maintained under normal experimental conditions for 4 h. For the ethanol-drinking behavior test, mice were provided with 10% (v/v) ethanol solution for 4 h, and their consumption was measured (Watanabe et al., 2014). The total amount of ethanol intake was represented as ethanol (g)/body weight (kg).

Ethanol CPP Test

The CPP apparatus was divided into two sections of equal size (14 × 14 cm) by a partition with a door (Melquest Co. Ltd, Toyama, Japan). One box was painted black with a smooth floor and the other was painted white with a textured floor. The CPP test was performed 1–2 days after the ethanol drinking behavior test according to a previously described procedure (Thanos et al., 2005). All mice were allowed to recover under normal conditions for 3–5 days before the conditioning phase. In the preconditioning phase (days 1–3), each mouse was placed in the CPP apparatus for 30 min. Data from the 3rd day was analyzed for any unconditioned box preference. In the conditioning phase (days 4–11), mice were given saline in the black box on days 4, 6, 8, and 10. On days 5, 7, 9, and 11, the same mice were given ethanol (2 g/kg i.p. 20% (v/v) EtOH in saline) in the opposite chamber (for 30 min/trial). In the test phase (day 12), mice were placed in the apparatus and allowed free access to all boxes for 30 min. Each test session (days 3 and 12) was automatically recorded using an animal movement analysis system (SCANET-40).

Statistical Analysis

Statistical analysis was performed using Stat View (SAS Institute, Cary, NC, USA). Significance of differences between two groups was calculated using Student's *t*-tests. Ethanol-drinking behavior and CPP test data were analyzed by two-way analyses of variance (ANOVA; Bonferroni's *post hoc* test). The alpha level was set to 0.05. Results of statistical analysis of RNA editing efficiency and behavioral tests were summarized in **Table 2**.

RESULTS

Conditional Knockout of ADAR2 in the NAc Using AAV-GFP/Cre

To generate NAc-specific *ADAR2* knockout mice with a C57BL/6J genetic background, AAV-GFP/Cre was injected into the NAc (core and shell) of $ADAR2^{flox/flox}$ mice (**Figure 1A**). Injection into the NAc showed about 500 μm diameter spread of AAV. Genomic PCR analysis showed that the recombination of $ADAR2^{flox/flox}$ gene by Cre recombinase occurred in the NAc of AAV-GFP/Cre-injected mice (**Figure 1B**). In immunohistochemical analysis, ADAR2 expression was not detected in AAV-infected GFP/Cre-positive neurons in the NAc, whereas both ADAR2 and GFP expression were observed in the control AAV-GFP-infected neurons (**Figure 1C**). The loss of ADAR2 expression in AAV-GFP/Cre-injected NAc was not due to cell death, because numbers of NeuN-positive cells were not reduced and both NeuN- and GFP-positive neurons were observed in control AAV-GFP-injected NAc (**Figure 2**). Four weeks after the injection, we examined the influence of *ADAR2*-knockout on RNA editing. Sections from the NAc were lysed, and cDNAs were synthesized. The RNA editing frequency of ADAR2-dependent RNA editing sites, GluA2 Q/R, 5-HT_{2C}R site D and CYFIP2 K/E, was measured by direct sequencing analysis of cDNAs. Compared with that of AAV-GFP-injected mice, the RNA editing frequency of the three editing sites was significantly lower in the NAc of NAc-specific *ADAR2* knockout mice (**Figures 3A,B**). The editing frequency of the ADAR2-mediated RNA editing sites 5-HT_{2C}R site D and CYFIP2 K/E site was reduced from 60% to 70% in controls to approximately 20% in *ADAR2* knockout mice. Editing frequency of the GluA2 Q/R site was also lower in *ADAR2* knockout mice (60%, compared with 100% in control mice). In contrast, the RNA editing frequency of all sites in the cortex was normal (**Figures 3A,B**). The body weight of control (GFP) and NAc-specific *ADAR2* knockout mice (Cre) was measured 4 weeks after AAV injection. All mice appeared to be healthy, and mean body weight was not significantly different between the groups (**Figure 3C**).

Analysis of Anxiety-Related Behaviors of NAc-Specific ADAR2 Knockout Mice

We characterized the behavioral phenotypes of NAc-specific *ADAR2* knockout mice 4 weeks after the AAV injection. Mice were subjected to a light/dark transition test, open field test and EPM test as analyses of anxiety-related behaviors. In

TABLE 2 | Summary of statistical analysis.

Tests and factors	*F* and *p* values	Statistical analysis
Ratio of RNA editing		
5-HT_{2C}R Site D NAc	$^{*}p = 0.0006$	*t*-test
5-HT_{2C}R Site D Cortex	$p = 0.8137$	*t*-test
CYFIP2 K/E NAc	$^{*}p = 0.00000006$	*t*-test
CYFIP2 K/E Cortex	$p = 0.6301$	*t*-test
GluA2 Q/R NAc	$^{*}p = 0.00001$	*t*-test
GluA2 Q/R Cortex	$p = 0.4893$	*t*-test
Body weight	$p = 0.7417$	*t*-test
Light/Dark test		
Transitions	$^{*}p = 0.0069$	*t*-test
Stay time in light	$p = 0.1986$	*t*-test
Latency to light	$p = 0.131$	*t*-test
Open field test		
Center time (s)	$p = 0.3882$	*t*-test
Center time (%)	$p = 0.4949$	*t*-test
Total distance	$^{*}p = 0.0058$	*t*-test
Elevated plus maze		
Number of entries	$p = 0.2828$	*t*-test
Entries into open arms	$p = 0.9678$	*t*-test
Time on open arms	$p = 0.7682$	*t*-test
Total distance	$p = 0.5778$	*t*-test
Spontaneous locomotor activity		
Light	$p = 0.6986$	*t*-test
Dark	$p = 0.1748$	*t*-test
Forced swim test	$p = 0.1571$	*t*-test
Tail suspension test	$p = 0.2692$	*t*-test
Sucrose test		
1% Sucrose consumption	$p = 0.2236$	*t*-test
Water	$p = 0.9768$	*t*-test
Total intake	$p = 0.2113$	*t*-test
Ethanol drinking test		
Ethanol vapor	$F_{(1,35)} = 6.687, p = 0.14$	ANOVA
Genotype	$F_{(1,35)} = 0.725, p = 0.4004$	ANOVA
Ethanol vapor × Genotype	$F_{(1,35)} = 4.911, ^{**}p = 0.0333$	ANOVA
Post hoc (Control vs. Ethanol vapor)		
GFP	$^{*}p = 0.0009$	Bonferroni
GFP/Cre	$p = 0.8068$	Bonferroni
CPP test (Control)		
Conditioning	$F_{(1,40)} = 0.734, p = 0.3968$	ANOVA
Genotype	$F_{(1,40)} = 0.11, p = 0.7423$	ANOVA
Conditioning × Genotype	$F_{(1,40)} = 0.18, p = 0.6736$	ANOVA
Post hoc (Control vs. Ethanol vapor)		
GFP	$p = 0.3478$	Bonferroni
GFP/Cre	$p = 0.7713$	Bonferroni
CPP test (Ethanol vapor)		
Conditioning	$F_{(1,34)} = 7.194, ^{**}p = 0.0112$	ANOVA
Genotype	$F_{(1,34)} = 0.042, p = 0.8392$	ANOVA
Conditioning × Genotype	$F_{(1,34)} = 0.63, p = 0.4328$	ANOVA
Post hoc (Control vs. Ethanol vapor)		
GFP	$^{**}p = 0.0222$	Bonferroni
GFP/Cre	$p = 0.1791$	Bonferroni

$^{*}p < 0.01$, $^{**}p < 0.05$.

the light/dark transition test, the total number of transitions between the light and dark chambers was significantly lower in NAc-specific *ADAR2* knockout mice ($p = 0.0069$; **Figure 4A**), while there were no significant differences in the stay time in the light chamber or latency to first enter the light chamber (**Figures 4B,C**). In the open field test, no significant difference between the two groups was observed in the time spent in the central area (**Figures 4D,E**). However, the distance traveled by NAc-specific *ADAR2* knockout mice was significantly lower than that of control mice ($p = 0.0058$; **Figure 4F**). In the EPM test, major differences were not seen in number and percentage of entries into the open arms, percentage of stay time in the open arm or the distance traveled (**Figures 4G–J**). A diurnal rhythm of spontaneous locomotor activity was recorded for 3 days. Both NAc-specific *ADAR2* knockout mice and control mice showed normal rhythms and similar gross levels of spontaneous locomotor activity (**Figure 5**).

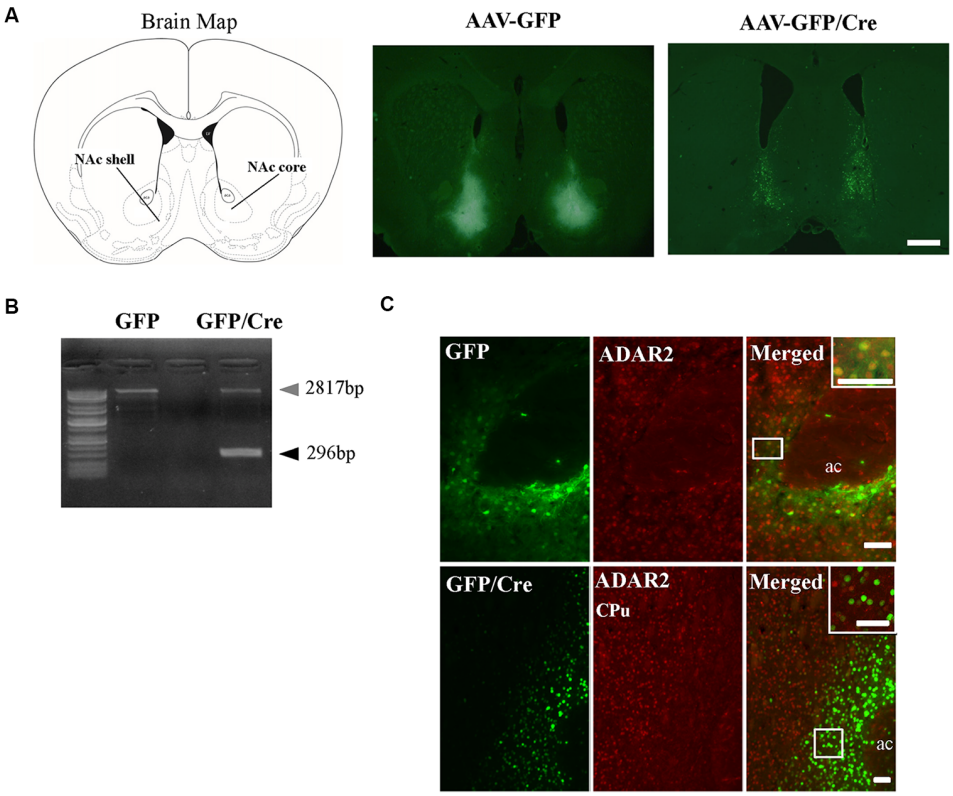


FIGURE 1 | Nucleus accumbens (NAc)-specific *ADAR2* knockout mice. **(A)** AAV-GFP or AAV-GFP/Cre was stereotaxically injected into the NAc of *ADAR2*$^{flox/flox}$ mice, based on the coordinates of Paxinos and Franklin (Paxinos and Franklin, 2004), and NAc sections were examined for GFP or GFP/Cre expression using anti-GFP antibody. Scale bar: 500 μm. **(B)** Knockout of the double-stranded RNA-specific adenosine deaminase *ADAR2* was validated by a genomic PCR assay. *ADAR2* was deleted by Cre-lox recombination (296 bp). **(C)** Ten micrometer coronal brain sections from *ADAR2*$^{flox/flox}$ mice injected with AAV-GFP (upper) or AAV-GFP/Cre (lower) into the NAc were immunohistochemically examined using anti-GFP (green) and anti-ADAR2 (red) antibodies. ac, anterior commissure; CPu, caudate putamen. Scale bars: 50 μm.

Analysis of Despair- and Anhedonia-Related Behaviors of NAc-Specific ADAR2 Knockout Mice

Next, we examined despair-related behaviors using the FST and TST. The results of the FST revealed no significant difference in immobility time between control and NAc-specific *ADAR2* knockout mice (**Figure 6A**). Similarly, there was no significant difference in immobility time in the TST (**Figure 6B**). Mice were subjected to the sucrose preference test as a measure of anhedonia-related behavior. Mice had the choice of either drinking 1% sucrose solution or water for 24 h. There were no differences in the consumption of sucrose solution or water between the two groups (**Figures 6C–E**). These results showed that despair- and anhedonia-related phenotypes of NAc-specific *ADAR2* knockout mice were normal.

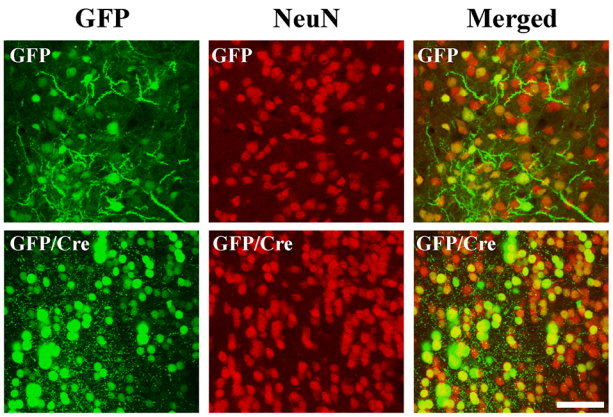


FIGURE 2 | Survival of ADAR2-knockout neurons in the NAc. AAV-GFP or AAV-GFP/Cre was stereotaxically injected into the NAc of *ADAR2*$^{flox/flox}$ mice. Coronal brain sections in the NAc were immunohistochemically examined using anti-GFP (green) and anti-NeuN (red) antibodies. Scale bars: 50 μm.

Ethanol-Drinking Behavior and Ethanol CPP Tests

GFP and Cre/GFP mice were exposed to ethanol vapor for 20 days prior to ethanol-drinking behavior and ethanol preference tests. Chronic ethanol vapor exposure significantly increased 10% ethanol intake in GFP mice (genotype × treatment: $F_{(1,35)} = 4.911$, $p = 0.0333$, two-way

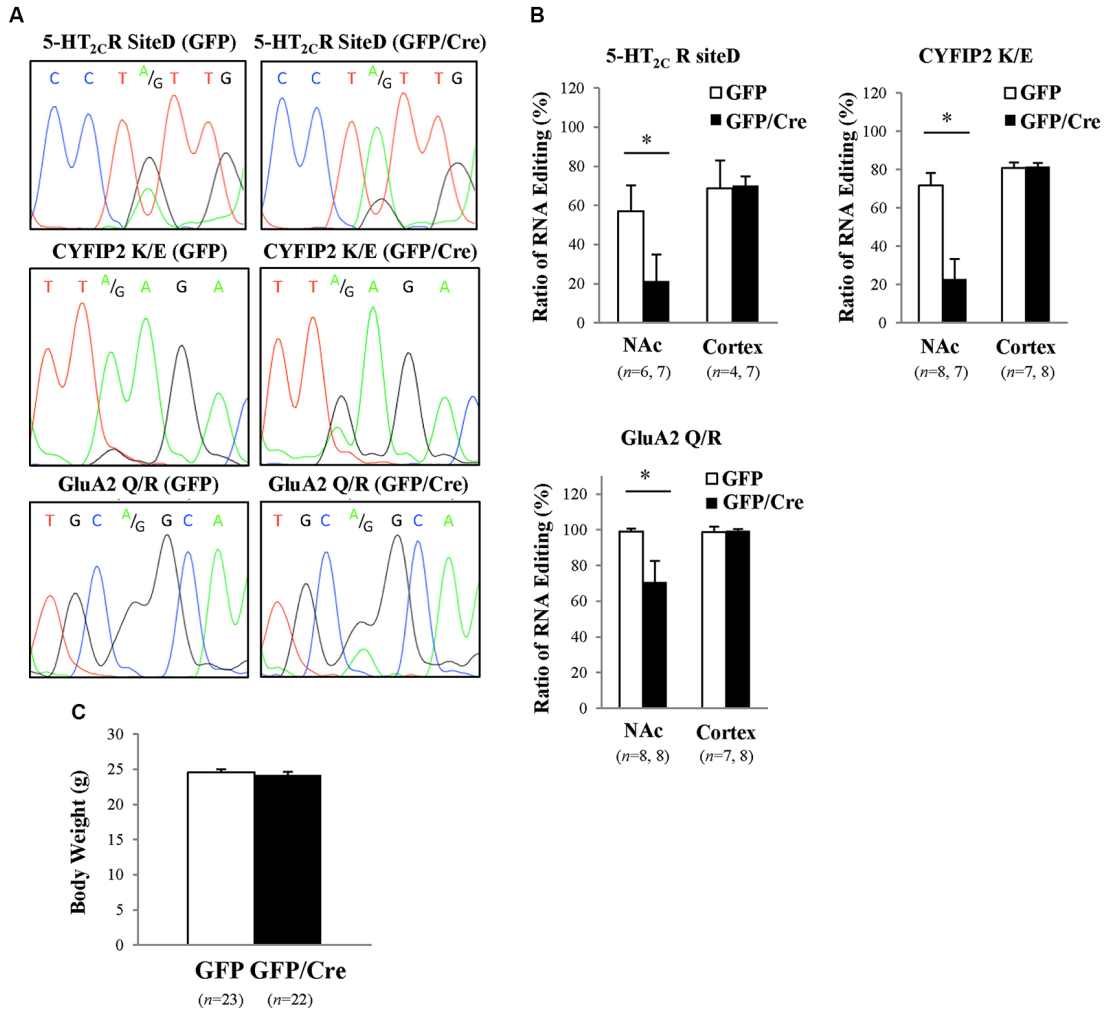


FIGURE 3 | Reduced RNA editing in the NAc of *ADAR2* knockout mice. **(A)** Suppression of ADAR2-mediated RNA editing; 5-HT$_{2C}$R site D (upper), CYFIP2 K/E site (middle), and GluA2 Q/R site (lower). Total RNA was prepared from the NAc of AAV-GFP- or AAV-GFP/Cre-injected mice. cDNAs were synthesized from total RNA, followed by direct sequencing analysis. Editing sites were indicated by A/G in waveform data. **(B)** Editing frequency was calculated based on sequence trace data. Samples were prepared from the NAc and prefrontal cortex of AAV-GFP- (open squares) or AAV-GFP/Cre- (closed squares) injected mice. *$p < 0.01$. **(C)** Body weights of AAV-GFP- or AAV-GFP/Cre-injected mice 4 weeks after AAV injection.

ANOVA; GFP-control vs. GFP-ethanol vapor: $p = 0.0009$, Bonferroni's *post hoc* test), while Cre/GFP mice did not show enhanced ethanol intake (**Figure 7A**). This result suggests that ADAR2-mediated RNA editing in the NAc is involved in ethanol-drinking behavior. Following the ethanol-drinking behavior test, ethanol preference was measured in these mice using the ethanol CPP test. In control mice, there was no significant difference in CPP score between GFP and Cre/GFP mice, and the mice in both cases did not develop ethanol CPP (**Figure 7B**). In contrast, there was a significant difference in the CPP scores of GFP mice after chronic ethanol exposure between the pre-conditioning and conditioning phases (**Figure 7C**; ethanol-conditioned: $F_{(1,34)} = 7.194$, $p = 0.0112$, two-way ANOVA; $p = 0.0222$ *post hoc* test), whereas there was no significant difference in the CPP scores of Cre/GFP mice after chronic ethanol exposure between the pre-conditioning and conditioning phases (**Figure 7C**). These results indicate that ADAR2-mediated RNA editing in the NAc is involved in the enhancement of ethanol preference induced by chronic ethanol exposure.

DISCUSSION

In the present study, we demonstrated that ADAR2-mediated RNA editing in the NAc underlies enhanced ethanol consumption and preference after chronic ethanol exposure. ADAR2 mediates RNA editing of various ion channels and receptors such as $Ca_V1.3$ calcium ion channel, $K_V1.1$ potassium ion channel, 5-HT$_{2C}$R, GluA2, and GABA$_A$ (Bhalla et al., 2004; Bazzazi et al., 2013; Behm and Öhman, 2016). We previously reported that 5-HT$_{2C}$R RNA editing is involved in voluntary alcohol consumption after chronic ethanol

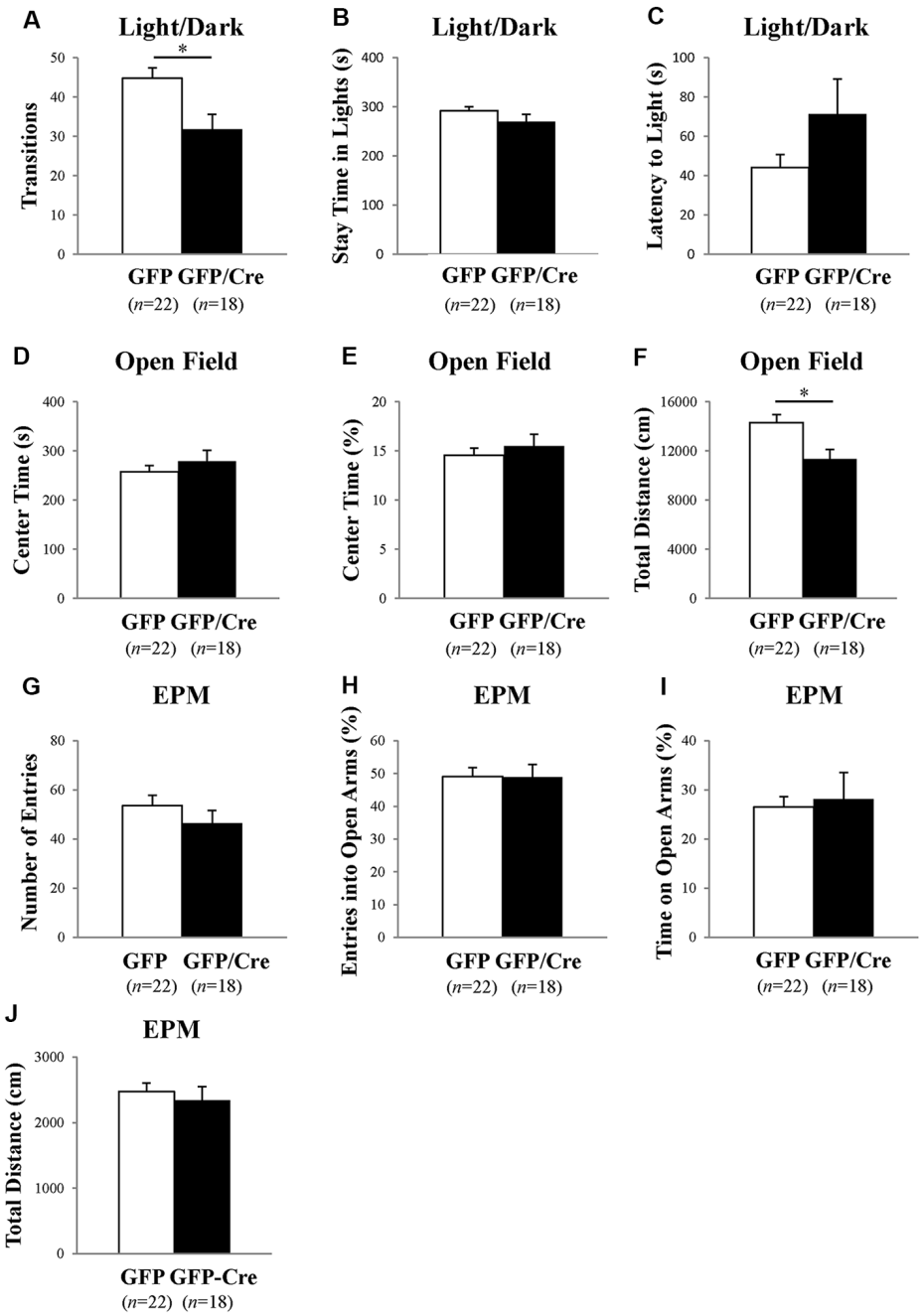


FIGURE 4 | Analysis of anxiety-like behaviors and locomotion in NAc-specific *ADAR2* knockout mice. AAV-GFP- (open squares) or AAV-GFP/Cre- (closed squares) injected mice were subjected to light/dark test **(A–C)**, open field test **(D,E)**, and elevated plus maze (EPM) test **(G–J)**. In the light/dark test, the number of light/dark transitions **(A)**, time spent in light compartment **(B)** and latency to enter the light compartment **(C)** were recorded. In the open field test, time spent in the center of the compartment **(D)** and total locomotor distance traveled **(E)** were recorded. In the EPM test, the number of arm entries **(F)**, percentage of entry into open arms **(G)**, time spent in open arms **(H)** and distance traveled **(I)** were recorded. Sample numbers are indicated in parentheses. Data are presented as mean ± SEM. *P* values were calculated using Student's *t*-test. $^{*}p < 0.01$.

vapor exposure (Watanabe et al., 2014). In C57BL/6J mice, chronic ethanol vapor exposure causes elevated 5-HT_{2C}R RNA editing in the NAc and DRN by upregulation of ADAR1 and ADAR2 expression. On the other hand, INI mice, in which 5-HT_{2C}R RNA editing was blocked by the deletion of the editing complementary sequence in intron 5 (160 bp), showed no enhanced ethanol consumption (Watanabe et al., 2014). In light of previous findings, our results may imply that a reduction in 5-HT_{2C}R RNA editing causes suppression of enhanced ethanol consumption and ethanol preference after

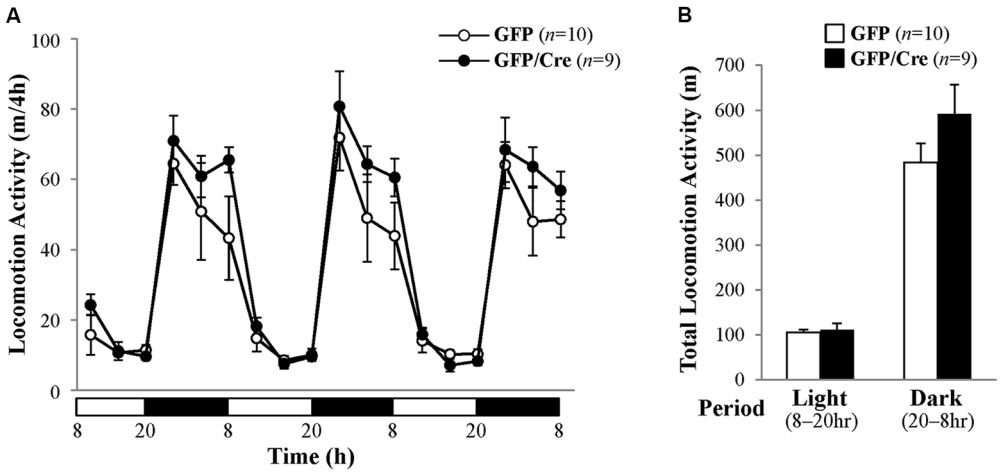


FIGURE 5 | Analysis of spontaneous locomotor activity. **(A)** Spontaneous locomotor activity of AAV-GFP- (n = 10) or AAV-GFP/Cre- (n = 9) injected mice was recorded for 3 days under 12 h light/dark cycle after familiarization to the new cage environment. Each point represents the 4-h average of locomotor activity. The day was divided into a 12-h light period (white bars) and a 12-h dark period (black bars). **(B)** Total locomotor activity for 3 days is presented as mean ± SEM. *P* values were calculated using Student's *t*-test.

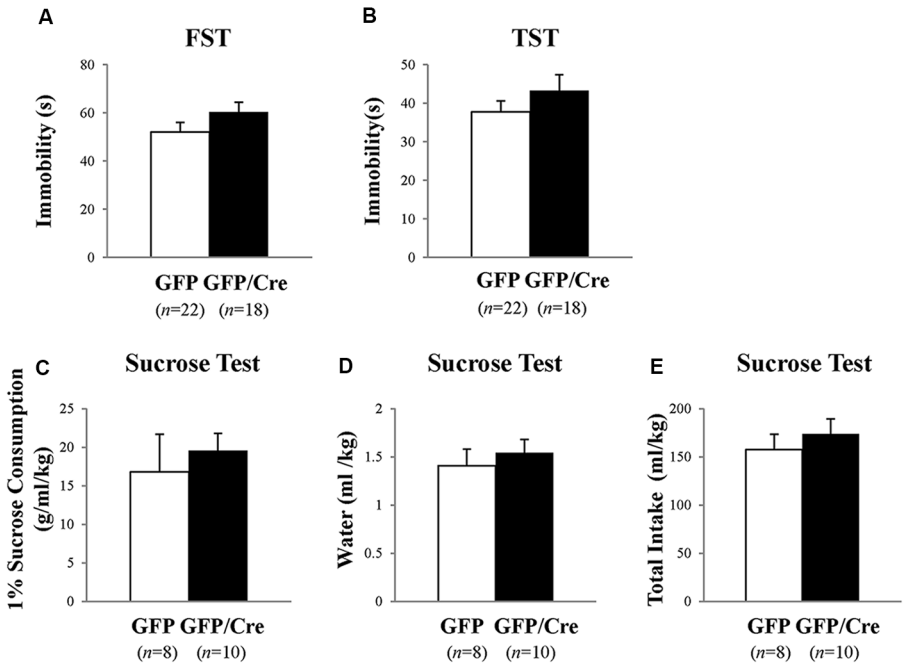


FIGURE 6 | Analysis of despair-like behaviors and anhedonia test. AAV-GFP- (open squares) or AAV-GFP/Cre- (closed squares) injected mice were subjected to forced swimming test (FST; **A**), tail suspension test (TST; **B**) and anhedonia test **(C–E)**. Immobility time was recorded **(A,B)**. Sucrose preference was measured as an indicator of anhedonia. Sucrose consumption **(C)**, water consumption **(D)** and total intake **(E)** were recorded for 24 h. Sample numbers are indicated in parentheses. Data are presented as mean ± SEM. *P* values were calculated using Student's *t*-test.

chronic ethanol vapor exposure in NAc-specific *ADAR2* knockout mice. Besides 5-HT_{2C}R RNA editing, RNA editing of other receptors and ion channels may be also involved in alcohol-related behaviors. For example, activation of $GABA_A$ receptor by RNA editing may enhance ethanol consumption and preference. An increase in GABAergic terminal density was found in the NAc of ethanol-preferring rats and high-ethanol-drinking rats (Hwang et al., 1990). The RNA-edited isoform of the $GABA_A$ α3 subunit exhibits greater GABA sensitivity and slower decay (Nimmich et al., 2009), suggesting that a reduction in $GABA_A$ α3 RNA editing may also cause suppression of enhanced ethanol consumption and ethanol

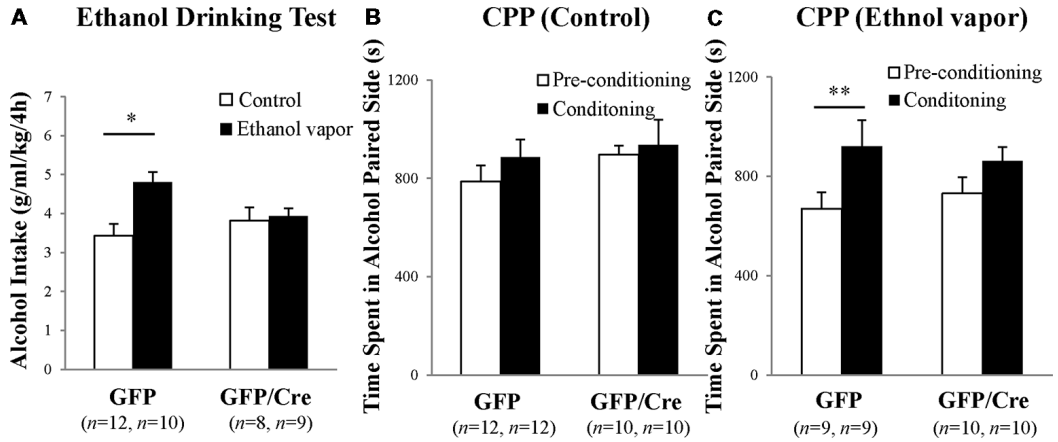


FIGURE 7 | Ethanol-drinking behavior and ethanol preference. AAV-GFP- (open squares) or AAV-GFP/Cre- (closed squares) injected mice were exposed to ambient air (Control) or ethanol vapor (EtOH) for 20 days. Ten percentage ethanol consumption within a 4-h period was recorded **(A)**. Data are presented as mean ± SEM. Statistical analyses were performed using two-way analyses of variance (ANOVA), followed by Bonferroni's *post hoc* test. Subsequently, these mice were subjected to the ethanol-induced conditioned place preference (CPP) test 1–2 days after the ethanol drinking test **(B,C)**. Time spent in the ethanol-paired side was measured in the pre-conditioning (open squares) and post-conditioning (closed squares) phase. Sample numbers are indicated in parentheses. Data are presented as mean ± SEM. Statistical analyses were performed using two-way ANOVA, followed by Bonferroni's *post hoc* test. $^{*}p < 0.01$ and $^{**}p < 0.05$.

preference in NAc-specific *ADAR2* knockout mice. Moreover, GluA2 RNA editing in the NAc may play a role in ethanol drinking behavior and ethanol preference. ADAR2-mediated RNA editing in the NAc is also involved in cocaine-seeking behavior. In contrast to ethanol preference, however, ADAR2-mediated RNA editing negatively affects cocaine seeking. Cocaine abstinence decreases ADAR2 expression in the NAc, resulting in reduction of GluA2 Q/R site editing, and unedited GluA2 promotes cocaine seeking (Schmidt et al., 2015). Furthermore, overexpression of ADAR2 in the NAc shell attenuates cocaine priming-induced reinstatement (Schmidt et al., 2015). The apparent discrepancy between the involvement of RNA editing in different types of addiction might be attributed to the distinct neuronal circuits and RNA-edited receptors underlying addiction to alcohol and cocaine. Collectively, the phenotype of accumbal *ADAR2* knockout mice in ethanol drinking and preference may be the total outcome of changes in multiple genes influenced by RNA editing, including 5-HT_{2C}R.

Neuropharmacological studies have shown that multiple 5-HT receptor subtypes, such as 5-$HT_{1A/1B}$R, 5-$HT_{2A/2C}$R, 5-HT_3R, and 5-HT_7R, are involved in the development of alcohol dependence (Sari et al., 2011; Yoshimoto et al., 2012; Hauser et al., 2015). Serotonergic system draws attention as a therapeutic target for alcohol dependence. Previously, we reported that wild type mice with a C57BL/6J genetic background predominantly express edited VXV isoforms of 5-HT_{2C}R (59%) in the NAc, including VNV, VGV and VSV isoforms (Watanabe et al., 2014). In the present study, the frequency of 5-HT_{2C}R RNA editing at site D was reduced to 20% in the NAc of *ADAR2* knockout mice (**Figure 3B**), meaning there was a reduction of expression of VXV isoforms. Compared with 5-HT_{2C}R VXV isoforms, other isoforms such as VNI and VGI, which are generated by blocking ADAR2-mediated RNA editing, have higher receptor activity (Burns et al., 1997; Niswender et al., 1999; Watanabe et al., 2014). 5-HT_{2C}R activity negatively modulates mesoaccumbens dopamine neurons through inhibition of dopamine release (De Deurwaerdère et al., 2004; Di Matteo et al., 2004). Dopaminergic dysfunction in the mesoaccumbens caused by long-term alcohol intake is known to be involved in alcoholism (Heinz, 2002). Thus, a reduction of accumbal dopamine signaling caused by enhanced RNA editing of 5-HT_{2C}R might contribute to an increase in ethanol drinking and preference after chronic ethanol exposure. Indeed, alcohol consumption influences levels of neurotransmitters, such as dopamine and 5-HT (Yoshimoto et al., 1996; Tattoli et al., 2001). The release of them in the NAc is altered during chronic consumption and withdrawal of alcohol, underlying the development of alcohol use disorder (Banerjee, 2014). Thus, the dopamine and 5-HT pathways might be influenced by the alteration of 5-HT_{2C}R RNA editing by chronic ethanol exposure, resulting in enhanced alcohol consumption.

It is thought that RNA editing is involved in many psychiatric disorders, including mood disorder and major depression (Tariq and Jantsch, 2012; Weissmann et al., 2016). Heterozygous *ADAR2* knockout mice showed a tendency of resistance to behavioral despair (Kubota-Sakashita et al., 2014). While we have previously reported that INI mice show a despair phenotype associated with accumbal NPY expression (Mombereau et al., 2010; Aoki et al., 2016), in this study, we did not observe this phenotype in NAc-specific *ADAR2* knockout mice (**Figure 6A**). A possible explanation for this difference is that the despair phenotype might be masked by the reduction of RNA editing of other gene transcripts such as AMPA receptors. Alternatively, further reduction of 5-HT_{2C}R RNA editing might be required to display severe phenotype since other editing sites of 5-HT_{2C}R which are mediated by ADAR1 is normal in NAc-specific *ADAR2* knockout mice.

In the present study, there were no significant difference in CPP scores between pre-conditioning and conditioning of control mice injected with GFP (**Figure 7B**). This might be due to the influence of the ethanol drinking test prior to the CPP test. All mice used in the CPP test had an experience of ethanol drinking for a short time. In further study, the will be performed using an independent cohort of mice.

In conclusion, in the present study, using NAc-specific *ADAR2* knockout mice, we have demonstrated that RNA editing in the NAc is involved in ethanol preference. Further study of the relationship between RNA editing and alcohol use disorder in humans will provide more insights, and could lead to a novel treatment for alcohol use disorder in the future.

AUTHOR CONTRIBUTIONS

MT and YW designed the experiments. TS and YW performed behavior tests, sequencing analysis, and statistical analysis. AT performed AAV production. MT and TS performed immunohistochemistry. SK provided advice for transgenic mice and sequencing analysis. TS, YW, and MT wrote the manuscript. TS, YW, SK, TY, NK, and MT discussed the results and commented on the manuscript.

FUNDING

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (grant numbers 25290014, 17H03553) to MT.

ACKNOWLEDGMENTS

We thank Dr. Miku Aoki for the help in behavioral tests. We also thank Melony Black, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

REFERENCES

Aoki, M., Watanabe, Y., Yoshimoto, K., Tsujimura, A., Yamamoto, T., Kanamura, N., et al. (2016). Involvement of serotonin 2C receptor RNA editing in accumbal neuropeptide Y expression and behavioural despair. *Eur. J. Neurosci.* 43, 1219–1228. doi: 10.1111/ejn.13233

Banerjee, N. (2014). Neurotransmitters in alcoholism: a review of neurobiological and genetic studies. *Indian J. Hum. Genet.* 20, 20–31. doi: 10.4103/0971-6866.132750

Bazzazi, H., Ben Johny, M., Adams, P. J., Soong, T. W., and Yue, D. T. (2013). Continuously tunable Ca(2+) regulation of RNA-edited CaV1.3 channels. *Cell Rep.* 5, 367–377. doi: 10.1016/j.celrep.2013.09.006

Behm, M., and Öhman, M. (2016). RNA editing: a contributor to neuronal dynamics in the mammalian brain. *Trends Genet.* 32, 165–175. doi: 10.1016/j.tig.2015.12.005

Bhalla, T., Rosenthal, J. J., Holmgren, M., and Reenan, R. (2004). Control of human potassium channel inactivation by editing of a small mRNA hairpin. *Nat. Struct. Mol. Biol.* 11, 950–956. doi: 10.1038/nsmb825

Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E., et al. (1997). Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature* 387, 303–308. doi: 10.1038/387303a0

De Deurwaerdère, P., Navailles, S., Berg, K. A., Clarke, W. P., and Spampinato, U. (2004). Constitutive activity of the serotonin2C receptor inhibits *in vivo* dopamine release in the rat striatum and nucleus accumbens. *J. Neurosci.* 24, 3235–3241. doi: 10.1523/JNEUROSCI.0112-04.2004

Di Matteo, V., Pierucci, M., and Esposito, E. (2004). Selective stimulation of serotonin2c receptors blocks the enhancement of striatal and accumbal dopamine release induced by nicotine administration. *J. Neurochem.* 89, 418–429. doi: 10.1111/j.1471-4159.2004.02337.x

Fitzgerald, L. W., Iyer, G., Conklin, D. S., Krause, C. M., Marshall, A., Patterson, J. P., et al. (1999). Messenger RNA editing of the human serotonin 5-HT_{2C} receptor. *Neuropsychopharmacology* 21, 82S–90S. doi: 10.1016/S0893-133X(99)00004-4

Gurevich, I., Englander, M. T., Adlersberg, M., Siegal, N. B., and Schmauss, C. (2002). Modulation of serotonin 2C receptor editing by sustained changes in serotonergic neurotransmission. *J. Neurosci.* 22, 10529–10532. doi: 10.1523/jneurosci.22-24-10529.2002

Hauser, S. R., Hedlund, P. B., Roberts, A. J., Sari, Y., Bell, R. L., and Engleman, E. A. (2015). The 5-HT7 receptor as a potential target for treating drug and alcohol abuse. *Front. Neurosci.* 8:448. doi: 10.3389/fnins.2014.00448

Heinz, A. (2002). Dopaminergic dysfunction in alcoholism and schizophrenia—psychopathological and behavioral correlates. *Eur. Psychiatry* 17, 9–16. doi: 10.1016/S0924-9338(02)00628-4

Hideyama, T., Yamashita, T., Suzuki, T., Tsuji, S., Higuchi, M., Seeburg, P. H., et al. (2010). Induced loss of ADAR2 engenders slow death of motor neurons from Q/R site-unedited GluR2. *J. Neurosci.* 30, 11917–11925. doi: 10.1523/JNEUROSCI.2021-10.2010

Hwang, B. H., Lumeng, L., Wu, J. Y., and Li, T. K. (1990). Increased number of GABAergic terminals in the nucleus accumbens is associated with alcohol preference in rats. *Alcohol. Clin. Exp. Res.* 14, 503–507. doi: 10.1111/j.1530-0277.1990.tb01188.x

Kaspar, B. K., Vissel, B., Bengoechea, T., Crone, S., Randolph-Moore, L., Muller, R., et al. (2002). Adeno-associated virus effectively mediates conditional gene modification in the brain. *Proc. Natl. Acad. Sci. U S A* 99, 2320–2325. doi: 10.1073/pnas.042678699

Kawahara, Y., Grimberg, A., Teegarden, S., Mombereau, C., Liu, S., Bale, T. L., et al. (2008). Dysregulated editing of serotonin 2C receptor mRNAs results in energy dissipation and loss of fat mass. *J. Neurosci.* 28, 12834–12844. doi: 10.1523/JNEUROSCI.3896-08.2008

Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., and Kwak, S. (2004). Glutamate receptors: RNA editing and death of motor neurons. *Nature* 427:801. doi: 10.1038/427801a

Kubota-Sakashita, M., Iwamoto, K., Bundo, M., and Kato, T. (2014). A role of ADAR2 and RNA editing of glutamate receptors in mood disorders and schizophrenia. *Mol. Brain* 7:5. doi: 10.1186/1756-6606-7-5

Martin, C. B., Ramond, F., Farrington, D. T., Aguiar, A. S. Jr., Chevarin, C., Berthiau, A. S., et al. (2013). RNA splicing and editing modulation of 5-HT_{2C} receptor function: relevance to anxiety and aggression in VGV mice. *Mol. Psychiatry* 18, 656–665. doi: 10.1038/mp.2012.171

Mombereau, C., Kawahara, Y., Gundersen, B. B., Nishikura, K., and Blendy, J. A. (2010). Functional relevance of serotonin 2C receptor mRNA editing in antidepressant- and anxiety-like behaviors. *Neuropharmacology* 59, 468–473. doi: 10.1016/j.neuropharm.2010.06.009

Nimmich, M. L., Heidelberg, L. S., and Fisher, J. L. (2009). RNA editing of the $GABA_A$ receptor α3 subunit alters the functional properties of recombinant receptors. *Neurosci. Res.* 63, 288–293. doi: 10.1016/j.neures.2009.01.003

Nishikura, K. (2016). A-to-I editing of coding and non-coding RNAs by ADARs. *Nat. Rev. Mol. Cell Biol.* 17, 83–96. doi: 10.1038/nrm.2015.4

Niswender, C. M., Copeland, S. C., Herrick-Davis, K., Emeson, R. B., and Sanders-Bush, E. (1999). RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. *J. Biol. Chem.* 274, 9472–9478. doi: 10.1074/jbc.274.14.9472

Niswender, C. M., Sanders-Bush, E., and Emeson, R. B. (1998). Identification and characterization of RNA editing events within the 5-HT_{2C} receptor. *Ann. N Y Acad. Sci.* 861, 38–48. doi: 10.1111/j.1749-6632.1998.tb10171.x

Paxinos, G., and Franklin, K. B. J. (2004). *The Mouse Brain in Stereotaxic Coordinates, Second Edition.* New York, NY: Elsevier.

Sari, Y., Johnson, V. R., and Weedman, J. M. (2011). Role of the serotonergic system in alcohol dependence: from animal models to clinics. *Prog.*

Mol. Biol. Transl. Sci. 98, 401–443. doi: 10.1016/B978-0-12-385506-0.00010-7

Schmidt, H. D., McFarland, K. N., Darnell, S. B., Huizenga, M. N., Sangrey, G. R., Cha, J. H., et al. (2015). ADAR2-dependent GluA2 editing regulates cocaine seeking. *Mol. Psychiatry* 20, 1460–1466. doi: 10.1038/mp.2014.134

Shirahase, T., Aoki, M., Watanabe, R., Watanabe, Y., and Tanaka, M. (2016). Increased alcohol consumption in relaxin-3 deficient male mice. *Neurosci. Lett.* 612, 155–160. doi: 10.1016/j.neulet.2015.12.014

Sommer, B., Köhler, M., Sprengel, R., and Seeburg, P. H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67, 11–19. doi: 10.1016/0092-8674(91)90568-j

Tariq, A., and Jantsch, M. F. (2012). Transcript diversification in the nervous system: a to I RNA editing in CNS function and disease development. *Front. Neurosci.* 6:99. doi: 10.3389/fnins.2012.00099

Tattoli, M., Cagiano, R., Gaetani, S., Ghiglieri, V., Giustino, A., Mereu, G., et al. (2001). Neurofunctional effects of developmental alcohol exposure in alcohol-preferring and alcohol-nonpreferring rats. *Neuropsychopharmacology* 24, 691–705. doi: 10.1016/S0893-133X(00)00225-6

Thanos, P. K., Dimitrakakis, E. S., Rice, O., Gifford, A., and Volkow, N. D. (2005). Ethanol self-administration and ethanol conditioned place preference are reduced in mice lacking cannabinoid CB1 receptors. *Behav. Brain Res.* 164, 206–213. doi: 10.1016/j.bbr.2005.06.021

Wang, Q., O'Brien, P. J., Chen, C. X., Cho, D. S., Murray, J. M., and Nishikura, K. (2000). Altered G protein-coupling functions of RNA editing isoform and splicing variant serotonin2C receptors. *J. Neurochem.* 74, 1290–1300. doi: 10.1046/j.1471-4159.2000.741290.x

Watanabe, Y., Tsujimura, A., Takao, K., Nishi, K., Ito, Y., Yasuhara, Y., et al. (2011). Relaxin-3-deficient mice showed slight alteration in anxiety-related behavior. *Front. Behav. Neurosci.* 5:50. doi: 10.3389/fnbeh.2011.00050

Watanabe, Y., Yoshimoto, K., Tatebe, H., Kita, M., Nishikura, K., Kimura, M., et al. (2014). Enhancement of alcohol drinking in mice depends on alterations in RNA editing of serotonin 2C receptors. *Int. J. Neuropsychopharmacol.* 17, 739–751. doi: 10.1017/S1461145713001545

Weissmann, D., van der Laan, S., Underwood, M. D., Salvetat, N., Cavarec, L., Vincent, L., et al. (2016). Region-specific alterations of A-to-I RNA editing of serotonin 2c receptor in the cortex of suicides with major depression. *Transl. Psychiatry* 6:e878. doi: 10.1038/tp.2016.121

Yamashita, T., and Kwak, S. (2014). The molecular link between inefficient GluA2 Q/R site-RNA editing and TDP-43 pathology in motor neurons of sporadic amyotrophic lateral sclerosis patients. *Brain Res.* 1584, 28–38. doi: 10.1016/j.brainres.2013.12.011

Yoshimoto, K., Watanabe, Y., Tanaka, M., and Kimura, M. (2012). Serotonin2C receptors in the nucleus accumbens are involved in enhanced alcohol-drinking behavior. *Eur. J. Neurosci.* 35, 1368–1380. doi: 10.1111/j.1460-9568.2012.08037.x

Yoshimoto, K., Yayama, K., Sorimachi, Y., Tani, J., Ogata, M., Nishimura, A., et al. (1996). Possibility of 5-HT3 receptor involvement in alcohol dependence: a microdialysis study of nucleus accumbens dopamine and serotonin release in rats with chronic alcohol consumption. *Alcohol. Clin. Exp. Res.* 20, 311A–319A. doi: 10.1111/j.1530-0277.1996.tb01164.x

Conflict of Interest Statement: 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: 05 February 2019
doi: 10.3389/fnbeh.2019.00009

Residual Information of Previous Decision Affects Evidence Accumulation in Current Decision

Farzaneh Olianezhad[1,2], *Sajjad Zabbah*[2], *Maryam Tohidi-Moghaddam*[2,3] *and Reza Ebrahimpour*[2,3]*

[1] Department of Electrical Engineering, Shahid Rajaee Teacher Training University, Tehran, Iran, [2] School of Cognitive Sciences, Institute for Research in Fundamental Sciences (IPM), Tehran, Iran, [3] Department of Computer Engineering, Shahid Rajaee Teacher Training University, Tehran, Iran

Bias in perceptual decisions can be generally defined as an effect which is controlled by factors other than the decision-relevant information (e.g., perceptual information in a perceptual task, when trials are independent). The literature on decision-making suggests two main hypotheses to account for this kind of bias: internal bias signals are derived from (a) the residual of motor signals generated to report a decision in the past, and (b) the residual of sensory information extracted from the stimulus in the past. Beside these hypotheses, this study suggests that making a decision in the past *per se* may bias the next decision. We demonstrate the validity of this assumption, first, by performing behavioral experiments based on the two-alternative forced-choice (TAFC) discrimination of motion direction paradigms and, then, we modified the pure drift-diffusion model (DDM) based on the accumulation-to-bound mechanism to account for the sequential effect. In both cases, the trace of the previous trial influences the current decision. Results indicate that the probability of being correct in the current decision increases if it is in line with the previously made decision even in the presence of feedback. Moreover, a modified model that keeps the previous decision information in the starting point of evidence accumulation provides a better fit to the behavioral data. Our findings suggest that the accumulated evidence in the decision-making process after crossing the bound in the previous decision can affect the parameters of information accumulation for the current decision in consecutive trials.

Keywords: perceptual decision, bias, accuracy, drift-diffusion model, sequential effect

OPEN ACCESS

Edited by:
Alicia Izquierdo, University of California, Los Angeles, United States

Reviewed by:
Kenway Louie, New York University, United States
Fuat Balcı, Koç University, Turkey

***Correspondence:**
Reza Ebrahimpour
rebrahimpour@srttu.edu

Received: *06 October 2018*
Accepted: *14 January 2019*
Published: *05 February 2019*

Citation:
Olianezhad F, Zabbah S, Tohidi-Moghaddam M and Ebrahimpour R (2019) Residual Information of Previous Decision Affects Evidence Accumulation in Current Decision. Front. Behav. Neurosci. 13:9. doi: 10.3389/fnbeh.2019.00009

INTRODUCTION

Perceptual decisions and their outcomes can be related to each other as a sequence (Hanks et al., 2011; Akaishi et al., 2014; Purcell and Kiani, 2016; Bornstein et al., 2017; Miller et al., 2017). This ability to merge the advance knowledge about choice alternatives with current evidence to make an appropriate decision is a hallmark of higher brain function (Cook and Maunsell, 2002; Roitman and Shadlen, 2002; Gold and Shadlen, 2007; Ratcliff et al., 2007; Churchland et al., 2008; Kiani et al., 2008; Heitz and Schall, 2012). Findings suggest that neural activities in brain areas involved in decision making process contain the history of previous decisions (Boettiger et al., 2007; Serences, 2008; Summerfield and Koechlin, 2008, 2010; Basten et al., 2010; Fleming et al., 2010a,b; Forstmann et al., 2010; Philiastides et al., 2010; Preuschhof et al., 2010; Scheibe et al., 2010; Mulder et al., 2012)

(Akaishi et al., 2014) and do not return to the initial value immediately after the time of decision (Cook and Maunsell, 2002; Roitman and Shadlen, 2002; Gold and Shadlen, 2007; Ratcliff et al., 2007; Churchland et al., 2008; Kiani et al., 2008; Heitz and Schall, 2012). Furthermore, there is a preference in humans to repeat their decision, especially when it was made about an ambiguous stimulus (Brehm, 1956; Izuma and Murayama, 2013; Akaishi et al., 2014), on the subsequent trial in the absence of response feedback. This interaction between the history of choices and sensory context, respectively called internal and external signals, is thought to cause the biased decisions about the sensory events (Albright, 2012; Awh et al., 2012; Carnevale et al., 2012; Akaishi et al., 2014).

The mechanism of decision bias as one of the most pervasive biases across many domains of cognitive science, however, remains obscure (Glimcher, 2003; Lauwereyns, 2010; Summerfield and Koechlin, 2010; White and Poldrack, 2014; Hanks and Summerfield, 2017; Kim et al., 2017). Two main hypotheses have been proposed to explain the reasons of this bias, although to date, none of them have been adequately supported. According to the first view, the residual of the sensory information of the previous stimulus causes internal bias signals (Becker, 2008; Pearson and Brascamp, 2008; Sigurdardottir et al., 2008; Albright, 2012; Carnevale et al., 2012). Therefore, a strong sensory signal in the previous trial affects the neural responses (increment in the baseline activity) in the brain sensory areas and the current decision is expected to be made under a larger bias. In the alternative view, the residual of motor response-related signals causes internal bias signals (Gold et al., 2008; Marcos et al., 2013); however, contrary to the first impression, the strength of the sensory signal in the previous trial does not seem to affect the decision-biasing. Akaishi et al. also suggest that, in the absence of response feedback, this bias is a mechanism to update the likelihood of a choice to be made (Akaishi et al., 2014).

Given previous work, we propose the following hypothesis: the residual decision evidence in the previous decision process affects evidence accumulation in the current decision even in the presence of feedback. We tested the validity of this claim using behavioral experiments based on the two-alternative forced-choice (TAFC) discrimination of motion direction and computational modeling. We revealed that, firstly, the probability of being correct in the current decision increases if it is in line with the previous decision, showing a trace from the previous trial on the current one. Secondly, this effect is evident in the presence of the feedback, and is independent of the correctness of the previous decision. Thirdly, excluding the strong stimuli from our analysis amplifies the observed effect. This observation could refer to the repulsive adaptation effect of these strong stimuli (Kohn, 2007). These last two eliminate the effect of the previous stimuli and merely include the decision.

Abbreviations: ms, millisecond; sd, standard deviation; BIC, Bayes Information Criterion; CDF, Cumulative Distribution Function; CRT, Cathode Ray Tube; DDM, Drift-Diffusion Model; GLM, Generalized Linear Model; PDE, Partial Differential Equation; R^2, R squared; SE, Standard Error; TAFC, Two-Alternative Forced-Choice.

Finally, in order to shed light on the plausible mechanism of the observed effect, we used one successful and the elaborate variant of decision-making models called "drift-diffusion" (Mazurek et al., 2003; Shadlen et al., 2006; Gold and Shadlen, 2007; Voss and Voss, 2007; Kiani et al., 2008; Voss et al., 2013; Tohidi-Moghaddam et al., 2016; Lerche and Voss, 2017; Dully et al., 2018). It has been shown that commitment to a choice is a consequence of a gradual increase in the activity of neurons selective for that specific choice. This gradual increment from a baseline activity is well explained, in this accumulation-to-bound model, by the accumulation of noisy evidence from a starting point which varies depending on the different parameters (Falmagne, 1965, 1968; Remington, 1969; Luce, 1986; Ratcliff et al., 1999; Bogacz et al., 2006; Forstmann et al., 2010; Rorie et al., 2010; Balci and Simen, 2014). In addition, improvement in the activity reaches a stereotyped threshold at decision end (Ratcliff, 1978, 2002; Bogacz et al., 2006; Ratcliff et al., 2016) which corresponds to reaching a specified bound in this model. Our results show that the model that keeps previous decision information in the starting point of accumulation provides a better fit to the behavioral data which support the idea that the activity of decision maker neurons (Gold and Shadlen, 2007) after crossing the bound, in the previous decision, may affect the process of information accumulation of those neurons for the current decision in consecutive trials.

MATERIALS AND METHODS

Participants

In this experiment, six adult participants, three males and three females, with normal or corrected-to-normal vision participated. All the participants, except for two of the middle authors, were unfamiliar with the design of the experiment. They signed informed written consent before attending the study. All experimental protocols were approved by the Iran University of Medical Sciences.

Visual Stimuli

Random dot motion stimuli are used in a large number of perceptual decision-making studies. These stimuli are movies in which some dots are randomly moving in different direction. In each frame, white dots (2×2 pixel, 0.088° per side) were displayed on a black background with a density of 16.7 dots/degree^2/s (Shadlen and Newsome, 2001; Roitman and Shadlen, 2002). The stimulus contained three interleaved sets of dots displayed on consecutive video frames. Each set was relocated three frames (40 ms) later while a fraction of dots had a coherent continuous motion toward a direction, and the rest of dots were resettled randomly. The stimulus strength was specified by the fraction of dots which moved coherently. Stimulus was presented using a psychophysics toolbox 3.0.12 (Brainard, 1997; Pelli, 1997) for MATLAB R2013a (MATLAB, 2013) on a computer with the operating system of Windows 7 (64-bit), Intel (R), Core (TM) i7, 16 GB internal storage, and NVIDIA Quadro K2000 GPU card.

Behavioral Task

All the experiments were carried out in a semi-dark and sound-proof room. The participants were seated in an adjustable chair at the distance of 57 cm from a cathode ray tube (CRT) display monitor (19 inch, with an 800 × 600 screen resolution, and 75 Hz refresh rate). An adjustable chin-rest had been appropriated to support the participant's chin and forehead. Each trial started with a red fixation point (FP, 0.3° diameter) at the center of the screen and two red choice targets (0.5° diameter) on the right and left side of the fixation point (10° eccentricity). The participants were asked to fix and maintain their gaze on the fixation point throughout the trial. After a 200 ms delay period, the random dots stimulus was displayed within a 5° circular aperture at the center of the screen for 120, 400, and 720 ms. The percentage of coherently moving dots was chosen from these following values: 0, 3.2, 6.4, 12.8, 25.6, and 51.2%. At the end of the stimulus presentation, a 120 ms delay period occurred. After the delay period, the Go signal cued the participants to respond by eliminating the fixation point. The participants were asked to report their decision, about the direction of motion, within 1 second after the Go signal by pressing a left or right key. Distinctive auditory feedback (e.g., beep) was delivered for 100 ms for correct responses, error responses, and missed trials. The type of feedback was chosen randomly for trials with 0% coherence. Trials have been separated by different gap durations: 0, 120, or 1,200 ms. Different gap durations were used to demonstrate their different effects on our results, but there was no significant difference between them, so we have pooled the data of the three gaps in all analysis. The arrangement of the motion direction, motion duration, gap duration, and motion strength varied randomly from trial to trial (**Figure 1**).

All possible types of trials were randomly interleaved in blocks with 150 trials. The participants were instructed to perform the experiments quickly and accurately to the possible extent. The overall probability of being correct was shown on the screen at the end of each block. Each participant performed one or two sessions (each session had four blocks) per day until 3,600 trials were collected. The participants completed at least one session on each day for six consecutive days. The results were consistent across all participants, but figures have collapsed the data across participants.

Data Analysis

For the purpose of this study, we focused our analysis on specific pairs of consecutive trials which will be explained along with their reasons in the following. First of all, in order to demonstrate the effect of previous stimulus strengths on the current decision, we picked out the pair of trials in which the first (previous) trial contained two groups of low (0 and 3.2%) and high (12.8 and 51.2%) motion strengths. This categorization is based on the subjects' performance. The performance in 0 and 51.2% is the minimum (50%) and the maximum (100%), correspondingly. The performance in 3.2% (∼65%) and 12.8% (∼85%) is 15% far from the minimum and the maximum, correspondingly. The second (current) trial consisted of low, middle and nearly high motion strength values (3.2, 6.4, and 12.8%) where the stimulus is not very strong. It also should be noted that in the preliminary analysis, we observed the same results of previous trials which had 25.6%, and 51.2% coherence. Furthermore, we probed previous trials with three different motion durations to illustrate the effect of previous stimulus durations on the current trials with constant motion durations (120 ms); however, no significant difference was found. Accordingly, we have pooled the data of the three motion durations of previous trials in further analysis.

A variety of logistic regression models were used to characterize the effect of different parameters on the probability of correct choice. The following models are fitted by using the generalized linear model (GLM) with binomial error structure. We use *Logit[P]* as a short form of $\log(\frac{P}{1-P})$, and β_i as fitted coefficients.

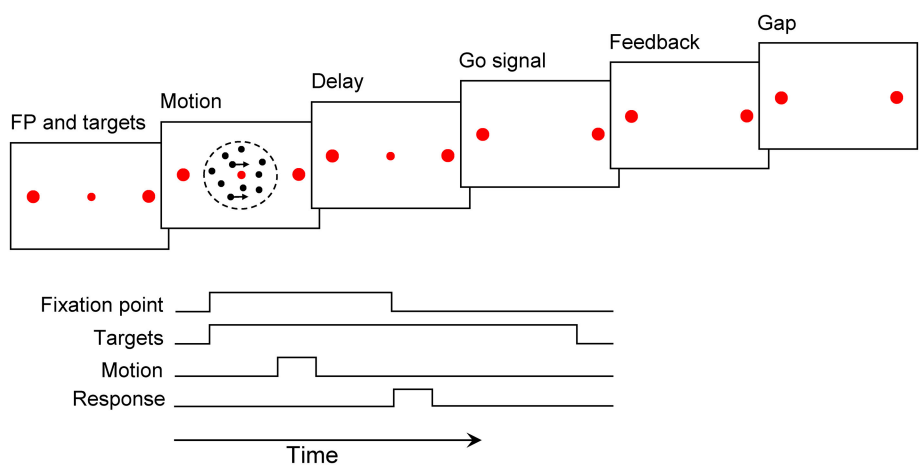


FIGURE 1 | Motion discrimination paradigm. A fixation point (FP) and two targets were presented for 200 ms. After that, the motion stimulus was shown for 120, 400, and 720 ms. The Go signal followed by a 120 ms delay period cued participants to report their decision, within 1 s, by pressing two specific keys. Auditory feedback was played for 100 ms. The following trial began after a gap of 0–1.2 s (see Materials and Methods).

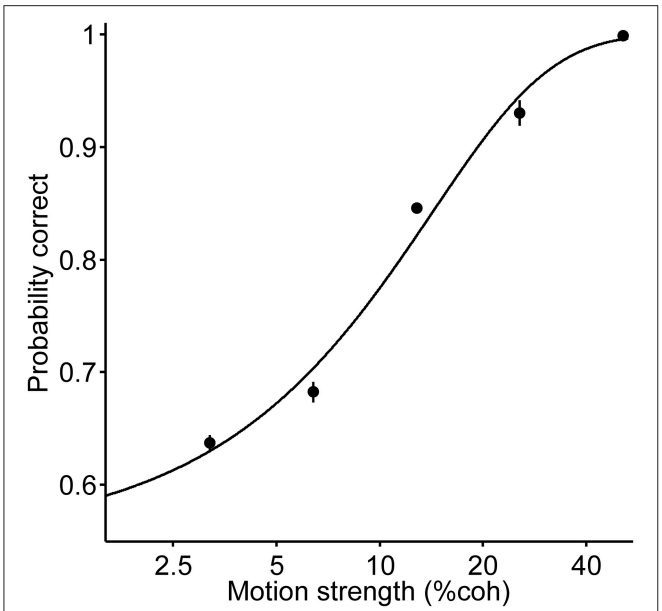


FIGURE 2 | Psychometric function of all the trials; each data point presents the performance of pooled data for all the three durations and two directions. The curve is the fit of a logistic regression to the data (Equation 1). Error bars indicate SE (Standard Error).

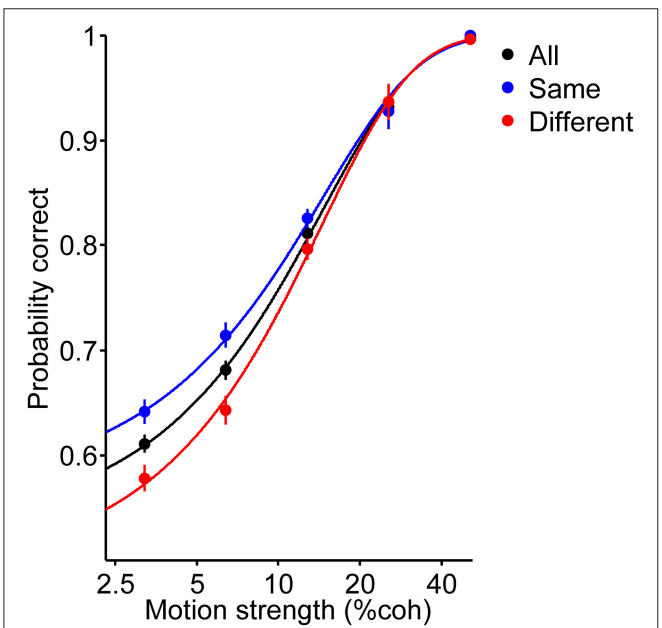


FIGURE 3 | Psychometric function of the current trials. Red and blue data points depict performance of participants for the different and same decision conditions, respectively. Black data points are pooled from these two conditions. Curves are the fit of the logistic regression to the data (Equation 1 for black curve and Equation 2 for red and blue curves). Error bars indicate SE (Standard Error).

The probability of a correct choice is defined by the following (to fit the psychometric function in **Figure 2** and for the black curve in **Figure 3**):

$$\text{Logit}\,[P_{\text{correct}}] = \beta_0 + \beta_c C_c \tag{1}$$

where C_c is motion strength. To evaluate the effect of the previous decision on the current choice accuracy, we fit the following: (to fit the psychometric function of same and different decision conditions in **Figure 3**):

$$\text{Logit}\,[P_{\text{Correct}}] = \beta_0 + \beta_s S + \beta_c C_c, S = \begin{cases} 0 & \text{different decision} \\ 1 & \text{same decision} \end{cases} \tag{2}$$

where C_c is the motion coherence of the current trials and S is an indicator variable for two successive decisions. The null hypothesis is that the current choice accuracy for same and different decision conditions are equal (*H0:* $\beta_s = 0$).

A modified version of Equation (2) was used to test whether the current choice accuracy was influenced by correctness of the previous trial:

$$\text{Logit}\,[P_{\text{Correct}}] = \beta_0 + \beta_s S + \beta_c C_c + \beta_e E,$$
$$S = \begin{cases} 0 & \text{different decision} \\ 1 & \text{same decision} \end{cases} \quad E = \begin{cases} 0 & \text{incorrect previous trial} \\ 1 & \text{correct previous trial} \end{cases} \tag{3}$$

where C_c is the motion coherence of the current trials. S and E are the indicator variables for two successive decisions and correctness of the previous trials, respectively. The null hypothesis is that the current choice accuracy does not depend on correctness of the previous trial (*H0:* $\beta_e = 0$).

To examine if the current choice accuracy was affected by two groups of low (0% and 3.2%) and high (12.8% and 51.2%) motion strengths of the previous trial, we altered Equation 2 as follows:

$$\text{Logit}\,[P_{\text{Correct}}] = \beta_0 + \beta_s S + \beta_c C_c + \beta_m M,$$
$$S = \begin{cases} 0 & \text{different decision} \\ 1 & \text{same decision} \end{cases},$$
$$M = \begin{cases} 0 & \text{high motion strength in previous trial} \\ 1 & \text{low motion strength in previous trial} \end{cases} \tag{4}$$

where C_c is the motion coherence of the current trials. S and M are the indicator variables for two successive decisions and motion strengths level of the previous trials, respectively. The null hypothesis is that the current choice accuracy does not depend on motion strengths level of the previous trial (*H0:* $\beta_m = 0$).

To assess the impact of the motion strength of the previous trial on the current choice accuracy we used the following regression:

$$\text{Logit}\,[P_{\text{Correct}}] = \beta_0 + \beta_p C_p + \beta_c C_c \tag{5}$$

where C_p and C_c are the motion coherence of the previous and current trials, respectively. The null hypothesis is that previous stimulus strength has no significant effect on current choice accuracy (*H0:* $\beta_p = 0$).

We compared the accuracy in the same decision condition (the blue curve in **Figure 3**) to the accuracy in different decision

condition (the red curve in **Figure 3**) using logistic regression, as follows:

$$\text{Logit}\,[P_s] = \beta + \text{Logit}\,[P_d] \quad (6)$$

where P_s and P_d are the probability correct in the same decision condition and different decision condition, respectively. The null hypothesis is that the accuracies in both conditions are equal (*H0:* $\beta = 0$).

All statistical analyses were performed in R version 3.3.1 (The R Foundation for Statistical Computing, www.R-project.org). The statistical analyses outcomes are presented in the RESULTS section.

Modeling

In order to investigate the mechanism of the last decision impact on the current decision, we used the drift-diffusion model (DDM) (Ratcliff, 1978; Ratcliff and McKoon, 2008) as implemented by Voss et al. in a computationally efficient, flexible and user-friendly program called fast-dm (Voss and Voss, 2007). Fast-dm estimated DDM's parameters using the partial differential equation (PDE) method through fast computations to calculate the cumulative distribution function (CDF) and the Chi-Square statistic (Voss et al., 2013; Lerche and Voss, 2017).

Undoubtedly, the diffusion model is a well-established model in the perceptual decision literature (Gold and Shadlen, 2007; Voss et al., 2013). This model consistently explains both neural and behavioral responses, and its different parameters can explain the process of commitment to a choice in the brain based on an accumulation-to-bound mechanism (Mazurek et al., 2003; Shadlen et al., 2006; Gold and Shadlen, 2007; Voss and Voss, 2007; Kiani et al., 2008). In the pure drift-diffusion model (DDM), momentary sensory evidence in favor of one of the choices starts to accumulate from a baseline point (z). Just after the integrated evidence over time (guided by drift rate v) hits a criterion level or bound (a), the decision process is terminated (Ratcliff, 1978; Ratcliff and McKoon, 2008; Ratcliff et al., 2016). Seven parameters that exist in the full DDM are divided into three categories: (1) the decision process parameters (decision bound a, mean baseline point z, and mean drift rate v), (2) the non-decision process parameter (non-decision time t_{ND}), (3) the variability across-trial parameters (variability in stimulus quality η, variability in baseline point sz, and variability in non-decision time st_{ND}) (Ratcliff, 1978; Ratcliff and Tuerlinckx, 2002; Ratcliff and McKoon, 2008). According to the proposed hypothesis in the present research, the previous decision can influence the current decision process in three possible ways: (a) the previous decision affects the rate of accumulated evidence (i.e., the drift rate, v) (Ashby, 1983; Ratcliff, 1985; Diederich and Busemeyer, 2006; Bornstein et al., 2017), (b) it changes the mean baseline point of evidence accumulation (z) (Edwards, 1965; Laming, 1968; Link and Heath, 1975; Ratcliff, 1985; Voss et al., 2004; Bogacz et al., 2006; Diederich and Busemeyer, 2006; Wagenmakers et al., 2008; Bornstein et al., 2017), or (c) it shifts the decision threshold (a) (Ratcliff and Rouder, 1998; Ratcliff and Smith, 2004; Bogacz et al., 2006; Simen et al., 2006; Goldfarb et al., 2012). The diffusion model along with a model comparison method (Smith and Spiegelhalter, 1980; Kass and Wasserman, 1995; Liddle, 2007) will be used to disentangle these three scenarios (Falmagne, 1965, 1968; Remington, 1969; Ratcliff, 1985; Luce, 1986; Ratcliff et al., 1999; Ratcliff and Smith, 2004).

RESULTS

Behavior

Six human participants reported the perceived direction of motion in trials with 120, 400 and 720 ms duration (**Figure 1**). The psychometric function for the participants is shown in **Figure 2**. The psychometric function of current trials separated in the three conditions is plotted in **Figure 3**. The first condition or the so called same decision condition, blue data points, shows the performance of current trials in which the participants have taken a decision similar to the previous trial. In the second condition or different decision condition, red data points, the participants' decisions in current trials are different from those in the previous trials. The third condition, black data points, is the performance of all current trials, independent of the decision in previous trials. Considering the black data points as a reference, an upward and a downward shift is obvious in the psychometric function of the same and different decision conditions, respectively. Generally, it can be said that upward and downward shifts which occurred in **Figure 3** are independent of the current stimuli with low and middle motion strength values (Equation 6, $\beta = 0.27 \pm 0.03$, $p = 3.2 \times 10^{-16,}$ positive β indicates accuracies in the same decision condition are higher than the accuracies in the different decision condition). This shift is not evident in the strong stimuli of the current trials (25.6 and 51.2%) because, in the salient stimuli which are not ambiguous, the decision is more dependent on the sensory information (Akaishi et al., 2014). Thus, detecting any kinds of bias is much more difficult in such stimuli. As a result, we focused our analysis on ambiguous stimuli in the current trials.

This difference between psychometric functions of the same and different decision conditions implies that not only does the probability of being correct in a decision depend on the stimulus strength, but also on the previous decision (Equation 2, $\beta_s = 0.25 \pm 0.09$, $p = 5.8 \times 10^{-8}$). Indeed, the probability of being correct in the current trial will increase (decrease) if the reported direction in the current decision and the chosen direction in the previous trial are alike (different).

One may conclude that this difference in performance is the effect of stimulus adaptation since the previous decision is itself correlated to the previous stimulus. Interestingly, the reported effect of the previous decision seems to be in contrast with the repulsive effect of adaptation. Taking the repulsive effect into account, we expect higher sensitivity for the perception of leftward (rightward) motion when it comes after a rightward (leftward) motion. As a result, the probability of being correct should be higher in the different decision condition compared to the same one. In what follows, we tried to elaborate on these two probable contradictory effects through further analysis.

It is worth noting that in case there is an adaptation effect in our paradigm, it should be stronger when the stimulus of the previous trial has high motion coherence. In order to investigate

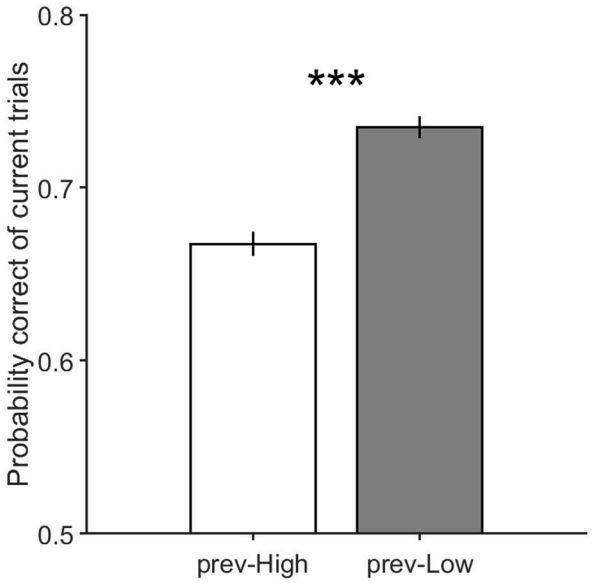


FIGURE 4 | The performance of the current trials which includes motion strengths of 3.2, 6.4, and 12.8% when their previous trials have high motion strengths of 12.8, 25.6, and 51.2% in prev-High, and have low motion strengths of 0 and 3.2% in prev-Low. Error bars indicate SE (Standard Error). Wilcoxon rank-sum test is used to test the significance of the difference, $^{***}p < 1E{-}3$.

whether there is any adaptation effect in our data, we separated trials with high and low motion strength in their previous trials and compared the performance of the current trials in these two conditions. As shown in **Figure 4**, the accuracy of trials which preceded by high coherence stimuli is significantly lower than the accuracy of those preceded by low coherence stimuli. This result supports the presence of the adaptation and its strong effect in trials preceded by high coherence stimuli. Therefore, to untwist the same/different decisions effect from the sensory adaptation effect, we separated high and low coherence stimuli from the preceding analysis, and calculated how the same and different decision conditions differ in performance.

Figure 5 illustrates the performance in current trials which includes motion strengths of 3.2, 6.4, and 12.8%, when previous trials have low motion strengths of 0 and 3.2% (**Figure 5A**, Equation 2, $\beta_s = 0.65 \pm 0.13$, $p = 4.3 \times 10^{-22}$) and high motion strengths of 12.8 and 51.2% (**Figure 5B**, Equation 2, $\beta_s = -0.14 \pm 0.13$, $p = 0.03$). As shown in this figure, the participants are significantly more likely to choose a correct decision in the different decision condition when the coherence of the previous trial is high, which is consistent with the repulsive adaptation effect. Whereas, the **Figure 5A** shows that a correct decision is more probable in the same decision condition when stimulus coherence in the previous trial is low (Equation 4, $\beta_m = 0.28 \pm 0.09$, $p = 1.5 \times 10^{-9}$). Moreover, this observed effect is significant even when previous trials have 0% coherence in which all dots move randomly, and minimizes the adaptation in any specific direction. However, as illustrated in **Figure 6**, the probability of being correct is greater in the same decision condition than in the different decision condition, even when there is lack of coherent motion (motion strengths of 0%) in the previous stimulus (Equation 2, $\beta_s = 0.98 \pm 0.19$, $p = 2.2 \times 10^{-23}$). Therefore, decreasing the effect of stimulus adaptation by excluding previous trials with high coherence stimulus seems to strengthen the effect of the previous decision presented in **Figure 3**.

Another salient point that may contribute to the current decision accuracy difference between the same and different decision conditions is the previous trial's feedback. As stated before, the feedback signal is different in the correct and incorrect trials, and may result in the observed effect. Here in **Figure 7**, by separating correct and incorrect previous trials in both the same and different decision conditions, we attempted to eliminate the influence of the feedback. As illustrated in this figure, the correctness of the previous decision does not remove the effect explained above (Equation 3, $\beta_e = -0.07 \pm 0.1$, $p = 0.19$). In other words, similar decision trials are significantly more probable to be correct than different decision trials, regardless of the previous decision to be correct (**Figure 7A**, Equation 2, $\beta_s = 0.37 \pm 0.17$, $p = 3.3 \times 10^{-5}$) or incorrect (**Figure 7B**, Equation 2, $\beta_s = 1.06 \pm 0.21$, $p = 3.6 \times 10^{-22}$).

Model Fits

As indicated previously, to investigate the underlying mechanism of the previous decision's effect on the probability of being correct in the current choice, we used the drift-diffusion model (DDM). Dependence of the model parameters on the previous decision gave us the chance to examine the effect of the previous decision on each parameter. To do so, besides the pure DDM, we ran three modified versions of it, and fit these four models to the behavioral data derived from experimental study to provide further intuition into the nature of the observed effect.

The first model (model_p) is the pure DDM in which the only dependent variable, drift-rate (v), depends on the current motion strength. In the second model (model_v), as a modified DDM, v depends on both current motion strength and previous decision (same and different decision conditions). The third one (model_z) is a drift-diffusion model in which the starting point of evidence accumulation (z) is dependent on the previous decision, and v depends on the current motion strength. The fourth DDM (model_a) modified by the dependence of the decision bound (a) on the previous decision, as well as v is dependent on the current motion strength.

Fitted parameters of each model are listed in **Tables 1–4** (mean±SE across participants). For each participant's details, see **Tables S1–S4**. Here, s and d indices respectively stand for the same and different decision conditions. As **Table 2** shows, based on the dependence of the drift-rate on both current motion strength and previous decision, there are six different drift-rates for three current stimulus coherences (3.2%, 6.4%, and 12.8%) and two conditions (same and different). Regarding the parameters of the third model in **Table 3**, there are two different starting points for the same and different decision conditions. As presented in **Table 4**, model_a has two different decision threshold related to two different decision conditions.

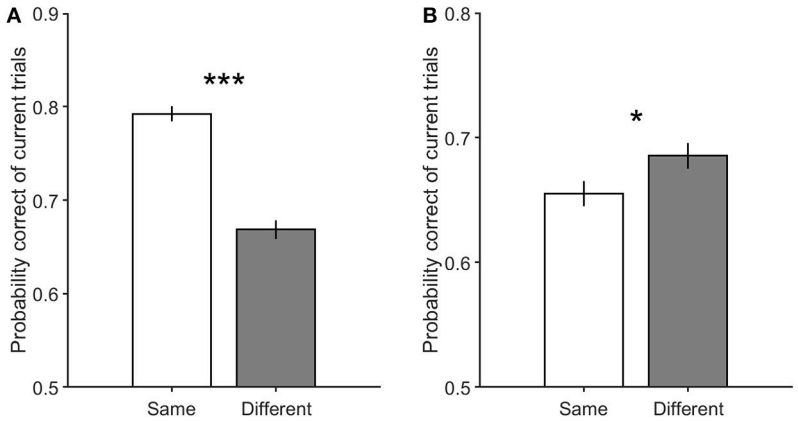


FIGURE 5 | The performance of the current trials with motion strengths of 3.2, 6.4, and 12.8%. **(A)** shows performance in the current trials when previous trials have low motion strengths of 0 and 3.2%. **(B)** illustrates performance in the current trials when previous trials have high motion strengths of 12.8 and 51.2%. Error bars indicate SE (Standard Error). Wilcoxon rank-sum test is used to test the significance of the differences, $^{*}p < 0.05$, $^{***}p <$ 1E−3.

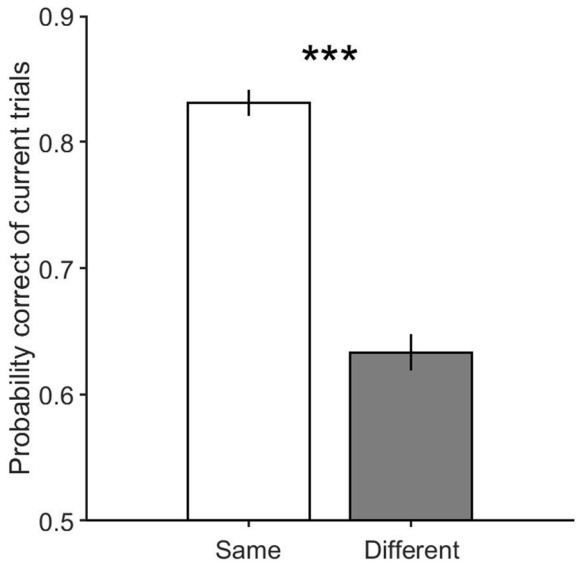


FIGURE 6 | The performance of the current trials which includes motion strengths of 3.2, 6.4, and 12.8% when their previous trials are 0%. Error bars indicate SE (Standard Error). Wilcoxon rank-sum test is used to test the significance of the difference, $^{***}p <$ 1E−3.

As we expected from behavioral results, which indicated the current decision had higher accuracy when the selected direction was similar to the reported one in the previous trial compared to when they were different, the drift-rate and starting point obtained bigger values in the same decision condition in comparison to the different decision condition provided that they are dependent on the previous decision. On the contrary, the decision threshold in the same decision condition is smaller than its value in the different decision condition.

Models have been compared using the Bayes Information Criterion (BIC) (Smith and Spiegelhalter, 1980; Kass and Wasserman, 1995; Liddle, 2007) for the different model fits which are exposed in **Table 5** (mean ± sd across participants). As shown in this table, the overall quality of the fits was good ($R^2 > 0.83$). For details of subjective scores, see **Tables S5–S9**.

Furthermore, BIC values were compared using a Student's *t*-test. Accordingly, the modified DDM with the dependent starting point, model_z, received the smallest BIC compared to the model_p ($p = 5.6 \times 10^{-3}$) and model_a ($p = 3.2 \times 10^{-4}$). Except for the first participant, all other five participants yielded the lower BIC for model_z than model_v (see **Tables S6**, **S7**). However, the comparison of overall BIC scores showed marginal significant lower BIC for model_z compared to model_v ($p = 0.051$). After excluding the first participant, the model_z led to a significant lower BIC value than model_v ($p = 0.03$). Eventually, we chose the model_z with the best explanation for how the current choice accuracy is influenced by previous decision.

In that case, we have provided more insight into the model_z through simulation. The model_z parameters were applied to obtain model performance individually for each of the conditions while the same order of the stimulus in the behavioral experiment was used as an input to this model. As illustrated in **Figure 8**, consistent with the behavioral results, the same decision condition in the model resulted in the greater accuracy of current trials compared to the different decision condition ($p = 2 \times 10^{-4}$).

In the latest step, we investigated the difference of the dependent parameter in different conditions for the winner model (model_z). As stated before, starting point gained higher value in the same decision condition (z_s) compared to the different decision condition (z_d), and it's consistent across all participants except participant 3 (for participants' details, see **Tables S3**). Focusing on the data of this participant, it seems that participant 3 is influenced by the sensory adaptation effect even in 3.2%. As shown in **Figure S1**, the accuracy of the current trials is higher in the same decision condition compared to the different decision condition only when previous trials have 0% motion strengths in which all dots had random movements. The

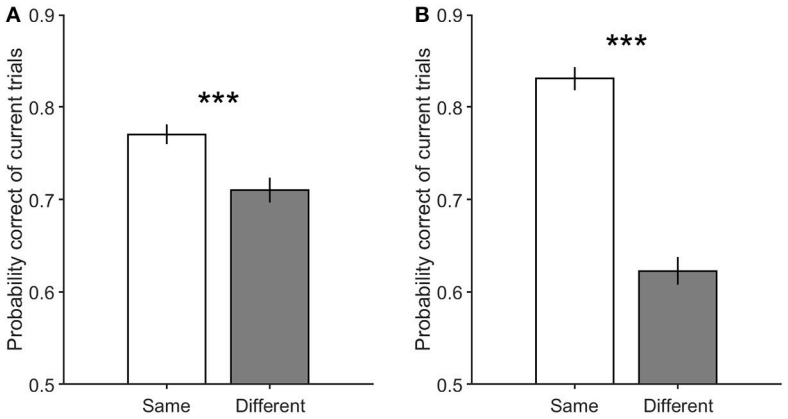


FIGURE 7 | The performance of the current trials which include motion strengths of 3.2, 6.4, and 12.8%. **(A)** is the performance of the current trials when their previous trials are correct with low motion strengths (0 and 3.2%). **(B)** is the performance of the current trials when their previous trials are incorrect with low motion strengths (0 and 3.2%). Error bars indicate SE (Standard Error). Wilcoxon rank-sum test is used to test the significance of the differences, ***$p < 1E{-}3$.

TABLE 1 | Fitted parameters (mean ± SE) of the pure DDM ($model_p$).

z	0.555 ± 0.015
a	0.681 ± 0.062
$v_{3.2}$	0.321 ± 0.081
$v_{6.4}$	0.780 ± 0.113
$v_{12.8}$	1.855 ± 0.155
t_{ND}	0.178 ± 0.008
st_{ND}	0.116 ± 0.012

TABLE 2 | Fitted parameters *(mean ± SE)* of the second DDM ($model_v$).

z	0.551 ± 0.013
a	0.690 ± 0.610
$v_{3.2\_s}$	0.488 ± 0.104
$v_{6.4\_s}$	1.023 ± 0.136
$v_{12.8\_s}$	1.907 ± 0.195
$v_{3.2\_d}$	0.201 ± 0.058
$v_{6.4\_d}$	0.525 ± 0.100
$v_{12.8\_d}$	1.748 ± 0.147
t_{ND}	0.176 ± 0.008
st_{ND}	0.120 ± 0.011

TABLE 3 | Fitted parameters *(mean ± SE)* of the third DDM ($model_z$).

z_s	0.566 ± 0.014
z_d	0.540 ± 0.013
a	0.688 ± 0.061
$v_{3.2}$	0.333 ± 0.070
$v_{6.4}$	0.755 ± 0.105
$v_{12.8}$	1.804 ± 0.135
t_{ND}	0.176 ± 0.008
st_{ND}	0.121 ± 0.011

TABLE 4 | Fitted parameters *(mean ± SE)* of the fourth DDM ($model_a$).

z	0.553 ± 0.013
a_s	0.685 ± 0.064
a_d	0.688 ± 0.059
$v_{3.2}$	0.324 ± 0.070
$v_{6.4}$	0.758 ± 0.104
$v_{12.8}$	1.806 ± 0.131
t_{ND}	0.176 ± 0.008
st_{ND}	0.120 ± 0.011

TABLE 5 | Model performance comparison via BIC and R^2 metrics (mean ± sd across participants).

Model	Total parameters	R^2	BIC
$Model_p$	7	0.836 ± 0.111	−26.726 ± 6.113
$Model_v$	10	0.951 ± 0.026	−29.902 ± 5.570
$Model_z$	8	0.965 ± 0.016	−34.858 ± 3.267
$Model_a$	8	0.843 ± 0.024	−25.406 ± 1.374

effect of decision bias will be twisted with the effect of the sensory adaptation through pooling the data of these two panels, and that is why the starting point in the same decision condition is not significantly higher than its value in the different decision condition. The significance of the differences between z_s and z_d was tested by the nonparametric bootstrap method (Efron and Tibshirani, 1994; Hinkley, 1998). These differences were quite significant ($p < 1.7 \times 10^{-6}$) for every five participants.

DISCUSSION

Our results showed, in sequential perceptual decisions, the probability of being correct in the current choice increases if it is similar to the previous one and conversely decreases when they are different. Although many studies suggested that sequential effects (Falmagne, 1965, 1968; Remington, 1969; Gold et al., 2008; Goldfarb et al., 2012) on decision processes are due to the motor response bias or sensory bias (Gold et al., 2008; Pearson and

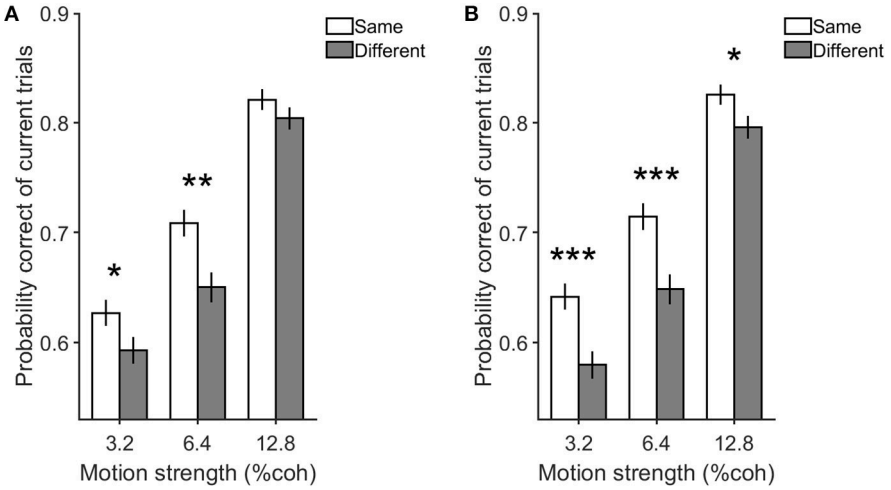


FIGURE 8 | Simulation and behavioral data for the previous decision effect on the current one in the same and different decision conditions. Either panel indicates the performance of the current trials with motion strengths of 3.2, 6.4, and 12.8% when previous trials have low motion strengths of 0 and 3.2%. **(A)** is the simulation data by model$_Z$ and **(B)** is the experimental data. Error bars indicate SE (Standard Error). Wilcoxon rank-sum test is used to test the significance of the differences, $^*p < 0.05$, $^{**}p <$ 1E−2, $^{***}p <$ 1E−3.

Brascamp, 2008; Albright, 2012; Carnevale et al., 2012; Marcos et al., 2013), Akaishi et al. showed that this decision history effect cannot be defined through these biases, as well as it can be explained by an autonomous learning rule to estimate the likelihood of a choice to be made (Akaishi et al., 2014). Besides, considering the fact that the firing rate of decision maker neurons cannot meet their baseline activity immediately after the decision (Cook and Maunsell, 2002; Roitman and Shadlen, 2002; Gold and Shadlen, 2007; Ratcliff et al., 2007; Churchland et al., 2008; Kiani et al., 2008; Heitz and Schall, 2012), we hypothesized that the bound crossing in the previous decision provides information which affects the state of decision variable in the subsequent decision.

To verify this assertion, we presented the results of a behavioral study of decision-making using 2AFC paradigm based on randomly moving dots with fixed duration and short interval time, focusing on sequences of two trials. To study the potentially plausible mechanisms accounted for the variations in the probability of correct due to the sequential effect (Falmagne, 1965, 1968; Remington, 1969; Ratcliff, 1985; Luce, 1986; Ratcliff et al., 1999; Ratcliff and Smith, 2004), we extended the pure DDM (Ratcliff, 1978, 2002; Ratcliff and Tuerlinckx, 2002; Bogacz et al., 2006). In the extended versions of DDM, different free parameters of the model were depended on the previous decision. We also indicated the model with dependent baseline has the best explanation for the observed changes in participants' performance for the same and different decision conditions. The results supported our hypothesis that the state of decision variable at the beginning of the information accumulation is being affected by the decision in the previous trial.

It should be noted that, to avoid increasing the time between consecutive decisions, we utilized fixed duration task which had fixed period for each part of a trial and limited Go signal. Indeed, we tried to prevent lengthening the time between previous bound crossing and start of the current decision process for the sake of preserving the previous decision effect. Nevertheless, we recorded the response time (time elapsed from Go signal onset to a hand key-press) besides the choice accuracy in our experiment. As shown in **Figure 9**, response times decreased with increasing strength of motions (Link, 1992; Roitman and Shadlen, 2002; Ratcliff and Smith, 2004), and were used as the input data of the models in addition to the current choice accuracy, current stimulus strength, and previous decision although there was no significant difference in them in different decision conditions due to fixed duration task.

With respect to all results elaborated on this study, in a comparative approach, we investigated the sequential effect on the probability of being correct in the current decision in contrast to what Akaishi et al. (2014) indicated the impact of the previous decision on the choice repetition probability. In addition, they did not use feedback in their experiments and declared that the mechanism which is associated with making an incorrect choice rather than recognition of an error is responsible for the decision bias (Akaishi et al., 2014), whereas we claimed that the decision, independent of the correctness and having positive or negative feedback, affects the probability of being correct in the next decision (as shown in **Figure 7**). Consequently, to support this statement we did another analysis by separating correct and incorrect previous trials with 0% motion strength in both the same and different conditions. Actually, we duplicated **Figure 7** only for 0% coherent motion of previous trials (See the **Figure S2**). In these trials, all dots had random movements which prevented the sensory bias in any particular direction and feedback was given randomly to the participants, independently of whether they pressed the left or the right key. So, the participants received positive feedback on 50% of the trials

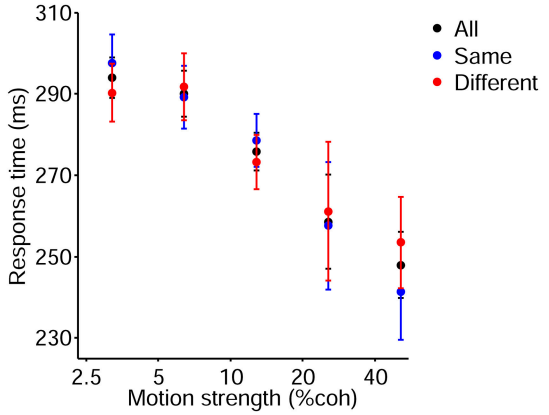


FIGURE 9 | Psychometric function of the current trials which includes motion strengths of 3.2, 6.4, 12.8, 25.6, and 51.2%. Red and blue data points depict response time of participants for the different and same decision conditions, respectively. Black data points are pooled from these two conditions. Error bars indicate SE (Standard Error).

(**Figure S2A**) and negative feedback on the other 50% of the trials (**Figure S2B**). As demonstrated in **Figure S2**, similar decision trials are significantly more probable to be correct than different decision trials regardless of the previously received feedback.

Since there is a clear feedback after each trial one may conclude that the main finding is due the win-stay lose-switch strategy where subjects tend to repeat their decision after a receiving a correct feedback and tend to switch their decision after receiving a wrong feedback. However, as shown in **Figure 7** same decisions have higher performance than different decisions for both correct and wrong previous trials. Thus, the effect is not due to the win-stay lose-switch strategy.

Our results can rule out the effect of sensory bias in three ways: (1) We discussed that there is a sensory bias in our results but that is in the opposite direction of our main effect. We showed that for the strong previous stimulus the effect is diminished. Moreover, the main effect is strongest when the previous stimulus is 0% which is not expected due to a sensory bias. (2) In the modeling part, we examined a model with different drift rates as a model for sensory bias but it cannot better explain the data than the other model. (3) We stated that the stimulus duration does not change the main effect which is in contrast to our expectation of the sensory bias.

Furthermore, we designed a control experiment to dissociate the effect of the previous decision from the motor response bias. In this experiment, the relationship between the decision and motor response is altered pseudorandomly across trials. Accordingly, one of the participants performed a version of the main task in which the right and leftward arrows were used above and below the fixation point as the choice targets (see the **Figure S3**). The arrangement of these two arrows changed pseudorandomly across trials. The participant was asked to report her decision about the direction of motion by pressing the upper and lower buttons, which arranged vertically and correspond to the position of the arrows, with the right middle and index fingers, respectively. Three thousand and six hundred trials (in 4 blocks $\times$ 6 sessions) were collected. As shown in **Figure S4**, the probability of being correct is significantly larger in the same decision condition compared to the different decision condition, even when the participant made a different motor response to report her perceived motion direction. In consequence, the motor response bias cannot account for the previous decisions' effect.

Although Equation 5, as a simple regression, illustrated that the strength of the stimulus in the previous trial does not affect the performance of current trial (Equation 5, $\beta_p = -0.003 \pm 0.002$, $p = 0.017$), separating high and low motion strengths in previous trials demonstrated that the probability correct of current trial is influenced by the previous decision. As a result, it suggests that the sequential effect should be considered in the perceptual decision-making tasks. For instance, the time between consecutive trials should be adjusted properly to keep down the previous decision effect. Two main contributions of the observed sequential effect are emphasized here. First, it originated from the previous decision which was made about the weak stimulus. The analysis of previous trials which consisted of 0% motion strength (**Figure S2**) showed that not only this sequential effect cannot be defined by the sensory bias, but also it stems from the previous decision which affects the parameters of evidence accumulation in the current decision. Second, the feedback did not play a key role in the effect of previous decisions, since the changes of current decision parameters were independent of the participants' awareness of their correct and incorrect previous decisions.

AUTHOR CONTRIBUTIONS

FO has contributed to the conception and study design, data collection, data analysis and interpretation, statistical analysis, modeling and drafting. MT-M has contributed to the study design, data collection, interpretation of data and drafting. SZ has contributed to the study design, drafting, modeling and interpretation of data. RE has contributed to the design of the work and interpretation of data. All authors have approved this final version of the manuscript to be published.

FUNDING

This work was partially supported by the Cognitive Sciences and Technologies Council under contract number 4608, and Institute for Research in Fundamental Sciences (IPM)-School of Cognitive Sciences (SCS). We are thankful to Amirhossein Farzmahdi, Hamed Nili, and Abdolhossein Vahabie for helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00009/full#supplementary-material

REFERENCES

Akaishi, R., Umeda, K., Nagase, A., and Sakai, K. (2014). Autonomous mechanism of internal choice estimate underlies decision inertia. *Neuron* 81, 195–206. doi: 10.1016/j.neuron.2013.10.018

Albright, T. D. (2012). On the perception of probable things: neural substrates of associative memory, imagery, and perception. *Neuron* 74, 227–245. doi: 10.1016/j.neuron.2012.04.001

Ashby, F. G. (1983). A biased random walk model for two choice reaction times. *J. Math. Psychol.* 27, 277–297. doi: 10.1016/0022-2496(83)90011-1

Awh, E., Belopolsky, A. V., and Theeuwes, J. (2012). Top-down versus bottom-up attentional control: a failed theoretical dichotomy. *Trends Cogn Sci.* 16, 437–443. doi: 10.1016/j.tics.2012.06.010

Balci, F., and Simen, P. (2014). Decision processes in temporal discrimination. *Acta Psychol.* 149, 157–168. doi: 10.1016/j.actpsy.2014.03.005

Basten, U., Biele, G., Heekeren, H. R., and Fiebach, C. J. (2010). How the brain integrates costs and benefits during decision making. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21767–21772. doi: 10.1073/pnas.0908104107

Becker, S. I. (2008). Can intertrial effects of features and dimensions be explained by a single theory? . *J. Exp. Psychol. Hum. Percept. Perform.* 34, 1414–1440. doi: 10.1037/a0011386

Boettiger, C. A., Mitchell, J. M., Tavares, V. C., Robertson, M., Joslyn, G., D'Esposito, M., et al. (2007). Immediate reward bias in humans: fronto-parietal networks and a role for the catechol-O-methyltransferase 158(Val/Val) genotype. *J. Neurosci.* 27, 14383–14391. doi: 10.1523/JNEUROSCI.2551-07.2007

Bogacz, R., Brown, E., Moehlis, J., Holmes, P., and Cohen, J. D. (2006). The physics of optimal decision making: a formal analysis of models of performance in two-alternative forced-choice tasks. *Psychol. Rev.* 113, 700–765. doi: 10.1037/0033-295X.113.4.700

Bornstein, A. M., Aly, M., Feng, S., Turk-Browne, N. B., Norman, K. A., and Cohen, J. D. (2017). Perceptual decisions result from the continuous accumulation of memory and sensory evidence. *bioRxiv [Preprint]*. doi: 10.1101/186817

Brainard, D. H. (1997). The psychophysics toolbox. *Spat. Vis.* 10, 433–436. doi: 10.1163/156856897X00357

Brehm, J. W. (1956). Postdecision changes in the desirability of alternatives. *J. Abnorm. Psychol.* 52, 384–389. doi: 10.1037/h0041006

Carnevale, F., de Lafuente, V., Romo, R., and Parga, N. (2012). Internal signal correlates neural populations and biases perceptual decision reports. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18938–18943. doi: 10.1073/pnas.1216799109

Churchland, A. K., Kiani, R., and Shadlen, M. N. (2008). Decision-making with multiple alternatives. *Nat. Neurosci.* 11, 693–702. doi: 10.1038/nn.2123

Cook, E. P., and Maunsell, J. H. (2002). Attentional modulation of behavioral performance and neuronal responses in middle temporal and ventral intraparietal areas of macaque monkey. *J. Neurosci.* 22, 1994–2004. doi: 10.1523/JNEUROSCI.22-05-01994.2002

Diederich, A., and Busemeyer, J. R. (2006). Modeling the effects of payoff on response bias in a perceptual discrimination task: bound-change, drift-rate-change, or two-stage-processing hypothesis. *Percept. Psychophys.* 68, 194–207. doi: 10.3758/BF03193669

Dully, J., McGovern, D. P., and O'Connell, R. G. (2018). The impact of natural aging on computational and neural indices of perceptual decision making: a review. *Behav. Brain Res.* 355, 48–55. doi: 10.1016/j.bbr.2018.02.001

Edwards, W. (1965). Optimal strategies for seeking information: models for statistics, choice reaction times, and human information processing. *J. Math. Psychol.* 2, 312–329. doi: 10.1016/0022-2496(65)90007-6

Efron, B., and Tibshirani, R. J. (1994). *An Introduction to the Bootstrap*. Boca Raton, FL: CRC press.

Falmagne, J. C. (1965). Stochastic models for choice reaction time with applications to experimental results. *J. Math. Psychol.* 12, 77–124. doi: 10.1016/0022-2496(65)90018-0

Falmagne, J. C. (1968). Note on a simple fixed-point property of binary mixtures. *Br. J. Math. Stat. Psychol.* 21, 131–132. doi: 10.1111/j.2044-8317.1968.tb00403.x

Fleming, S. M., Thomas, C. L., and Dolan, R. J. (2010a). Overcoming status quo bias in the human brain. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6005–6009. doi: 10.1073/pnas.0910380107

Fleming, S. M., Whiteley, L., Hulme, O. J., Sahani, M., and Dolan, R. J. (2010b). Effects of category-specific costs on neural systems for perceptual decision-making. *J. Neurophysiol.* 103, 3238–3247. doi: 10.1152/jn.01084.2009

Forstmann, B. U., Brown, S., Dutilh, G., Neumann, J., and Wagenmakers, E. J. (2010). The neural substrate of prior information in perceptual decision making: a model-based analysis. *Front. Hum. Neurosci.* 4:40. doi: 10.3389/fnhum.2010.00040

Glimcher, P. W. (2003). The neurobiology of visual-saccadic decision making. *Annu. Rev. Neurosci.* 26, 133–179. doi: 10.1146/annurev.neuro.26.010302.081134

Gold, J. I., Law, C. T., Connolly, P., and Bennur, S. (2008). The relative influences of priors and sensory evidence on an oculomotor decision variable during perceptual learning. *J. Neurophysiol.* 100, 2653–2668. doi: 10.1152/jn.90629.2008

Gold, J. I., and Shadlen, M. N. (2007). The neural basis of decision making. *Annu. Rev. Neurosci.* 30, 535–574. doi: 10.1146/annurev.neuro.29.051605.113038

Goldfarb, S., Wong-Lin, K., Schwemmer, M., Leonard, N. E., and Holmes, P. (2012). Can post-error dynamics explain sequential reaction time patterns? *Front. Psychol.* 3:213. doi: 10.3389/fpsyg.2012.00213

Hanks, T. D., Mazurek, M. E., Kiani, R., Hopp, E., and Shadlen, M. N. (2011). Elapsed decision time affects the weighting of prior probability in a perceptual decision task. *J. Neurosci.* 31, 6339–6352. doi: 10.1523/JNEUROSCI.5613-10.2011

Hanks, T. D., and Summerfield, C. (2017). Perceptual decision making in rodents, monkeys, and humans. *Neuron* 93, 15–31. doi: 10.1016/j.neuron.2016.12.003

Heitz, R. P., and Schall, J. D. (2012). Neural mechanisms of speed-accuracy tradeoff. *Neuron* 76, 616–628. doi: 10.1016/j.neuron.2012.08.030

Hinkley, D. V. (1998). Bootstrap methods. *J. R. Stat. Soc. Series B Stat. Methodol.* 50, 321–337.

Izuma, K., and Murayama, K. (2013). Choice-induced preference change in the free-choice paradigm: a critical methodological review. *Front. Psychol.* 4:41. doi: 10.3389/fpsyg.2013.00041

Kass, R., and Wasserman, L. (1995). A reference Bayesian test for nested hypotheses and its relationship to the Schwarz criterion. *J. Am. Stat. Assoc.* 90, 928–934. doi: 10.1080/01621459.1995.10476592

Kiani, R., Hanks, T. D., and Shadlen, M. N. (2008). Bounded integration in parietal cortex underlies decisions even when viewing duration is dictated by the environment. *J. Neurosci.* 28, 3017–3029. doi: 10.1523/JNEUROSCI.4761-07.2008

Kim, T. D., Kabir, M., and Gold, J. I. (2017). Coupled decision processes update and maintain saccadic priors in a dynamic environment. *J. Neurosci.* 37, 3632–3645. doi: 10.1523/JNEUROSCI.3078-16.2017

Kohn, A. (2007). Visual adaptation: physiology, mechanisms, and functional benefits. *J. Neurophysiol.* 97, 3155–3164. doi: 10.1152/jn.00086.2007

Laming, D. R. J. (1968). *Information Theory of Choice-Reaction Times*. New York, NY: Academic Press

Lauwereyns, J. (2010). *The Anatomy of Bias: How Neural Circuits Weigh The Options*. Cambridge, MA: MIT Press.

Lerche, V., and Voss, A. (2017). Retest reliability of the parameters of the Ratcliff diffusion model. *Psychol. Res.* 81, 629–652. doi: 10.1007/s00426-016-0770-5

Liddle, A. R. (2007). Information criteria for astrophysical model selection. *Mon. Not. R. Astron. Soc. Lett.* 377, L74–L78. doi: 10.1111/j.1745-3933.2007.00306.x

Link, S. W. (1992). *The Wave Theory of Difference And Similarity*. Hillsdale, NJ: Psychology Press.

Link, S. W., and Heath, R. A. (1975). A sequential theory of psychological discrimination. *Psychometrika* 40, 77–105.

Luce, R. D. (1986). *Response Times*. New York, NY: Oxford University Press.

Marcos, E., Pani, P., Brunamonti, E., Deco, G., Ferraina, S., and Verschure, P. (2013). Neural variability in premotor cortex is modulated by trial history and predicts behavioral performance. *Neuron* 78, 249–255. doi: 10.1016/j.neuron.2013.02.006

MATLAB, R. (2013). *Version 8.1. 0.604 (R2013a)*. Natrick, MA: The MathWorks Inc.

Mazurek, M. E., Roitman, J. D., Ditterich, J., and Shadlen, M. N. (2003). A role for neural integrators in perceptual decision making. *Cereb. Cortex* 13, 1257–1269. doi: 10.1093/cercor/bhg097

Miller, K., Shenhav, A., and Ludvig, E. (2017). Habits without values. *bioRxiv [Preprint]*. doi: 10.1101/067603

Mulder, M. J., Wagenmakers, E. J., Ratcliff, R., Boekel, W., and Forstmann, B. U. (2012). Bias in the brain: a diffusion model analysis of prior probability and potential payoff. *J. Neurosci.* 32, 2335–2343. doi: 10.1523/JNEUROSCI.4156-11.2012

Pearson, J., and Brascamp, J. (2008). Sensory memory for ambiguous vision. *Trends Cogn. Sci.* 12, 334–341. doi: 10.1016/j.tics.2008.05.006

Pelli, D. G. (1997). The videotoolbox software for visual psychophysics: transforming numbers into movies. *Spat. Vis.* 10, 437–442. doi: 10.1163/156856897X00366

Philiastides, M. G., Biele, G., and Heekeren, H. R. (2010). A mechanistic account of value computation in the human brain. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9430–9435. doi: 10.1073/pnas.1001732107

Preuschhof, C., Schubert, T., Villringer, A., and Heekeren, H. R. (2010). Prior Information biases stimulus representations during vibrotactile decision making. *J. Cogn. Neurosci.* 22, 875–887. doi: 10.1162/jocn.2009.21260

Purcell, B. A., and Kiani, R. (2016). Neural mechanisms of post-error adjustments of decision policy in parietal cortex. *Neuron* 89, 658–671. doi: 10.1016/j.neuron.2015.12.027

Ratcliff, R. (1978). A theory of memory retrieval. *Psychol. Rev.* 85, 59–108. doi: 10.1037/0033-295X.85.2.59

Ratcliff, R. (1985). Theoretical interpretations of the speed and accuracy of positive and negative responses. *Psychol. Rev.* 92, 212–225. doi: 10.1037/0033-295X.92.2.212

Ratcliff, R. (2002). A diffusion model account of response time and accuracy in a brightness discrimination task: fitting real data and failing to fit fake but plausible data. *Psychon. Bull. Rev.* 9, 278–291. doi: 10.3758/BF03196283

Ratcliff, R., Hasegawa, Y. T., Hasegawa, R. P., Smith, P. L., and Segraves, M. A. (2007). Dual diffusion model for single-cell recording data from the superior colliculus in a brightness-discrimination task. *J. Neurophysiol.* 97, 1756–1774. doi: 10.1152/jn.00393.2006

Ratcliff, R., and McKoon, G. (2008). The diffusion decision model: theory and data for two-choice decision tasks. *Neural Comput.* 20, 873–922. doi: 10.1162/neco.2008.12-06-420

Ratcliff, R., and Rouder, J. (1998). Modeling response times for two-choice decisions. *Psychol.Sci.* 9, 347–356. doi: 10.1111/1467-9280.00067

Ratcliff, R., and Smith, P. L. (2004). A comparison of sequential sampling models for two-choice reaction time. *Psychol. Rev.* 111, 333–367. doi: 10.1037/0033-295X.111.2.333

Ratcliff, R., Smith, P. L., Brown, S. D., and McKoon, G. (2016). Diffusion decision model: current issues and history. *Trends Cogn. Sci.* 20, 260–281. doi: 10.1016/j.tics.2016.01.007

Ratcliff, R., and Tuerlinckx, F. (2002). Estimating parameters of the diffusion model: approaches to dealing with contaminant reaction times and parameter variability. *Psychon. Bull. Rev.* 9, 438–481. doi: 10.3758/BF03196302

Ratcliff, R., Van Zandt, T., and McKoon, G. (1999). Connectionist and diffusion models of reaction time. *Psychol. Rev.* 106, 261–300. doi: 10.1037/0033-295X.106.2.261

Remington, R. J. (1969). Analysis of sequential effects in choice reaction times. *J. Exp. Psychol.* 82, 250–257. doi: 10.1037/h0028122

Roitman, J. D., and Shadlen, M. N. (2002). Response of neurons in the lateral intraparietal area during a combined visual discrimination reaction time task. *J. Neurosci.* 22, 9475–9489. doi: 10.1523/JNEUROSCI.22-21-09475.2002

Rorie, A. E., Gao, J., McClelland, J. L., and Newsome, W. T. (2010). Integration of sensory and reward information during perceptual decision-making in lateral intraparietal cortex (LIP) of the macaque monkey. *PLoS ONE* 5:e9308. doi: 10.1371/journal.pone.0009308

Scheibe, C., Ullsperger, M., Sommer, W., and Heekeren, H. R. (2010). Effects of parametrical and trial-to-trial variation in prior probability processing revealed by simultaneous electroencephalogram/functional magnetic resonance imaging. *J. Neurosci.* 30, 16709–16717. doi: 10.1523/JNEUROSCI.3949-09.2010

Serences, J. T. (2008). Value-based modulations in human visual cortex. *Neuron* 60, 1169–1181. doi: 10.1016/j.neuron.2008.10.051

Shadlen, M. N., Hanks, T. D., Churchland, A. K., Kiani, R., and Yang, T. (2006). "The speed and accuracy of a simple perceptual decision: a mathematical primer," in *Bayesian Brain: Probabilistic Approaches to Neural Coding*, eds K. Doya, S. Ishii, A. Pouget, and R. Rao (Cambridge, MA: MIT Press), 209–237.

Shadlen, M. N., and Newsome, W. T. (2001). Neural basis of a perceptual decision in the parietal cortex (area LIP) of the rhesus monkey. *J. Neurophysiol.* 86, 1916–1936. doi: 10.1152/jn.2001.86.4.1916

Sigurdardottir, H. M., Kristjánsson, Á., and Driver, J. (2008). Repetition streaks increase perceptual sensitivity in brief visual search displays. *Vis. Cogn.* 16, 643–658. doi: 10.1080/13506280701218364

Simen, P., Cohen, J. D., and Holmes, P. (2006). Rapid decision threshold modulation by reward rate in a neural network. *Neural Netw.* 19, 1013–1026. doi: 10.1016/j.neunet.2006.05.038

Smith, A. F., and Spiegelhalter, D. J. (1980). Bayes factors and choice criteria for linear models. *J. R. Stat. Soc. Series B Stat. Methodol.* 42, 213–220. doi: 10.1111/j.2517-6161.1980.tb01122.x

Summerfield, C., and Koechlin, E. (2008). A neural representation of prior information during perceptual inference. *Neuron* 59, 336–347. doi: 10.1016/j.neuron.2008.05.021

Summerfield, C., and Koechlin, E. (2010). Economic value biases uncertain perceptual choices in the parietal and prefrontal cortices. *Front. Hum. Neurosci.* 4:208. doi: 10.3389/fnhum.2010.00208

Tohidi-Moghaddam, M., Zabbah, S., and Ebrahimpour, R. (2016). The role of the primary information on importance of the last information in decision making. *Neurosci. J. Shefaye Khatam.* 4, 26–34. doi: 10.18869/acadpub.shefa.4.4.26

Voss, A., Nagler, M., and Lerche, V. (2013). Diffusion models in experimental psychology: a practical introduction. *Exp. Psychol.* 60, 385–402. doi: 10.1027/1618-3169/a000218

Voss, A., Rothermund, K., and Voss, J. (2004). Interpreting the parameters of the diffusion model: an empirical validation. *Mem. Cognit.* 32, 1206–1220. doi: 10.3758/BF03196893

Voss, A., and Voss, J. (2007). Fast-dm: a free program for efficient diffusion model analysis. *Behav. Res. Methods* 39, 767–775. doi: 10.3758/BF03192967

Wagenmakers, E. J., Ratcliff, R., Gomez, P., and McKoon, G. (2008). A diffusion model account of criterion shifts in the lexical decision task. *J. Mem. Lang.* 58, 140–159. doi: 10.1016/j.jml.2007.04.006

White, C. N., and Poldrack, R. A. (2014). Decomposing bias in different types of simple decisions. *J. Exp. Psychol. Learn. Mem. Cogn.* 40, 385–398. doi: 10.1037/a0034851

Conflict of Interest Statement: 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: 15 February 2019
doi: 10.3389/fnbeh.2019.00027

Environmental Enrichment During Adulthood Reduces Sucrose Binge-Like Intake in a High Drinking in the Dark Phenotype (HD) in C57BL/6J Mice

*Elisa Rodríguez-Ortega[1], Manuel Alcaraz-Iborra[1], Leticia de la Fuente[1], Enedina de Amo[1] and Inmaculada Cubero[1,2]**

[1]*Departamento de Psicología, Faculty of Education Sciences, University of Almería, Almería, Spain,* [2]*CERNEP, Universidad de Almería, Almería, Spain*

Repetitive binge episodes favor transition to binge-eating disorders. Experimental evidence points to positive influence of environmental enrichment (EE) on drug/food addiction, although far less is known regarding EE effects over binge-like consumption. Here, we evaluate the following: (1) whether switching from nonenriched standard environment (SE) to EE housing conditions during adulthood alters a stable pattern of voluntary sucrose (10% w/v) binge-like intake in high (HD) vs. low (LD) drinking phenotypes under a drinking in the dark (DID) schedule; and (2) sucrose binge-like intake in a DID task in response to a pharmacological challenge with an OXr1 antagonist in HD/LD subpopulations after long-term exposure to SE or EE conditions. Adolescent (postnatal day 21; PND21) mice were housed in SE conditions. At PND65, all animals were long-term exposed to sucrose DID. On the first episode of DID (PND65), animals were divided into HD vs. LD subpopulations according to their sucrose intake. On PND85, an OXr1 antagonist test was conducted on HD and LD mice with SB-334867 (SB) administration. On PND95, HD and LD subpopulations were again randomly allocated into two subgroups, resulting in the following experimental conditions: HD-SE, HD-EE, LD-SE and LD-EE. Sucrose binge-like intake continued until PND116, when a second SB test was conducted. The main findings are: (1) a single 2 h episode of sucrose binge drinking in a DID procedure consistently segregates two behavioral subpopulations, HD and LD; (2) when adult mice in standard conditions and long-term exposed to sucrose DID were switched to EE conditions, an immediate reduction in sucrose binge-like intake was observed in HD mice, pointing to a therapeutic role of EE exposure; and (3) administration of the OXr1 antagonist caused an acute reduction in sucrose binge-like intake in HD and LD mice exposed to SE conditions. Importantly, exposure to EE conditions blunted the inhibitory effect of SB on sucrose binge consumption in both behavioral phenotypes, indirectly suggesting a potential EE/OXr1 signaling interaction. We propose the hypothesis that EE might regulate OX-dependent anxiety/compulsivity brain systems, which might secondarily modulate sucrose binge-like intake.

Keywords: environmental enrichment, drinking in the dark, sucrose, HD/LD phenotypes, orexin receptor 1

OPEN ACCESS

Edited by:
Walter Adriani,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
Amy Lasek,
University of Illinois at Chicago, United States
Marise B. Parent,
Georgia State University, United States

****Correspondence:***
Inmaculada Cubero
icubero@ual.es

Received: *24 September 2018*
Accepted: *30 January 2019*
Published: *15 February 2019*

Citation:
Rodríguez-Ortega E, Alcaraz-Iborra M, de la Fuente L, de Amo E and Cubero I (2019) Environmental Enrichment During Adulthood Reduces Sucrose Binge-Like Intake in a High Drinking in the Dark Phenotype (HD) in C57BL/6J Mice. Front. Behav. Neurosci. 13:27. doi: 10.3389/fnbeh.2019.00027

INTRODUCTION

Binge eating disorder (BED) is a severe, life-threatening, addiction spectrum disorder (American Psychiatric Association, 2013) included in the Diagnostic and Statistical Manual of Mental Disorders (DMS-V) that is characterized by recurrent episodes of eating large quantities of food in a discrete period of time (American Psychiatric Association, 2013). BED is the most common eating disorder in the United States, and its study is of major concern given BED comorbidity with obesity (Grucza et al., 2007). Repetitive binge episodes are a common pattern exhibited during early stages in the addiction cycle (Koob and Volkow, 2009), and it has been proposed that repetitive binge eating episodes might favor transition to binge-eating disorders and "food addiction" (Avena, 2010; Cowin et al., 2011; Smith and Robbins, 2013). Therefore, it is important a further knowledge of the neurobehavioral and neurochemical mechanisms involved in repetitive binge eating in order to develop innovative behavioral and pharmacological strategies that may protect binge eaters from transition to eating disorders and food addiction.

Recent growing experimental evidence has showed that environmental enrichment (EE), a preclinical model where animals are exposed to housing conditions with access to exercise, novelty and social interaction (Crofton et al., 2015), which facilitates motor, cognitive and sensory stimulation, may have positive consequences for multiple pathological processes, such as drug addiction (Nithianantharajah and Hannan, 2006; Stairs and Bardo, 2009; Solinas et al., 2010), ethanol consumption, ethanol self-administration (SA) and ethanol reward (Deehan et al., 2007, 2011; de Carvalho et al., 2010; Lopez et al., 2011; Li et al., 2015; Lopez and Laber, 2015; Bahi, 2017; Marianno et al., 2017). Additionally, recent studies suggests that EE might also protect and have therapeutic modulatory effects over the emergence of binge-like consumption. Thus, social and EE reduced ethanol binge-like consumption and preference in C57BL/6 mice in a modified two-bottle choice drinking in the dark task (DID-2BC; Marianno et al., 2017), and 40 days of social housing after weaning caused decreased ethanol binge-like intake in C57BL/6J mice in a DID-2BC schedule when compared with mice in isolated conditions (Lopez et al., 2011). Similarly, EE exposure effectively blunted excessive ethanol binge-like consumption in C57BL/6J mice chronically housed in social isolation conditions (Lopez and Laber, 2015). Our laboratory showed that rearing mice in EE during adolescence effectively reduces high ethanol binge-like intake during adulthood, as measured by an intermittent DID (iDID) procedure, along with reducing anxiety-, compulsivity-like responses and novelty-seeking behaviors, as measured by the hole board (HB) and the elevated plus maze (EPM) tests (Rodríguez-Ortega et al., 2018). Additionally, EE shows therapeutic effects in drug intake by dependent animals (for review, see Solinas et al., 2010). EE also shows protective and therapeutic effects in ethanol binge-like consumption (Lopez et al., 2011; Lopez and Laber, 2015; Marianno et al., 2017; Rodríguez-Ortega et al., 2018).

Recent studies have provided additional evidence that enriched housing conditions modulate consumption of sucrose, a highly palatable substance. Thus, Lister hooded rats reared in social conditions significantly consumed less sucrose in a 2BC schedule than their littermates reared in isolation (Hall et al., 1997); Sprague-Dawley rats housed in isolation conditions significantly drunk more sucrose in 2BC paradigm than their counterparts housed in enriched or social conditions (Brenes and Fornaguera, 2008); sucrose conditioned place preference (CPP) was higher in Wistar rats housed in isolation conditions when compared with rats in social housing conditions (Van den Berg et al., 1999); EE housed Sprague-Dawley rats showed less sensitization to sucrose paired with a cue-light reinforcer that rats housed under social or isolation conditions (Gill and Cain, 2011). Similarly, EE housed Sprague-Dawley rats exhibited enhanced extinction of sucrose-maintained lever presses than rats housed in isolation as measured by a continuous reinforcement paradigm (Stairs et al., 2006). Furthermore, EE exposure reduced sucrose seeking in Long-Evans rats trained for sucrose SA (Grimm et al., 2008); Long-Evans rats chronically or acutely exposure to EE conditions showed blunted sucrose consumption and cue reactivity (Grimm et al., 2013, 2016). Taken together, evidences from recent studies have shown that EE could modulate sucrose seeking and consumption; nevertheless, little is known about EE effects over sucrose binge-like consumption.

Given experimental evidence indicating that EE ameliorates sucrose preference, sucrose intake and sucrose seeking in preclinical models (Stairs et al., 2006; Brenes and Fornaguera, 2008; Grimm et al., 2008, 2013, 2016; Gill and Cain, 2011), we hypothesized that EE might also have a preventive and therapeutic impact on sucrose binge-like consumption as measured by a DID procedure. The first objective in this study addresses the ability of EE to modulate sucrose binge-like intake, as measured by a long-term DID procedure. It is important to highlight that the selected long-term DID procedure fulfills the criteria for preclinical models of binge eating. Thus, the selected DID procedure triggers binge-like consumption of sucrose and saccharin (Lowery et al., 2008, 2010; Kaur et al., 2012) in *ad libitum-fed* animals in such a way that over a period of 2 h, mice drunk on average more than 50% of the total sucrose they usually consume over 24 h, under a 2BC unlimited access schedule (Sparta et al., 2008; Kaur et al., 2012; Alcaraz-Iborra et al., 2014). Preclinical models have been developed based on the DSM-5 criteria for human binge episodes, including eating during a short, discrete period of time larger amounts of food than the normal quantity of food consumed under the same circumstances during the same period of time (Corwin and Babbs, 2012; American Psychiatric Association, 2013). The scientific literature has additionally considered a 2 h binge episode to be an adequate amount of time to assess binging in both human (Wolfe et al., 2009) and rodent studies (Valdivia et al., 2014); during this period, binging animals should consume at least a twofold increase in calories compared with the control group (Perello et al., 2014). Finally, repetitive binge episodes should also be part of

animal models of binge eating, since they are a hallmark of BED or BN disorders (American Psychiatric Association, 2013; Perello et al., 2014).

It is worth noting that even though many individuals exposed to addictive substances might exhibit spontaneous binge-like episodes, only a small number of them would develop drug/food addiction and compulsive seeking behaviors after repeated binge-withdrawal-relapse cycles (Belin et al., 2016). Several authors have pointed to the utility of High vs. Low consumer subpopulations in preclinical and clinical studies for a better understanding of the neurobehavioral mechanisms underlying drug addiction and BEDs (for reviews, see Ramoz et al., 2007; Monteleone and Maj, 2008; Hutson et al., 2018). In this regard, we have provided recent behavioral, pharmacological and molecular evidence pointing to the relevance of using the High/Low drinker (HD/LD) subpopulations stemming from DID procedures to explore neurobehavioral processes underlying ethanol binge intake (Alcaraz-Iborra et al., 2017). We defend here the idea that DID might also successfully segregate two behavioral phenotypes (HD/LD) for sucrose binge-like intake. In the study, we comparatively evaluate whether switching from nonenriched standard environment (SE) to enriched environmental housing conditions during adulthood alters a stable pattern of voluntary sucrose binge-like intake as measured by the DID procedure in HD vs. LD subpopulations.

Recent research has underscored the capacity of orexin (OX) in mediating "food addiction," binge-like eating (Avena et al., 2008; Avena, 2010; Avena and Bocarsly, 2012), compulsive consumption of palatable food (Smith and Robbins, 2013) and binge-like intake of palatable caloric and noncaloric substances (Alcaraz-Iborra et al., 2014). Moreover, it has been suggested the involvement of OXr1 signaling in food-reinforced behaviors (Nair et al., 2008; Borgland et al., 2009; Cason and Aston-Jones, 2013), food seeking (Cason and Aston-Jones, 2013, 2014; Kay et al., 2014) and compulsive eating (see Merlo Pich and Melotto, 2014 ; for review, Piccoli et al., 2012). Our laboratory has provided additional evidence that OXr1 blockade significantly decreases sucrose and saccharin binge-like intake in a DID test in nondeprived animals; furthermore, exposure during 4 days to 2 h episodes of sucrose or saccharin binge-like intake significantly decreases expression of OX mRNA in the lateral hypothalamus (LH; Alcaraz-Iborra et al., 2014), indicating the role of OXr1 signaling in sucrose binge-like consumption. Bearing in mind experimental evidence suggesting a key role for OXr1 signaling in sucrose binge-like consumption in nondependent animals, the third objective in the study was aimed to provide preliminary pharmacological evidence regarding a potential EE/OX system interaction. For this purpose, we evaluated sucrose binge-like intake in a DID task in response to a pharmacological challenge with an OXr1 antagonist in HD/LD mice exposed long-term to EE vs. SE.

MATERIALS AND METHODS

Animals and Housing

Male young C57BL/6J mice (Charles River Laboratories, Spain S.A., Barcelona, Spain) arrived to the laboratory immediately after weaning (PND21) and were housed in in polycarbonate cages (50 × 15 × 25 cm) in groups of four animals (standard housing condition, SE) with stainless steel wire mesh lids and sawdust bedding. To help individual following across the study, each animal's tail was marked daily with a nontoxic marker. During the entire study, animals had *ad libitum* access to water and rodent chow. Room temperature was set at 21 ± 2°C in a 12:12 h light/dark schedule (lights on from 7 pm to 7 am). All procedures were approved by the Bioethical Animal Care Committee at the University of Almeria, Spain following the animal care guidelines established by the Spanish Royal Decree 53/2013 for reducing animal pain and discomfort.

Drugs

The OXr1 antagonist SB-334867 (SB; 1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloridre; Tocris, Bristol, UK) was suspended in 1.5% dimethyl sulfoxide (DMSO), 20% 2-hydroxypropyl-b-cyclodextrin (HBC) and sterile water. For the pharmacological tests, 30 min before the test 5 mg/kg were given intraperitoneally (ip) in a volume of 10 ml/kg (Alcaraz-Iborra et al., 2017). The vehicle solution was prepared with saline and 1.5% DMSO and given in a 10 ml/kg volume. The selected dose of SB effectively reduces ethanol binge-like drinking (Alcaraz-Iborra et al., 2017) and sucrose binge-like drinking (unpublished observation).

Behavioral Testing

Sucrose Binge-Like DID Procedure and Segregation of HD/LD Phenotypes

On PND65, animals were exposed to 20 consecutive episodes of 10% (w/v) sucrose binge-like DID as follows: 3 h into the dark phase, on days 1–20 animals were transported to individual polycarbonate cages, and a single bottle of 10% (w/v) sucrose was placed on cages for 2 h (see **Figure 1**). On day 1, HD/LD subpopulations (behavioral phenotypes) were identified in the DID session and segregated by sucrose consumption group median (above median for HD and below median for LD; Alcaraz-Iborra et al., 2017). During sessions, mice had *ad libitum* access to rodent chow, which was daily weighed. To subtract possible fluid spillage from total consumption, an empty cage on each self was used to place dummy bottles to measure lost fluid. Once each DID session ended, mice were returned to their house cages. Every 3 days, body weight (BW) was measured and recorded.

After exposure to 20 episodes of sucrose binge-like DID (PND85), a first pharmacological challenge (Test 1) with an intraperitoneal (ip) administration of the OXr1 antagonist SB (5 mg/kg) or vehicle was performed. Then, all the animals continued a daily sucrose binge-like DID schedule for 10 additional days (drug washout period from PND86 to PND95; see **Figure 1**).

Environmental Enrichment

Once the washout period ended (PND95), HD and LD mice were divided into two subgroups, according to sucrose solution intake, and assigned to one of two possible environmental housing conditions: enriched environment group (EE;

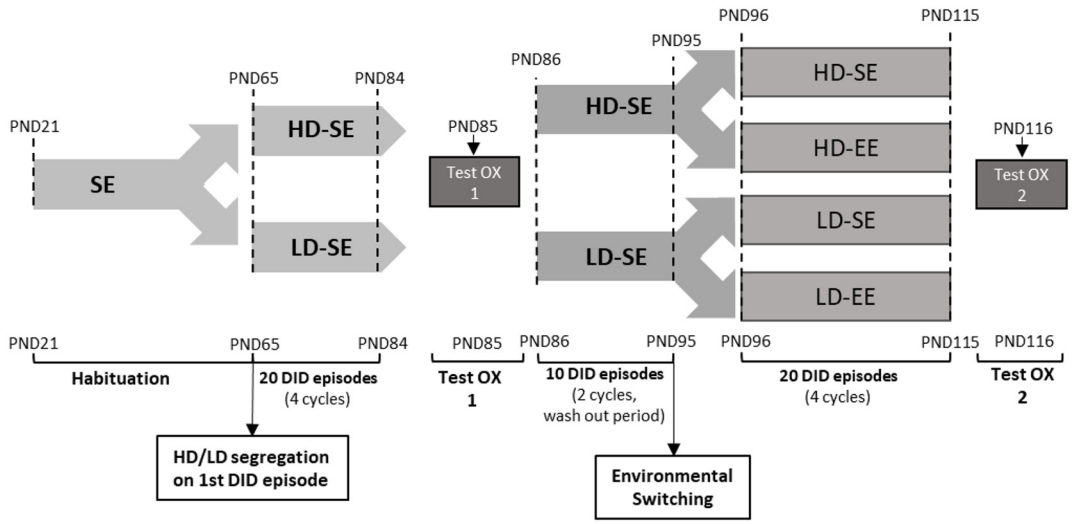


FIGURE 1 | The experimental design over time in the study. After arrival, on PND21, animals were housed in standard environment (SE) until adulthood (PND65). At PND65, mice were exposed to four consecutive drinking in the dark (DID) cycles (from PND65 to PND84). Each DID cycle is composed of five consecutive DID sessions, each one consisting of 2 h access to 10% (w/v) sucrose, 3 h into the dark stage. These DID cycles are repeated consecutively during the whole experiment. Importantly, the first DID session mice segregated High vs. Low DID subpopulations, according to whether their sucrose intake was above or below the median. High/Low drinker (HD/LD) segregation was maintained throughout the rest of the experiment. At PND85, a pharmacological test with SB (5 mg/kg) or vehicle was performed (test OX 1). The drug washout period, from PND86 to PND95, consisted of two additional cycles of DID. At the end of the washout period, on PND95, half of the animals switched their environmental conditions, and half maintained their original housing conditions, leading to four experimental groups: HD-SE, HD-environmental enrichment (EE), LD-SE and LD-EE. From PND96 to PND115, the animal experienced four after-switching additional cycles of sucrose binge-like DID. Finally, on PND116, a new pharmacological challenge with SB (5 mg/kg) or vehicle was performed in all four groups (test OX 2).

N = 14) and SE group (N = 16). Thus, four experimental groups, EE-HD; EE-LD; SE-HD and SE-LD, emerged, and HD/LD subpopulations were exposed to 20 additional consecutive episodes of sucrose binge-like DID in two different environmental housing conditions, EE vs. SE. Enriched conditions (EE) supposed social housing (four mice per cage) and included a plastic shelter and a mice running wheel permanently available. To maintain novelty, sets of three objects were included every 5 days. Objects consisted in carton tubes and cotton for nesting purposes, checkers tiles, plastic drinking glasses, ping pong balls and PVC tubes. Therefore, EE conditions consisted in access to 5 objects: two of them permanently available and three of them changed every 5 days. On the other hand, the SE maintained previous housing conditions from PND21 to PND95, which consisted of four mice in a cage that contained a single carton tube for nesting purposes.

Finally, on PND116 a second pharmacological challenge (Test 2) with the OX antagonist SB (5 mg/kg, ip) was administered to comparatively evaluate potential EE/OX interaction in HD vs. LD subpopulations (see **Figure 1** for details).

Data Analysis

In the present study, HD/LD subpopulations were selected according to the median of sucrose binge-like intake scores (g/kg/2 h) obtained during the first session of a DID procedure. Thus, mice whose sucrose consumption was above the median constituted the HD subpopulation, and mice whose sucrose consumption was below the median constituted the LD subpopulation (Alcaraz-Iborra et al., 2017). A one-way analyses of variance (ANOVA) evaluated subpopulation differences in sucrose consumption. Data for the average sucrose binge-like intake (g/kg/2 h) and average kilocalories ingested (kcal/g/2 h; chow + sucrose solution) that was obtained both before and after providing EE DID sessions to HD/LD animals were collapsed into 5-day cycles, except for the 10 days of the washout period that were not collapsed in order to evaluate SB washout daily progression. HD and LD sucrose binge-like intakes obtained during the first four cycles of DID before the pharmacological challenge were analyzed as data from before providing EE. Average sucrose binge-like intake and average kilocalories consumed during the additional 5-day cycles of DID after providing EE housing conditions on PND95 were also analyzed to evaluate the differential impact of EE introduction on sucrose binge-like DID consumption by HD and LD subpopulations. Subpopulation differences in average sucrose binge-like intake and kilocalorie consumption during DID sessions while housed in SE conditions prior to environmental switching were analyzed by independent repeated measures (2 × 4; subpopulation × cycle) ANOVAs (from PND65 to PND84; see **Figure 1**). Independent repeated measures (2 × 10; subpopulation × time) ANOVAs were conducted for sucrose binge-like intake during the washout period (from PND86 to PND95) in order to track consumption by HD/LD animals during that period. To compare sucrose/kcal intake before vs. after EE access, a time point represented by average sucrose/kcal consumed during the last day of the

washout period (PND95) was included in the analysis as a before EE estimation. Thus, to evaluate the impact of switching to an enriched environment on sucrose binge-like intake and kilocalorie consumption by HD/LD subpopulations (from PND96 to PND115; see **Figure 1**), independent repeated measures (2 × 2 × 5; environment × subpopulation × cycle) ANOVAs were performed. BW (g) was also recorded every 3 days, and independent repeated measures ANOVAs were performed for data from before EE (2 × 4; subpopulation × cycle) and after environmental switching (2 × 2 × 4; environment × subpopulation × cycle). All data collected in this study are presented as the mean ± SEM, and differences between and within groups were analyzed using ANOVA procedures. We assessed the assumptions of the ANOVA models on each set of data. The Shapiro-Wilk test of normality and Levene's test of homoscedasticity were used in all cases; Mauchly's test of sphericity was used when repeated measures variables were involved in the ANOVA procedure. Thus, when assumptions were violated, the Greenhouse-Geisser correction over the degrees of freedom was used to estimate both the repeated measures and interaction effects. For these results, we only report the violations of the assumptions, indicating the alternative test used when needed. Statistically significant interaction effects were analyzed by the simple effects analysis, and pairwise comparisons of means were performed using the Bonferroni correction. Additionally, in order to evaluate temporal dynamics of consumption over time, planned *t*-tests were performed across key cycles within each group, which allowed a general sight of sucrose consumption progression across the cycles in each group. Thus, we compared cycle 1 vs. 2 and cycle 2 vs. 4 for before EE conditions. We also compared the last washout time-point (PND95) vs. cycle 1, the washout time-point (PND95) vs. cycle 4, and cycle 2 vs. 4 during the conditions after environmental switching. Different error terms were used for each set of comparisons. Additional planned *t*-test comparisons compared binge-like sucrose consumption by vehicle- and SB-injected mice in HD/LD groups in Test 1 and Test 2. In all cases, $p < 0.05$ (two-tailed) was used as the level of statistical significance. Effect size estimates by η^2 (eta-squared) are reported for the significant effects; the effect size indicators provide an estimate of the magnitude of the relationship between the IV and DV that is independent of the sample size. All analyses were made using IBM SPSS (v.22).

RESULTS

Sucrose Binge-Like Intake by HD and LD Phenotypes in a Continued DID Schedule

Figure 2A depicts individual sucrose intake data (g/kg/2 h) in the first DID session relative to the median value. The DID procedure clearly segregated the population into two subpopulations for sucrose binge-like consumption, high vs. low drinkers; the one-way ANOVA conducted showed that HD mice consumed significantly more sucrose than their LD counterparts ($F_{(1,30)} = 68.845$, $p < 0.0001$).

Figure 2B shows sucrose binge-like intake by HD and LD subpopulations during the first four 5-day cycles, prior to providing EE, as measured in a DID procedure in SE housing condition (from PND65 to PND84). The assumptions analysis for mixed 2 × 4 ANOVA (subpopulation × cycle) showed that the assumption of sphericity, assessed by Mauchly's test of sphericity, had been violated ($\chi^2_{(5)} = 0.449$, $p = 0.001$). The results indicated statistically significant differences for the "cycle" main factor ($F_{(2.30,64.59)} = 30.61$, $p < 0.0001$; $\eta^2 = 0.52$) and the "subpopulation" main factor ($F_{(1,28)} = 7.15$, $p = 0.01$; $\eta^2 = 0.20$), indicating a stable lower sucrose consumption in LD vs. HD subpopulation over time.

An additional set of planned *t*-test comparisons permitted us to analyze the temporal consumption dynamic over cycles in each experimental group. Thus, the HD group showed statistically significant differences in sucrose consumption in cycle 1 vs. 2 ($t = -5.07$, $p < 0.001$; $df = 13$) but not for cycle 2 vs. 4 ($t = 0.69$,

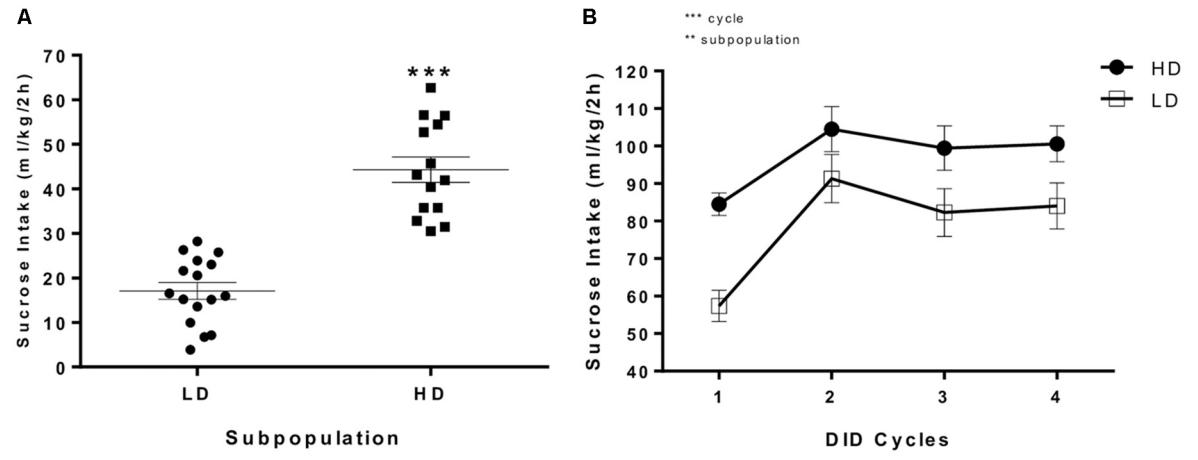


FIGURE 2 | (A) Individual 10% (w/v) sucrose binge-like intake (ml/kg/2 h) during the first DID session at PND65 in which HD and LD subpopulations were segregated and **(B)** average (means ± SEM) 10% (w/v) sucrose binge-like intake (ml/kg/2 h) during the first four DID cycles before providing EE (from PND65 to PND84) by HD and LD mice under SE housing conditions. $^{**}p < 0.01$, $^{***}p < 0.001$.

$p = 0.5$; $df = 13$), indicating that sucrose binge-like consumption in HD mice increased from cycle 1–2, reaching a plateau at this time point. For the LD group, planned *t*-tests showed statistically significant differences for cycle 1 vs. 2 ($t = -7.52$, $p < 0.001$; $df = 15$) but not for cycle 2 vs. 4 ($t = 1.73$, $p = 0.1$; $df = 15$), indicating that sucrose binge-like intake by LD mice also increased from cycle 1–2 and then stabilized.

For the kilocalorie intake data, Mauchly's test for the repeated measures ANOVA 2 × 4 (subpopulation × cycle) showed violation of the sphericity assumption (Mauchly's test: $\chi^2_{(5)} = 0.626$, $p = 0.02$). When animals were housed in SE conditions during four sucrose DID cycles (from PND65 to PND86), the ANOVA conducted on kilocalorie data consumed during the DID tests revealed that only the main factor "cycle" attained statistically significant differences ($F_{(2.28,63.88)} = 9.21$, $p < 0.0001$; $\eta^2 = 0.24$), indicating increased kilocalorie intake over time in both HD and LD mice (**Table 1**).

When BW was analyzed for the first four cycles, Mauchly's test for the repeated measures ANOVA 2 × 4 (subpopulation × cycle) showed violation of the sphericity assumption (Mauchly's test: $\chi^2_{(5)} = 0.122$, $p < 0.0001$). The results showed statistically significant differences for the main factor "cycle" ($F_{(1.46,41.06)} = 33.44$, $p < 0.0001$; $\eta^2 = 0.54$) and for the "cycle" × "subpopulation" interaction ($F_{(1.46,41.06)} = 4.28$, $p = 0.03$; $\eta^2 = 0.13$). Additional Bonferroni *post hoc* analyses for the interaction showed a progressive increase in BW in HD mice over time (cycle 1 vs. 2, $p = 0.01$; cycle 1 vs. 3, $p < 0.0001$; cycle 1 vs. 4, $p < 0.0001$). For BW in LD mice, the Bonferroni test showed differences in BW in cycle 1 vs. 2 ($p < 0.0001$) and 1 vs. 3 ($p < 0.0001$) but not 1 vs. 4, indicating that BW progressively increased in the HD but not the LD group over time (**Table 2**).

TABLE 1 | Average (means ± SEM) kilocalorie intake (g/kg) before and after providing environmental enrichment (EE) housing conditions in both High/Low drinker (HD and LD) subpopulations.

	DID cycles before providing EE			
Group	**1**	**2**	**3**	**4**
HD	1.91 ± 0.1	1.85 ± 0.12	2.11 ± 0.14	2.26 ± 0.09
LD	1.75 ± 0.1	1.67 ± 0.07	1.89 ± 0.1	1.99 ± 0.11
	DID cycles after providing EE			
	1	**2**	**3**	**4**
HD-SE	2.17 ± 0.15	2.03 ± 0.19	2.45 ± 0.23	2.25 ± 0.23
HD-EE	2.02 ± 0.16	1.45 ± 0.12	1.79 ± 0.12	1.55 ± 0.15
LD-SE	1.98 ± 0.23	1.77 ± 0.21	1.99 ± 0.19	1.75 ± 0.22
LD-EE	1.83 ± 0.21	1.42 ± 0.25	1.7 ± 0.27	1.55 ± 0.32

Top panel: average (means ± SEM) kilocalorie intake (g/kg) of HD and LD groups during the four drinking in the dark (DID) cycles before providing EE. Bottom panel: average (means ± SEM) kilocalorie intake of HD-standard environment (SE), HD-EE, LD-SE and LD-EE groups during the four DID cycles after providing EE.

TABLE 2 | Average (means ± SEM) body weight (g) before and after providing EE housing conditions in both HD and LD mice.

	DID cycles before providing EE			
Group	**1**	**2**	**3**	**4**
HD	26.32 ± 0.48	26.71 ± 0.48	27.37 ± 0.49	27.84 ± 0.5
LD	26.95 ± 0.41	27.29 ± 0.42	27.68 ± 0.42	27.68 ± 0.34
	DID cycles after providing EE			
	1	**2**	**3**	**4**
HD-SE	29.24 ± 0.89	29.57 ± 0.95	29.84 ± 1.02	29.77 ± 0.81
HD-EE	29.26 ± 0.47	29.31 ± 0.34	29.28 ± 0.69	30.03 ± 0.49
LD-SE	28.91 ± 0.72	29.42 ± 0.72	29.38 ± 0.84	30.36 ± 0.8
LD-EE	28.93 ± 0.52	29.3 ± 0.47	29.49 ± 0.48	29.93 ± 0.48

Top panel: average (means ± SEM) body weight (g) of HD and LD groups during four DID cycles before providing EE. Bottom panel: average (means ± SEM) body weight (g) of HD-SE, HD-EE, LD-SE and LD-EE groups during four DID cycles after providing EE.

Effects of Switching to an Enriched Environment on Sucrose Binge-Like Intake by HD and LD Phenotypes

Figures 3A,B depicts HD and LD sucrose binge-like intake by SE and EE groups during four 5-day DID cycles, from PND96 to PND115. Average sucrose binge-like intake during the last day of the washout period following SB administration (PND95) was included in the analysis as a before EE time-point to analyze the immediate impact of switching to EE on sucrose consumption.

The assumptions analysis for the mixed 2 × 2 × 5 ANOVA (subpopulation × environment × cycle) showed violation of the sphericity assumption (Mauchly's test: $\chi^2_{(9)} = 0.043$; $p = 0.000$). The results showed statistically significant differences for the "cycle" ($F_{(1.52,39.67)} = 4.96$, $p = 0.01$; $\eta^2 = 0.16$) and "environment" ($F_{(1,26)} = 4.64$, $p = 0.04$; $\eta^2 = 0.15$) main factors, as well as for their interaction (environment × cycle; $F_{(1.52,39.67)} = 4.71$, $p = 0.02$; $\eta^2 = 0.15$). The second order interaction factor (subpopulation × environment × cycle) also attained statistical significance ($F_{(1.52,39.67)} = 4.13$, $p = 0.03$; $\eta^2 = 0.13$). Thus, sucrose binge-like intake for each subpopulation, HD and LD, was analyzed separately. For HD mice, an independent repeated measures ANOVA (2 × 5; environment × cycle) revealed statistically significant differences for factors interaction ($F_{(1.45,17.46)} = 6.34$, $p = 0.01$; $\eta^2 = 0.34$; **Figure 3A**); additional *post hoc* analyses revealed higher sucrose binge-like intake in HD-SE mice compared to HD-EE mice in cycle 2 ($p = 0.02$), cycle 3 ($p = 0.007$) and cycle 4 ($p = 0.02$). Additional planned *t*-test comparisons within HD-SE and HD-EE groups allowed us to evaluate consumption dynamics of each group. Thus, the HD-SE group showed no statistically significant differences in PND95 (before EE time-point) vs. cycle 1 ($t = -0.343$, $p = 0.74$; $df = 6$), in PND95 vs. cycle 2 ($t = -4.42$, $p = 0.67$; $df = 6$), or in cycle 2 vs. 4 ($t = -2.18$, $p = 0.07$; $df = 6$), showing that the HD-SE group had similar sucrose binge-like intake during the periods of the experiment before and after switching. When the HD-EE group was analyzed, planned *t*-test comparisons showed statistically significant differences for PND95 vs. cycle 1 ($t = 6.05$, $p = 0.001$; $df = 6$) and PND95 vs. cycle 2 ($t = 5.44$, $p = 0.002$; $df = 6$), but not for cycle 1 vs. 2 ($t = 1.91$, $p = 0.1$; $df = 6$) or cycle 2 vs. 4 ($t = -0.81$, $p = 0.44$; $df = 6$), showing that EE access immediately and effectively reduced sucrose binge-like intake in cycle 1 in HD mice. Furthermore, reduced sucrose binge-like intake was maintained for the rest of the experiment.

On the other hand, the analysis performed on sucrose binge-like intake data in LD mice did not indicate statistically

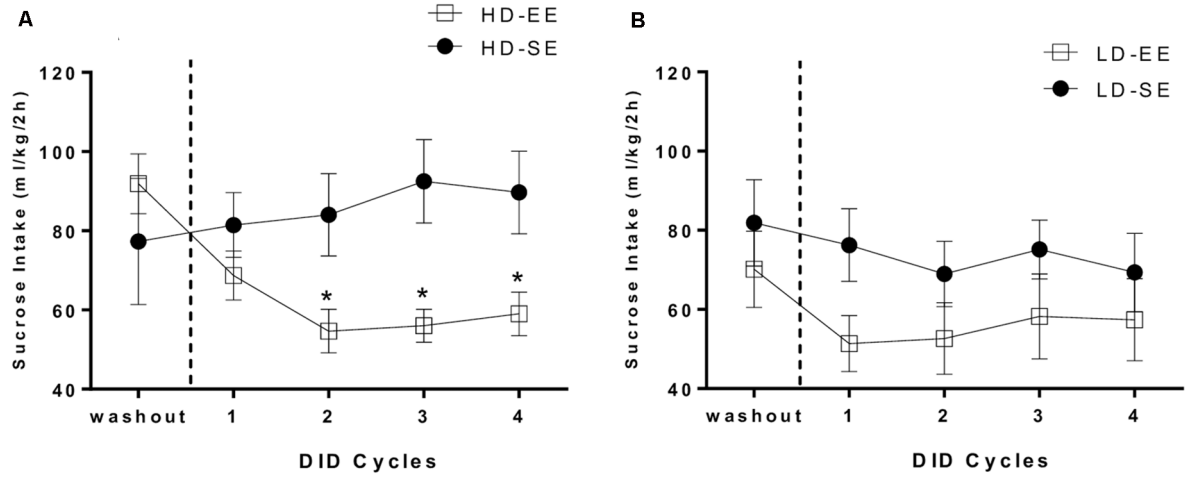


FIGURE 3 | (A) HD group average (means ± SEM) 10% (w/v) sucrose binge-like intake (ml/kg/2 h) during the last day of the washout period (PND95) and four DID cycles after providing EE (from PND96 to PND115) and **(B)** LD group average (means ± SEM) 10% (w/v) sucrose binge-like intake (ml/kg/2 h) during the last day of the washout period (PND95) and four DID cycles after providing EE (from PND96 to PND115). Vertical dotted lines indicate the switching time point of the environmental housing conditions. *$P < 0.05$ relative to HD-SE group.

significant effects for the "cycle" ($F_{(1.57,22.06)} = 3.56$, $p = 0.06$; $\eta^2 = 0.2$) or "environment" ($F_{(1,14)} = 1.77$, $p = 0.2$; $\eta^2 = 0.11$) factors or for their interaction, indicating that introducing EE housing conditions to LD subpopulation did not significantly alter sucrose binge-like intake (**Figure 3B**).

For kilocalorie intake, Mauchly's test for the repeated measures ANOVA (2 × 2 × 5; subpopulation × environment × cycle) showed violation of the sphericity assumption (Mauchly's test: $\chi^2_{(9)} = 0.068$, $p < 0.0001$). The analysis conducted on kilocalorie intake data after environmental switching in HD and LD mice showed statistical significance for the main factor "cycle" ($F_{(1.82,47.34)} = 8.17$, $p = 0.001$; $\eta^2 = 0.23$) and the interaction "cycle" × "environment" ($F_{(1.82,47.34)} = 3.94$, $p = 0.02$; $\eta^2 = 0.13$). Additional Bonferroni *post hoc* analyses revealed higher kilocalorie intake by SE animals than by EE mice in cycle 3 ($p = 0.03$; **Table 1**).

For BW data analysis, Mauchly's test for the repeated measures ANOVA (2 × 2 × 5; subpopulation × environment × cycle) showed violation of the sphericity assumption (Mauchly's test: $\chi^2_{(5)} = 0.438$, $p = 0.001$). The analysis conducted on BW data from after environmental switching only showed a statistically significant difference for the "cycle" main factor ($F_{(2.15,56.14)} = 20.96$, $p < 0.0001$; $\eta^2 = 0.44$), indicating that BW increased over time, regardless of the subpopulation or housing conditions (**Table 2**).

Effects of SB-334867 (5 mg/kg) on Sucrose Binge-Like Intake by HD and LD Phenotypes in SE vs. EE Conditions

SB Challenge Before Introduction of EE Conditions (PND85; Test OX 1)

Figure 4A shows average sucrose binge-like intake by HD and LD subpopulations on PND85 (SE, before providing EE housing conditions) as a result of a pharmacological challenge with ip SB (5 mg/kg) or vehicle administration (test OX 1, see **Figure 1**). An independent (2 × 2; subpopulation × treatment) ANOVA was conducted on sucrose binge-like intake data in response to administration of SB or vehicle by HD and LD subpopulations pre-exposed to four cycles of sucrose DID. The results showed statistically significant differences for "treatment" ($F_{(1,26)} = 24.88$; $p = 0.000$; $\eta^2 = 0.49$) and "subpopulation" main factors ($F_{(1,26)} = 8.32$; $p = 0.008$; $\eta^2 = 0.24$) but not for their interaction ($F_{(1,26)} = 0.44$, $p = 0.51$; **Figure 4A**), indicating that SB (5 mg/kg) administration significantly reduced sucrose binge-like intake both in HD and LD subpopulations.

To control the temporal dynamics of sucrose binge consumption during the washout period following OXr1 antagonist administration, daily sucrose binge-like intake during the 10 days of the washout period was analyzed in a repeated measures ANOVA 2 × 10 (subpopulation × time; **Table 3**) and showed violation of the sphericity assumption (Mauchly's test: $\chi^2_{(44)} = 0.002$, $p < 0.0001$). The ANOVA conducted on sucrose binge-like intake data by HD and LD subpopulations revealed statistically significant differences for the main factor "time" ($F_{(3.88,93.34)} = 2.97$, $p = 0.02$; $\eta^2 = 0.11$) and "subpopulation" ($F_{(1,24)} = 7.45$, $p = 0.01$; $\eta^2 = 0.23$), indicating that differences in sucrose consumption by HD vs. LD persisted over the washout period.

SB Challenge After Providing EE Conditions (PND116): Test OX 2

Figure 4B depicts after-switching sucrose binge-like intake by HD/LD subpopulations in SE vs. EE housing conditions as a result of SB (5 mg/kg) and vehicle administration on PND116 (test OX 2, see **Figure 1**). The assumptions analysis carried out for the independent three-way (2 × 2 × 2; subpopulation × environment × treatment) ANOVA on sucrose binge-like intake in OX test 2 showed compliance with both normality and homoscedasticity assumptions for

TABLE 3 | Average (means ± SEM) sucrose binge-like intake of HD and LD groups during the 10 days of washout period after test OX 1 with SB-334867 administration.

		Washout day				
Group	**Treatment**	**1**	**2**	**3**	**4**	**5**
HD	Veh	112.12 ± 6.26	95.82 ± 7.82	94.22 ± 5.34	85.17 ± 6.09	79.02 ± 9.53
	SB	99.34 ± 5.29	90.83 ± 8.93	99.57 ± 8.92	87.33 ± 10.01	93.56 ± 9.79
LD	Veh	75.67 ± 8.82	68.83 ± 8.19	63.05 ± 6.85	54.65 ± 9.31	63.69 ± 10.95
	SB	67.01 ± 11.18	76.34 ± 12.44	87.79 ± 10.52	78.67 ± 14.74	70.93 ± 14
		6	**7**	**8**	**9**	**10**
HD	Veh	81.8 ± 7.66	82.68 ± 5.21	69.99 ± 14.77	82.6 ± 8.98	88.37 ± 6.89
	SB	87.2 ± 12.29	90.38 ± 10.48	76.69 ± 15.07	84.46 ± 12.87	80.78 ± 16.68
LD	Veh	52.86 ± 10.81	51.16 ± 8.1	58.34 ± 9.55	59.63 ± 6.82	62.79 ± 7.09
	SB	67.78 ± 13.76	75.26 ± 11.5	73.25 ± 11.09	92.48 ± 12.03	90.26 ± 12.53

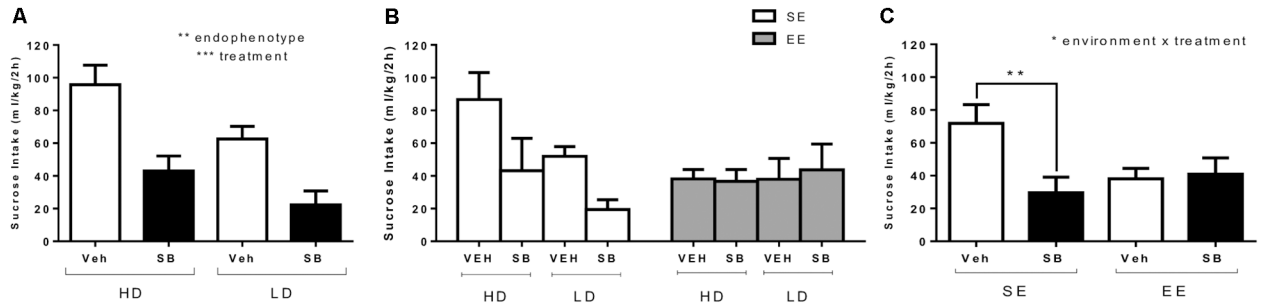


FIGURE 4 | (A) Average (means ± SEM) 10% (w/v) sucrose binge-like intake (ml/kg/2 h) by HD and LD mice in response to a pharmacological challenge with SB (5 mg/kg) or vehicle (test 1, PND85) performed after the first four cycles of sucrose DID; **(B)** average (means ± SEM) 10% (w/v) sucrose binge-like intake (ml/kg/2 h) by HD and LD subpopulations in SE/EE conditions in response to a pharmacological challenge with SB (5 mg/kg) or vehicle (test 2, PND116); and **(C)** average (means ± SEM) 10% (w/v) sucrose binge-like intake (ml/kg/2 h) by SE and EE mice in response to a pharmacological challenge with SB (5 mg/kg) or vehicle (test 2, PND116), right after complete four after-switching cycles of sucrose binge-like DID. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

all of the experimental design conditions. The (2 × 2 × 2) ANOVA performed on sucrose binge-like intake data showed statistically significant differences only for the interaction "environment" × "treatment" ($F_{(1,22)} = 4.61$; $p = 0.04$; $\eta^2 = 0.17$), indicating similar sucrose consumption patterns in response to SB by HD and LD mice but differences in response to treatment, depending upon the environmental housing conditions. Thus, since the "subpopulation" factor did not reach statistical significance, HD and LD data were collapsed (**Figure 4C**). Additional Bonferroni *post hoc* analysis revealed that compared to vehicle, SB reduced sucrose binge-like intake in SE ($p = 0.01$) but not EE mice ($p = 0.87$) in both HD and LD subpopulations (**Figure 4C**). Additional planned *t*-test comparisons showed higher sucrose intake by SE vs. EE vehicle-injected mice ($t = 2.65$, $p = 0.02$; $df = 13$) but not SB-injected mice ($t = -0.82$, $p = 0.42$; $df = 13$), confirming that EE mice consumed significantly less sucrose than their SE counterparts.

DISCUSSION

The relevant findings in the current study are the following: (1) a single 2 h episode of sucrose binge drinking in a DID procedure segregates two behavioral subpopulations, HD and LD, in iC57BL/6J mice; (2) when adult mice reared in SE conditions and long-term exposed to sucrose DID (4, 5-day cycles) were switched to EE conditions, an immediate significant reduction in sucrose binge-like intake was observed in HD mice, suggesting the therapeutic role of EE exposure for HD mice showing spontaneous high sucrose binge-like intake; and (3) administration of the OXr1 antagonist SB caused an acute reduction in sucrose binge-like intake in HD and LD mice exposed to SE conditions. Importantly, exposure to EE conditions blunted the inhibitory effect of SB on sucrose binge consumption in both subpopulations, indirectly suggesting that EE exposure might impact the OX system.

Importantly, the therapeutic effects of EE over sucrose consumption from our study were found when compared with control SE housed mice. Interestingly, previous studies have showed that isolated rats (as the EE control condition) had greater sucrose consumption (Hall et al., 1997; Brenes and Fornaguera, 2008). Altogether, current and previous studies results pointing to an isolation and SE conditions correlation with sucrose intake, suggest that social housing (as in our SE group) is a necessary but not sufficient factor to blunt sucrose binge-like intake in mice. However, different methodological variables such as differences in EE housing items or conditions, time to EE and sucrose consumption exposure, together with other factors such as species, sex or strains used for the studies could all influence behavioral results in studies employing EE paradigms (Crofton et al., 2015). Accordingly, disparity between laboratories outcomes as to the EE modulation of sucrose intake might be explained.

Due to both the utility of subpopulations in preclinical and clinical studies for a better understanding of vulnerability to development of drug addiction and BEDs (for reviews, see Ramoz et al., 2007; Monteleone and Maj, 2008; Hutson et al., 2018) as well as our recent behavioral, pharmacological and molecular evidence pointing to the existence of High/Low ethanol drinking (HD/LD) subpopulations stemming from DID procedures (Alcaraz-Iborra et al., 2017), the first objective in the present study addressed the ability of DID to segregate two independent behavioral HD/LD subpopulations for sucrose binge-like consumption, as it does for ethanol binge-like consumption. The first relevant finding is that a single 2 h episode of DID sucrose effectively segregated two behavioral phenotypes, HD and LD that last for four cycles and the entire washout period. Thus, under SE conditions, HD mice showed significantly higher sucrose binge-like intake compared to their LD counterparts before switching to EE. Taken into account that C57BL/6J mice share same genetic load, it is reasonable that HD/LD subpopulations could result from an epigenetic mechanism. Consistently, previous studies have showed elevated variation of phenotypes for ethanol binge-like intake (Crabbe et al., 2010; Barkley-Levenson and Crabbe, 2014) and obesity induced by diet in C57BL/6J mice (Koza et al., 2006). Present results extend our previous observations that DID segregates two behavioral HD/LD phenotypes for spontaneous ethanol binge-like drinking. Moreover, we provide here experimental evidence that HD/LD segregation successfully predicts sucrose consumption in response to environmental variations, as we discuss later. In our laboratory we are currently conducting studies aiming to further characterize HD/LD subpopulations from a molecular and behavioral approach to extend our knowledge on neurobehavioral phenotypes underlying spontaneous high binge intake of caloric palatable food.

The second main consideration in the study is the therapeutic effect of EE access during adulthood on sucrose binge-like intake in HD animals. When adult mice housed during their adolescence in SE conditions and long-term exposed to repetitive sucrose binge episodes in a DID task (4, 5-day cycles) were exposed to EE, an instantaneous decrease in sucrose binge-like intake by HD mice was observed. Thus, HD animals exposed to EE conditions (HD-EE) showed a dramatic and immediate reduction in sucrose binge-like intake, as early as cycle 2, when compared to animals in SE conditions (HD-SE). Moreover, sucrose binge-like consumption by HD-EE animals was reduced immediately and long-term, matching that exhibited by LD (both LD-EE and LD-SE) mice, which confirms and extends previous observations suggesting a therapeutic effect of EE on sucrose consumption and sucrose seeking. EE access had a dramatic effect on both extinction and the response to a sucrose SA-associated cue in rats (Grimm et al., 2008); brief exposure to EE reduced sucrose cue-reactivity and consumption in rats after 1 or 30 days of compelled abstinence from SA (Grimm et al., 2013), and either acute or chronic EE access decreased c-Fos levels in relapse-related rat brain regions (Grimm et al., 2016). Additionally, current results are in concordance with previous preclinical studies supporting therapeutic effects of EE housing conditions on addiction to several drugs such as cocaine (Solinas et al., 2008; Chauvet et al., 2009, 2011, 2012; Thiel et al., 2009), heroin (Galaj et al., 2016), methamphetamine (Hajheidari et al., 2015, 2017) and ethanol (Lopez et al., 2011; Lopez and Laber, 2015; Marianno et al., 2017).

More importantly, our present observations extend and complement recent results from our laboratory showing that EE access during adulthood reduces ethanol binge-like consumption by animals housed in SE conditions during adolescence (Rodríguez-Ortega et al., 2018). Taking together, our present and previous results demonstrate that EE exposure successfully modulates high binge-like intake of a rewarding stimulus, such as ethanol and sucrose, characteristic of the early and transient stages of the addiction cycle (Koob and Volkow, 2009). Additionally, present findings highlight the relevance of segregating behavioral HD/LD subpopulations stemming from DID procedures to evaluate and characterize the therapeutic effects of EE in preclinical models of binge-like intake.

Total kilocalories (sucrose + chow) during DID sessions along with BW were assessed in the study. Despite HD-SE mice consuming more sucrose and total kilocalorie during the study, however, no significant differences were found in BW compared to HD-EE mice or LD mice. Present BW data are in agreement with previous evidence in preclinical studies showing that sweet or fat bingeing procedures fail to induce overweight (Corwin and Babbs, 2012), probably due to a compensatory reduction of chow intake between binge episodes (Avena, 2007; Cowin et al., 2011; Hone-Blanchet and Fecteau, 2014). Furthermore, BW data are consistent with human research showing that only 35% of binge eaters are overweight or obese (Cowin et al., 2011) as well as human studies indicating the existence of a compensatory mechanism triggered in BED in which BW is successfully balanced by undereating after binge episodes (American Psychiatric Association, 2013). In our study, we cannot rule out that longer exposure to sucrose consumption might eventually trigger increased BW in HD-SE mice. Additional studies extending the number of sucrose DID episodes would clarify whether BW consistently varies in parallel with sucrose and caloric consumption under different housing conditions.

The third relevant result is focused on the potential EE/OX interaction. When HD and LD mice housed in SE conditions and pre-exposed long-term to sucrose binge intake were pharmacologically challenged with the OXr1 antagonist SB (test OX 1), sucrose binge-like intake was reduced in both subpopulations to approximately one-half of that exhibited by SE vehicle-injected mice. Interestingly, switching to EE blunted the inhibitory effect of SB, both in HD and LD subpopulations. Although spontaneous sucrose binge intake was low in EE exposed mice at the time of SB second challenge (OX test 2), a floor effect is unlikely since SB successfully reduced sucrose consumption during the OX test 1 in LD mice showing similar levels of intake to that exhibited by EE mice during the OX test 2. Present findings showing an inhibitory modulation of sucrose binge intake by antagonizing OXr1 are in agreement with previous studies supporting a key role for the OX system in food-reward behaviors (Cason and Aston-Jones,

2013, 2014), binge intake (Alcaraz-Iborra et al., 2014) and food overconsumption (Piccoli et al., 2012; Merlo Pich and Melotto, 2014). It is difficult to form any consistent conclusions about the lack of SB effect during the OX test 2. Our observation that EE exposure blunts the inhibitory effect of the OXr1 antagonist SB on sucrose binge intake during a DID test indirectly suggests a role for OXr1 signaling in EE effects. Given the role of OX in anxiety/compulsivity (Piccoli et al., 2012; Merlo Pich and Melotto, 2014) and binge-like consumption (Alcaraz-Iborra et al., 2014), as well as evidence that EE modulates anxiety-like responses (Benaroya-Milshtein et al., 2004; Peña et al., 2006, 2009; Sztainberg et al., 2010; Ragu Varman and Rajan, 2015; Bahi, 2017; Rodríguez-Ortega et al., 2018) and compulsivity (Bechard and Lewis, 2016; Bechard et al., 2016; Rodríguez-Ortega et al., 2018), a working hypothesis is that EE interacts with the OX brain system to primarily modulate anxiety/compulsivity responses; in turn, these responses secondarily reduce binge-like consumption during early, pre-dependent stages of the addiction cycle. Nonetheless, whether present findings indicate the existence of a causal link between EE-driven altered responses to OXr1 antagonism and reduced sucrose consumption by HD mice remains unknown and needs further exploration.

In summary, first, our findings highlight the importance of HD/LD segregation resulting from DID procedures to further evaluate neurobehavioral processes governing the early and transient phases of the food addiction cycle and BED development in preclinical models. Current results underpin the therapeutic value of EE on sucrose binge-like intake in HD mice in a long-term DID procedure that successfully models human binge-like consumption. When HD mice had access to EE housing conditions, sucrose binge-like intake was reduced immediately and in the long term, matching the level of intake exhibited by LD mice. Interestingly, EE blunted the inhibitory effect of an OXr1 antagonist on sucrose consumption, providing preliminary indirect evidence of EE/OX interaction. We suggest the preliminary hypothesis that a primary interaction of EE is on OX-dependent compulsivity/anxiety brain systems, which, secondarily, influence sucrose binge-like consumption during the pre-dependent phases of the addiction cycle.

AUTHOR CONTRIBUTIONS

IC provided the overall coordination and supervision for the study. IC and ER-O were responsible for the study concept and design and wrote the manuscript. ER-O, EA and MA-I conducted behavioral characterization. LF was responsible for statistical analyses. All authors critically reviewed the content and approved the final version for publication.

FUNDING

This work was supported by FEDER co-funding and Ministerio de Economia y Competitividad (Spain; grant PSI2015-64460-R to IC); Feder grant FEDERUALM05-23-006 (to IC).

REFERENCES

Alcaraz-Iborra, M., Carvajal, F., Lerma-cabrera, J. M., Miguel, L., and Cubero, I. (2014). Binge-like consumption of caloric and non-caloric palatable substances in *ad libitum* -fed C57BL/6J mice: pharmacological and molecular evidence of orexin involvement. *Behav. Brain Res.* 272, 93–99. doi: 10.1016/j.bbr.2014.06.049

Alcaraz-Iborra, M., Navarrete, F., Rodríguez-Ortega, E., de La Fuente, L., Manzanares, J., and Cubero, I. (2017). Different molecular/behavioral endophenotypes in C57BL/6J mice predict the impact of OX1 receptor blockade on binge-like ethanol intake. *Front. Behav. Neurosci.* 11:186. doi: 10.3389/fnbeh.2017.00186

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders*. 5th Edn. Washington, DC: American Psychiatric Association.

Avena, N. M. (2007). Examining the addictive-like properties of binge eating using an animal model of sugar dependence. *Exp. Clin. Psychopharmacol.* 15, 481–491. doi: 10.1037/1064-1297.15.5.481

Avena, N. M. (2010). The study of food addiction using animal models of binge eating. *Appetite* 55, 734–737. doi: 10.1016/j.appet.2010.09.010

Avena, N. M., and Bocarsly, M. E. (2012). Dysregulation of brain reward systems in eating disorders: neurochemical information from animal models of binge eating, bulimia nervosa and anorexia nervosa. *Neuropharmacology* 63, 87–96. doi: 10.1016/j.neuropharm.2011.11.010

Avena, N. M., Rada, P., and Hoebel, B. G. (2008). Evidence for sugar addiction: behavioral and neurochemical effects of intermittent, excessive sugar intake. *Neurosci. Biobehav. Rev.* 32, 20–39. doi: 10.1016/j.neubiorev.2007.04.019

Bahi, A. (2017). Environmental enrichment reduces chronic psychosocial stress-induced anxiety and ethanol-related behaviors in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 77, 65–74. doi: 10.1016/j.pnpbp.2017.04.001

Barkley-Levenson, A. M., and Crabbe, J. C. (2014). High drinking in the dark mice: a genetic model of drinking to intoxication. *Alcohol* 48, 217–223. doi: 10.1016/j.alcohol.2013.10.007

Bechard, A. R., Cacodcar, N., King, M. A., and Lewis, M. H. (2016). How does environmental enrichment reduce repetitive motor behaviors? Neuronal activation and dendritic morphology in the indirect basal ganglia pathway of a mouse model. *Behav. Brain Res.* 15, 122–131. doi: 10.1016/j.bbr.2015.11.029

Bechard, A. R., and Lewis, M. H. (2016). Transgenerational effects of environmental enrichment on repetitive motor behavior development. *Behav. Brain Res.* 307, 145–149. doi: 10.1016/j.bbr.2016.04.005

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., et al. (2004). Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. *Eur. J. Neurosci.* 20, 1341–1347. doi: 10.1111/j.1460-9568.2004.03587.x

Belin, D., Belin-Rauscent, A., Everitt, B. J., and Dalley, J. W. (2016). In search of predictive endophenotypes in addiction: insights from preclinical research. *Genes Brain Behav.* 15, 74–88. doi: 10.1111/gbb.12265

Borgland, S. L., Ungless, M. A., and Bonci, A. (2009). Convergent actions of orexin/hypocretin and CRF on dopamine neurons: emerging players in addiction. *Brain Res.* 1314, 139–144. doi: 10.1016/j.brainres.2009.10.068

Brenes, J. C., and Fornaguera, J. (2008). Effects of environmental enrichment and social isolation on sucrose consumption and preference: associations with depressive-like behavior and ventral striatum dopamine. *Neurosci. Lett.* 436, 278–282. doi: 10.1016/j.neulet.2008.03.045

Cason, A. M., and Aston-Jones, G. (2013). Role of orexin/hypocretin in conditioned sucrose-seeking in rats. *Psychopharmacology* 226, 155–165. doi: 10.1007/s00213-012-2902-y

Cason, A. M., and Aston-Jones, G. (2014). Role of orexin/hypocretin in conditioned sucrose-seeking in female rats. *Neuropharmacology* 86, 97–102. doi: 10.1016/j.neuropharm.2014.07.007

Chauvet, C., Goldberg, S. R., Jaber, M., and Solinas, M. (2012). Effects of environmental enrichment on the incubation of cocaine craving.

Neuropharmacology 63, 635–641. doi: 10.1016/j.neuropharm.2012.05.014

Chauvet, C., Lardeux, V., Goldberg, S. R., Jaber, M., and Solinas, M. (2009). Environmental enrichment reduces cocaine seeking and reinstatement induced by cues and stress but not by cocaine. *Neuropsychopharmacology* 34, 2767–2778. doi: 10.1038/npp.2009.127

Chauvet, C., Lardeux, V., Jaber, M., and Solinas, M. (2011). Brain regions associated with the reversal of cocaine conditioned place preference by environmental enrichment. *Neuroscience* 184, 88–96. doi: 10.1016/j.neuroscience.2011.03.068

Cowin, R. L., Avena, N. M., and Boggiano, M. M. (2011). Feeding and reward: perspectives from three rat models of binge eating. *Physiol. Behav.* 104, 87–97. doi: 10.1016/j.physbeh.2011.04.041

Corwin, R. L. W., and Babbs, R. K. (2012). Rodent models of binge eating: are they models of addiction? *ILAR J.* 53, 23–34. doi: 10.1093/ilar.53.1.23

Crabbe, J. C., Phillips, T. J., and Belknap, J. K. (2010). The complexity of alcohol drinking: studies in rodent genetic models. *Behav. Genet.* 40, 737–750. doi: 10.1007/s10519-010-9371-z

Crofton, E. J., Zhang, Y., and Green, T. A. (2015). Inoculation stress hypothesis of environmental enrichment. *Neurosci. Biobehav. Rev.* 49, 19–31. doi: 10.1016/j.neubiorev.2014.11.017

de Carvalho, C. R., Pandolfo, P., Pamplona, F. A., and Takahashi, R. N. (2010). Environmental enrichment reduces the impact of novelty and motivational properties of ethanol in spontaneously hypertensive rats. *Behav. Brain Res.* 208, 231–236. doi: 10.1016/j.bbr.2009.11.043

Deehan, G. A., Cain, M. E., and Kiefer, S. W. (2007). Differential rearing conditions alter operant responding for ethanol in outbred rats. *Alcohol. Clin. Exp. Res.* 31, 1692–1698. doi: 10.1111/j.1530-0277.2007.00466.x

Deehan, G. A., Palmatier, M. I., Cain, M. E., and Kiefer, S. W. (2011). Differential rearing conditions and alcohol-preferring rats: consumption of and operant responding for ethanol. *Behav. Neurosci.* 125, 184–193. doi: 10.1037/a0022627

Galaj, E., Manuszak, M., and Ranaldi, R. (2016). Environmental enrichment as a potential intervention for heroin seeking. *Drug Alcohol Depend.* 163, 195–201. doi: 10.1016/j.drugalcdep.2016.04.016

Gill, M. J., and Cain, M. E. (2011). Effects of satiety on operant responding in rats raised in enrichment. *Behav. Pharmacol.* 22, 40–48. doi: 10.1097/FBP.0b013e3283425a86

Grimm, J. W., Barnes, J. L., Koerber, J., Glueck, E., Ginder, D., Hyde, J., et al. (2016). Effects of acute or chronic environmental enrichment on regional Fos protein expression following sucrose cue-reactivity testing in rats. *Brain Struct. Funct.* 221, 2817–2830. doi: 10.1007/s00429-015-1074-z

Grimm, J. W., Osincup, D., Wells, B., Manaois, M., Fyall, A., and Harkness, J. H. (2008). Environmental enrichment attenuates cue-induced reinstatement of sucrose seeking in rats. *Program* 19, 777–785. doi: 10.1097/FBP.0b013e32831c3b18

Grimm, J. W., Weber, R., Barnes, J., Koerber, J., Dorsey, K., and Glueck, E. (2013). Brief exposure to novel or enriched environments reduces sucrose cue-reactivity and consumption in rats after 1 or 30 days of forced abstinence from self-administration. *PLoS One* 8:e54164. doi: 10.1371/journal.pone.0054164

Grucza, R. A., Przybeck, T. R., and Cloninger, R. (2007). Prevalence and correlates of binge eating disorder in a community sample. *Compr. Psychiatry* 48, 124–131. doi: 10.1016/j.comppsych.2006.08.002

Hajheidari, S., Miladi-Gorji, H., and Bigdeli, I. (2015). Effect of the environmental enrichment on the severity of psychological dependence and voluntary methamphetamine consumption in methamphetamine withdrawn rats. *Neurosci. Lett.* 584, 151–155. doi: 10.1016/j.neulet.2014.10.017

Hajheidari, S., Miladi-gorji, H., and Bigdeli, I. (2017). Environmental enrichment prevents methamphetamine-induced spatial memory deficits and obsessive-compulsive behavior in rats. *Iran. J. Psychiatry* 12, 8–14.

Hall, F. S., Humby, T., Wilkinson, L. S., and Robbins, T. W. (1997). The effects of isolation-rearing on sucrose consumption in rats. *Physiol. Behav.* 62, 291–297. doi: 10.1016/s0031-9384(97)00116-9

Hone-Blanchet, A., and Fecteau, S. (2014). Overlap of food addiction and substance use disorders definitions: analysis of animal and human studies. *Neuropharmacology* 85, 81–90. doi: 10.1016/j.neuropharm.2014.05.019

Hutson, P. H., Balodis, I. M., and Potenza, M. N. (2018). Binge-eating disorder: clinical and therapeutic advances. *Pharmacol. Ther.* 182, 15–27. doi: 10.1016/j.pharmthera.2017.08.002

Kaur, S., Li, J., Stenzel-Poore, M. P., and Ryabinin, A. E. (2012). Corticotropin-releasing factor acting on corticotropin-releasing factor receptor type 1 is critical for binge alcohol drinking in mice. *Alcohol. Clin. Exp. Res.* 36, 369–376. doi: 10.1111/j.1530-0277.2011.01610.x

Kay, K., Parise, E. M., Lilly, N., and Williams, D. L. (2014). Hindbrain orexin 1 receptors influence palatable food intake, operant responding for food and food-conditioned place preference in rats. *Psychopharmacology* 231, 419–427. doi: 10.1007/s00213-013-3248-9

Koob, G. F., and Volkow, N. D. (2009). Neurocircuitry of addiction. *Neuropsychopharmacology* 35, 217–238. doi: 10.1038/npp.2009.110

Koza, R. A., Nikonova, L., Hogan, J., Rim, J. S., Mendoza, T., Faulk, C., et al. (2006). Changes in gene expression foreshadow diet-induced obesity in genetically identical mice. *PLoS Genet.* 2:e81. doi: 10.1371/journal.pgen.0020081

Li, X., Meng, L., Huang, K., Wang, H., and Li, D. (2015). Environmental enrichment blocks reinstatement of ethanol-induced conditioned place preference in mice. *Neurosci. Lett.* 599, 92–96. doi: 10.1016/j.neulet.2015.05.035

Lopez, M. F., Doremus-Fitzwater, T. L., and Becker, H. C. (2011). Chronic social isolation and chronic variable stress during early development induce later elevated ethanol intake in adult C57BL/6J mice. *Alcohol* 45, 355–364. doi: 10.1016/j.alcohol.2010.08.017

Lopez, M. F., and Laber, K. (2015). Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. *Physiol. Behav.* 148, 151–156. doi: 10.1016/j.physbeh.2014.11.012

Lowery, E. G., Spanos, M., Navarro, M., Lyons, A. M., Hodge, C. W., and Thiele, T. E. (2010). CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. *Neuropsychopharmacology* 35, 1241–1252. doi: 10.1038/npp.2009.209

Lowery, E. G., Sparrow, A. M., Breese, G. R., Knapp, D. J., and Thiele, T. E. (2008). The CRF-1 receptor antagonist, CP-154,526, attenuates stress-induced increases in ethanol consumption by BALB/cJ mice. *Alcohol. Clin. Exp. Res.* 32, 240–248. doi: 10.1111/j.1530-0277.2007.00573.x

Marianno, P., Abrahao, K. P., and Camarini, R. (2017). Environmental enrichment blunts ethanol consumption after restraint stress in C57BL/6 mice. *PLoS One* 12:e0170317. doi: 10.1371/journal.pone.0170317

Monteleone, P., and Maj, M. (2008). Genetic susceptibility to eating disorders: associated polymorphisms and pharmacogenetic suggestions. *Pharmacogenomics* 9, 1487–1520. doi: 10.2217/14622416.9.10.1487

Merlo Pich, E. M., and Melotto, S. (2014). Orexin 1 receptor antagonists in compulsive behavior and anxiety: possible therapeutic use. *Front. Neurosci.* 8:26. doi: 10.3389/fnins.2014.00026

Nair, S. G., Golden, S. A., and Shaham, Y. (2008). Differential effects of the hypocretin 1 receptor antagonist SB 334867 on high-fat food self-administration and reinstatement of food seeking in rats. *Br. J. Pharmacol.* 1, 406–416. doi: 10.1038/bjp.2008.3

Nithianantharajah, J., and Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat. Rev. Neurosci.* 7, 697–709. doi: 10.1038/nrn1970

Peña, Y., Prunell, M., Dimitsantos, V., Nadal, R., and Escorihuela, R. M. (2006). Environmental enrichment effects in social investigation in rats are gender dependent. *Behav. Brain Res.* 174, 181–187. doi: 10.1016/j.bbr.2006.07.007

Peña, Y., Prunell, M., Rotllant, D., Armario, A., and Escorihuela, R. M. (2009). Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. *Psychoneuroendocrinology* 34, 1390–1404. doi: 10.1016/j.psyneuen.2009.04.019

Perello, M., Valdivia, S., García Romero, G., and Raingo, J. (2014). Considerations about rodent models of binge eating episodes. *Front. Psychol.* 5:372. doi: 10.3389/fpsyg.2014.00372

Piccoli, L., Micioni Di Bonaventura, M. V., Cifani, C., Costantini, V. J. A., Massagrande, M., Montanari, D., et al. (2012). Role of orexin-1 receptor mechanisms on compulsive food consumption in a model of binge eating

in female rats. *Neuropsychopharmacology* 37, 1999–2011. doi: 10.1038/npp.2012.48

Ragu Varman, D., and Rajan, K. E. (2015). Environmental enrichment reduces anxiety by differentially activating serotonergic and neuropeptide Y (NPY)-ergic system in indian field mouse (*Mus booduga*): an animal model of post-traumatic stress disorder. *PLoS One* 10:e0127945. doi: 10.1371/journal.pone.0127945

Ramoz, N., Versini, A., and Gorwood, P. (2007). Eating disorders: an overview of treatment responses and the potential impact of vulnerability genes and endophenotypes. *Expert Opin. Pharmacother.* 8, 2029–2044. doi: 10.1517/14656566.8.13.2029

Rodríguez-Ortega, E., de la Fuente, L., de Amo, E., and Cubero, I. (2018). Environmental enrichment during adolescence acts as a protective and therapeutic tool for ethanol binge-drinking, anxiety-like, novelty seeking and compulsive-like behaviors in C57BL/6J mice during adulthood. *Front. Behav. Neurosci.* 12:177. doi: 10.3389/fnbeh.2018.00177

Smith, D. G., and Robbins, T. W. (2013). The neurobiological underpinnings of obesity and binge eating: a rationale for adopting the food addiction model. *Biol. Psychiatry* 73, 804–810. doi: 10.1016/j.biopsych.2012.08.026

Solinas, M., Chauvet, C., Thiriet, N., El Rawas, R., and Jaber, M. (2008). Reversal of cocaine addiction by environmental enrichment. *Proc. Natl. Acad. Sci. U S A* 105, 17145–17150. doi: 10.3410/f.1127001.584126

Solinas, M., Thiriet, N., Chauvet, C., and Jaber, M. (2010). Prevention and treatment of drug addiction by environmental enrichment. *Prog. Neurobiol.* 92, 572–592. doi: 10.1016/j.pneurobio.2010.08.002

Sparta, D. R., Sparrow, A. M., Lowery, E. G., Fee, J. R., Knapp, D. J., and Thiele, T. E. (2008). Blockade of the corticotropin releasing factor type 1 receptor attenuates elevated ethanol drinking associated with drinking in the dark procedures. *Alcohol. Clin. Exp. Res.* 32, 259–265. doi: 10.1111/j.1530-0277.2007.00575.x

Stairs, D. J., and Bardo, M. T. (2009). Neurobehavioral effects of environmental enrichment and drug abuse vulnerability. *Pharmacol. Biochem. Behav.* 92, 377–382. doi: 10.1016/j.pbb.2009.01.016

Stairs, D. J., Klein, E. D., and Bardo, M. T. (2006). Effects of environmental enrichment on extinction and reinstatement of amphetamine self-administration and sucrose-maintained responding. *Behav. Pharmacol.* 17, 597–604. doi: 10.1097/01.fbp.0000236271.72300.0e

Sztainberg, Y., Kuperman, Y., Tsoory, M., Lebow, M., and Chen, A. (2010). The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1. *Mol. Psychiatry* 15, 905–917. doi: 10.1038/mp.2009.151

Thiel, K. J., Sanabria, F., Pentkowski, N. S., and Neisewander, J. L. (2009). Anti-craving effects of environmental enrichment. *Int. J. Neuropsychopharmacol.* 12, 1151–1156. doi: 10.1017/S1461145709990472

Valdivia, S., Patrone, A., Reynaldo, M., and Perello, M. (2014). Acute high fat diet consumption activates the mesolimbic circuit and requires orexin signaling in a mouse model. *PLoS One* 9:e87478. doi: 10.1371/journal.pone.0087478

Van den Berg, C. L., Pijlman, F. T. A., Koning, H. A. M., Diergaarde, L., Van Ree, J. M., and Spruijt, B. M. (1999). Isolation changes the incentive value of sucrose and social behaviour in juvenile and adult rats. *Behav. Brain Res.* 106, 133–142. doi: 10.1016/s0166-4328(99)00099-6

Wolfe, B. E., Baker, C. W., Smith, A. T., and Kelly-Weeder, S. (2009). Validity and utility of the current definition of binge eating. *Int. J. Eat. Disord.* 42, 674–686. doi: 10.1002/eat.20728

Conflict of Interest Statement: 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: 05 March 2019
doi: 10.3389/fnbeh.2019.00023

Sex Differences in the Behavioral and Synaptic Consequences of a Single *in vivo* Exposure to the Synthetic Cannabimimetic WIN55,212-2 at Puberty and Adulthood

Milene Borsoi[1,2], *Antonia Manduca*[1,2], *Anissa Bara*[1,2], *Olivier Lassalle*[1,2], *Anne-Laure Pelissier-Alicot*[1,2,3,4] *and Olivier J. Manzoni*[1,2]*

[1]Aix Marseille Université, Institut National de la Santé et de la Recherche Médicale (INSERM), Institut de Neurobiologie de la Méditerranée (INMED), Marseille, France, [2]Cannalab, Cannabinoids Neuroscience Research International Associated Laboratory, INSERM-Indiana University, Marseille, France, [3]Assistance Publique Hôpitaux de Marseille (APHM), CHU Conception, Service de Psychiatrie, Marseille, France, [4]Assistance Publique Hôpitaux de Marseille (APHM), CHU Timone Adultes, Service de Médecine Légale, Marseille, France

Heavy cannabis consumption among adolescents is associated with significant and lasting neurobiological, psychological and health consequences that depend on the age of first use. Chronic exposure to cannabinoid agonists during the perinatal period or adolescence alters social behavior and prefrontal cortex (PFC) activity in adult rats. However, sex differences on social behavior as well as PFC synaptic plasticity after acute cannabinoid activation remain poorly explored. Here, we determined that the consequences of a single *in vivo* exposure to the synthetic cannabimimetic WIN55,212-2 differently affected PFC neuronal and synaptic functions after 24 h in male and female rats during the pubertal and adulthood periods. During puberty, single cannabinoid exposure (SCE) reduced play behavior in females but not males. In contrast, the same treatment impaired sociability in both sexes at adulthood. General exploration and memory recognition remained normal at both ages and both sexes. At the synaptic level, SCE ablated endocannabinoid-mediated synaptic plasticity in the PFC of females of both ages and heightened excitability of PFC pyramidal neurons at adulthood, while males were spared. In contrast, cannabinoid exposure was associated with impaired long-term potentiation (LTP) specifically in adult males. Together, these data indicate behavioral and synaptic sex differences in response to a single *in vivo* exposure to cannabinoid at puberty and adulthood.

Keywords: prefrontal cortex, adolescence, cannabis, sexual differences, social behavior, CB1 receptor, synaptic plasticity, endocannabinoid

OPEN ACCESS

Edited by:
Walter Adriani,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
Carla Cannizzaro,
Università degli Studi di Palermo, Italy
Adriana Caballero,
University of Illinois at Chicago, United States

***Correspondence:**
Olivier J. Manzoni
olivier.manzoni@inserm.fr

Received: *12 September 2018*
Accepted: *30 January 2019*
Published: *05 March 2019*

Citation:
Borsoi M, Manduca A, Bara A, Lassalle O, Pelissier-Alicot A-L and Manzoni OJ (2019) Sex Differences in the Behavioral and Synaptic Consequences of a Single in vivo Exposure to the Synthetic Cannabimimetic WIN55,212-2 at Puberty and Adulthood. Front. Behav. Neurosci. 13:23. doi: 10.3389/fnbeh.2019.00023

INTRODUCTION

Cannabis is the most frequently and widely used illicit drug among adolescents in developed countries (Gowing et al., 2015). Heavy cannabis consumption among adolescents is associated with significant and lasting neurobiological, psychological and health consequences developing in a dose-dependent fashion which are influenced by age of first use (Iede et al., 2017;

Levine et al., 2017; Lisdahl et al., 2018). Chronic adolescent exposure to cannabinoids is linked to persistent adverse effects such as poor cognitive and psychiatric outcomes in adulthood (Levine et al., 2017) and regular cannabis use is associated with psychosocial impairment even in users without cannabis use disorder (Foster et al., 2017).

The primary psychoactive compound of the plant *Cannabis sativa*, Δ-9-tetrahydrocannabinol (THC), as well as the main endogenous cannabinoids (eCBs) anandamide and 2-arachidonoylglycerol, all engage the same primary target in the central nervous system: the G-protein coupled cannabinoid receptor type 1 (CB1R). The eCB system consists of CB1R and other eCB receptors (e.g., CB2R, TRPV1R), eCB, and the enzymatic machinery for eCB synthesis and degradation (Hu and Mackie, 2015). It participates in neuronal development and synaptic plasticity in most brain areas (Gaffuri et al., 2012; Manduca et al., 2012; Lu and Mackie, 2016).

Social relationships during adolescence are essential for the maturation of adult social and cognitive skills (Casey et al., 2008). Disruptions in social exchanges participate to the etiology of neuropsychiatric and neurodevelopmental disorders (Hankin et al., 1998). In rodents, the eCB system modulates specific brain circuits underlying social behavior (Manduca et al., 2015, 2016; Wei et al., 2017) and rather unsurprisingly, exposure to cannabinoid agonists during adolescence alters social behavior at short- (Trezza and Vanderschuren, 2008a, 2009) and long-term time points (Rubino et al., 2008; Schneider, 2008; Schneider et al., 2008; Renard et al., 2016b). Current evidences suggest that "among the vast and complex neural networks involved in social behavior, the prefrontal cortex (PFC) and its massive reciprocal connections constitute a top-down modulatory system for social behavior" (Ko, 2017).

Sex differences in the eCB system (Cooper and Craft, 2018) appear early (Krebs-Kraft et al., 2010) and hormonal regulation affects eCB activity at adulthood: brain CB1R expression (Rodríguez de Fonseca et al., 1994; González et al., 2000) and eCB content (Bradshaw et al., 2006) are cycle-dependent in female rodents. Human studies demonstrate sex differences in cannabis use, acute and long-term effects, dependence and withdrawal. Males are more likely to initiate cannabis use at a younger age than female, and men use higher quantities more frequently than women who more frequently report nausea and anxiety during the withdrawal period (Stinson et al., 2006; Cuttler et al., 2016). During adolescence, women present a faster transition from initiation of cannabis use to regular use than men (Schepis et al., 2011). In rodents, the effects of cannabis also differ between sexes. Female rodents are more sensitive to the biphasic effects of cannabinoids on locomotion, have more impairments in spatial learning and are more sensitive to the reinforcing effects of cannabinoids than males (for review see Craft et al., 2013).

Many sex differences appear at adolescence during the maturation of adult social and cognitive skills (Casey et al., 2008; Rubino et al., 2008; Marusich et al., 2015; Rubino and Parolaro, 2015; Silva et al., 2016; Wiley et al., 2017). Adolescence is a period of profound morphological, neurodevelopmental and behavioral maturation. Brain volumes, sex steroids, and cortical morphometry all contribute to sex influences on developmental trajectories which are accompanied by changes in the behavioral repertoire normally observed in this transitional period from infancy to adulthood. Puberty is characterized by external physical signs and hormonal alterations whose onset is signaled by gonadotropin-releasing hormone (Spear, 2000; Harris and Levine, 2003; Ojeda et al., 2003). This period is elicited through the complex interaction of endogenous and environmental factors (Sisk and Foster, 2004). Both adolescence and puberty are essential periods of postnatal brain maturation and are characterized by heightened susceptibility to mental disorders (Schneider, 2013). Specifically, changes in puberty onset are associated with increased risk for depression, anxiety (Stice et al., 2001; Kaltiala-Heino et al., 2003) and substance use (Hummel et al., 2013).

Although the consequences of chronic exposure to cannabinoids during the adolescent period have been intensely studied (Liu et al., 2010; Cass et al., 2014; Lovelace et al., 2015; Rubino and Parolaro, 2015, 2016; Renard et al., 2016b), the neuronal and behavioral consequences of cannabis initiation, i.e., the first exposure to the drug, are less clear. Endocannabinoids are widespread mediators of synaptic plasticity, a phenomenon critical to normal function of neural circuits in several brain regions and experience-dependent adaptations (Castillo et al., 2012). As reviewed recently, cannabis has a brain-wide impact on synaptic functions and behavior including the PFC (Zlebnik and Cheer, 2016; Araque et al., 2017). We and others have previously revealed how a single exposure to THC *in vivo* ablates eCB-mediated synaptic plasticity (i.e., short and long-term depression, LTD) in the accumbens and hippocampus (Mato et al., 2004) but not hippocampal CA1 long-term potentiation (LTP; Hoffman et al., 2007) or eCB-LTD at VTA GABA synapses (Friend et al., 2017). Additionally, acute cannabinoid exposure impaired LTP in the ventral subiculum-accumbens pathway (Abush and Akirav, 2012). Thus, it appears that the effects of a single cannabinoid exposure (SCE) greatly depend on the brain area.

An important caveat is that most of the aforementioned studies used adolescent rats which range in age is between 25 and 45 days-old and do not take into account the pubertal period, i.e., its onset or completion. This interval is comprised of the different phases of adolescence which are common for males and females: early-, mid- and late-adolescence. However, mid-adolescence, when the physical markers of puberty typically appear, differs between sexes: females reach puberty around post-natal day (PND) 30–40 while puberty takes place in males later at approximately PND 40–50 (Schneider, 2008; Vetter-O'Hagen and Spear, 2012; Burke et al., 2017). Thus, based on the developmental profile of the eCB system and the sensitivity of the pubertal period, we reasoned that two factors, pubertal period and sex, may further complicate the effects of acute exposure to exogenous cannabinoids. The present study focuses on pubescent and adult rats of both sexes that were tested for social and cognitive behaviors as well as neuronal and synaptic parameters in pyramidal neurons of the PFC 24 h after a single *in vivo* exposure to the synthetic cannabimimetic WIN55,212-2.

MATERIALS AND METHODS

Animals

Wistar rats bred in our animal facility were weaned from the mother at PND 21 and housed in groups of five individuals of the same sex with 12 h light/dark cycles and *ad libitum* access to food and water. All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health Guide for the care and use of laboratory animals. The protocol "Synaptopathies mesocorticales" n°2015121715284829-V4 n°#3279 was approved by Comité d'Ethique de Marseille. All behavioral and electrophysiological experiments were performed on pubescent and adult rats from both sexes. Male and female rats do not reach puberty at the same time (Schneider, 2013), thus experiments in pubescent animals were performed in male rats between 47 and 51 and female rats between 34 and 37 days of age. Male and female rats were considered adult at PND 90–120. All animals were experimentally naïve and used only once. The number of animals per group is indicated in the corresponding figure legends.

Drugs

The mixed cannabinoid agonist WIN55,212–2 (WIN; 2 mg/kg) was dissolved in 10% polyethylene glycol/10% Tween80/saline and injected subcutaneously (s.c.) 24 h before the behavioral and electrophysiological essays. Control animals (Sham group) received vehicle. Solutions were freshly prepared on the day of the experiment and were administered in a volume of 2 mL/kg for rats weighing <150 g and 1 mL/kg for adult rats. WIN is a cannabimimetic with a higher affinity for CBRs than THC (Lawston et al., 2000). In rodents, WIN mimics most of the effects elicited by marijuana (Richardson et al., 2002; Viveros et al., 2005). It is estimated that the average content of THC in a joint is 3 mg/kg (Zamberletti et al., 2012). However, as the degree of CB1/CB2 activation after WIN administration at this same dose would be much greater compared to THC, we decided to use a slightly smaller dose. The 2 mg/kg dose chosen for single exposure is within the 1.2–3 mg/kg range that reliably causes behavioral and neuronal effects when given chronically (Tagliaferro et al., 2006; Wegener and Koch, 2009).

Behavioral Paradigms

Experiments were performed 24 h after WIN or vehicle administration in a sound attenuated chamber under dim light conditions (15–25 lux). Animals were handled two consecutive days before starting the behavioral tests and adapted to the room laboratory conditions 1 h before the tests. They were tested in a 45 × 45 cm Plexiglass arena with ±2 cm of wood shavings covering the floor. Drug treatments were counterbalanced by cage (mates were allocated to different treatment groups). Behavioral procedures were performed between 10:00 am and 3:00 pm. All sessions were recorded using a video camera using the Ethovision XT 13.0 video tracking software (Noldus, Netherlands) and analyzed by a trained observer who was unaware of treatment condition.

Social Behavior in Pubescent and Adult Rats

The social behavior test was performed as previously published (Manduca et al., 2015). The animals of each pair were equally treated (WIN or vehicle), did not differ more than 10 g in body weight and were sex and age mates but not cage mates. Pubescent or adult rats of both sexes were individually habituated to the test cage daily for either 10 (pubescent) or 5 min (adult) 2 days prior to testing. At the end of the second day of habituation (24 h before the test), the rats received the treatment. To enhance their social motivation and thus facilitate the expression of social behaviors, pubescent and adult animals were socially isolated before testing for 3.5 and 24 h, respectively (Niesink and Van Ree, 1989). The test consisted of placing two equally treated rats into the test cage for either 15 min (pubescent) or 10 min (adult).

In pubescent rats, we scored: 1/Social behavior related to play: pouncing (one animal is soliciting the other to play by attempting to nose or rub the nape of its neck) and pinning (one animal lying with its dorsal surface on the floor with the other animal standing over it). This is the most characteristic posture in social play in rats; it occurs when one animal is solicited to play by its test partner and rotates to its dorsal surface (Panksepp and Beatty, 1980; Trezza et al., 2010) and 2/Social behavior unrelated to play (assessed as a measure of general social interest): sniffing (when the rat sniff, licking, or grooms any part of the body of the test partner).

In adult rats we scored: 1/Play-related behaviors: pouncing, pinning and boxing and 2/Social behaviors unrelated to play: sniffing, social grooming (the rat licks and chews the fur of the conspecific, while placing its forepaws on the back or the neck of the other rat), following/chasing (walking or running in the direction of the partner which stays where it is or moves away), crawling under/over (one animal crawls underneath or over the partner's body, crossing it transversely from one side to the other), kicking (the rat kicks backwards at the conspecific with one or both hind paws).

The parameters were analyzed grouped and considered as *total social exploration*, calculated as the sum of social behaviors. Aggressive behavior was also scored but not considered in the calculation of *total social exploration*.

Open Field

The test was performed as previously described (Manduca et al., 2017). Each animal was transferred to the center of the arena and allowed to freely explore for 10 min. The floor was cleaned between each trial to avoid olfactory clues. Numbers of rearing and grooming were manually scored. A video tracking system (Ethovision XT, Noldus Information Technology) recorded the total distance traveled and time spent in the central zone (21 × 21 cm) of the apparatus.

Novel Object Recognition Test

The test comprised two phases: training (acquisition trial) and test. Each session lasted 5 min. During the acquisition trial, the rat was placed into the arena containing two identical sample objects (A1 and A2) placed near the two corners at either end of one side of the arena (8 cm from each adjacent wall). Thirty

minutes later, the rat returned to the apparatus containing two objects, one of them was a copy to the object used in the acquisition trial (A3), and the other one was novel (B). The objects in the test were placed in the same positions as during the acquisition trial. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploration was scored when the animal was observed sniffing or touching the object with the nose and/or forepaws. Sitting on objects was not considered to indicate exploratory behavior. The apparatus and the objects were cleaned thoroughly with 50% ethanol between trials to ensure the absence of olfactory cues. The recognition index was calculated as follow: time spent by each animal exploring the novel object divided by the total time spent exploring both objects. Recognition index higher than 0.5 indicates preferable object recognition memory. Number of rearing and grooming were registered during the acquisition trial.

Slice Preparation

Twenty-four hours after WIN or vehicle administration, rats were anesthetized with isoflurane and decapitated according to institutional regulations. The brain was sliced (300 μm) in the coronal plane with a vibratome (Integraslice, Campden Instruments, Loughborough, UK) in a sucrose-based solution at 4°C (values in mM: 87 NaCl, 75 sucrose, 25 glucose, 5 KCl, 21 $MgCl_2$, 0.5 $CaCl_2$, and 1.25 NaH_2PO_4). Slices were allowed to recover for 60 min at ±32°C in a low calcium artificial cerebrospinal fluid (aCSF; in mM: 126 NaCl, 2.5 KCl, 2.4 $MgCl_2$, 1.2 $CaCl_2$, 18 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose, equilibrated with 95% O_2/5% CO_2. Slices were maintained at room temperature until recording.

Electrophysiology

Whole-cell patch-clamp and extra-cellular field recordings were made from layer 5 pyramidal cells of the prelimbic cortex (mPFC; Kasanetz et al., 2013; Martin et al., 2016). For recording, slices were superfused (1.5–2 mL/min) with aCSF containing picrotoxin (100 μM) to block $GABA_A$ receptors. All experiments were performed at 32 ± 2°C. To evoke synaptic currents, 100–200 μs stimuli were delivered at 0.1 Hz through an aCSF-filled glass electrode positioned dorsal to the recording electrode in layer 5. Patch-clamp recordings were performed with a potassium gluconate based intracellular solution (values mM: 143 potassium gluconate, 3 NaCl, 1 $MgCl_2$, 0.3 $CaCl_2$, 1 EGTA, 0.2 cAMP, 0.3 NaGTP, 2 NaATP, 10 HEPES, pH 7.25, osmolarity 290–300 mol/L). Patch pipettes had a resistance between 3 and 5 MΩ. Cells were clamped at −70 mV (without junction potential correction). During recordings holding currents, series and input resistances and the membrane time constant (τ) were monitored. If the series resistance exceeded 25 MΩ or varied by >20% during the experiment the recording was rejected.

Current-voltage (*I–V*) curves were made by a series of hyperpolarizing to depolarizing current steps immediately after breaking into the cell. Membrane resistance was estimated from the *I–V* curve around resting membrane potential (Thomazeau et al., 2014).

For extracellular field experiments, the recording pipette was filled with aCSF. The glutamatergic nature of the field excitatory postsynaptic potential (fEPSP) was systematically confirmed at the end of the experiments using the ionotropic glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 20 μM), that specifically blocked the synaptic component without altering the non-synaptic component (data not shown). Example EPSPs and fEPSPs are single sweeps from the indicated time points, for clarity the stimulation artifact was removed from the fEPSP.

Data Analysis

The magnitude of plasticity was calculated 35–40 min after and compared to the average of baseline response. fEPSP were analyzed with Clampfit 10.6.2.2 (Molecular Devices, LLC). Statistical analysis of data was performed with Prism 6 (GraphPad Software) using tests indicated in the main text after outlier subtraction (ROUT's test). The number of outliers is indicated in the main text. Statistical tests were chosen based on normality distribution according to D'Agostino-Pearson normality test. Graph values are given as mean ± SEM and table values are given as median and interquartiles ranges. Statistical significance was set at $p < 0.05$ (two-tailed).

RESULTS

ROUT's test analysis indicates outliers' subjects present in some experimental groups as follows. Social behavior test (adults): one subject removed from female WIN group for number of pouncing, number of pinning and number of total social interactions; open field test (adults): one subject removed from Sham Male group for total distance traveled; novel object recognition test (adults): one subject removed from Sham female group for index discrimination; patch-clamp experiments (pubescents): three cells from two different rats removed from Sham female group, one cell removed from WIN female group and two cells from one rat removed from Sham male group for resting potential. Patch-clamp experiments (adults): one cell removed from Sham female group and one cell removed from Sham male group for resting potential. All these cells were then considered as outliers for other parameters such as IV-curve, rheobase and number of spikes.

All the experiments were performed using the mixed synthetic CB1/CB2 receptor agonist WIN55,212-2 that mimics most of the effects elicited by marijuana or THC in rodents (Richardson et al., 2002; Viveros et al., 2005). The 2 mg/kg dose chosen for single exposure is within the 1.2–3 mg/kg range that reliably causes behavioral and neuronal effects when given chronically (Tagliaferro et al., 2006; Wegener and Koch, 2009). The subcutaneous route of administration was chosen to minimize stress.

Single Exposure to WIN Alters Social Behavior in a Sex- and Age-Dependent Manner

We compared distinct behavioral elements related to the social repertoire of rodents in male and female rats at different ages

(puberty and adulthood) previously exposed to a single dose (2 mg/kg) of the synthetic cannabinoid agonist WIN55,212-2 (WIN). In contrast with previous studies where animals were tested shortly after WIN administration, i.e., 30 min after 0.1–1 mg/kg (Trezza and Vanderschuren, 2008a), 0.3 mg/kg (Trezza and Vanderschuren, 2008b) and 1.2 mg/kg (Schneider et al., 2008), the behavioral and synaptic tests were performed 24 h after WIN administration.

At puberty, male rats exhibited normal social play behavior 24 h after a SCE: the number of pouncing (**Figure 1A**: $U = 44$, $p = 0.696$, Mann-Whitney U-test) and pinning (**Figure 1C**: $U = 42$, $p = 0.588$, Mann-Whitney U-test) behaviors were unaltered. Accordingly, the total time spent exploring the partner during the test was unaffected (**Figure 1E**: $U = 42$, $p = 0.602$, Mann-Whitney U-test). In contrast, pubescent female rats showed significant reductions on parameters related to play behavior evidenced by a marked reduction in the number of play solicitations, i.e., pouncing (**Figure 1B**: $U = 13.5$, $p = 0.008$, Mann-Whitney U-test) and play responses, i.e., pinning (**Figure 1D**: $U = 9$, $p = 0.001$, Mann-Whitney U-test) observed 24 h after WIN administration. On the other hand, the total time spent exploring the social partner was comparable to that of the Sham group (**Figure 1F**: $U = 27$, $p = 0.156$, Mann-Whitney U-test), indicating a specific impairment on social play behavior in this group. When comparing the male and female Sham groups, a significant difference was found in the number of pinning behaviors ($U = 11$, $p = 0.001$, Mann-Whitney test, data not shown). Females presented higher levels of pinning than males (21.70 ± 2.47 and 8.81 ± 2.10, respectively) which may be attributed to either the sex difference *per se* or the age difference at which puberty appears in both sexes.

In contrast to pubescent rats, both male and female adult rats showed reduced social interest 24 h after SCE. Adult male rats administered WIN presented reduced general social exploration (**Figure 2A**: $U = 7$, $p = 0.003$, Mann-Whitney U-test) as well as reduced sniffing exploration (**Figure 2C**: $U = 3.5$,

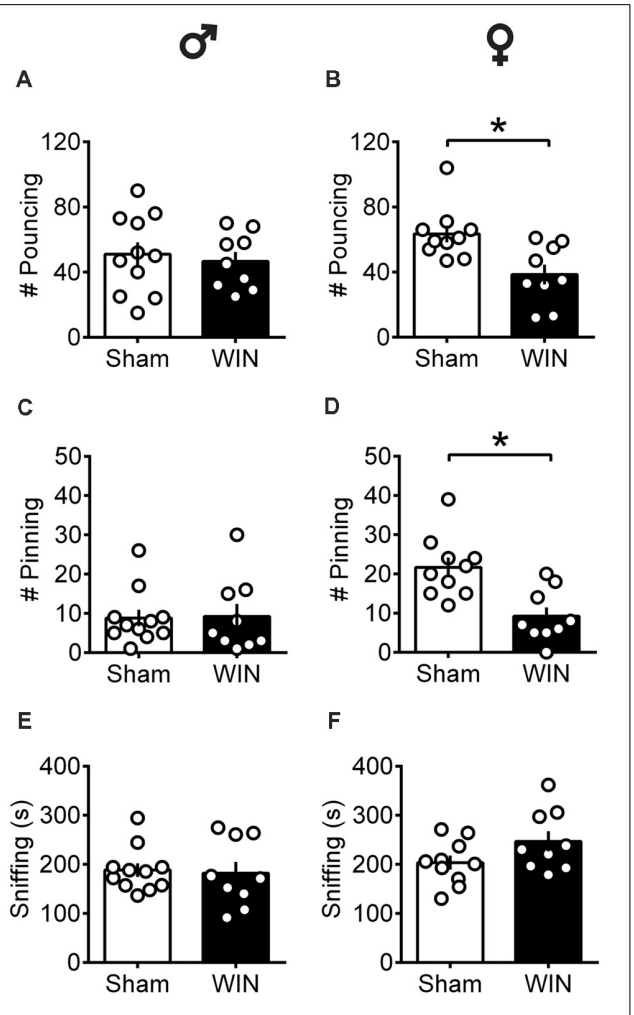


FIGURE 1 | Sex-specific alteration of play behavior in pubescent rats 24 h after a single *in vivo* cannabinoid exposure. Twenty-four hours following a single exposure to WIN55,212-2 (WIN, 2 mg/kg, s.c.), pouncing was normal in male pubescent rats **(A)** in contrast to female littermates **(B)** whom displayed a marked reduction in the number of pouncing compared to Sham animals. Twenty-four hours following WIN exposure, pinning was similar to that of Sham animals in males **(C)** but was largely reduced in female littermates **(D)**. WIN-exposed rats of both sexes (**E**, Male; **F**, Female) spent similar time sniffing the congener compared to their respective Sham groups. Data represent mean ± SEM. Scatter dot plot represents a pair of animals. *$p < 0.05$, Mann-Whitney U-test. ♂Males (Sham $n = 11$ pairs; WIN $n = 9$ pairs); ♀Females (Sham $n = 10$ pairs; WIN $n = 9$ pairs).

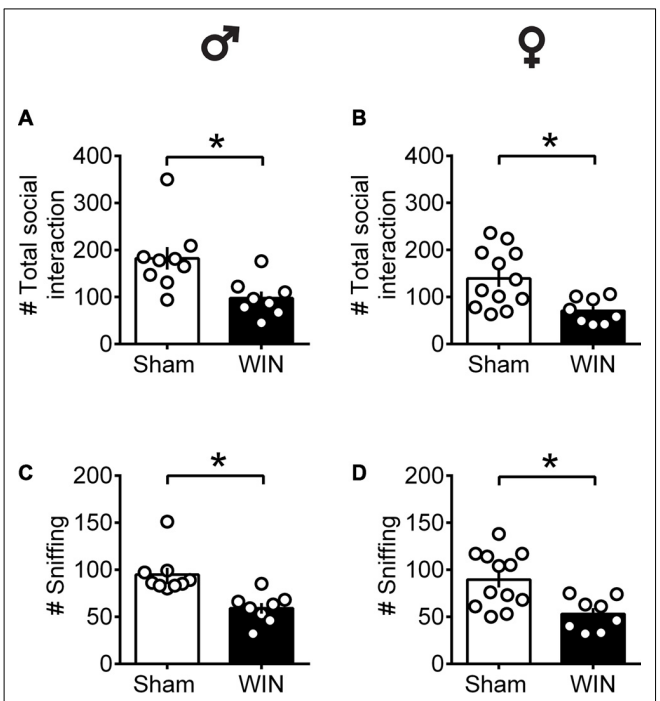


FIGURE 2 | Social interactions are diminished in adult rats of both sex 24 h after a single *in vivo* cannabinoid exposure. Adult male **(A)** and female **(B)** rats had fewer social contacts with their congeners 24 h following a single exposure to WIN55,212-2 (2 mg/kg, s.c.). Similarly, sniffing was reduced in both adult male **(C)** and female **(D)** rats 24 h following a single exposure WIN, compared to control animals. Data represent mean ± SEM. Scatter dot plot represents a pair of animals. *$p < 0.05$, Mann-Whitney U-test. ♂Males (Sham $n = 8$ pairs; WIN $n = 8$ pairs); ♀Females (Sham $n = 12$ pairs; WIN $n = 8$ pairs).

$p < 0.001$, Mann-Whitney U-test) compared to the Sham group. Similarly, adult cannabinoid-exposed females had less social contact (**Figure 2B**: $U = 14.5$, $p = 0.007$, Mann-Whitney U-test) and sniffing events (**Figure 2D**: $U = 15.5$, $p = 0.010$, Mann-Whitney U-test) with congeners. In addition, SCE did not elicit aggressive behavior in any of the tested groups (data not shown).

Together, these data show that during puberty, SCE is sufficient to alter social behavior in a sex-specific manner: play behavior was specifically reduced in females while males were spared. In adults, SCE caused a general impairment in sociability, exhibited by a reduced number of events related to the total social contacts and sniffing in both male and female rats.

Additional groups of pubescent and adult rats, from either sex, were evaluated in the open field test 24 h after WIN administration. As shown in **Figure 3**, previous WIN administration did not alter pubescent rats' behavior in the open field test, since there was no change in the total distance traveled in either male or female groups treated with win (**Figure 3A**, male: $U = 20$, $p = 0.138$; female: $U = 39$, $p = 0.435$; Mann-Whitney U-test). Interestingly, parameters that would suggest alterations on anxiety levels in WIN-treated pubescent rats such as the time spent in the central part of the arena (**Figure 3B**, male: $U = 34$, $p = 0.888$; female: $U = 39$, $p = 0.435$; Mann Whitney U-test) or in the time spent in the peripheral zone (**Figure 3C**, Male: $U = 34$, $p = 0.888$; female: $U = 39$, $p = 0.436$) remained unchanged 24 h after WIN administration. Furthermore, the number of rearing (male: $U = 29.5$, $p = 0.138$; female: $U = 43$, $p = 0.615$; Mann-Whitney U-test) and grooming behaviors (male Sham: 1.62 ± 0.49, male WIN: 2.77 ± 0.49; female Sham: 4.10 ± 1.49, female WIN: 2.60 ± 0.49; mean ± SEM. $U = 28.5$, $p = 0.487$; female: $U = 45$, $p = 0.723$; Mann-Whitney U-test; data not shown) was similar between Sham and WIN treated groups of both sexes.

Locomotion was reduced in the adult female group 24 h after WIN administration (**Figure 3D**, $U = 24$, $p = 0.029$; Mann-Whitney U-test), but no change in the time spent in the central part of the apparatus (**Figure 3E**, $U = 44$, $p = 0.710$; Mann Whitney U-test) or in the time spent in the peripheral zone (**Figure 3F**, $U = 44$, $p = 0.710$; Mann Whitney U-test) was observed. Rearing ($U = 33.5$, $p = 0.136$; Mann-Whitney U-test) and grooming ($U = 32$, $p = 0.101$; Mann Whitney U-test) were not affected by previous WIN exposure in this group (data not shown). Adult male rats exhibited no alteration in the total distance traveled (**Figure 3D**, $U = 29$, $p = 0.211$; Mann Whitney U-test) nor in the time spent in the central (**Figure 3E**, $U = 26$, $p = 0.133$; Mann Whitney U-test) or peripheral zones of the apparatus (**Figure 3F**, $U = 26$, $p = 0.133$; Mann Whitney U-test). As observed in adult females, rearing behavior ($U = 33.5$, $p = 0.136$; Mann-Whitney U-test) and grooming ($U = 25$, $p = 0.298$; Mann Whitney U-test) were not affected in adult males previously exposed to WIN (data not shown). Taken together, these data do not suggest a major contribution of WIN-induced anxiety to the reduction in sociability observed in adult rats.

Intact Memory Recognition in Pubescent and Adult Rats of Both Sexes After Single Cannabinoid Exposure

In humans (Walsh et al., 2017) and rodents (Wegener et al., 2008; Han et al., 2012; Galanopoulos et al., 2014), cannabinoids rapidly

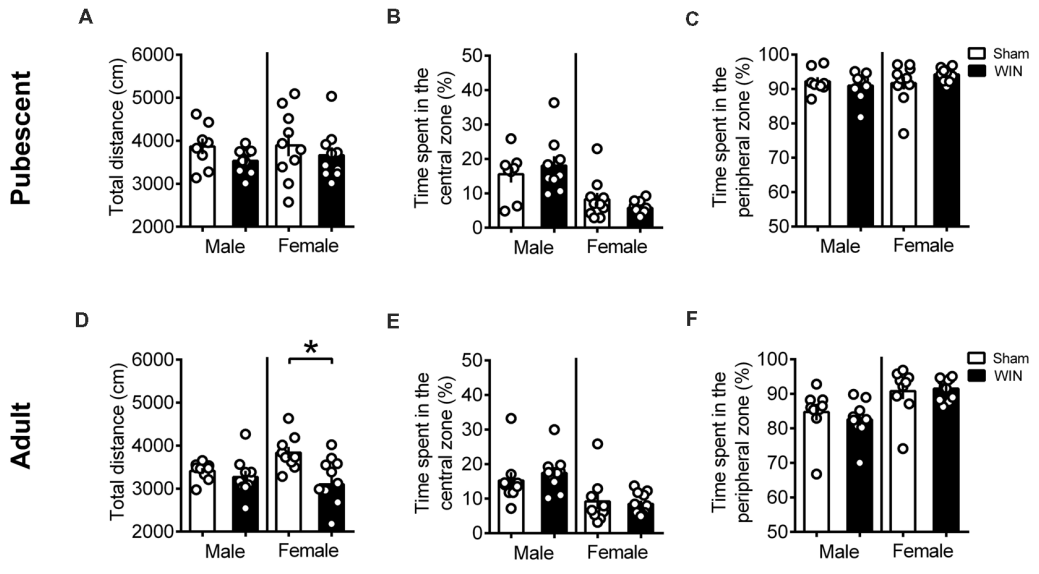


FIGURE 3 | Sex-specific effects in locomotion 24 h after single *in vivo* cannabinoid exposure. Single administration of WIN55,212-2 (WIN, 2 mg/kg, s.c.) 24 h before did not alter the distance traveled **(A)** nor the time spent in the central **(B)** or peripheral zone **(C)** of the apparatus in pubescent rats of both sexes during the open field test. At adulthood, WIN-exposed females had a reduction in the locomotion, while males were not affected **(D)**. Time spent in the central **(E)** or peripheral zone **(F)** remain unchanged in adult rats of both sexes. Data represent mean ± SEM. Scatter dot plot represents one animal. *$p < 0.05$, Mann-Whitney U-test. Pubescent Males: Sham $n = 8$ and WIN $n = 9$; Pubescent Females: Sham = 10 and WIN = 10; Adult Males: Sham = 9 and WIN = 10; Adult Females: Sham $n = 9$ and WIN $n = 11$.

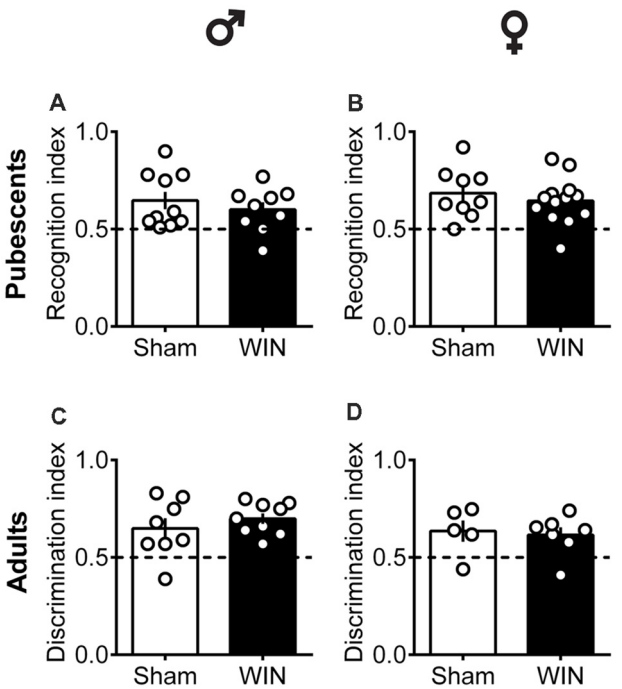


FIGURE 4 | Intact memory discrimination in the novel object recognition test 24 h after single *in vivo* cannabinoid exposure in both pubescent and adult male and female rats. Animals were tested 24 h after a single exposure to WIN55,212-2 (WIN, 2 mg/kg, s.c.). Recognition index between the novel and familiar objects were similar in male **(A)** and female **(B)** WIN-treated pubescent rats compared to their respective Sham groups. Similarly, no differences were observed in recognition index in male **(C)** and female **(D)** adult rats treated with WIN. Data represent mean ± SEM. Scatter dot plot represents one animal. Mann-Whitney *U*-test. ♂Males (Pubescents: Sham $n = 10$ and WIN $n = 9$; Adults: Sham $n = 8$ and WIN $n = 9$); ♀Females (Pubescents: Sham $n = 9$ and WIN $n = 13$; Adults: Sham = 5 and WIN = 7).

impair recent memory. Social behavior requires emotional control and cognitive abilities (Trezza et al., 2014). Thus, we used the Novel Object Recognition test to evaluate the consequences of SCE on rats of our sex and age groups. Twenty four hours after SCE, pubescent male (**Figure 4A**: $U = 17$, $p = 0.999$, Mann-Whitney *U*-test) and female (**Figure 4B**: $U = 52$, $p = 0.682$, Mann-Whitney *U*-test) rats presented normal short-term memory. Furthermore, recognition indexes were similar in both adult male and female Sham- and WIN-treated rats (**Figure 4C**: male, $U = 29.5$, $p = 0.557$; **Figure 4D**: female, $U = 15$, $p = 0.755$; Mann-Whitney *U*-test). The total time spent exploring the objects during the acquisition trial was not altered in any of the tested groups (Pubescent Males: Sham vs. WIN, $U = 31$, $p = 0.277$; Pubescent Females: Sham vs. WIN, $U = 42$, $p = 0.292$; Adult Males: Sham vs. WIN, $U = 31$, $p = 0.673$; Adult Females: Sham vs. WIN, $U = 5$, $p = 0.082$; Mann-Whitney *U*-test; data not shown). Similarly, none of the parameters linked to exploration and emotionality such as rearing and grooming were altered after WIN administration during the acquisition trial of the test (**Table 1**).

Single *in vivo* Cannabinoid Exposure Leads to Sex-Specific Ablation of Prefrontal eCB Plasticity

The central position of the PFC and eCB system in the regulation of social behavior and the important role of synaptic plasticity in this structure in mediating experience-dependent adaptations are well-documented (for review see Araque et al., 2017). At the synaptic level, activity-dependent plasticity in the PFC—including eCB-mediated LTD and NMDAR-mediated LTP—is a common target in animal models of neuropsychiatric diseases (Scheyer et al., 2017). We compared the LTD mediated by the eCB system (eCB-LTD) in the PFC between Sham- and WIN-treated rats of both sexes at different ages, specifically pubescence and adulthood.

Low-frequency stimulation of layer 5 PFC synapses induced comparable LTD in both control and cannabinoid-exposed pubescent male rats (**Figure 5A**: Sham: $t_{(6)} = 5.596$, $p = 0.001$; WIN: $t_{(4)} = 3.190$, $p = 0.033$; Paired *t*-test). Similar results were observed in adult males with or without prior *in vivo* cannabinoid exposure (**Figure 5B**: Sham, $t_{(6)} = 3.116$, $p = 0.020$; WIN, $t_{(6)} = 2.787$, $p = 0.031$; Paired *t*-test). In the male rat PFC, eCB-LTD is not affected 24 h after *in vivo* cannabinoid administration. Strikingly, eCB-LTD was ablated in PFC slices obtained from female rats in both age groups. **Figure 5C** shows the lack of LTD in PFC slices from cannabinoid-treated pubescent (Sham, $t_{(4)} = 5.021$, $p = 0.007$; WIN, $t_{(4)} = 1.129$, $p = 0.322$; paired *t*-test) and adult female rats (**Figure 5D**: Sham, $t_{(4)} = 2.979$, $p = 0.040$; WIN, $t_{(7)} = 1.003$, $p = 0.349$; paired *t*-test).

TABLE 1 | Statistical report for rearing and grooming events in pubescent and adult rats from both sexes tested 24 h after a single *in vivo* exposure to WIN55,212-2 (2 mg/kg, s.c.).

		Sham			WIN				
		Median	**Quartiles**	***n***	**Median**	**Quartiles**	***n***	***p***	***U***
Rearing	♂Pubescent	37.50	32.75/46.00	10	38.00	31.00/43.50	9	0.826	42
	♂Adult	35.50	25.25/44.00	8	36.00	16.50/40.00	9	0.524	29
	♀Pubescent	35.00	25.00/40.50	9	37.00	30.50/45.00	13	0.502	48
	♀Adult	22.00	19.50/30.50	5	28.00	23.00/44.00	7	0.162	8.5
Grooming	♂Pubescent	0.50	0/2.25	10	2.00	1.00/3.00	9	0.129	26.5
	♂Adult	1.00	0.25/1.75	8	1	1.00/3.00	9	0.395	26.5
	♀Pubescent	1.00	0/2.50	9	1	1.00/5.00	13	0.732	53
	♀Adult	2.00	0.50/4.50	5	3.00	0/5.00	7	0.977	17

The number of rearing and grooming was counted during the acquisition trial in the novel object recognition test. Quartiles, 25 and 75% percentiles; n, number of animals; Mann-Whitney U-test; ♂males; ♀females.

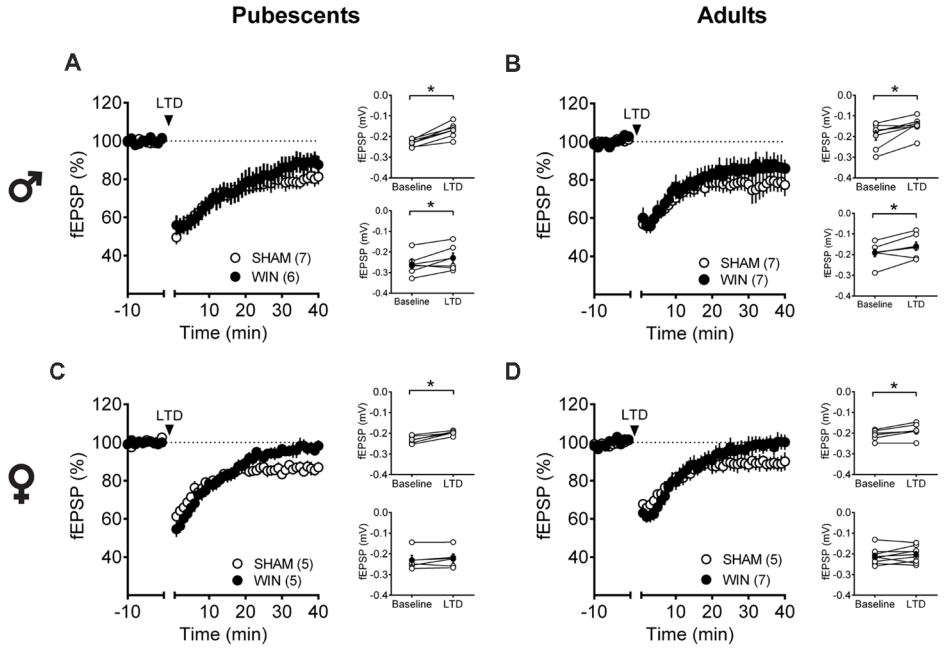


FIGURE 5 | Sex-specific effects of a single *in vivo* cannabinoid exposure on prefrontal cortex (PFC) endogenous cannabinoid (eCB)-long-term depression (LTD). Rats were exposed to WIN55,212-2 (WIN, 2 mg/kg, s.c.) 24 h before. Average time-courses of mean field excitatory postsynaptic potentials (fEPSPs) showing that low-frequency stimulation (indicated by arrow) induced LTD at mPFC synapses in both Sham- (white circles, $n = 7$) and WIN- (black circles, $n = 6$) exposed pubescent males **(A)**. Similarly, LTD was identical in Sham- (white circles, $n = 7$) and WIN- (black circles, $n = 7$) exposed adult males **(B)**. In contrast, LTD was ablated in mPFC slices obtained from both pubescent (**C**, Sham, white circles, $n = 5$; WIN, black circles, $n = 5$) and adult (**D**, Sham, white circles, $n = 5$; WIN, black circles, $n = 7$) females 24 h after a single exposure to WIN. Adjacent to the time-course figures individual experiments (white circles) and group average (Sham, gray circles; WIN, black circles) before (baseline) and after (35–40 min) LTD induction are showed. LTD is present in WIN-treated male rats at both ages: pubescent (**A**, on the right) and adulthood (**B**, on the right). In contrast, LTD was absent in both pubescent (**C**, on the right) and adult (**D**, on the right) females previously treated with WIN. Error bars indicate SEM, n = individual rats, $^*p < 0.05$, Paired t-test. ♂Males; ♀Females.

Age- and Sex-Dependent Ablation of LTP After *in vivo* Single Exposure to Cannabinoid

Considering that the extensive repertoire of synaptic plasticity expressed by medial PFC synapses is sensitive to various regimens of exposure to drugs of abuse (Kasanetz et al., 2013; van Huijstee and Mansvelder, 2015; Lovelace et al., 2015; Renard et al., 2016a; Cannady et al., 2017), we assessed a second type of plasticity in the PFC which is frequently related to endophenotypes of neuropsychiatric disorders (Thomazeau et al., 2014; Neuhofer et al., 2015; Iafrati et al., 2016; Labouesse et al., 2017; Manduca et al., 2017), the NMDAR-dependent LTP (NMDAR-LTP). NMDAR-LTP was ablated in adult male rats while pubescent males were spared. **Figures 5A,B** show comparable LTP between Sham and cannabinoid-treated pubescent male rats (**Figure 6A**: Sham, $t_{(6)} = 9.676$, $p < 0.001$; WIN, $t_{(7)} = 3.677$, $p = 0.007$; Paired t-test), but not in adult male rats (**Figure 6B**: Sham, $t_{(8)} = 5.560$, $p < 0.001$; WIN, $t_{(6)} = 2.062$, $p = 0.084$; Paired t-test). In contrast, in both age groups, NMDAR-LTP was comparable in Sham- and cannabinoid-treated female rats: both pubescent (**Figure 6C**: Sham, $t_{(6)} = 8.424$, $p < 0.001$; WIN, $t_{(6)} = 3.369$, $p = 0.015$; Paired t-test) and adult rats (**Figure 6D**: Sham, $t_{(4)} = 4.349$, $p = 0.012$; WIN, $t_{(7)} = 3.133$, $p = 0.016$; Paired t-test) had normal NMDAR-LTP 24 h following *in vivo* cannabinoid exposure.

Single *in vivo* Exposure to WIN Causes Age- and Sex-Specific Modifications in Intrinsic Pyramidal Neuron Properties

Independent of sex, all recorded PFC neurons in pubescent rats showed similar membrane reaction profiles in response to a series of somatic current steps 24 h after SCE (**Figure 7A**: male, $F_{(\text{interaction } 10,440)} = 1.551$, $p = 0.118$; **Figure 7B**: female, $F_{(\text{interaction } 10,270)} = 0.499$, $p = 0.889$; two-way repeated-measures ANOVA). The resting membrane potential (**Figure 7C**: male, $U = 230$, $p = 0.627$; **Figure 7D**: female, $U = 99.5$, $p = 0.854$; Mann-Whitney U-test), as well as the rheobase (**Figure 7E**: male, $U = 194.5$, $p = 0.198$; **Figure 7F**: female, $U = 68$, $p = 0.115$; Mann-Whitney U-test), were comparable between Sham- and WIN-treated pubescent rats from both sexes. Also, no changes in excitability were observed since the number of actions potentials in response to somatic currents steps were comparable in both control and WIN-treated pubescent rats of both sexes (**Figure 7G**: male, $F_{(\text{interaction } 12,492)} = 1.189$, $p = 0.287$; **Figure 7H**: female, $F_{(\text{interaction } 12,324)} = 3.624$, $p < 0.001$ and $F_{(\text{treatment } 1,27)} = 0.389$, $p = 0.537$; two-way repeated measures ANOVA).

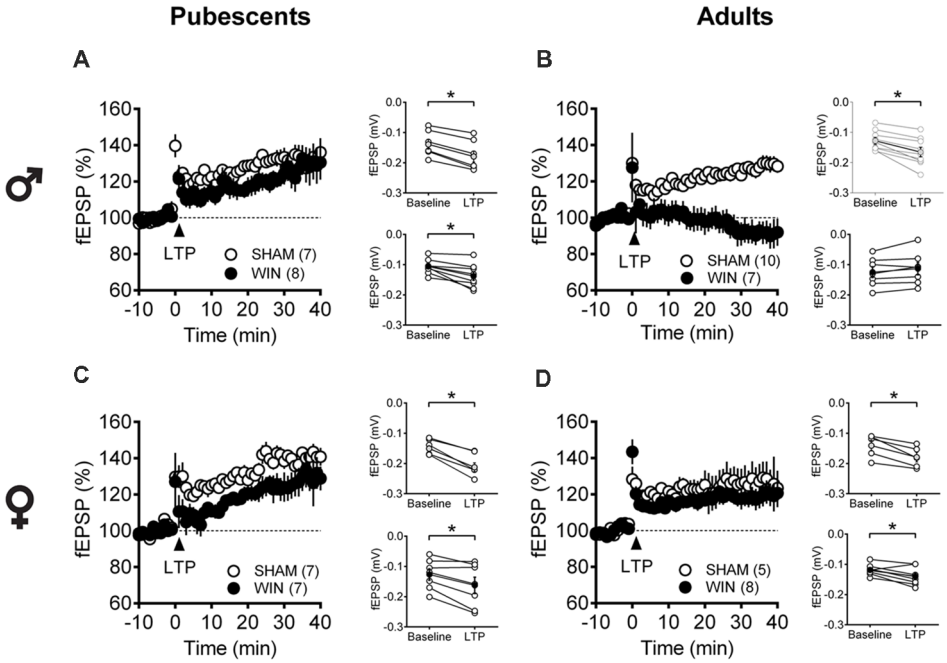


FIGURE 6 | Age- and sex-dependent ablation of long-term potentiation (LTP) in the rat PFC 24 h after *in vivo* cannabinoid exposure. Rats were exposed to WIN55,212-2 (WIN, 2 mg/kg, s.c.) 24 h before. Average time-courses of mean fEPSPs showing that theta-burst stimulation (indicated arrow) induced a LTP at mPFC synapses in both Sham- (white circles, $n = 7$) and WIN- (black circles, $n = 8$) exposed pubescent males **(A)** but not WIN-treated adults (**B**, Sham: white circles, $n = 10$; WIN: black circles, $n = 7$). In contrast, LTP was present in mPFC slices obtained from both pubescent (**C**, Sham: white circles, $n = 7$; WIN: black circles, $n = 7$) and adult (**D**, Sham: white circles, $n = 5$; WIN: black circles, $n = 8$) WIN-treated females. Adjacent to the time-course figures are showed individual experiments (white circles) and group average (Sham, gray circles; WIN, black circles) before (baseline) and after (35–40 min) LTP induction showing that, in males, LTP is present in pubescent (**A**, on the right) but not in adults (**B**, on the right). In contrast, LTP was present in both pubescent (**C**, on the right) and adult (**D**, on the right) females previously treated with WIN. Error bars indicate SEM, n = individual rats, $^*p < 0.05$, Paired t-test. ♂Males; ♀Females.

In adult rats however, sex-specific modifications of the excitability of pyramidal neurons sampled from females were observed following a single *in vivo* cannabinoid exposure. Intrinsic properties of layer V PFC pyramidal neurons (I/V curve **Figure 8A**: $F_{(\text{interaction } 9,225)} = 1.907$, $p = 0.052$, two-way repeated measures ANOVA), resting membrane potentials (**Figure 8C**: $U = 79$, $p = 0.614$, Mann-Whitney U-test; rheobase **Figure 8E**: $U = 79$, $p = 0.614$, Mann-Whitney U-test) and the number of action potentials in response to increasing depolarizing current (**Figure 8G**: $F_{(\text{interaction } 10,250)} = 1.417$, $p = 0.173$, two-way repeated measures ANOVA) were comparable in control and WIN-treated male rats. In striking contrast, a single *in vivo* cannabinoid exposure increased the excitability of PFC pyramidal neurons of adult females. Thus, we observed an alteration of the membrane reaction profile in response to a series of somatic current steps (**Figure 8B**: $F_{(\text{interaction } 9,369)} = 3.480$, $p < 0.001$ and $F_{(\text{treatment } 1,41)} = 5.576$, $p = 0.023$, two-way repeated measures ANOVA) and a marked reduction of the rheobase (**Figure 8F**: $U = 137.5$, $p = 0.023$, Mann-Whitney U-test) accompanying an increased number of action potential in response to increasing depolarizing current (**Figure 8H**: $F_{(\text{interaction } 10,410)} = 3.038$, $p = 0.001$ and $F_{(\text{treatment } 1,41)} = 8.041$, $p = 0.007$, two-way repeated measures ANOVA). The resting membrane potentials were similar to that of control female rats (**Figure 8D**, $U = 166.5$, $p = 0.124$, Mann-Whitney U-test). Taken together, these data suggest an overall increase in the excitability of PFC pyramidal neurons in adult females 24 h after SCE.

DISCUSSION

We found that 24 h after a single *in vivo* exposure to a cannabinoid, the behavioral, neuronal and synaptic consequences differ depending on the sex and age of the rat. The current data indicate a heightened sensitivity of females, especially during pubescence. Specifically, social behavior and eCB-mediated LTD showed strong deficits in exposed pubescent females while age-matched male littermates were spared. During adulthood, although reduced social interactions were observed in both sexes, eCB-mediated synaptic plasticity was ablated specifically in females and NMDAR-dependent LTP specifically in males.

Stimulation of CB1R acutely modulates social play in adolescent rats (Trezza and Vanderschuren, 2008a). We showed that a single exposure to the synthetic cannabinoid WIN (2 mg/kg), at a dose reported to acutely decrease social interactions in male rats (Schneider et al., 2008; Trezza and Vanderschuren, 2008a,b) has sex-specific effects as long as 24 h after *in vivo* exposure. In the pubescent

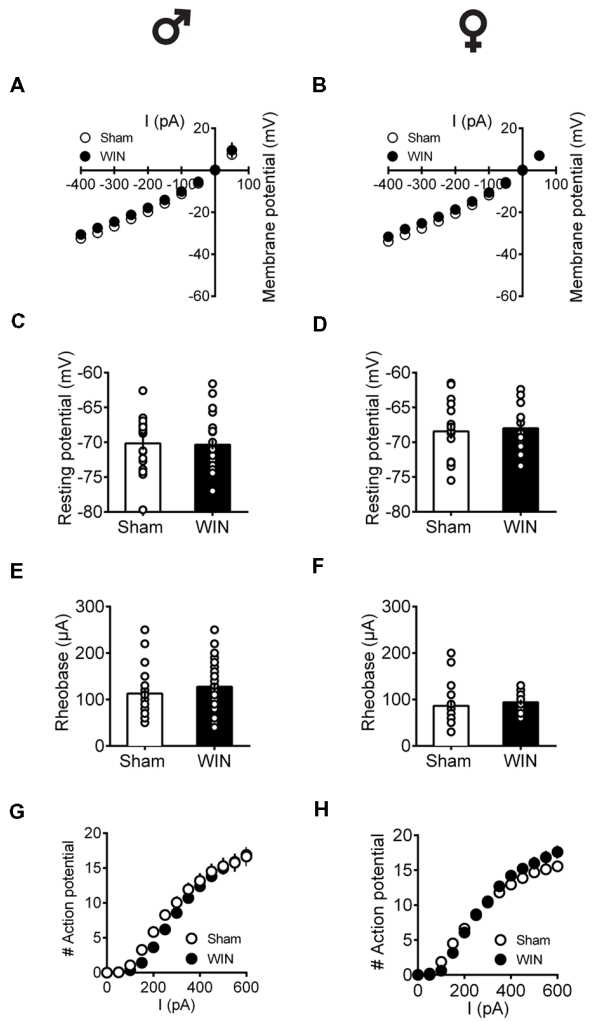


FIGURE 7 | Intrinsic properties of PFC pyramidal neurons are not altered by single *in vivo* exposure to cannabinoid in pubescent rats. Rats received a single administration of WIN55,212-2 (WIN, 2 mg/kg, s.c.) 24 h before. Current–voltage plot from visually identified pyramidal neurons recorded from pubescent rats showing similar cell voltage in response to current steps between Sham and WIN of both male **(A)** and female **(B)** groups. No change was observed in the resting membrane potential 24 h after WIN treatment in both male **(C)** and female **(D)** pubescent rats. Quantification of neuronal spiking properties indicated no change in the rheobase of either males **(E)** or females **(F)** 24 h after single WIN. The number of evoked action potentials in response to increasing depolarizing current steps was similar in Sham and WIN-treated male **(G)** and female **(H)** pubescent rats. Males: Sham, $n = 15$ cells/6 rats; WIN, $n = 28$ cells/10 rats. Females: Sham, $n = 17$ cells/7 rats; WIN, $n = 13$ cells/7 rats. Scatter dot plot represents one cell. Data represent mean ± SEM. ♂Males; ♀Females.

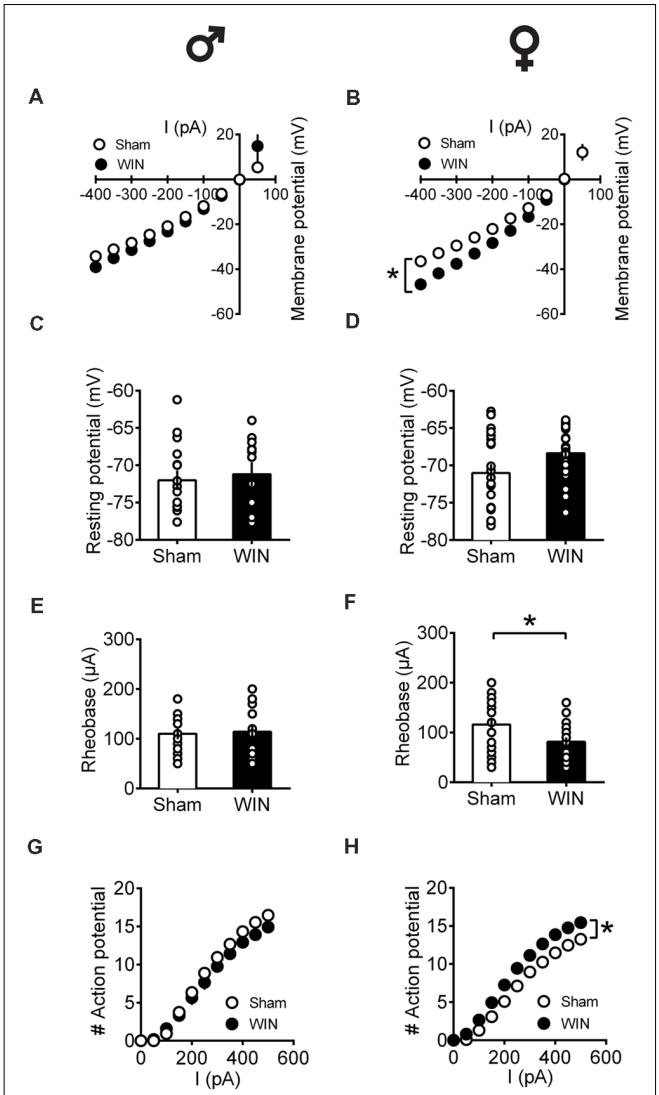


FIGURE 8 | Sex-specific alteration of pyramidal neurons' intrinsic properties in adult rats 24 h following single *in vivo* cannabinoid administration. Rats received a single administration of WIN55,212-2 (WIN, 2 mg/kg, s.c.) 24 h before. Current–voltage plot from visually identified pyramidal neurons recorded from adult rats showing no difference in cell voltage in response to current steps between Sham and WIN groups of adult male rats **(A)**. In contrast, membrane potentials were altered in adult WIN-treated females compared to control group **(B)**. The resting membrane potentials were similar to that of control in adult males **(C)** and females **(D)** 24 h following single WIN exposure. Quantification of neuronal spiking properties showed no change in the rheobase of males **(E)**, but a marked reduction in the female WIN-treated group **(F)**. The number of evoked action potentials in response to increasing depolarizing current step was similar in Sham and WIN-treated males **(G)**. In contrast, females showed a higher number of action potentials 24 h after WIN treatment **(H)**. Male: Sham, $n = 16$ cells/6 rats; WIN, $n = 12$ cells/5 rats; Female: Sham, $n = 16$ cells/6 rats; WIN, $n = 20$ cells/7 rats. Scatter dot plot represents one cell. Data represent mean ± SEM. $^*p < 0.05$, Mann-Whitney test (B), Bonferroni's multiple comparisons test (F). ♂Males; ♀Females.

group, cannabinoid-treated females exhibited less social play behavior but normal social investigation and locomotion while the sociability of pubescent males exposed to WIN was indistinguishable from that of sham rats.

Sham pubescent females presented higher levels of play than sham pubescent males. This observation can be explained by the difference in the age range at which males and females were herein tested: the objective was verifying the effect of acute cannabinoid exposure during pubescence. Thus, as females reach puberty before males (Schneider, 2008), we used females at an earlier PND of development than males. The frequency and

intensity of play behaviors peak between PNDs 28–40 regardless of sex (Panksepp, 1981) and decline thereafter when rats reach sexual maturity. Because the latter differs between sexes, it is expected that males and females display different levels of play during puberty. Thus, considering that in our conditions the play behavior of pubescent females was reduced 24 h after SCE to the same levels of those observed in control males, it is tempting to hypothesize that SCE induces a "masculinization" of female social play behavior. Interestingly, a recent study showed that the activation of both CB1 and CB2 receptors (as that observed following exposure to WIN) is implicated in the masculinization of play behavior of pre-pubertal female rats (Argue et al., 2017), reinforcing the idea of sex-dependent modulation of social behaviors that arises early in life. Potential mechanisms include dendrites and spines' morphological—and presumably functional—alterations (Argue et al., 2017) and/or altered levels of circulating eCB (Craft et al., 2013). Future studies of anhedonia in the current particular experimental condition will provide insights into the rewarding component of social interactions and, for example, rule out putative depressive effect of SCE. Taken together these data confirm and extend those of Craft et al. (2013) who showed that females are more affected by exogenous cannabinoids during pubescence than males.

Gonadal steroids hormones seem to be involved in the sexual differentiation of cannabinoid sensitivity. Importantly, rat hormonal status (i.e., estrous cycle phase) has been reported to significantly influence sex differences for cannabinoid effects (revised from Cooper and Craft, 2018). Indeed, sex differences are not entirely consistent across studies regarding differences in CB1R mRNA or binding affinity and eCB content (Reich et al., 2009; Riebe et al., 2010; Castelli et al., 2014; Weed et al., 2016), supporting the important role of hormonal status in these differences. As cannabinoid dose, administration route, post-administration intervals and rat strains are not consistent among studies, methodological details may help explain this divergence. Comparing the effect of a single administration of WIN during the different phases of the female cycle may help in better understanding the subtle effects reported in the current study.

It is well described that social interactions during adolescence are crucial for the development of social competence at adulthood (Douglas et al., 2004; Vanderschuren and Trezza, 2014) and that modification of rat adolescent social activity alters neurobehavioral parameters related to pain processing, anxiety, depression and substance abuse (reviewed from Burke et al., 2017). Among the several forms of sociability in rodents, play behavior is thought to be of principal importance for social development. When developing rats are deprived from play with sex- and age-matched conspecifics, abnormal patterns of social, sexual and aggressive behaviors are observed once they reach adulthood (Vanderschuren and Trezza, 2014). Future experiments will aid in determining if the deficits caused by SCE are long lasting and whether they can be reversed with pharmacological intervention.

As rats reach sexual maturation, neural alterations of the "social brain" are differently regulated according to age and sex. Thus, the social behavior repertoire differs between pubescent and adult rats (Panksepp and Beatty, 1980; Pellis and Pellis, 2007; Graham and Burghardt, 2010). Accordingly, we showed that acute WIN administration in adult rats triggered different consequences on behavior. In contrast to the pubescent groups, a unique exposure to WIN perturbed social behavior in both sexes at adulthood. There is strong evidence of an anxiolytic/anxiogenic component which influences adult social behaviors. Namely, a decrease in social interactions represents an anxiogenic response while increased social interaction followed by unchanged motor activity indicates an anxiolytic effect (File and Seth, 2003). Parameters that would suggest alterations on anxiety levels in our study such as time spent in the central part of the arena remained unchanged in both males and females treated with WIN. The reduced locomotion observed in adult WIN-treated females could be indicative of a slight sedative effect of WIN, which could in turn be implicated in the reduced sociability herein observed.

Although sex differences on cannabinoids' effects on cognition have been reported (Rubino et al., 2008; Trezza and Vanderschuren, 2009; Rubino and Parolaro, 2015; Silva et al., 2016; Wiley et al., 2017), some studies demonstrated no changes in learning ability 24 h after cannabinoid administration (water maze, object location and object recognition tasks, WIN 1.2 mg/kg, i.p.; Abush and Akirav, 2012). In the present work, novel object recognition memory was unaffected in either sex, in favor of the idea that the deficits are not generalized but rather selective to the social behavior.

We observed impairments on synaptic plasticity in a sex- and age-dependent manner. Surprisingly, we showed that 24 h after SCE, pubescent males did not display behavioral or synaptic changes, while adult rats did. On the other hand, female rats of both ages were negatively impacted by previous WIN administration, in agreement with the literature showing that pubescent females are the more vulnerable group (Rubino and Parolaro, 2011, 2016; Craft et al., 2013; Renard et al., 2016a). The eCB signaling machinery is positioned in a way to influence PFC communication and control other brain regions (Domenici et al., 2006; Hill et al., 2007). Chronic cannabinoid exposure significantly impairs synaptic plasticity throughout the brain (Renard et al., 2016b; Araque et al., 2017), while the synaptic plasticity deficits resulting from acute cannabinoid exposure largely depend on the brain area. For example, a single exposure to THC (3 mg/kg; 15–20 h before) ablated eCB-mediated synaptic plasticity in the adult mouse NAc and hippocampus (Mato et al., 2004) but not hippocampal CA1 LTP (10 mg/kg; 24 h before; Hoffman et al., 2007) nor eCB-LTD at VTA GABA synapses (Friend et al., 2017). In rats, an acute single injection of WIN (1.2 mg/kg; 24 h before) impaired LTP in the ventral subiculum-accumbens pathway (Abush and Akirav, 2012) and in the Schaffer collateral-CA1 projection (WIN 0.5 mg/kg; 30 min before; Abush and Akirav, 2010). In a dose-dependent manner, WIN (0.5–2 mg/kg, i.p.) impaired short-term plasticity and LTP at perforant path dentate gyrus synapses in adult rats (Colangeli et al., 2017). It is important to highlight that in the aforementioned studies only male rodents were

evaluated. Here, we showed that, male rats of both ages showed normal eCB-LTD, the latter was ablated in female rats 24 h after SCE regardless of the age. Furthermore, only adult females exhibited altered neuronal excitability. These results suggest impaired cannabinoid signaling with possible mechanisms ranging from the reduction of presynaptic mobility of surface CB1R receptors as shown by our group 24 h after WIN *in vitro* (Mikasova et al., 2008), sex-differential CB1R desensitization (Farquhar et al., 2019), modified interactions with adaptor proteins (e.g., GASP and AP-3; for review see Howlett et al., 2010) or CB1R interacting proteins (e.g., SGIP1 and CRIP1, Howlett et al., 2010; Hájková et al., 2016) and altered functions of the enzymes controlling circulating eCB.

Sex differences in the eCB system may be involved in the aforementioned effects. Cortical CB1R expression and function are higher in juvenile male rats (PND 28–35) as compared to adolescent sex-matched subjects (PND 40), and CB1R levels decrease thereafter towards young adult levels (PND 70; Heng et al., 2011). Compared to females, male rats have a higher density of CB1R. However, a higher G-protein activation after CB1R stimulation is observed in adolescent females in several brain areas (Rubino et al., 2008; Burston et al., 2010). Additional molecular mechanisms may help explain the observed sex differences. Sexual differences in the eCB system appear early in development in rodents (Craft et al., 2013). Sexually dimorphic regulation of synaptic plasticity or intrinsic neuronal activity in the amygdala (Fendt et al., 2013; Chen et al., 2014; Bender et al., 2017), hippocampus (Huang and Woolley, 2012; Inoue et al., 2014; Harte-Hargrove et al., 2015; Qi et al., 2016) and PFC (Nakajima et al., 2014; Li et al., 2016) has been described. Female rats exhibit greater concentrations of the metabolic enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) as early as PND 4 compared to males (Krebs-Kraft et al., 2010). Moreover, CB1R expression reaches its peak earlier in females (PND 30) than in males (PND 40; Romero et al., 1997), whereas at adulthood, CB1R density is lower in the PFC and amygdala of cycling females (Castelli et al., 2014). Finally, it was recently shown that the CB1 agonist CP 55, 940 has widespread effects on the brain lipidome in adolescent female mice (Leishman et al., 2018). Thus, females' eCB systems appear more sensitive to the deleterious influence of exogenous cannabinoids.

While eCB-LTD in both pubescent and adult males was unaffected by a single WIN exposure, NMDAR-LTP was selectively ablated in adult males. Regarding pubescent rats, both males and females were spared. These findings show that cannabinoid-induced impairments on synaptic plasticity are not generalized in the PFC. Altered CB1R activity induced by SCE may be involved in the altered NMDAR-LTP found in adult males. The eCB system controls NMDAR activity through mechanisms involving signaling pathways and/or direct physical coupling between CB1R and NMDAR NR1 subunits (Rodríguez-Muñoz et al., 2016). Some evidence indicates that NMDAR activity may be differently modulated according to sex. Gonadectomy alters mouse behavioral responses to the NMDAR agonist MK-801 in males but not females; male mice have higher NMDAR density in the NAc, motor and cingulate cortices (van den Buuse et al., 2017) and gonadal hormonal status influences both LTP induction and NMDAR function in male rats (Moradpour et al., 2013). Thus, SCE causes similar behavioral deficits in both male and female rats but triggered different alterations of PFC synapses.

Along with sex-specific responses to cannabinoids, sex differences in drug pharmacokinetics may be involved in the reported behavioral and electrophysiological effects. As reviewed by Rubino and Parolaro (2011), dimorphism in the eCB system and in cannabinoid metabolism may explain the different sensitivity between sexes found in which females seem to be more vulnerable to exogenous cannabinoid exposure. Furthermore, potential age-specific differences in the pharmacokinetics of cannabinoids might also explain why pubescent males respond differently to WIN when compared to adults. Even using the same drug dose and administration route, the subsequent mechanisms involved in drug absorption, distribution (specially in brain tissue), metabolism and elimination may not be equivalent between young and adult rodents. In humans, pharmacokinetics parameters such as absorption, volume of distribution, drug bioavailability and clearance are age-related (Fernandez et al., 2011). While age-dependent differences in cannabinoid metabolism cannot completely be ruled out, there is to the best of our knowledge, no report of such an occurrence in the literature.

The present model of subcutaneous WIN injection was chosen over the more common intraperitoneal route to minimize stress to the animals (Stuart and Robinson, 2015). Unfortunately, the pharmacokinetics of WIN and other cannabimimetics after subcutaneous administration remains poorly described (Fox et al., 2001; Carlier et al., 2018). In particular, the presence and distribution of potential active metabolites is not known. Available data show that the half-life of WIN is limited following intraperitoneal injection (Barna et al., 2009) and it is unlikely that the previous results can be explained by ongoing occupation of CB1R by WIN 24 h after subcutaneous exposure. Further experiments during precise stages of the estrous cycle will be necessary to further establish the relationship between CBR activation and gonadal hormones. Finally, taking into consideration the rewarding component of social interactions (Vanderschuren et al., 2016), investigating the role of WIN in the hedonic valence of social behavior will help elucidate the bases of the phenotype observed here.

Together, our results reveal behavioral and synaptic sex differences in response to a single *in vivo* exposure to a cannabinoid. Further analyses of both electrophysiological function and its molecular underpinnings associated with the heightened sensitivity of females to a single *in vivo* exposure to cannabinoid may reveal long-term consequences of these early life drug-induced alterations.

AUTHOR CONTRIBUTIONS

MB, AM, AB, A-LP-A and OM designed the research. MB, AM, AB and OL performed the research. MB analyzed the data. MB,

A-LP-A and OM wrote the article. OM and A-LP-A supervised the entire project.

FUNDING

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Brazil, process 200843/2015-0 to MB); Agence National de la Recherche (ANR «Cannado» to A-LP-A); Fondation pour la Recherche Médicale (Equipe FRM 2015 to OM) and the National Institutes of Health (NIH; R01DA043982).

ACKNOWLEDGMENTS

We are grateful to Dr. Pascale Chavis for helpful discussions, Dr. Andrew Scheyer for critical reading of the manuscript and to the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (Rockville, MD, USA) for providing CNQX.

REFERENCES

Abush, H., and Akirav, I. (2010). Cannabinoids modulate hippocampal memory and plasticity. *Hippocampus* 20, 1126–1138. doi: 10.1002/hipo.20711

Abush, H., and Akirav, I. (2012). Short- and long-term cognitive effects of chronic cannabinoids administration in late-adolescence rats. *PloS One* 7:e31731. doi: 10.1371/journal.pone.0031731

Araque, A., Castillo, P. E., Manzoni, O. J., and Tonini, R. (2017). Synaptic functions of endocannabinoid signaling in health and disease. *Neuropharmacology* 124, 13–24. doi: 10.1016/j.neuropharm.2017.06.017

Argue, K. J., VanRyzin, J. W., Falvo, D. J., Whitaker, A. R., Yu, S. J., and McCarthy, M. M. (2017). Activation of both CB1 and CB2 endocannabinoid receptors is critical for masculinization of the developing medial amygdala and juvenile social play behavior. *eNeuro* 4:ENEURO.0344-16.2017. doi: 10.1523/eneuro.0344-16.2017

Barna, I., Till, I., and Haller, J. (2009). Blood, adipose tissue and brain levels of the cannabinoid ligands WIN-55,212 and SR-141716A after their intraperitoneal injection in mice: compound-specific and area-specific distribution within the brain. *Eur. Neuropsychopharmacol.* 19, 533–541. doi: 10.1016/j.euroneuro.2009.02.001

Bender, R. A., Zhou, L., Vierk, R., Brandt, N., Keller, A., Gee, C. E., et al. (2017). Sex-dependent regulation of aromatase-mediated synaptic plasticity in the basolateral amygdala. *J. Neurosci.* 37, 1532–1545. doi: 10.1523/jneurosci.1532-16.2016

Bradshaw, H. B., Rimmerman, N., Krey, J. F., and Walker, J. M. (2006). Sex and hormonal cycle differences in rat brain levels of pain-related cannabimimetic lipid mediators. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R349–R358. doi: 10.1152/ajpregu.00933.2005

Burke, A. R., McCormick, C. M., Pellis, S. M., and Lukkes, J. L. (2017). Impact of adolescent social experiences on behavior and neural circuits implicated in mental illnesses. *Neurosci. Biobehav. Rev.* 76, 280–300. doi: 10.1016/j.neubiorev.2017.01.018

Burston, J. J., Wiley, J. L., Craig, A. A., Selley, D. E., and Sim-Selley, L. J. (2010). Regional enhancement of cannabinoid CB1 receptor desensitization in female adolescent rats following repeated Δ^9-tetrahydrocannabinol exposure. *Br. J. Pharmacol.* 161, 103–112. doi: 10.1111/j.1476-5381.2010.00870.x

Cannady, R., McGonigal, J. T., Newsom, R. J., Woodward, J. J., Mulholland, P. J., and Gass, J. T. (2017). Prefrontal cortex KCa^2 channels regulate mGlu5-dependent plasticity and extinction of alcohol-seeking behavior. *J. Neurosci.* 37, 4359–4369. doi: 10.1523/jneurosci.2873-16.2017

Carlier, J., Wohlfarth, A., Salmeron, B. D., Scheidweiler, K. B., Huestis, M. A., and Baumann, M. H. (2018). Pharmacodynamic effects, pharmacokinetics and metabolism of the synthetic cannabinoid AM-2201 in male rats. *J. Pharmacol. Exp. Ther.* 367, 543–550. doi: 10.1124/jpet.118.250530

Casey, B. J., Getz, S., and Galvan, A. (2008). The adolescent brain. *Dev. Rev.* 28, 62–77. doi: 10.1016/j.dr.2007.08.003

Cass, D. K., Flores-Barrera, E., Thomases, D. R., Vital, W. F., Caballero, A., and Tseng, K. Y. (2014). CB1 cannabinoid receptor stimulation during adolescence impairs the maturation of GABA function in the adult rat prefrontal cortex. *Mol. Psychiatry* 19, 536–543. doi: 10.1038/mp.2014.14

Castelli, M. P., Fadda, P., Casu, A., Spano, M. S., Casti, A., Fratta, W., et al. (2014). Male and female rats differ in brain cannabinoid CB1 receptor density and function and in behavioural traits predisposing to drug addiction: effect of ovarian hormones. *Curr. Pharm. Des.* 20, 2100–2113. doi: 10.2174/13816128113199990430

Castillo, P. E., Younts, T. J., Chávez, A. E., and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron* 76, 70–81. doi: 10.1016/j.neuron.2012.09.020

Chen, L.-S., Tzeng, W.-Y., Chuang, J.-Y., Cherng, C. G., Gean, P.-W., and Yu, L. (2014). Roles of testosterone and amygdaloid LTP induction in determining sex differences in fear memory magnitude. *Horm. Behav.* 66, 498–508. doi: 10.1016/j.yhbeh.2014.07.008

Colangeli, R., Pierucci, M., Benigno, A., Campiani, G., Butini, S., and Di Giovanni, G. (2017). The FAAH inhibitor URB597 suppresses hippocampal maximal dentate afterdischarges and restores seizure-induced impairment of short and long-term synaptic plasticity. *Sci. Rep.* 7:11152. doi: 10.1038/s41598-017-11606-1

Cooper, Z. D., and Craft, R. M. (2018). Sex-dependent effects of cannabis and cannabinoids: a translational perspective. *Neuropsychopharmacology* 43, 34–51. doi: 10.1038/npp.2017.140

Craft, R. M., Marusich, J. A., and Wiley, J. L. (2013). Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? *Life Sci.* 92, 476–481. doi: 10.1016/j.lfs.2012.06.009

Cuttler, C., Mischley, L. K., and Sexton, M. (2016). Sex differences in cannabis use and effects: a cross-sectional survey of cannabis users. *Cannabis Cannabinoid Res.* 1, 166–175. doi: 10.1089/can.2016.0010

Domenici, M. R., Azad, S. C., Marsicano, G., Schierloh, A., Wotjak, C. T., Dodt, H.-U., et al. (2006). Cannabinoid receptor type 1 located on presynaptic terminals of principal neurons in the forebrain controls glutamatergic synaptic transmission. *J. Neurosci.* 26, 5794–5799. doi: 10.1523/jneurosci.0372-06.2006

Douglas, L. A., Varlinskaya, E. I., and Spear, L. P. (2004). Rewarding properties of social interactions in adolescent and adult male and female rats: impact of social versus isolate housing of subjects and partners. *Dev. Psychobiol.* 45, 153–162. doi: 10.1002/dev.20025

Farquhar, C. E., Breivogel, C. S., Gamage, T. F., Gay, E. A., Thomas, B. F., Craft, R. M., et al. (2019). Sex, THC, and hormones: effects on density and sensitivity of CB_1 cannabinoid receptors in rats. *Drug Alcohol Depend.* 194, 20–27. doi: 10.1016/j.drugalcdep.2018.09.018

Fendt, M., Imobersteg, S., Peterlik, D., Chaperon, F., Mattes, C., Wittmann, C., et al. (2013). Differential roles of mGlu(7) and mGlu(8) in amygdala-dependent behavior and physiology. *Neuropharmacology* 72, 215–223. doi: 10.1016/j.neuropharm.2013.04.052

Fernandez, E., Perez, R., Hernandez, A., Tejada, P., Arteta, M., and Ramos, J. T. (2011). Factors and mechanisms for pharmacokinetic differences between pediatric population and adults. *Pharmaceutics* 3, 53–72. doi: 10.3390/pharmaceutics3010053

File, S. E., and Seth, P. (2003). A review of 25 years of the social interaction test. *Eur. J. Pharmacol.* 463, 35–53. doi: 10.1016/s0014-2999(03)01273-1

Foster, K. T., Arterberry, B. J., Iacono, W. G., McGue, M., and Hicks, B. M. (2017). Psychosocial functioning among regular cannabis users with and without cannabis use disorder. *Psychol. Med.* 48, 1–9. doi: 10.1017/s0033291717003361

Fox, A., Kesingland, A., Gentry, C., McNair, K., Patel, S., Urban, L., et al. (2001). The role of central and peripheral Cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain* 92, 91–100. doi: 10.1016/s0304-3959(00)00474-7

Friend, L., Weed, J., Sandoval, P., Nufer, T., Ostlund, I., and Edwards, J. G. (2017). CB1-dependent long-term depression in ventral tegmental area

GABA neurons: a novel target for marijuana. *J. Neurosci.* 37, 10943–10954. doi: 10.1523/jneurosci.0190-17.2017

Gaffuri, A.-L., Ladarre, D., and Lenkei, Z. (2012). Type-1 cannabinoid receptor signaling in neuronal development. *Pharmacology* 90, 19–39. doi: 10.1159/000339075

Galanopoulos, A., Polissidis, A., Georgiadou, G., Papadopoulou-Daifoti, Z., Nomikos, G. G., Pitsikas, N., et al. (2014). WIN55,212-2 impairs non-associative recognition and spatial memory in rats via CB1 receptor stimulation. *Pharmacol. Biochem. Behav.* 124, 58–66. doi: 10.1016/j.pbb.2014.05.014

González, S., Bisogno, T., Wenger, T., Manzanares, J., Milone, A., Berrendero, F., et al. (2000). Sex steroid influence on cannabinoid CB_1 receptor mRNA and endocannabinoid levels in the anterior pituitary gland. *Biochem. Biophys. Res. Commun.* 270, 260–266. doi: 10.1006/bbrc.2000.2406

Gowing, L. R., Ali, R. L., Allsop, S., Marsden, J., Turf, E. E., West, R., et al. (2015). Global statistics on addictive behaviours: 2014 status report. *Addiction* 110, 904–919. doi: 10.1111/add.12899

Graham, K. L., and Burghardt, G. M. (2010). Current perspectives on the biological study of play: signs of progress. *Q. Rev. Biol.* 85, 393–418. doi: 10.1086/656903

Hájková, A., Techlovská, Š., Dvořáková, M., Chambers, J. N., Kumpošt, J., Hubálková, P., et al. (2016). SGIP1 alters internalization and modulates signaling of activated cannabinoid receptor 1 in a biased manner. *Neuropharmacology* 107, 201–214. doi: 10.1016/j.neuropharm.2016.03.008

Han, J., Kesner, P., Metna-Laurent, M., Duan, T., Xu, L., Georges, F., et al. (2012). Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. *Cell* 148, 1039–1050. doi: 10.1016/j.cell.2012.01.037

Hankin, B. L., Abramson, L. Y., Moffitt, T. E., Silva, P. A., McGee, R., and Angell, K. E. (1998). Development of depression from preadolescence to young adulthood: emerging gender differences in a 10-year longitudinal study. *J. Abnorm. Psychol.* 107, 128–140. doi: 10.1037//0021-843x.107.1.128

Harris, G. C., and Levine, J. E. (2003). Pubertal acceleration of pulsatile gonadotropin-releasing hormone release in male rats as revealed by microdialysis. *Endocrinology* 144, 163–171. doi: 10.1210/en.2002-220767

Harte-Hargrove, L. C., Varga-Wesson, A., Duffy, A. M., Milner, T. A., and Scharfman, H. E. (2015). Opioid receptor-dependent sex differences in synaptic plasticity in the hippocampal mossy fiber pathway of the adult rat. *J. Neurosci.* 35, 1723–1738. doi: 10.1523/jneurosci.0820-14.2015

Heng, L., Beverley, J. A., Steiner, H., and Tseng, K. Y. (2011). Differential developmental trajectories for CB1 cannabinoid receptor expression in limbic/associative and sensorimotor cortical areas. *Synapse* 65, 278–286. doi: 10.1002/syn.20844

Hill, E. L., Gallopin, T., Férézou, I., Cauli, B., Rossier, J., Schweitzer, P., et al. (2007). Functional CB1 receptors are broadly expressed in neocortical GABAergic and glutamatergic neurons. *J. Neurophysiol.* 97, 2580–2589. doi: 10.1152/jn.00603.2006

Hoffman, A. F., Oz, M., Yang, R., Lichtman, A. H., and Lupica, C. R. (2007). Opposing actions of chronic Delta9-tetrahydrocannabinol and cannabinoid antagonists on hippocampal long-term potentiation. *Learn. Mem.* 14, 63–74. doi: 10.1101/lm.439007

Howlett, A. C., Blume, L. C., and Dalton, G. D. (2010). CB1 cannabinoid receptors and their associated proteins. *Curr. Med. Chem.* 17, 1382–1393. doi: 10.2174/092986710790980023

Hu, S. S.-J., and Mackie, K. (2015). Distribution of the endocannabinoid system in the central nervous system. *Handb. Exp. Pharmacol.* 231, 59–93. doi: 10.1007/978-3-319-20825-1_3

Huang, G. Z., and Woolley, C. S. (2012). Estradiol acutely suppresses inhibition in the hippocampus through a sex-specific endocannabinoid and mGluR-dependent mechanism. *Neuron* 74, 801–808. doi: 10.1016/j.neuron.2012.03.035

Hummel, A., Shelton, K. H., Heron, J., Moore, L., and van den Bree, M. B. M. (2013). A systematic review of the relationships between family functioning, pubertal timing and adolescent substance use. *Addict. Abingdon Engl.* 108, 487–496. doi: 10.1111/add.12055

Iafrati, J., Malvache, A., Gonzalez Campo, C., Orejarena, M. J., Lassalle, O., Bouamrane, L., et al. (2016). Multivariate synaptic and behavioral profiling reveals new developmental endophenotypes in the prefrontal cortex. *Sci. Rep.* 6:35504. doi: 10.1038/srep35504

Iede, M. A., Nunn, K., Milne, B., and Fitzgerald, D. A. (2017). The consequences of chronic cannabis smoking in vulnerable adolescents. *Paediatr. Respir. Rev.* 24, 44–53. doi: 10.1016/j.prrv.2016.11.001

Inoue, S., Shikanai, H., Matsumoto, M., Hiraide, S., Saito, Y., Yanagawa, Y., et al. (2014). Metaplastic regulation of the median raphe nucleus via serotonin 5-HT1A receptor on hippocampal synaptic plasticity is associated with gender-specific emotional expression in rats. *J. Pharmacol. Sci.* 124, 394–407. doi: 10.1254/jphs.13237fp

Kaltiala-Heino, R., Marttunen, M., Rantanen, P., and Rimpelä, M. (2003). Early puberty is associated with mental health problems in middle adolescence. *Soc. Sci. Med.* 57, 1055–1064. doi: 10.1016/s0277-9536(02)00480-x

Kasanetz, F., Lafourcade, M., Deroche-Gamonet, V., Revest, J.-M., Berson, N., Balado, E., et al. (2013). Prefrontal synaptic markers of cocaine addiction-like behavior in rats. *Mol. Psychiatry* 18, 729–737. doi: 10.1038/mp.2012.59

Ko, J. (2017). Neuroanatomical substrates of rodent social behavior: the medial prefrontal cortex and its projection patterns. *Front. Neural. Circuits* 11:41. doi: 10.3389/fncir.2017.00041

Krebs-Kraft, D. L., Hill, M. N., Hillard, C. J., and McCarthy, M. M. (2010). Sex difference in cell proliferation in developing rat amygdala mediated by endocannabinoids has implications for social behavior. *Proc. Natl. Acad. Sci. U S A* 107, 20535–20540. doi: 10.1073/pnas.1005003107

Labouesse, M. A., Lassalle, O., Richetto, J., Iafrati, J., Weber-Stadlbauer, U., Notter, T., et al. (2017). Hypervulnerability of the adolescent prefrontal cortex to nutritional stress via reelin deficiency. *Mol. Psychiatry* 22, 961–971. doi: 10.1038/mp.2016.193

Lawston, J., Borella, A., Robinson, J. K., and Whitaker-Azmitia, P. M. (2000). Changes in hippocampal morphology following chronic treatment with the synthetic cannabinoid WIN55,212–2. *Brain Res.* 877, 407–410. doi: 10.1016/s0006-8993(00)02739-6

Leishman, E., Murphy, M., Mackie, K., and Bradshaw, H. B. (2018). Δ^9-Tetrahydrocannabinol changes the brain lipidome and transcriptome differentially in the adolescent and the adult. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1863, 479–492. doi: 10.1016/j.bbalip.2018.02.001

Levine, A., Clemenza, K., Rynn, M., and Lieberman, J. (2017). Evidence for the risks and consequences of adolescent cannabis exposure. *J. Am. Acad. Child Adolesc. Psychiatry* 56, 214–225. doi: 10.1016/j.jaac.2016.12.014

Li, K., Nakajima, M., Ibañez-Tallon, I., and Heintz, N. (2016). A cortical circuit for sexually dimorphic oxytocin-dependent anxiety behaviors. *Cell* 167, 60.e11–72.e11. doi: 10.1016/j.cell.2016.08.067

Lisdahl, K. M., Sher, K. J., Conway, K. P., Gonzalez, R., Feldstein Ewing, S. W., Nixon, S. J., et al. (2018). Adolescent brain cognitive development (ABCD) study: overview of substance use assessment methods. *Dev. Cogn. Neurosci.* 32, 80–96. doi: 10.1016/j.dcn.2018.02.007

Liu, Z., Han, J., Jia, L., Maillet, J.-C., Bai, G., Xu, L., et al. (2010). Synaptic neurotransmission depression in ventral tegmental dopamine neurons and cannabinoid-associated addictive learning. *PLoS One* 5:e15634. doi: 10.1371/journal.pone.0015634

Lovelace, J. W., Corches, A., Vieira, P. A., Hiroto, A. S., Mackie, K., and Korzus, E. (2015). An animal model of female adolescent cannabinoid exposure elicits a long-lasting deficit in presynaptic long-term plasticity. *Neuropharmacology* 99, 242–255. doi: 10.1016/j.neuropharm.2015.04.034

Lu, H.-C., and Mackie, K. (2016). An introduction to the endogenous cannabinoid system. *Biol. Psychiatry* 79, 516–525. doi: 10.1016/j.biopsych.2015.07.028

Manduca, A., Bara, A., Larrieu, T., Lassalle, O., Joffre, C., Layé, S., et al. (2017). Amplification of mglu5-endocannabinoid signaling rescues behavioral and synaptic deficits in a mouse model of adolescent and adult dietary polyunsaturated fatty acid imbalance. *J. Neurosci.* 37, 6851–6868. doi: 10.1523/JNEUROSCI.3516-16.2017

Manduca, A., Campolongo, P., and Trezza, V. (2012). Cannabinoid modulation of mother-infant interaction: is it just about milk? *Rev. Neurosci.* 23, 707–722. doi: 10.1515/revneuro-2012-0074

Manduca, A., Lassalle, O., Sepers, M., Campolongo, P., Cuomo, V., Marsicano, G., et al. (2016). Interacting cannabinoid and opioid receptors in the nucleus accumbens core control adolescent social play. *Front. Behav. Neurosci.* 10:211. doi: 10.3389/fnbeh.2016.00211

Manduca, A., Morena, M., Campolongo, P., Servadio, M., Palmery, M., Trabace, L., et al. (2015). Distinct roles of the endocannabinoids anandamide and 2-arachidonoylglycerol in social behavior and emotionality at different

developmental ages in rats. *Eur. Neuropsychopharmacol.* 25, 1362–1374. doi: 10.1016/j.euroneuro.2015.04.005

Martin, H. G. S., Lassalle, O., Brown, J. T., and Manzoni, O. J. (2016). Age-dependent long-term potentiation deficits in the prefrontal cortex of the Fmr1 knockout mouse model of fragile X syndrome. *Cereb. Cortex* 26, 2084–2092. doi: 10.1093/cercor/bhv031

Marusich, J. A., Craft, R. M., Lefever, T. W., and Wiley, J. L. (2015). The impact of gonadal hormones on cannabinoid dependence. *Exp. Clin. Psychopharmacol.* 23, 206–216. doi: 10.1037/pha0000027

Mato, S., Chevaleyre, V., Robbe, D., Pazos, A., Castillo, P. E., and Manzoni, O. J. (2004). A single *in vivo* exposure to delta 9THC blocks endocannabinoid-mediated synaptic plasticity. *Nat. Neurosci.* 7, 585–586. doi: 10.1038/nn1251

Moradpour, F., Fathollahi, Y., Naghdi, N., Hosseinmardi, N., and Javan, M. (2013). Prepubertal castration causes the age-dependent changes in hippocampal long-term potentiation. *Synapse* 67, 235–244. doi: 10.1002/syn.21636

Nakajima, M., Görlich, A., and Heintz, N. (2014). Oxytocin modulates female sociosexual behavior through a specific class of prefrontal cortical interneurons. *Cell* 159, 295–305. doi: 10.1016/j.cell.2014.09.020

Neuhofer, D., Henstridge, C. M., Dudok, B., Sepers, M., Lassalle, O., Katona, I., et al. (2015). Functional and structural deficits at accumbens synapses in a mouse model of Fragile X. *Front. Cell. Neurosci.* 9:100. doi: 10.3389/fncel.2015.00100

Niesink, R. J., and Van Ree, J. M. (1989). Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. *Neuropharmacology* 28, 411–418. doi: 10.1016/0028-3908(89)90038-5

Ojeda, S. R., Prevot, V., Heger, S., Lomniczi, A., Dziedzic, B., and Mungenast, A. (2003). Glia-to-neuron signaling and the neuroendocrine control of female puberty. *Ann. Med.* 35, 244–255. doi: 10.1080/07853890310005164

Panksepp, J. (1981). The ontogeny of play in rats. *Dev. Psychobiol.* 14, 327–332. doi: 10.1002/dev.420140405

Panksepp, J., and Beatty, W. W. (1980). Social deprivation and play in rats. *Behav. Neural Biol.* 30, 197–206. doi: 10.1016/s0163-1047(80)91077-8

Pellis, S., and Pellis, V. (2007). Rough-and-tumble play and the development of the social brain. *Curr. Dir. Psychol. Sci.* 16, 95–98. doi: 10.1111/j.1467-8721.2007.00483.x

Qi, X., Zhang, K., Xu, T., Yamaki, V. N., Wei, Z., Huang, M., et al. (2016). Sex differences in long-term potentiation at temporoammonic-CA1 synapses: potential implications for memory consolidation. *PLoS One* 11:e0165891. doi: 10.1371/journal.pone.0165891

Reich, C. G., Taylor, M. E., and McCarthy, M. M. (2009). Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. *Behav. Brain Res.* 203, 264–269. doi: 10.1016/j.bbr.2009.05.013

Renard, J., Rushlow, W. J., and Laviolette, S. R. (2016a). What can rats tell us about adolescent cannabis exposure? Insights from preclinical research. *Can. J. Psychiatry* 61, 328–334. doi: 10.1177/0706743716645288

Renard, J., Vitalis, T., Rame, M., Krebs, M.-O., Lenkei, Z., Le Pen, G., et al. (2016b). Chronic cannabinoid exposure during adolescence leads to long-term structural and functional changes in the prefrontal cortex. *Eur. Neuropsychopharmacol.* 26, 55–64. doi: 10.1016/j.euroneuro.2015.11.005

Richardson, G. A., Ryan, C., Willford, J., Day, N., and Goldschmidt, L. (2002). Prenatal alcohol and marijuana exposure: effects on neuropsychological outcomes at 10 years. *Neurotoxicol. Teratol.* 24, 309–320. doi: 10.1016/s0892-0362(02)00193-9

Riebe, C. J. N., Hill, M. N., Lee, T. T. Y., Hillard, C. J., and Gorzalka, B. B. (2010). Estrogenic regulation of limbic cannabinoid receptor binding. *Psychoneuroendocrinology* 35, 1265–1269. doi: 10.1016/j.psyneuen.2010.02.008

Rodríguez de Fonseca, F., Cebeira, M., Ramos, J. A., Martín, M., and Fernández-Ruiz, J. J. (1994). Cannabinoid receptors in rat brain areas: sexual differences, fluctuations during estrous cycle and changes after gonadectomy and sex steroid replacement. *Life Sci.* 54, 159–170. doi: 10.1016/0024-3205(94)00585-0

Rodríguez-Muñoz, M., Sánchez-Blázquez, P., Merlos, M., and Garzón-Niño, J. (2016). Endocannabinoid control of glutamate NMDA receptors: the therapeutic potential and consequences of dysfunction. *Oncotarget* 7, 55840–55862. doi: 10.18632/oncotarget.10095

Romero, J., Garcia-Palomero, E., Berrendero, F., Garcia-Gil, L., Hernandez, M. L., Ramos, J. A., et al. (1997). Atypical location of cannabinoid receptors in white matter areas during rat brain development. *Synapse* 26, 317–323. doi: 10.1002/(SICI)1098-2396(199707)26:3<317::AID-SYN12>3.0.CO;2-S

Rubino, T., and Parolaro, D. (2011). Sexually dimorphic effects of cannabinoid compounds on emotion and cognition. *Front. Behav. Neurosci.* 28:64. doi: 10.3389/fnbeh.2011.00064

Rubino, T., and Parolaro, D. (2015). Sex-dependent vulnerability to cannabis abuse in adolescence. *Front. Psychiatry* 6:56. doi: 10.3389/fpsyt.2015.00056

Rubino, T., and Parolaro, D. (2016). The impact of exposure to cannabinoids in adolescence: insights from animal models. *Biol. Psychiatry* 79, 578–585. doi: 10.1016/j.biopsych.2015.07.024

Rubino, T., Vigano', D., Realini, N., Guidali, C., Braida, D., Capurro, V., et al. (2008). Chronic delta 9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. *Neuropsychopharmacology* 33, 2760–2771. doi: 10.1038/sj.npp.1301664

Schepis, T. S., Desai, R. A., Cavallo, D. A., Smith, A. E., McFetridge, A., Liss, T. B., et al. (2011). Gender differences in adolescent marijuana use and associated psychosocial characteristics. *J. Addict. Med.* 5, 65–73. doi: 10.1097/adm.0b013e3181d8dc62

Scheyer, A. F., Manzoni, O. J., and Martin, H. G. S. (2017). "The endocannabinoid system in prefrontal synaptopathies," in *Endocannabinoids and Lipid Mediators in Brain Functions*, ed. M. Melis (Cham: Springer), 171–210.

Schneider, M. (2008). Puberty as a highly vulnerable developmental period for the consequences of cannabis exposure. *Addict. Biol.* 13, 253–263. doi: 10.1111/j.1369-1600.2008.00110.x

Schneider, M. (2013). Adolescence as a vulnerable period to alter rodent behavior. *Cell Tissue Res.* 354, 99–106. doi: 10.1007/s00441-013-1581-2

Schneider, M., Schömig, E., and Leweke, F. M. (2008). Acute and chronic cannabinoid treatment differentially affects recognition memory and social behavior in pubertal and adult rats. *Addict. Biol.* 13, 345–357. doi: 10.1111/j.1369-1600.2008.00117.x

Silva, L., Black, R., Michaelides, M., Hurd, Y. L., and Dow-Edwards, D. (2016). Sex and age specific effects of delta-9-tetrahydrocannabinol during the periadolescent period in the rat: the unique susceptibility of the prepubescent animal. *Neurotoxicol. Teratol.* 58, 88–100. doi: 10.1016/j.ntt.2016.02.005

Sisk, C. L., and Foster, D. L. (2004). The neural basis of puberty and adolescence. *Nat. Neurosci.* 7, 1040–1047. doi: 10.1038/nn1326

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Stice, E., Presnell, K., and Bearman, S. K. (2001). Relation of early menarche to depression, eating disorders, substance abuse and comorbid psychopathology among adolescent girls. *Dev. Psychol.* 37, 608–619. doi: 10.1037//0012-1649.37.5.608

Stinson, F. S., Ruan, W. J., Pickering, R., and Grant, B. F. (2006). Cannabis use disorders in the USA: prevalence, correlates and co-morbidity. *Psychol. Med.* 36, 1447–1460. doi: 10.1017/s0033291706008361

Stuart, S. A., and Robinson, E. S. (2015). Reducing the stress of drug administration: implication for the 3Rs. *Sci. Rep.* 5:14288. doi: 10.1038/srep14288

Tagliaferro, P., Javier Ramos, A., Onaivi, E. S., Evrard, S. G., Lujilde, J., and Brusco, A. (2006). Neuronal cytoskeleton and synaptic densities are altered after a chronic treatment with the cannabinoid receptor agonist WIN 55,212–2. *Brain Res.* 1085, 163–176. doi: 10.1016/j.brainres.2005.12.089

Thomazeau, A., Lassalle, O., Iafrati, J., Souchet, B., Guedj, F., Janel, N., et al. (2014). Prefrontal deficits in a murine model overexpressing the down syndrome candidate gene dyrk1a. *J. Neurosci.* 34, 1138–1147. doi: 10.1523/jneurosci.2852-13.2014

Trezza, V., Baarendse, P. J. J., and Vanderschuren, L. J. M. J. (2010). The pleasures of play: pharmacological insights into social reward mechanisms. *Trends Pharmacol. Sci.* 31, 463–469. doi: 10.1016/j.tips.2010.06.008

Trezza, V., Baarendse, P. J. J., and Vanderschuren, L. J. M. J. (2014). On the interaction between drugs of abuse and adolescent social behavior. *Psychopharmacology* 231, 1715–1729. doi: 10.1007/s00213-014-3471-z

Trezza, V., and Vanderschuren, L. J. M. J. (2008a). Bidirectional cannabinoid modulation of social behavior in adolescent rats. *Psychopharmacology* 197, 217–227. doi: 10.1007/s00213-007-1025-3

Trezza, V., and Vanderschuren, L. J. M. J. (2008b). Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. *Eur. Neuropsychopharmacol.* 18, 519–530. doi: 10.1016/j.euroneuro.2008.03.001

Trezza, V., and Vanderschuren, L. J. M. J. (2009). Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. *J. Pharmacol. Exp. Ther.* 328, 343–350. doi: 10.1124/jpet.108.141069

van den Buuse, M., Low, J. K., Kwek, P., Martin, S., and Gogos, A. (2017). Selective enhancement of NMDA receptor-mediated locomotor hyperactivity by male sex hormones in mice. *Psychopharmacology* 234, 2727–2735. doi: 10.1007/s00213-017-4668-8

van Huijstee, A. N., and Mansvelder, H. D. (2015). Glutamatergic synaptic plasticity in the mesocorticolimbic system in addiction. *Front. Cell. Neurosci.* 8:466. doi: 10.3389/fncel.2014.00466

Vanderschuren, L. J., Achterberg, E. J., and Trezza, V. (2016). The neurobiology of social play and its rewarding value in rats. *Neurosci. Biobehav. Rev.* 70, 86–105. doi: 10.1016/j.neubiorev.2016.07.025

Vanderschuren, L. J. M. J., and Trezza, V. (2014). What the laboratory rat has taught us about social play behavior: role in behavioral development and neural mechanisms. *Curr. Top. Behav. Neurosci.* 16, 189–212. doi: 10.1007/7854_2013_268

Vetter-O'Hagen, C. S., and Spear, L. P. (2012). Hormonal and physical markers of puberty and their relationship to adolescent-typical novelty-directed behavior. *Dev. Psychobiol.* 54, 523–535. doi: 10.1002/dev.20610

Viveros, M. P., Llorente, R., Moreno, E., and Marco, E. M. (2005). Behavioural and neuroendocrine effects of cannabinoids in critical developmental periods. *Behav. Pharmacol.* 16, 353–362. doi: 10.1097/00008877-200509000-00007

Walsh, Z., Gonzalez, R., Crosby, K., S Thiessen, M., Carroll, C., and Bonn-Miller, M. O. (2017). Medical cannabis and mental health: a guided systematic review. *Clin. Psychol. Rev.* 51, 15–29. doi: 10.1016/j.cpr.2016.10.002

Weed, P. F., Filipeanu, C. M., Ketchum, M. J., and Winsauer, P. J. (2016). Chronic Δ9-Tetrahydrocannabinol during adolescence differentially modulates striatal cb1 receptor expression and the acute and chronic effects on learning in adult rats. *J. Pharmacol. Exp. Ther.* 356, 20–31. doi: 10.1124/jpet.115.227181

Wegener, N., and Koch, M. (2009). Behavioural disturbances and altered Fos protein expression in adult rats after chronic pubertal cannabinoid treatment. *Brain Res.* 1253, 81–91. doi: 10.1016/j.brainres.2008.11.081

Wegener, N., Kuhnert, S., Thüns, A., Roese, R., and Koch, M. (2008). Effects of acute systemic and intra-cerebral stimulation of cannabinoid receptors on sensorimotor gating, locomotion and spatial memory in rats. *Psychopharmacology* 198, 375–385. doi: 10.1007/s00213-008-1148-1

Wei, D., Allsop, S., Tye, K., and Piomelli, D. (2017). Endocannabinoid signaling in the control of social behavior. *Trends Neurosci.* 40, 385–396. doi: 10.1016/j.tins.2017.04.005

Wiley, J. L., Lefever, T. W., Marusich, J. A., and Craft, R. M. (2017). Comparison of the discriminative stimulus and response rate effects of Δ9-tetrahydrocannabinol and synthetic cannabinoids in female and male rats. *Drug Alcohol Depend.* 172, 51–59. doi: 10.1016/j.drugalcdep.2016.11.035

Zlebnik, N. E., and Cheer, J. F. (2016). Drug-induced alterations of endocannabinoid-mediated plasticity in brain reward regions. *J. Neurosci.* 36, 10230–10238. doi: 10.1523/jneurosci.1712-16.2016

Zamberletti, E., Prini, P., Speziali, S., Gabaglio, M., Solinas, M., Parolaro, D., et al. (2012). Gender-dependent behavioral and biochemical effects of adolescent delta-9-tetrahydrocannabinol in adult maternally deprived rats. *Neuroscience* 204, 245–257. doi: 10.1016/j.neuroscience.2011.11.038

Conflict of Interest Statement: 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: 05 April 2019
doi: 10.3389/fnbeh.2019.00069

Latency to Reward Predicts Social Dominance in Rats: A Causal Role for the Dopaminergic Mesolimbic System

Laura Lozano-Montes, Simone Astori, Sonia Abad, Isabelle Guillot de Suduiraut, Carmen Sandi and Ioannis Zalachoras**

Laboratory of Behavioral Genetics, Brain Mind Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

Reward signals encoded in the mesolimbic dopaminergic system guide approach/seeking behaviors to all varieties of life-supporting stimuli (rewards). Differences in dopamine (DA) levels have been found between dominant and submissive animals. However, it is still unclear whether these differences arise as a consequence of the rewarding nature of the acquisition of a dominant rank, or whether they preexist and favor dominance by promoting reward-seeking behavior. Given that acquisition of a social rank determines animals' priority access to resources, we hypothesized that differences in reward-seeking behavior might affect hierarchy establishment and that modulation of the dopaminergic system could affect the outcome of a social competition. We characterized reward-seeking behaviors based on rats' latency to get a palatable-reward when given temporary access to it. Subsequently, rats exhibiting short (SL) and long (LL) latency to get the rewards cohabitated for more than 2 weeks, in order to establish a stable hierarchy. We found that SL animals exhibited dominant behavior consistently in social competition tests [for palatable-rewards and two water competition tests (WCTs)] after hierarchy was established, indicating that individual latency to rewards predicted dominance. Moreover, because SL animals showed higher mesolimbic levels of DA than LL rats, we tested whether stimulation of mesolimbic DA neurons could affect the outcome of a social competition. Indeed, a combination of optical stimulation of mesolimbic DA neurons during individual training and during a social competition test for palatable rewards resulted in improved performance on this test.

Keywords: social dominance, reward seeking, latency to rewards, ventral tegmental area, optogenetic activations, dopamine and mesolimbic system

OPEN ACCESS

Edited by:
Liana Fattore, Italian National Research Council (CNR), Italy

Reviewed by:
Marcello Solinas, Institut National de la Santé et de la Recherche Médicale (INSERM), France
Fabrizio Sanna, University of Cagliari, Italy
Kim Huhman, Georgia State University, United States

****Correspondence:***
Laura Lozano-Montes
laura.lozanomontes@unifr.ch
Ioannis Zalachoras
ioannis.zalachoras@epfl.ch

Received: *20 January 2019*
Accepted: *18 March 2019*
Published: *05 April 2019*

Citation:
Lozano-Montes L, Astori S, Abad S, Guillot de Suduiraut I, Sandi C and Zalachoras I (2019) Latency to Reward Predicts Social Dominance in Rats: A Causal Role for the Dopaminergic Mesolimbic System. Front. Behav. Neurosci. 13:69. doi: 10.3389/fnbeh.2019.00069

INTRODUCTION

Social inequalities derived from hierarchy establishment have an important impact on individual's health (Hoebel et al., 2017); in despotic hierarchies most subordinate individuals exhibit a higher prevalence of health problems such as basal hypertension, pathogenic cholesterol profile and increased vulnerability to the atherogenic effects of high-fat diet (Sapolsky, 2005). The establishment of social hierarchies often requires competition between animals to arrange

themselves in a priority order (i.e., rank) for the division of resources such as territory, food, water, or sexual partners (Alcock and Rubenstein, 1989; Sapolsky, 2005). Despite the societal and health implications of social status (Sapolsky, 2005; Wilkinson and Pickett, 2006), very little is known about the factors that contribute to the determination of social dominance rank.

Although genetic (van der Kooij and Sandi, 2015) and environmental factors, such as exposure to stressors (Cordero and Sandi, 2007), are known to contribute to the determination of social dominance rank and aggressive behaviors, behavioral dimensions such as individual differences in trait anxiety or motivational processes have been hypothesized to play a key mediating role (van der Kooij and Sandi, 2015). Indeed, recent work has emphasized the involvement of trait anxiety in defining social competitiveness in both rodents (Hollis et al., 2015; Larrieu et al., 2017) and humans (Goette et al., 2015). Moreover, high-dominance individuals have been shown to be faster in decision-making, both in competitive and non-competitive settings, suggesting a general cognitive pattern related to dominance trait (Santamaría-García et al., 2013, 2015; da Cruz et al., 2018).

Laboratory rats form social hierarchies when living in groups, thus they provide an excellent model to study the neuronal mechanisms underlying social behaviors, such as social dominance (Davis et al., 2009; Wang et al., 2014). In the wild, social hierarchies among rodents are established based on displays of offensive behaviors, competitive access to food and water, marking the territory and grooming behaviors among others (Alcock and Rubenstein, 1989; Berdoy et al., 1995). In a laboratory setting, several tests have been designed in order to measure social rank focusing on the natural behaviors that affect hierarchy formation in the wild. Many studies have measured dominance in rats by performing social dominance tests or water and food competition tests. In these tests, animals compete for a new territory (typically through the display of offensive behaviors) or for limited access to water and food, respectively (Peres and Leite, 2002; Cordero and Sandi, 2007; Akers et al., 2008; Timmer and Sandi, 2010; Timmer et al., 2011; Hollis et al., 2015; Larrieu et al., 2017; van der Kooij et al., 2018). Therefore, there is ecological validity for laboratory competition tasks, as in the wild, rats with higher status have more access to natural resources (food, water, among others) while rats with a lower position in the hierarchy often experience reduced access to resources (Berdoy et al., 1995).

Currently, only a few studies have investigated a possible role for individual differences in reward-seeking behavior on dominance (Davis et al., 2009; Balconi and Vanutelli, 2016). For instance, dominant rats on a visible burrow system (VBS) showed higher reward-seeking behavior; however, such behavior was studied after hierarchy establishment, which makes conclusions about causality difficult (Davis et al., 2009). Recent human studies have indicated a possible role of a personality trait, the high Behavioral Activation System (BAS; associated with reward-seeking behaviors), on social competition outcome (Carver and White, 1994; Balconi and Vanutelli, 2016). High-BAS individuals had better performance during an interpersonal competitive task. However, the BAS questionnaires were answered after the individuals had completed the interpersonal competitive phase. This makes it difficult to judge about causality, as participants' BAS score might have been influenced by the recent performance on the competition task.

Reward-seeking behavior is defined as an activation of the instinctual emotional appetitive state evolved to induce organisms to search and/or approach all varieties of life-supporting stimuli (Alcaro et al., 2007). Reward-seeking is a major modulator of animal behavior; animals will learn to repeat actions that bring them closer to the rewards (Lechner et al., 2000; Hills et al., 2004; Roitman et al., 2004; Wise, 2004). Motivational aspects, including reward-seeking behaviors, have been extensively related to dopamine (DA) projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc; Alcaro et al., 2007; Nicola, 2007; Arias-Carrión et al., 2010; Luciana et al., 2012; Russo and Nestler, 2013; Ichinose et al., 2017). Reduction of accumbal dopaminergic function has been shown to reduce animals' exertion of effort to obtain rewards and even to cause failure to respond to reward-predictive cues (Nicola, 2007, 2010; Salamone and Correa, 2012). Furthermore, recent studies have shown that chemogenetic activation of DA neurons in the VTA increased initiation of reward-seeking actions (Boekhoudt et al., 2018). Additionally, optogenetic studies showed a causal role for phasic activation of VTA neurons (mainly projecting to the NAc) on reward-seeking behaviors, social behaviors and motivated behaviors (Adamantidis et al., 2011; Chaudhury et al., 2013; Steinberg et al., 2013; Tye et al., 2013; Gunaydin et al., 2014). These studies have highlighted the role of specific patterns of dopaminergic activity during time-precise behavioral events (such as reward delivery) on the behavioral outcomes (Adamantidis et al., 2011; Steinberg et al., 2013).

In addition, there is indirect evidence from gene expression studies in human (Martinez et al., 2010) and non-human primates (Morgan et al., 2002; Miller-Butterworth et al., 2008; Nader et al., 2012) linking dopaminergic function with social dominance. Dominant monkeys were found to display higher levels of DA metabolites in cerebrospinal fluid samples than subordinate ones (Kaplan et al., 2002). Recent studies from our lab have suggested activation on the dopaminergic projections from the VTA to the NAc and subsequent DA release in the NAc as the underlying neurobiological mechanism by which anxiolytic drugs increase dominance on a social dominance test (van der Kooij et al., 2018).

Here, we examined whether differences in reward-seeking behavior could predict the outcome of a social competition in male rats. We focused on male rats, as they exhibit higher aggression and competitiveness compared to female rats. We hypothesized that individual differences in reward-seeking behavior might predispose high reward-seeking individuals to become dominant when competing for natural resources. We then asked whether these individual differences in reward-seeking behavior are accompanied by differences in the function of the mesolimbic dopaminergic system and whether boosting the dopaminergic output could promote dominance. To this aim,

we optically activated DA neurons during a social competition for a limited reward.

Our data showed that reward-seeking behaviors predicted social rank after hierarchy establishment and animals with different reward-seeking behaviors presented different accumbal DA levels. Finally, the outcome of a social competition could be modulated by activating the dopaminergic mesolimbic system in a time-precise manner.

MATERIALS AND METHODS

Animals

Heterozygous transgenic rats expressing Cre recombinase under the control of tyrosine hydroxylase promoter (TH::Cre) on a Long-Evans background were obtained from K. Deisseroth (Witten et al., 2011; McCutcheon et al., 2014). TH::Cre transgenic rats were bred in our animal house at the EPFL by mating Cre-positive founders to wild-type rats; TH::Cre offspring were used in all optogenetic experiments and their wild-type littermates in the rest of experiments. Only male rats aged 12–15 weeks at the initiation of behavioral experiments were used. Animals were housed in a 12 h standard light-dark cycle (lights on from 07:00 to 19:00 h), and food and water were available *ad libitum* (except for water competition task, see below). After weaning, all animals were housed with same-sex littermates. We assumed that this resulted in a very mild dominance relationship; previous studies showed that group housing of littermates had no effects on individual behaviors that were modulated after social defeats (Arakawa, 2006). At the beginning of the experiment, rats were single-housed for a minimum of 7 days, during which handling and habituation phases to the social competition for palatable-reward (SCPR) test (see below) took place. All experiments were carried out in non-food-deprived rats. Thereafter, rats were housed in pairs, with the exception of animals for optogenetics that were single-housed throughout the experiment. Then, social rank established between the two cohabitating rats was determined through several social competition tests as indicated in the timeline scheme (**Figure 1A**; see also **Supplementary Table S1** for an overview of the experiments). All experiments were performed with the approval of the Cantonal Veterinary Authorities (Vaud, Switzerland) and carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU).

Test for Anxiety: Elevated Plus Maze (EPM)

In order to assess trait anxiety, animals underwent the elevated plus maze (EPM) test before starting the individual training for the social competition for palatable rewards (see below; Herrero et al., 2006). This test consists of two opposing open arms (45 × 10 cm) and two opposing closed arms (45 × 10 cm with walls 50 cm high) that extend from a central platform (10 × 10 cm) elevated 65 cm above the floor. Rats were placed on the central platform facing the same closed arm and allowed to explore the maze freely for 5 min. EPM test was performed in the morning, between 9:00 am and 12:00. The behavior of each rat was video recorded and analyzed using a computerized tracking system (Ethovision 3.1.16, Noldus IT, Netherlands). Time spent in the open and closed arms were measured in order to evaluate trait anxiety. Total distance walked in the EPM and the total number of arm entries were measured to assess locomotor activity.

Open Field Test

In order to assess locomotion differences between SL and LL groups or due to phasic activations of VTA dopaminergic neurons, animals underwent an open field test. The open field consisted of a black circular arena (1 m in diameter, surrounded by walls 32 cm high). For analysis, the total distance walked was calculated. Animals were placed in the center of the arena and their behavior was monitored for 10 min using a video camera that was mounted from the ceiling above the center of the arena. The light was adjusted to 8–10 lx in the center of the arena. Open field test was performed in the morning, between 9:00 am and 12:00. Total distance walked was calculated using Ethovision (Noldus). To test the locomotion of animals receiving optogenetic stimulation, we split the test into two epochs. The first 5 min were without stimulation (light off), while the second epoch (min 5–10) rats received optogenetic stimulation (light on).

Social Competition for Palatable-Reward (SCPR) Test

The SCPR test was based on a protocol described by Akers et al. (2008) with minor modifications. The apparatus consisted of a testing box made of Plexiglas (52 × 32 × 65 cm). It contained a narrow runway with the reward located at the end of it (**Supplementary Figure S1A**). The same boxes were used for habituation, individual training and social competition sessions. To habituate rats to the reward, a small amount of melted chocolate (Nutella) was applied with a cotton-tip on the wall of animals' homecage once a day for three consecutive days. After familiarization with the reward, animals were habituated individually to the box for 3 days. The habituation session consisted of a first phase of 2 min during which animals were confined to one-half of the cage. An opaque divider, that prevented access to the runway, was then removed and rats could explore the whole apparatus for 5 min, accessing the narrow runway and getting the reward. Habituation to both the palatable-reward and the competition box took place always while animals were single-housed.

Individual Training Without Competition

Rats were trained individually to enter the runway and consume a small drop of the palatable-reward at the end of the runway. Individual training started around 15:00. On each training day, rats performed six trials. In each trial, following 2 min during which animals were placed in the main area of the box, the divider was removed and animals had 30 s to enter the runway and consume the reward (except for the very first trial in which animals were given 60 s to complete the trial). Rats were trained for a minimum of 5 days. Animals were considered to have learned the task when they missed a maximum of one trial per session. When they missed more than

one trial, they received further training sessions until criterion was reached (for a maximum of two extra days). Animals were always single-housed during the individual training period. Only animals that reached the criterion were involved in further experiments. Based on the results from the last day of individual training animals were matched on dyads in order to perform the SCPR test. Wild-type animals were matched for opposite latency to rewards and put to cohabitate together in order to establish a hierarchy. However, in experiments involving TH::Cre animals, they were matched to form pairs with similar latency to rewards and did not cohabitate before the SCPR test. Furthermore, animals in each dyad were matched for age, weight and anxiety levels as measured in the EPM.

SCPR Test

Pairs of rats were tested on 12 consecutive trials in a single session. SCPR tests started around 15:00. Rats were marked with either black or blue coloring on the sides of their bodies to distinguish the two rats of each pair. Both rats were introduced to the testing cage simultaneously. Competition testing was conducted in the same way as during individual training.

Measurements

The latency to begin reward consumption and the time spent consuming the reward were manually scored by an experimenter blind to the experimental conditions using "Clicker v1.13" software (Toledo-Rodriguez and Sandi, 2007). If a rat did not consume the reward, a latency equal to the maximum trial length (i.e., 30 s) was scored. The latency to begin reward consumption and the time spent consuming the reward were considered for the analysis of the individual training and social competition test (**Supplementary Table S2**). For classification purposes, the average latency per animal to consume the reward during the six trials of the last training session was considered; animals with average latency below 2.25 s or above 5 s, were classified as short latency to reward (SL) and long latency to reward (LL), respectively. These cut off values were established based on the distribution of the animals' latencies from the first group we used (**Supplementary Figure S1B**, left panel). We tried to consider cut-off values that would split the distribution into three subpopulations (LL animals were those falling over the 75th percentile and SL animals were those falling under the 40th percentile), with the intention to study the two extreme subpopulations. This method had the advantage of clearly separating LL rats and splitting the rest (which fell in a shorter range of latencies) almost in two groups containing equal numbers of rats. The distribution of all animals used in the study was, based on our cut-off values, approximately 27% for LL rats, 36% for intermediate latencies (>2.25 and <5 s) and 37% for SL (**Supplementary Figure S1B**, right panel), suggesting that groups were fairly consistent between different cohorts.

Water Competition Test (WCT)

Same pairs of cohabitating rats that performed the SCPR test (described above) underwent water competition tests (WCTs) 23 days after SCPR test completion. The pairs of rats were deprived of water mainly during the dark cycle from 00:00 to 08:00. Then, a 5-min WCT was performed by placing a bottle of water in the feeder holder of their homecage during the light cycle in the housing room. WCT tests were manually scored by an experimenter blind to the conditions with the aid of Clicker v1.13 software (Toledo-Rodriguez and Sandi, 2007). Latency to first drink and the duration of drinking behavior for each rat within each dyad were measured. The animal in the dyad that drank more was considered the dominant rat, as previously reported (Baenninger, 1970; Lucion and Vogel, 1994; Cordero and Sandi, 2007).

High Performance Liquid Chromatography (HPLC) Analysis of Monoamine Level in Brain Samples

Animals were decapitated and their brains were quickly removed, frozen in isopentane on dry ice, at a temperature between −50 and −40°C, and stored at −80°C until further processing. Coronal sections (200-μm thick) were punched to obtain the brain tissue of NAc region as previously described (Guitart et al., 2000). Brain samples were briefly sonicated in Eppendorf vials containing 120 μl of 0.1 M perchloric acid (PCA) and centrifuged at 16,000 *g* for 10 min at 4°C. The supernatant was collected and used for high performance liquid chromatography (HPLC) analysis. Two samples of SL rats were lost during the extraction process. Levels of DA, as well as DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were assessed by reverse-phase HPLC with electrochemical detection (HPLC-ECD stand-alone system, HTEC-500). Using a mobile phase, consisting of 20% methanol 8.85 g/l citric acid monohydrate, 200 mg/l octane-1-sulfonic acid sodium salt, 5 mg/l EDTA, 3.11 g/l sodium acetate dissolved in Milli-Q water, the different catecholamines were separated in a reversed phase separation column EICOMPACK SC-3ODS.

Virus Injection and Implantation of Optical Fibers

Standard stereotaxic procedures were used to infuse the virus, as previously described (Witten et al., 2011). TH::Cre transgenic rats received unilateral VTA injections of AAV5 (1.5×10^{12} particles/ml) with the following constructs from the University of North Carolina Vector Core: EF1a-DIO-hChR2(H134R)-EYFP for cre-inducible expression of channelrhodopsin 2 (ChR2) or EF1a-DIO- EYFP as control.

Two small burr holes were drilled unilaterally over the VTA at the following coordinates: AP −5.3 and −6.3; ML ± 0.7 (the hemisphere of injection was randomly selected), as previously described (Tye et al., 2013). A custom-made 26 gauge infuser was used to deliver 1.0 μl of virus at two depths in each hole (DV −8.2 and −7.0, all coordinates from skull surface) for a total of 4.0 μl virus delivered unilaterally to the VTA. Each 1.0 μl of virus was infused at a speed of 0.1 μl per minute using a syringe pump (Harvard Apparatus). The virus infuser was left in place for an additional 10 min following each injection before it was slowly removed.

Four weeks after viral infusion was performed, animals underwent a second surgery to insert the optic fibers into the

VTA (AP −5.8; ML ± 0.7; DV −7.5), as previously described (Tye et al., 2013). The implanted optic fiber was 240 μm outer diameter (Doric lenses) and was secured to the skull surface with five stainless steel screws and dental cement. All behavioral tests were conducted >5 weeks post- viral injection surgery.

Optical Activations

A 200-μm patch cord was connected to the external portion of the chronically implantable optical fiber with a zirconia sleeve (Doric Lenses). Patch cords were attached through a rotatory joint (Doric lenses) to a 473-nm blue laser diode (Dreamlasers), and light pulses were generated through a stimulator (Agilent technologies). Optical-fiber light power from the patch cord was measured using a light sensor (Thorlabs) before each animal was connected to a patch cord, in order to check that laser intensity was constant among animals. Light intensity ranged from 1.5 to 2 mW at the end of the patch cord for each session. For effects of phasic stimulation of DA neurons during individual training, both ChR2-expressing rats and control animals were optically stimulated during the 30 s of reward availability by 20 pulses at 20 Hz, 5 ms pulse duration every 5 s. During SCPR experiments the phasic protocol of stimulation lasted the whole test and was 20 Hz, 20 pulses, 5 ms pulse duration every 5 s.

Intracranial Self-Stimulation (ICSS) Test

In order to validate our optogenetic activation protocol, we aimed to induce intracranial self-stimulation (ICSS) by phasically activating DA neurons in the VTA. This behavioral effect in response to optogenetic activations has been previously reported (Witten et al., 2011). Experimental sessions were conducted in operant conditioning chambers (25.4 × 30.5 × 25.4 cm^3; Coulbourn Instruments, Bilaney Consultants) contained within sound-attenuating cubicles. The left panel was fitted with two nosepoke ports, each with three LED lights at the rear. Prior to training sessions, rats were gently attached to patchcord cables for optical stimulation. Optical stimulation was controlled by a computer running Graphic State 2 (Coulbourn instruments) software, which also recorded responses at both nosepoke ports. The protocol followed was the same as previously described by Witten et al. (2011). For all test sessions, the start of a session was indicated to the rat by the illumination of a white house light. During the first day of training, both active and inactive nosepoke ports were baited with a chocolate cereal treat to facilitate the initial investigation. Rats were given four daily sessions of 2 h each in which they could respond freely at either nosepoke port. For all rats (ChR2 and controls), a response at the active port resulted in the delivery of a 1 s train of light pulses (20 Hz, 20 pulses, 5 ms duration). Concurrently, the LED lights in the recess of the active port were illuminated, providing a visible cue whenever stimulation was delivered. Responses at the active port made during the 1 s period when the light train was being delivered were recorded but had no consequence. Responses at the inactive port were always without consequence.

Histology

Specificity of ChR2-EYFP expression in DA neurons and viral injections and optic fiber placements were verified *post hoc*. Animals were decapitated, brains were rapidly isolated and transferred to a 4% Paraformaldehyde (PFA) solution at 4°C for 24 h. Subsequently, the brains were transferred to a 30% sucrose solution at 4°C for 48 h. Then brains were frozen in isopentane on dry ice and finally stored at −20°C. Immunohistochemical detection of eYFP and tyrosine hydroxylase was performed similarly as previously described (Witten et al., 2011). Free-floating coronal sections of 40 μm were washed with TBS 1× 0.3% triton. Subsequently, brain sections were blocked with 20% donkey serum for 2 h. Goat polyclonal anti TH antibody (1:1,000 dilution, ab101853 from Abcam) was incubated overnight at 4°C. Sections were then washed with TBS 1× 0.3% triton and incubated with (1:600 Alexa 568 donkey antigoat) for 2 h. Finally, sections were mounted onto microscope slides in phosphate-buffered water and coverslipped with Vectashield mounting medium that contained a DAPI nuclear counterstain (Reactolab SA). ChR2-YFP expression and fiber optic placement into the VTA were checked in all rats used for behavioral experiments. No animal needed to be removed due to lack of viral expression or optic fiber misplacement within the VTA.

Statistical Analyses

Sample sizes (n) are indicated in figure legends and represent biological replicates. Sample sizes were calculated based on pilot experiments and animals were allocated to different groups based on characterization criteria (short latency to rewards and low latency to rewards) or randomly allocated to a group (ChR2 or control). Behavioral scoring and experimental assessment were performed by experimenters blind to treatment groups. Unpaired two-tailed Student's t-tests were used to compare sets of data obtained from independent groups of animals. Welch correction was used for the analysis of the latency to reward during the last day of individual training, as the samples did not have equal standard deviations. Paired two-tailed Student's t-tests were used to compare behavioral data within dyads. In the data sets from the WCTs, the percentage of drinking was compared using one-sample t-test against the level of chance (50%). Two-way ANOVA was used in order to analyze the individual data on the training sessions between days in both SL/LL and ChR2/Control groups. Two-tailed Mann-Whitney test with Bonferroni correction was used in order to analyze the responses to the active and inactive ports during the ICSS test. All data were analyzed using Prism version 5.01 (Graphpad Software, San Diego, CA, USA). Data are presented as the mean ± SEM and statistical significance is considered at $p < 0.05$.

RESULTS

Individual Differences in Latency to Approach a Reward Predict Social Rank

Single-housed, rats were exposed to five training days (at least) and classified according to their average latency to obtain a palatable-reward during the last day of individual training, as animals with either short latency (SL; latency below 2.25 s) or long latency to reward (LL; latency above 5 s; **Figure 1B**). During the last day of individual training, SL animals had significantly lower latencies to rewards than LL animals (t = 6.477, df = 9,

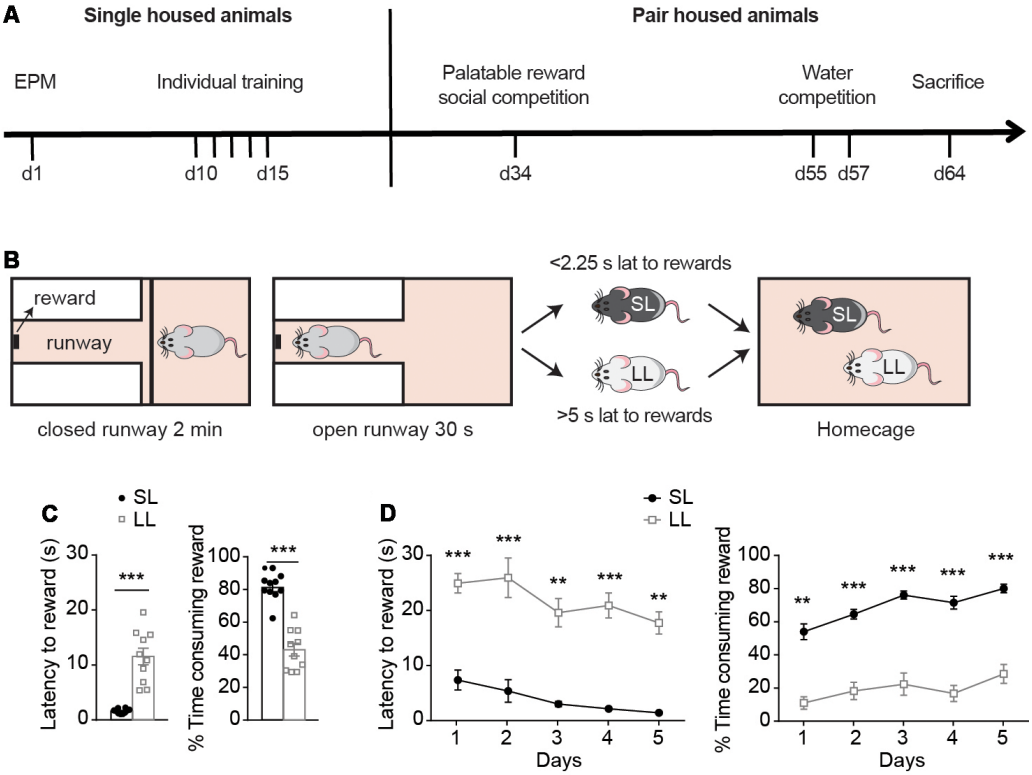


FIGURE 1 | Reward-seeking characterization before hierarchy establishment. **(A)** Experimental timeline, with the battery of tests to which animals were exposed while they were single-housed or pair-housed. **(B)** Criteria used for establishment of LL, SL groups were based on animals' average latency to rewards during the last day of individual training, when animals had already learned the task. Animals with average latency below 2.25 s or above 5 s, were classified as short latency to reward (SL) and long latency to reward (LL), respectively. **(C)** Behavioral measurements (mean ± SEM) of SL (n = 10) and LL (n = 10) animals before cohabitation were analyzed. During their last day of individual training, SL rats had lower latency to rewards and spent more time consuming the rewards compared to LL rats. Animals that missed more than one reward on the 5th day of training were re-trained (for two extra days maximum) until they did not miss more than one reward. **(D)** Average latency to rewards and average percentage of time consuming the reward during the first 5 days of individual training of SL and LL animals. $^{**}p < 0.01$, $^{***}p < 0.001$.

$p < 0.0001$; **Figure 1C**) and spent more time consuming the rewards ($t = 8.069$, $df = 18$; $p < 0.0001$; **Figure 1C**). As indicated in "Materials and Methods" section, the study only involved animals that readily consumed the food reward in their homecage (**Supplementary Figure S1C**) and learned the task during individual training. Training criterion was to miss a maximum of one trial per session. When animals missed more than one trial, they received further training sessions until the criterion was reached (**Supplementary Figure S1D**, for a maximum of two extra days). Animals classified as either SL or LL on the basis of their latencies on the last training day, showed as well different latencies to the reward and in the percentage of time spent consuming the reward across the different training days. Two way ANOVA followed by Bonferroni post-tests indicated differences between SL and LL animals on their latency to rewards (Group effect: $F_{(1,18)} = 113.2$, $p < 0.0001$, $t = 4.421$, $p < 0.01$; $t = 4.756$, $p < 0.001$; $t = 5.526$, $p < 0.001$; $t = 5.632$, $p < 0.001$; $t = 5.292$, $p < 0.001$, for days 1–5 respectively); and in the time spent consuming rewards (Group effect $F_{(1,18)} = 131.3$, $p < 0.0001$, $t = 4.421$, $p < 0.01$; $t = 4.756$, $p < 0.001$; $t = 5.526$, $p < 0.001$; $t = 5.632$, $p < 0.001$; $t = 5.292$, $p < 0.001$, **Figure 1D**) on each of the first 5 days of training. These differences were also present when comparing the last 5 days of individual training of each animal (**Supplementary Figure S1E**, $p < 0.001$, $t = 5.2305$, $p < 0.001$, $t = 6.9468$, $p < 0.001$, $t = 6.9504$, $p < 0.001$, $t = 6.6204$, $p = 0.0034$, $t = 3.5221$, for latency the last 5 days of training (1–5, respectively) and, $p < 0.001$, $t = 6.4513$, $p < 0.001$, $t = 6.9865$, $p < 0.001$, $t = 10.075$, $p < 0.001$, $t = 8.8382$, $p < 0.001$, $t = 6.0973$ for % time consuming rewards, in the last five of individual training (1–5, respectively).

Following this behavioral characterization, pairs constituted of one SL and one LL rat were matched for similar body weight and anxiety levels (**Supplementary Figure S1F**; both traits have been shown to affect social dominance; Barnet, 1958; Hollis et al., 2015). Behavioral measurements from the EPM and open field tests were analyzed, confirming that there were no differences in anxiety levels ($p > 0.05$, $t = 0.934$ for % time in open arms, $p > 0.05$, $t = 0.732$ for latency to open arms and $p > 0.05$, $t = 0.440$ for total number of arm entries), or total locomotion between SL and LL animals ($p > 0.05$, $t = 0.523$ for distance moved in the EPM and $p > 0.05$, $t = 1.309$ for distance moved in the open field) that could represent an advantage for the social competition tests (**Supplementary Figures S1F,G**). Pairs of SL and LL animals were then left to cohabitate for 17 days.

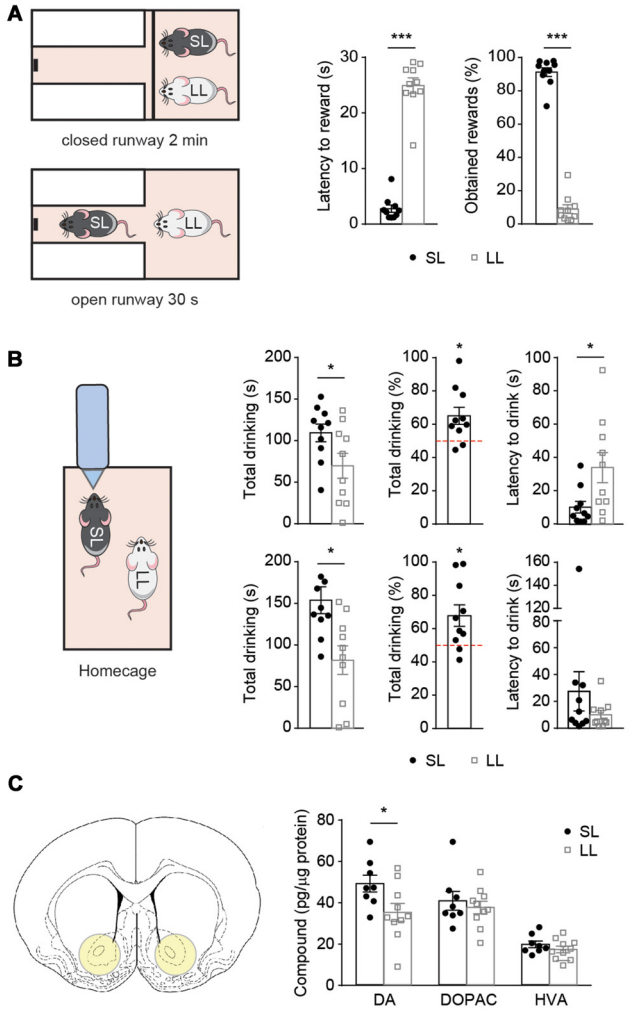


FIGURE 2 | Latency to reward predicts the outcome of several social competition tests after hierarchy establishment. Short and long latency animals show differences on accumbal dopamine (DA) levels. **(A)** Pairs of SL-LL animals (n = 10) performed the social competition for palatable-reward (SCPR) test after cohabitation. SL rats had lower latency to rewards and spent more time consuming the rewards compared to LL rats. **(B)** Same pairs of SL-LL animals performed two water competition tests (WCTs). SL rats drank more than LL rats and their water consumption was significantly higher than chance level. They also had lower latency to the initial drink compared to LL rats. In a second WCT, SL rats drank more, also compared to chance level, but without having differences in latency to initial drink (mean ± SEM). **(C)** Schematic of accumbal tissue collection for neurotransmitter analysis. Accumbal levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA; mean ± SEM) in SL (n = 8) and LL (n = 10) animals used for previous social competition experiments were analyzed here. SL rats had higher DA content in the nucleus accumbens (NAc) compared to LL rats. No difference was observed in DOPAC or HVA content between the groups. $^{*}p < 0.05$, $^{***}p < 0.001$.

Subsequently, we evaluated if cohabitating SL and LL animals would differ in their ability to reach the palatable reward when competing for its access (**Figure 2**). SL animals retained lower latencies to reward (t = 11.74, df = 9, p = 0.0001; **Figure 2A**) and obtained a higher percentage of rewards (t = 15.83, df = 9, p < 0.0001; **Figure 2A**, p = 0.0020; **Figure 2A**) during the competition test than their LL counterparts.

In order to assess whether the advantage of SL rats to win a competition could be extended to competitions involving rewards beyond the one used for the group stratification and a test where the fastest animal is not necessarily the dominant one, the same pairs of animals were subsequently submitted to two WCTs. Before each test, the pair of cohabitating animals was water-deprived overnight. Then, they were given access to a single water bottle for 5 min, the bottle access was not restricted, therefore the slower animal could also approach the bottle and push away the animal that was drinking (**Figure 2B**). In both tests, SL animals displayed an advantage over LL ones in accessing water, displaying lower latencies to drink (t = 2.547, df = 9, p = 0.0314; **Figure 2B**) and spending more time drinking than their LL counterparts (t = 3.149, df = 9, p = 0.0118; **Figure 2B**). We also verified that the percentage of drinking by SL rats was different from chance level (50%; one sample t-test t = 2.928, df = 9, p = 0.0168; **Figure 2B**). Similar results were obtained on a subsequent WCT performed 2 days afterwards; in terms of time drinking (t = 2.551, df = 9, p = 0.0311; **Figure 2B**; test against chance level (t = 2.742, df = 9, p = 0.0228; **Figure 2B**), except for the latency to drink that was not significantly different (t = 1.101, df = 9, p = 0.2995; **Figure 2B**).

These data suggest that reward-seeking behavior plays an important role in hierarchy establishment, as indicated by the outcome of social competition tests that involved different sensory-motor functions. Importantly, the results of the second WCT, where LL animals did not differ in the latency to drink, but they still spent less time drinking than the SL rats, indicate that the establishment of social hierarchy did not merely depend on how fast animals could access the resource, but that SL animals could use other strategies to become dominant, such as pushing the LL animals away in order to ensure prolonged access to the resource.

Individual Differences in Latency to Reward Are Accompanied by Differences in Accumbal Dopamine Levels

Given that DA has been implicated in the locomotor approach to reward-associated cues (Nicola, 2010), we compared levels of accumbal DA and its metabolites between SL and LL animals. NAc tissue punches were taken at basal conditions 1 week after the last WCT. We found higher DA levels in SL than in LL rats (t = 2.296, df = 16, p = 0.0355; **Figure 2C**), but no differences in the levels of DOPAC and HVA (t = 0.6110, df = 16, p = 0.5498; t = 1.100, df = 16, p = 0.2877, for DOPAC and HVA, respectively; **Figure 2C**). As we could only measure DA levels in the NAc post-mortem, we aimed to subsequently study the causal effects of DA modulation on the outcome of a SCPR test.

Optogenetic Activation of Mesolimbic Dopamine Neurons Increases Social Dominance

Phasic optogenetic activation of VTA neurons mainly projecting to the NAc has been causally linked to reward-seeking

behaviors (Adamantidis et al., 2011; Steinberg et al., 2013). Furthermore, in the same transgenic TH::Cre rat model as used here, phasic activation (20 Hz) of VTA DA neurons was shown to increase DA levels in the NAc and induced ICSS (Witten et al., 2011). Here, TH::Cre rats were infused with a Cre-inducible AAV expressing ChR2-eYFP into the VTA (**Supplementary Figure S2A**). Control animals were TH::Cre rats that received an infusion of Cre-inducible AAV expressing only eYFP into the VTA. Histological analyses confirmed that ChR2-eYFP expression was restricted to the VTA (**Supplementary Figure S2A**).

We first aimed to verify the feasibility of our optogenetic approach by replicating the optogenetic induction of self-stimulation in an operant task (Witten et al., 2011). Rats were given the opportunity to freely respond at two identical ports (**Supplementary Figure S2B**). Nosepokes at the active port induced phasic activations of VTA DA neurons, whereas nosepokes at the inactive port had no consequence. ChR2 expressing rats made significantly more responses in the active port than control animals did during each one of the 4 days of training ($U = 0$, $p = 0.0079$; $U = 0$, $p = 0.0119$; $U = 0$, $p = 0.0079$; $U = 0$, $p = 0.0357$; **Supplementary Figure S2C**). This indicates that phasic dopaminergic stimulation was correctly delivered in our experiments as it induces vigorous ICSS, as previously reported (Witten et al., 2011).

Then, we aimed to study if phasic activation of VTA DA neurons during reward acquisition would be sufficient to change the outcome of a SCPR test. We first assessed whether optogenetic activation of DA neurons in the VTA during individual training in one of the two subsequently competing animals would affect the subsequent outcome of the dyadic competition (**Figure 3A**). Animals performed 3 days of training without any stimulation. Then, during the last 2 days of training, animals were given phasic optogenetic stimulation while they accessed the rewards (**Figure 3B**). Optogenetic stimulation did not result in differences in performance during training. Two way ANOVA followed by Bonferroni post-tests indicated no differences between control and ChR2 animals on their latency to rewards (Group effect: $F_{(1,39)} = 0.141$, $p = 0.71$, $t = 1.148$, $p > 0.99$; $t = 0.184$, $p > 0.99$; $t = 0.118$, $p > 0.99$; $t = 0.309$, $p > 0.99$; $t = 0.037$, $p > 0.99$, for days 1–5 respectively); or in the time spent consuming rewards (Group effect $F_{(1,39)} = 2.122$, $p = 0.153$, $t = 0.906$, $p > 0.99$; $t = 0.721$, $p > 0.99$; $t = 0.734$, $p > 0.99$; $t = 0.511$, $p > 0.99$; $t = 0.395$, $p > 0.99$) on any training day (**Figure 3C**). We found no effect of the treatment when pairs of formerly stimulated ChR2 and control rats were allowed to compete for the palatable-reward (**Figure 3D**), as indicated by equivalent latency to reward ($t = 0.7325$, $df = 4$, $p = 0.5045$; **Figure 3E**) and percentage of obtained rewards ($t = 0.6319$, $df = 4$, $p = 0.5618$; **Figure 3E**). Subsequently, in the same pair of rats that had undergone optogenetic activation of DA neurons during the last 2 days of individual training, we performed a second SCPR test in which animals received phasic DA activations (**Figure 3F**). We found lower latencies to reward ($t = 5.384$, $df = 4$, $p = 0.0058$; **Figure 3G**) and a higher percentage of obtained rewards ($t = 5.522$, $df = 4$, $p = 0.0053$; **Figure 3G**) in ChR2 than in control animals.

Given that the same pair of animals had previously competed against each other, a carry-over effect in the last part of the experiment could not be ruled out. Therefore, we performed a further experiment in which new pairs of animals from the same cohort were matched based on their similar latency to rewards during the last training day and were put together for the first time for an SCPR test while DA neurons of the ChR2 rats were phasically activated and control rats received only the same light pulses into their VTA. As hypothesized, ChR2 rats displayed lower latency to reward ($t = 2.911$, $df = 4$, $p = 0.0436$; **Figure 3H**) and obtained a higher percentage of rewards ($t = 2.884$, $df = 4$, $p = 0.0448$) than controls (**Figure 3H**).

In order to study whether DA activations during competition were sufficient to change the outputs of the SCPR test, we performed an additional experiment in a different group of animals in which activation of DA neurons took place only during the SCPR test was not affected (**Figure 4A**). Animals underwent individual training for the SCPR test without DA activations (**Figure 4B**). There were no differences between control and ChR2 animals' performance on their last day of individual training. Control and ChR2 animals did not differ on their latency to rewards ($t = 0.3418$, $df = 12$, $p = 0.7384$; **Figure 4C**) or in the time they spent consuming the rewards ($t = 1.250$, $df = 12$, $p = 0.2352$). Afterward, pairs of ChR2 and control animals underwent optical activations of the VTA during the SCPR test, as previously described, in order to study if DA activations during the competition were enough to modulate the outputs of the SCPR test (**Figure 4D**). We found no effect of the treatment when pairs of ChR2 and control rats were allowed to compete for the palatable-reward (**Figure 4E**), as indicated by equivalent latency to reward ($t = 0.5522$, $df = 6$, $p = 0.6007$; **Figure 4E**) and percentage of obtained rewards ($t = 0.8439$, $df = 6$, $p = 0.4311$; **Figure 4E**). Subsequently, in the same pair of rats that had undergone optogenetic activation of DA neurons during the SCPR test, we performed a second SCPR test in which animals also received phasic DA activations, in order to investigate whether several days of DA activations were required to have the effects on SCPR outputs (**Figure 4F**). We found no effect of the treatment when pairs of ChR2 and control rats were allowed to compete for the palatable-reward (**Figure 4E**), as indicated by equivalent latency to reward ($t = 0.3251$, $df = 6$, $p = 0.7561$; **Figure 4F**) and percentage of obtained rewards ($t = 0.8154$, $df = 6$, $p = 0.4460$; **Figure 4F**). Therefore, our results indicated that a combination of phasic stimulation during both training and the SCPR test were required in order to enhance animals' competitiveness in this test.

DISCUSSION

Our results are in line with previous studies showing that dominant rats present higher reward-seeking behaviors (Davis et al., 2009) and recent human studies indicating that high reward-seeking improves performance of a competitive task (Balconi and Vanutelli, 2016). However, these studies measured reward seeking behaviors after hierarchy has already been established, making difficult to conclude if differences in reward-

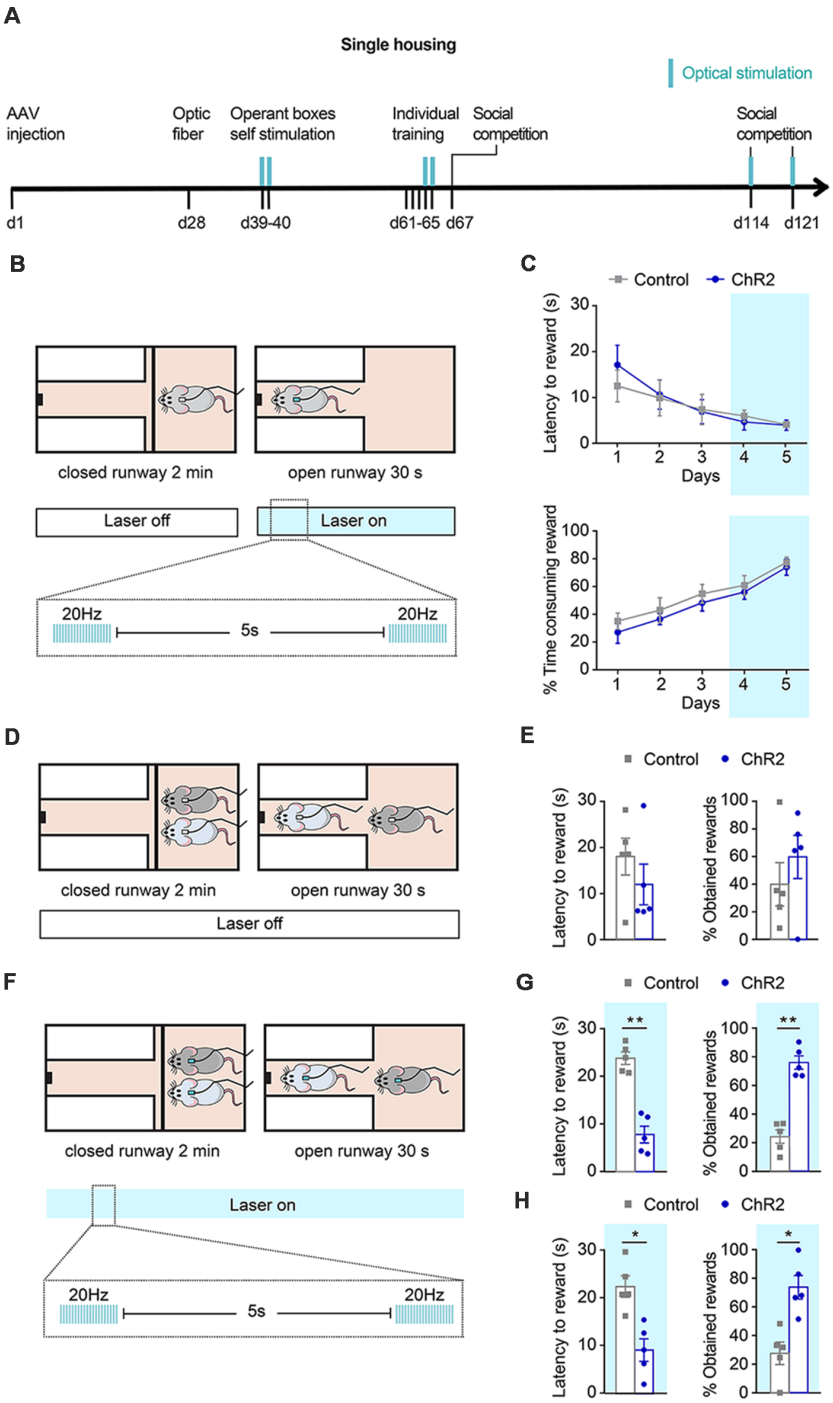


FIGURE 3 | Effects of phasic DA during individual training and social competition in dominance. **(A)** Experimental timeline followed by a group of channelrhodopsin 2 (ChR2; $n = 5$) and control ($n = 5$) animals to study the effects of phasic DA activations during individual training and competition on the SCPR test outcomes. Blue marks indicate sessions in which optical stimulation was applied. **(B)** Schematic representation of illumination pattern used during the last 2 days of the individual training in a group of ChR2 ($n = 5$) and control ($n = 5$) animals. **(C)** Average latency to rewards and percentage of time consuming the reward (mean ± SEM) during each day of individual training for ChR2 and control animals. **(D)** Animals underwent a social competition test without optical activation. **(E)** No difference in average latency to rewards and percentage of obtained rewards (mean ± SEM) for ChR2 and control animals during a social competition test. **(F)** Animals underwent two further social competition tests while their ventral tegmental area (VTA) DA neurons were phasically activated. **(G)** Average latency to rewards was lower in ChR2 rats and percentage of obtained rewards was higher compared to control rats (mean ± SEM) for the same pairs of ChR2 and control animals. **(H)** Average latency to rewards and percentage of obtained rewards (mean ± SEM) from new pairs of ChR2 and control animals ($n = 5$), which had never encountered each other before, during a social competition test. Average latency to rewards was lower in ChR2 rats and percentage of obtained rewards was higher compared to control rats (mean ± SEM). Blue background on the graphs indicates sessions during which optical activation was used. $^*p < 0.05$, $^{**}p < 0.01$.

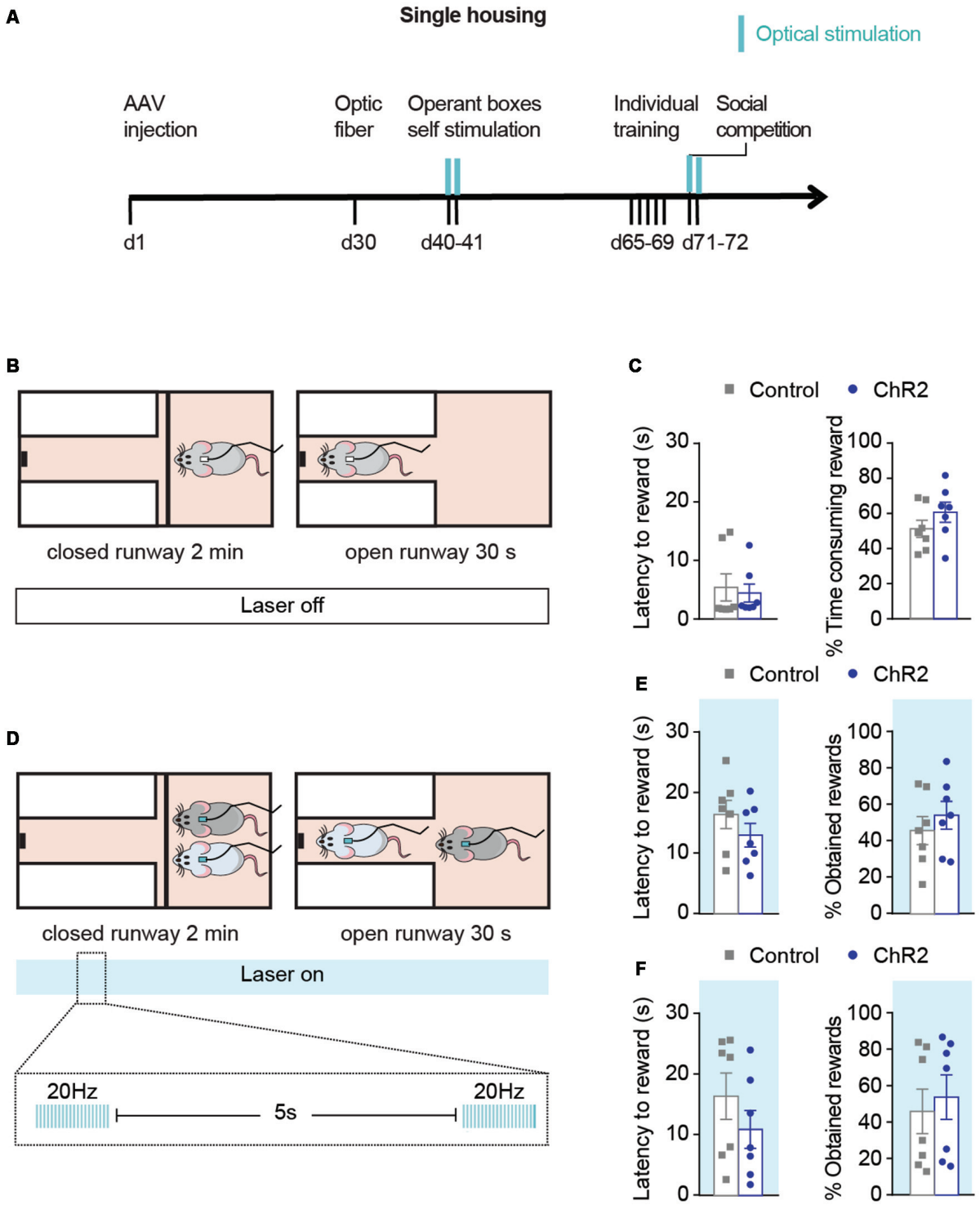


FIGURE 4 | Optical activation during social competition test only does not modulate the outcome of the competition. **(A)** Experimental timeline followed by a group of ChR2 (n = 7) and control (n = 7) animals to study the effects of phasic DA activations during competition on the SCPR test outcomes. Blue marks indicate sessions in which optical stimulation was applied. **(B)** Rats underwent individual training without optical activations. **(C)** Latency to rewards and percentage of obtained rewards (mean ± SEM) on day 5 of individual training for ChR2 (n = 7) and controls (n = 7). **(D)** During the SCPR test, animals underwent optical activations of VTA dopaminergic neurons. **(E)** Latency to rewards and percentage of obtained rewards (mean ± SEM) on the same animals during SCPR test while phasic activations of dopaminergic neurons in the VTA. No differences were observed between the groups. **(F)** Latency to rewards and percentage of obtained rewards (mean ± SEM) on the same animals during a second SCPR test while phasic activations of dopaminergic neurons in the VTA. No differences were observed between the two groups.

seeking behaviors influence the social rank acquisition or if the high social rank acquisition produces changes in reward-seeking behaviors. Here, we show that differences in reward-seeking behaviors prior to hierarchy establishment can predict the outcome of several social competition tests after hierarchy has been established. Hence, we provide evidence in support of reward-seeking behavior functioning as a personality trait in rats related to dominance and the formation of social hierarchies. Our data suggest that this trait is related to accumbal DA content, thus it may be important to use latency to approach a reward for the characterization of individual differences in outbred rat populations, together with assessments of anxiety or exploratory behavior.

Animals were classified based on their latency to get a palatable-reward when given temporary access to it, individually. The selection of latencies to reward as a measurement of reward-seeking behavior is supported by human studies showing that high reward-seeking individuals have lower latencies to react to pleasant pictures (Aluja et al., 2015). Furthermore, deficits in reward-seeking behavior of animals treated with DA receptor antagonists were due to an increased latency to initiate approach behavior towards the reward (Nicola, 2010). Conversely, high dominance trait in humans is accompanied by faster decision making (da Cruz et al., 2018). As our animals were classified based on the latency to acquire rewards, we additionally used a different kind of social competition test in which the latency to arrive to the reward does not play a role in dominance. This is the case of the WCT, in which the access to the bottle is not limited and therefore the animal arriving second can still push the other one away and drink. On the second WCT we did not see any differences between SL and LL animals in the latencies to drink, however, the SL animals pushed the LL away and still drank more.

In addition, our results showed increased levels of accumbal DA in the SL animals, as measured after the hierarchy was established. Consistently, a great number of studies have indicated a role for the dopaminergic reward system in dominance (Grant et al., 1998; Overli et al., 1999; Kaplan et al., 2002; Morgan et al., 2002; Martinez et al., 2010; Hollis et al., 2015). However, most of these studies (including ours) measured DA levels after hierarchy establishment making difficult any assumption on its causality on dominance. However, individual differences in dopaminergic responses to a new situation (presence of a competitor) during a social competition might determine the chances of becoming dominant. Accordingly, previous studies from our lab have shown that activation of accumbal neurons expressing the D1 receptor during social competition was highly correlated with animals' performance (highlighting that activation of DA neurons during competition might play a critical role; van der Kooij et al., 2018).

It is important to mention that even though we found differences in accumbal DA levels, we did not find differences in DA metabolite levels (DOPAC and HVA), which could suggest lack of differences in DA signaling or turnover. Since our measurement of DA and its metabolites is limited only to analysis under basal conditions, without multiple time points, we cannot make any inferences regarding DA signaling/turnover. However, other explanations may also exist: first, we measured total neurotransmitter levels, not solely extracellular levels, therefore, it is not possible to draw any conclusions regarding levels of DA release. Thus, even though SL rats had higher DA levels compared to LL rats, this may represent DA that can be released upon presentation of a certain stimulus (i.e., social competition or involvement in a task for the acquisition of a reward), rather than constitutive high levels of DA release. Secondly, DOPAC and HVA levels do not depend solely on DA levels, but rather to a combination of factors including DA synthesis rate, levels of DA release and utilization and expression levels of the metabolizing enzymes (Monoamine oxidase for DOPAC and Monoamine oxidase and Catechol-O-methyltransferase for HVA). Therefore, an 1-1 relationship between DA and metabolite levels cannot always be found (Sharp et al., 1986; Soares-Da-Silva and Garrett, 1990).

In accordance with our data, previous studies using rats selected for different traits have often found relations between the activity of the dopaminergic system and individual differences in behaviors related to reward-seeking or motivation. For example, work done in rats selectively bred for their locomotor response to novelty has shown that "high-responders" (individuals displaying high locomotor response to novelty) also display higher sensation-seeking and risk-taking behaviors, while they also display a hyperdopaminergic state, compared to "low responders" (individuals displaying low locomotor response to novelty; Flagel et al., 2014). Similarly, Roman high- (RHA) and low-avoidance rats (RLA) display differences in their dopaminergic signaling. In this model of rats selectively bred for rapid or poor acquisition of active avoidance (Driscoll et al., 1998), RHA rats display higher preference and intake of palatable food and higher DA release in response to cocaine, compared to RLA rats (Giorgi et al., 2007). These findings, including ours, suggest that there may be a direct association between increased dopaminergic tone and reward-seeking behaviors oriented towards drug consumption, palatable food intake or even sexual behavior (Melis et al., 2018). Given that reward-seeking behaviors (strongly associated with DA) predicted hierarchy establishment, in our experiments, we hypothesized that mesolimbic dopaminergic responses (during training and/or competition session) might have a causal role on the SCPR outcome.

Recently it has been proven that phasic DA stimulation of the VTA unilaterally delivered on a time-precise manner (during reward delivery) was sufficient to improve the learning of the reward acquisition paradigm (Steinberg et al., 2013). Similar phasic DA activations of the VTA have been shown to modulate reward seeking, motivation and social behaviors, closely related to dominance (Adamantidis et al., 2011; Tye et al., 2013; Gunaydin et al., 2014). Furthermore, synaptic DA release in the NAc depends on neuronal activity in the VTA (Sombers et al., 2009) and phasic unilateral activation of VTA DA neurons has been shown to increase DA levels on the NAc by using voltammetry *in vivo* (Witten et al., 2011) and even lead to sustained DA release for several

minutes, as measured by microdialysis (Lohani et al., 2018). It is important to note that in our study we used unilateral stimulation of VTA neurons, which was shown to be effective in previous studies.

Previous studies have highlighted the role of individual differences in DA responses in reversal learning and flexible approaches in relation to reward acquisition (Nicola, 2010; Klanker et al., 2015; Boekhoudt et al., 2018). Moreover, individual differences in phasic DA release evoked by the new rewards predicted reversal learning behavior (Klanker et al., 2015). Individuals that have an elevated DA response associated with the reward acquisition have also higher chances to develop a flexible approach (such as reverse learning) in order to improve the chances of reward acquisition in a new situation in which a different behavior is required in order to get the reward. Therefore, we studied whether an enhancement of the endogenous DA release during the acquisition of the palatable rewards on the last 2 days of individual training could also increase the chances of the animals to get more rewards during the SCPR test, perhaps by developing a flexible strategy (such as pushing the other animal away).

Our phasic activations were time-locked to the reward acquisition epochs (30 s where the animal could access the palatable reward) in order to enhance their endogenous levels of DA to the reward acquisition. We did not observe any changes on their average latencies to rewards or the time-consuming rewards during the two last sessions where DA activations occurred. This could have been because animal latencies were already very low and it was not possible to decrease further. Furthermore, previous studies have shown that DA is required for reward-seeking behaviors only when the specific actions to obtain the reward vary (when different actions are required to reach the reward), or when animals need to develop a new behavioral strategy to acquire the reward (van der Meer and Redish, 2009; Nicola, 2010), which was not the case for the last two training sessions.

Our findings indicated that the phasic DA activations during the last 2 days of training were not sufficient to fully shift the outcome of the SCPR in favor of the ChR2 rat in all competing pairs. Nevertheless, in the majority of pairs, the ChR2 rat won the competition even in the SCPR test without phasic DA activations. Furthermore, when adding a previous cohort of animals that received optical stimulation during the last 2 days of individual training and performed SCPR test without optical activations, we also observed a similar pattern of only a few animals that did not win the SCPR test (**Supplementary Figure S3**). It is worth noting that in the ChR2 rats that did not win the competition following phasic DA activations only during training, we could not identify any issues with viral delivery/expression, optic fiber implantation or any other issue that would result to exclusion of these rats.

In line with a previous study indicating that the accumbal neurons that fired during reward delivery also fired when animals decided which actions to take during flexible (but not inflexible) approach (van der Meer and Redish, 2009), our findings indicated that a combination of phasic DA activations during the last 2 days of individual training and during SCPR test were sufficient to enhance dominance (**Figure 3G**) and shift the outcome of the competition in favor of the ChR2 rat in all pairs. Importantly, when we performed a new SCPR test with new pairs, we found the same effect of optical activation, suggesting that the result could not be attributed to a carry-over effect of the first SCPR test done with the same pairs.

In contrast, phasic activations of DA neurons only during the SCPR test were not enough to shift dominance in favor of the ChR2 group in the SCPR test (**Figure 4E**), highlighting the critical effect of the phasic DA activations during training. The lack of effect of the phasic DA activations during a second SCPR test suggests that the effect of the combination of phasic DA activations during individual training and during the SCPR test is not merely due to the repeated activation of DA neurons, but that the timing and context of the activations is crucial. Finally, the comparable performance of the ChR2 and control animal in the Open Field test with and without phasic dopaminergic activation (**Supplementary Figure S2D**), suggests that the observed effects in the SCPR test could not have been solely dependent on non-specific changes in the rats' locomotor output.

A limitation of our study was the use of only one stimulation protocol. It has been previously shown that tonic stimulation of dopaminergic neurons in the VTA results in inhibition of reward consummatory behaviors (Mikhailova et al., 2016) and that tonic but not phasic stimulation reduces ethanol self-administration (Bass et al., 2013). Therefore, it would be interesting to test different stimulation protocols in future studies (e.g., tonic vs. phasic) in relation to their effects on reward-seeking and social competition behaviors.

A remaining question is how the results of this study concerning dominance relationships in dyads could be generalized in the context of the more complex hierarchies and interactions in ethologically relevant (and more naturalistic) settings. Results from such a setting, namely the VBS, in which male and female rats are housed together and social hierarchies are formed rapidly (Blanchard et al., 1995), have suggested that following exposure to the VBS, a subgroup of subordinate males develop alterations in DA activity in mesolimbic structures (Lucas et al., 2004). In addition, dominant males in the VBS display increased responding for food rewards, suggesting higher engagement in reward-seeking behaviors (Davis et al., 2009). Based on this evidence, we could hypothesize that SL rats could possibly assume dominant positions in such naturalistic context, whereas LL rats would most likely be subordinate. However, this needs to be tested experimentally.

In summary, our results provide new insights into the role of reward-seeking behavior in hierarchy establishment. Our study is the first one to highlight that the individual trait seeking behavior, measured by the latency to obtain rewards can be predictive of the outcome of social competition tests after hierarchy has been established. Moreover, our findings suggest a key role for accumbal DA levels in relation to trait reward-seeking behavior after hierarchy establishment. Finally, modulation of dopaminergic neuron firing in the VTA (shown to enhance reward-seeking behaviors), and

for the first time, that levels of DA associated to reward acquisition, individually, could determine the outcome of an SCPR test, indicating that reward-seeking behavior and responses to social competition may impinge on the same neuronal circuits.

ETHICS STATEMENT

All experiments were performed with the approval of the Cantonal Veterinary Authorities (Vaud, Switzerland) and carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU).

AUTHOR CONTRIBUTIONS

CS and LL-M conceived and planned the experiments. IZ, SAb, IGS, and LL-M carried out the experiments. LL-M and CS contributed to the interpretation of the results. LL-M took the lead in writing the manuscript under the supervision of IZ. SAs and IZ provided critical feedback and helped shape the research, analysis and manuscript. All authors gave their inputs to the final version of the manuscript.

REFERENCES

Adamantidis, A. R., Tsai, H.-C., Boutrel, B., Zhang, F., Stuber, G. D., Budygin, E. A., et al. (2011). Optogenetic interrogation of dopaminergic modulation of the multiple phases of reward-seeking behavior. *J. Neurosci.* 31, 10829–10835. doi: 10.1523/JNEUROSCI.2246-11.2011

Akers, K. G., Yang, Z., DelVecchio, D. P., Reeb, B. C., Romeo, R. D., McEwen, B. S., et al. (2008). Social competitiveness and plasticity of neuroendocrine function in old age: influence of neonatal novelty exposure and maternal care reliability. *PLoS One* 3:e2840. doi: 10.1371/journal.pone.0002840

Alcaro, A., Huber, R., and Panksepp, J. (2007). Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. *Brain Res. Rev.* 56, 283–321. doi: 10.1016/j.brainresrev.2007.07.014

Alcock, J., and Rubenstein, D. R. (1989). *Animal Behavior*. Sunderland, MA: Sinauer Associates.

Aluja, A., Blanch, A., Blanco, E., and Balada, F. (2015). Affective modulation of the startle reflex and the reinforcement sensitivity theory of personality: the role of sensitivity to reward. *Physiol. Behav.* 138, 332–339. doi: 10.1016/j.physbeh.2014.09.009

Arakawa, H. (2006). Changes in the pattern of exploratory behavior are associated with the emergence of social dominance relationships in male rats. *Dev. Psychobiol.* 48, 39–47. doi: 10.1002/dev.20114

Arias-Carrión, O., Stamelou, M., Murillo-Rodríguez, E., Menéndez-González, M., and Pöppel, E. (2010). Dopaminergic reward system: a short integrative review. *Int. Arch. Med.* 3:24. doi: 10.1186/1755-7682-3-24

Baenninger, L. P. (1970). Social dominance orders in the rat: "Spontaneous," food, and water competition. *J. Comp. Physiol. Psychol.* 71:202. doi: 10.1037/h0029162

Balconi, M., and Vanutelli, M. E. (2016). Competition in the brain. the contribution of EEG and fNIRS modulation and personality effects in social ranking. *Front. Psychol.* 7:1587. doi: 10.3389/fpsyg.2016.01587

Barnet, S. A. (1958). *A Century of Darwin*. Cambridge: Harvard University Press.

Bass, C. E., Grinevich, V. P., Gioia, D., Day-Brown, J., Bonin, K. D., Stuber, G. D., et al. (2013). Optogenetic stimulation of VTA dopamine neurons reveals that tonic but not phasic patterns of dopamine transmission reduce ethanol self-administration. *Front. Behav. Neurosci.* 7:173. doi: 10.3389/fnbeh.2013.00173

FUNDING

This work was supported by grants from the Swiss National Science Foundation (31003A-152614); NCCR Synapsy (5INF40-158776 and 51NF40_185897) and intramural funding from the École Polytechnique Fédérale de Lausanne to CS. IZ was supported by an EMBO long-term fellowship (ALTF 1537-2015), co-funded by Marie Curie actions (LTFCOFUND2013, GA-2013-609409).

ACKNOWLEDGMENTS

We thank J. Grosse, E. Schranz and O. Zanoletti for experimental assistance. Drs E. Tsika and E. O'Conner for their great technical support on HPLC and optogenetic techniques, respectively and Dr K. Deisseroth for the generous gift of the TH::Cre rats to start our breeding.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00069/full#supplementary-material

Berdoy, M., Smith, P., and MacDonald, D. W. (1995). Stability of social status in wild rats: age and the role of settled dominance. *Behaviour* 132, 193–212. doi: 10.1163/156853995x00694

Blanchard, D. C., Spencer, R. L., Weiss, S. M., Blanchard, R. J., McEwen, B., and Sakai, R. R. (1995). Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. *Psychoneuroendocrinology* 20, 117–134. doi: 10.1016/0306-4530(94)e0045-b

Boekhoudt, L., Wijbrans, E. C., Man, J. H., Luijendijk, M. C., de Jong, J. W., van der Plasse, G., et al. (2018). Enhancing excitability of dopamine neurons promotes motivational behaviour through increased action initiation. *Eur. Neuropsychopharmacol.* 28, 171–184. doi: 10.1016/j.euroneuro.2017.11.005

Carver, C. S., and White, T. L. (1994). Behavioral inhibition, behavioral activation and affective responses to impending reward and punishment: the BIS/BAS scales. *J. Pers. Soc. Psychol.* 67, 319–333. doi: 10.1037//0022-3514.67.2.319

Chaudhury, D., Walsh, J. J., Friedman, A. K., Juarez, B., Ku, S. M., Koo, J. W., et al. (2013). Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature* 493, 532–536. doi: 10.1038/nature11713

Cordero, M. I., and Sandi, C. (2007). Stress amplifies memory for social hierarchy. *Front. Neurosci.* 1, 175–184. doi: 10.3389/neuro.01.1.1.013.2007

da Cruz, J., Rodrigues, J., Thoresen, J. C., Chicherov, V., Figueiredo, P., Herzog, M. H., et al. (2018). Dominant men are faster in decision-making situations and exhibit a distinct neural signal for promptness. *Cereb. Cortex* 28, 3740–3751. doi: 10.1093/cercor/bhy195

Davis, J., Krause, E., Melhorn, S., Sakai, R., and Benoit, S. (2009). Dominant rats are natural risk takers and display increased motivation for food reward. *Neuroscience* 162, 23–30. doi: 10.1016/j.neuroscience.2009.04.039

Driscoll, P., Escorihuela, R., Fernández-Teruel, A., Giorgi, O., Schwegler, H., Steimer, T., et al. (1998). Genetic selection and differential stress responses. the Roman lines/strains of rats. *Ann. N Y Acad. Sci.* 851, 501–510. doi: 10.1111/j.1749-6632.1998.tb09029.x

Flagel, S. B., Waselus, M., Clinton, S. M., Watson, S. J., and Akil, H. (2014). Antecedents and consequences of drug abuse in rats selectively bred for high and low response to novelty. *Neuropharmacology* 76, 425–436. doi: 10.1016/j.neuropharm.2013.04.033

Giorgi, O., Piras, G., and Corda, M. G. (2007). The psychogenetically selected Roman high-and low-avoidance rat lines: a model to study the individual vulnerability to drug addiction. *Neurosci. Biobehav. Rev.* 31, 148–163. doi: 10.1016/j.neubiorev.2006.07.008

Goette, L., Bendahan, S., Thoresen, J., Hollis, F., and Sandi, C. (2015). Stress pulls us apart: anxiety leads to differences in competitive confidence under stress. *Psychoneuroendocrinology* 54, 115–123. doi: 10.1016/j.psyneuen.2015.01.019

Grant, K. A., Shively, C. A., Nader, M. A., Ehrenkaufer, R. L., Line, S. W., Morton, T. E., et al. (1998). Effect of social status on striatal dopamine D2 receptor binding characteristics in cynomolgus monkeys assessed with positron emission tomography. *Synapse* 29, 80–83. doi: 10.1002/(sici)1098-2396(199805)29:1<80::aid-syn7>3.0.co;2-7

Guitart, X., Méndez, R., Ovalle, S., Andreu, F., Carceller, A., Farré, A. J., et al. (2000). Regulation of ionotropic glutamate receptor subunits in different rat brain areas by a preferential sigma 1 receptor ligand and potential atypical antipsychotic. *Neuropsychopharmacology* 23, 539–546. doi: 10.1016/s0893-133x(00)00142-1

Gunaydin, L. A., Grosenick, L., Finkelstein, J. C., Kauvar, I. V., Fenno, L. E., Adhikari, A., et al. (2014). Natural neural projection dynamics underlying social behavior. *Cell* 157, 1535–1551. doi: 10.1016/j.cell.2014.05.017

Herrero, A. I., Sandi, C., and Venero, C. (2006). Individual differences in anxiety trait are related to spatial learning abilities and hippocampal expression of mineralocorticoid receptors. *Neurobiol. Learn. Mem.* 86, 150–159. doi: 10.1016/j.nlm.2006.02.001

Hills, T., Brockie, P. J., and Maricq, A. V. (2004). Dopamine and glutamate control area-restricted search behavior in *Caenorhabditis elegans*. *J. Neurosci.* 24, 1217–1225. doi: 10.1523/jneurosci.1569-03.2004

Hoebel, J., Maske, U. E., Zeeb, H., and Lampert, T. (2017). Social inequalities and depressive symptoms in adults: the role of objective and subjective socioeconomic status. *PLoS One* 12:e0169764. doi: 10.1371/journal.pone.0169764

Hollis, F., van der Kooij, M. A., Zanoletti, O., Lozano, L., Cantó, C., and Sandi, C. (2015). Mitochondrial function in the brain links anxiety with social subordination. *Proc. Natl. Acad. Sci. U S A* 112, 15486–15491. doi: 10.1073/pnas.1512653112

Ichinose, T., Tanimoto, H., and Yamagata, N. (2017). Behavioral modulation by spontaneous activity of dopamine neurons. *Front. Syst. Neurosci.* 11:88. doi: 10.3389/fnsys.2017.00088

Kaplan, J. R., Manuck, S. B., Fontenot, M. B., and Mann, J. J. (2002). Central nervous system monoamine correlates of social dominance in cynomolgus monkeys (*Macaca fascicularis*). *Neuropsychopharmacology* 26, 431–443. doi: 10.1016/S0893-133X(01)00344-X

Klanker, M., Sandberg, T., Joosten, R., Willuhn, I., Feenstra, M., and Denys, D. (2015). Phasic dopamine release induced by positive feedback predicts individual differences in reversal learning. *Neurobiol. Learn. Mem.* 125, 135–145. doi: 10.1016/j.nlm.2015.08.011

Larrieu, T., Cherix, A., Duque, A., Rodrigues, J., Lei, H., Gruetter, R., et al. (2017). Hierarchical status predicts behavioral vulnerability and nucleus accumbens metabolic profile following chronic social defeat stress. *Curr. Biol.* 27, 2202–2210. doi: 10.1016/j.cub.2017.06.027

Lechner, H. A., Baxter, D. A., and Byrne, J. H. (2000). Classical conditioning of feeding in Aplysia: II. Neurophysiological correlates. *J. Neurosci.* 20, 3377–3386. doi: 10.1523/jneurosci.20-09-03377.2000

Lohani, S., Martig, A. K., Underhill, S. M., DeFrancesco, A., Roberts, M. J., Rinaman, L., et al. (2018). Burst activation of dopamine neurons produces prolonged post-burst availability of actively released dopamine. *Neuropsychopharmacology* 43, 2083–2092. doi: 10.1038/s41386-018-0088-7

Lucas, L., Celen, Z., Tamashiro, K., Blanchard, R., Blanchard, D., Markham, C., et al. (2004). Repeated exposure to social stress has long-term effects on indirect markers of dopaminergic activity in brain regions associated with motivated behavior. *Neuroscience* 124, 449–457. doi: 10.1016/j.neuroscience.2003.12.009

Luciana, M., Wahlstrom, D., Porter, J. N., and Collins, P. F. (2012). Dopaminergic modulation of incentive motivation in adolescence: age-related changes in signaling, individual differences and implications for the development of self-regulation. *Dev. Psychol.* 48, 844–861. doi: 10.1037/a0027432

Lucion, A., and Vogel, W. H. (1994). Effects of stress on defensive aggression and dominance in a water competition test. *Integr. Psychol. Behav. Sci.* 29, 415–422. doi: 10.1007/BF02691361

Martinez, D., Orlowska, D., Narendran, R., Slifstein, M., Liu, F., Kumar, D., et al. (2010). Dopamine type 2/3 receptor availability in the striatum and social status in human volunteers. *Biol. Psychiatry* 67, 275–278. doi: 10.1016/j.biopsych.2009.07.037

McCutcheon, J. E., Cone, J. J., Sinon, C. G., Fortin, S. M., Kantak, P. A., Witten, I. B., et al. (2014). Optical suppression of drug-evoked phasic dopamine release. *Front. Neural Circuits* 8:114. doi: 10.3389/fncir.2014.00114

Melis, M. R., Sanna, F., and Argiolas, A. (2018). Rats selectively bred for showing divergent behavioral traits in response to stress or novelty or spontaneous yawning with a divergent frequency show similar changes in sexual behavior: the role of dopamine. *Rev. Neurosci.* doi: 10.1515/revneuro-2018-0058 [Epub ahead of print].

Mikhailova, M. A., Bass, C. E., Grinevich, V. P., Chappell, A. M., Deal, A. L., Bonin, K. D., et al. (2016). Optogenetically-induced tonic dopamine release from VTA-nucleus accumbens projections inhibits reward consummatory behaviors. *Neuroscience* 333, 54–64. doi: 10.1016/j.neuroscience.2016.07.006

Miller-Butterworth, C. M., Kaplan, J. R., Shaffer, J., Devlin, B., Manuck, S. B., and Ferrell, R. E. (2008). Sequence variation in the primate dopamine transporter gene and its relationship to social dominance. *Mol. Biol. Evol.* 25, 18–28. doi: 10.1093/molbev/msm219

Morgan, D., Grant, K. A., Gage, H. D., Mach, R. H., Kaplan, J. R., Prioleau, O., et al. (2002). Social dominance in monkeys: dopamine D2 receptors and cocaine self-administration. *Nat. Neurosci.* 5, 169–174. doi: 10.1038/nn798

Nader, M. A., Nader, S. H., Czoty, P. W., Riddick, N. V., Gage, H. D., Gould, R. W., et al. (2012). Social dominance in female monkeys: dopamine receptor function and cocaine reinforcement. *Biol. Psychiatry* 72, 414–421. doi: 10.1016/j.biopsych.2012.03.002

Nicola, S. M. (2007). The nucleus accumbens as part of a basal ganglia action selection circuit. *Psychopharmacology* 191, 521–550. doi: 10.1007/s00213-006-0510-4

Nicola, S. M. (2010). The flexible approach hypothesis: unification of effort and cue-responding hypotheses for the role of nucleus accumbens dopamine in the activation of reward-seeking behavior. *J. Neurosci.* 30, 16585–16600. doi: 10.1523/jneurosci.3958-10.2010

Overli, O., Harris, C. A., and Winberg, S. (1999). Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. *Brain Behav. Evol.* 54, 263–275. doi: 10.1159/000006627

Peres, R. C., and Leite, J. R. (2002). The influence of competitive status (winner/loser) on the behavior of male rats in three models of anxiety. *Aggres. Behav.* 28, 164–171. doi: 10.1002/ab.90018

Roitman, M. F., Stuber, G. D., Phillips, P. E., Wightman, R. M., and Carelli, R. M. (2004). Dopamine operates as a subsecond modulator of food seeking. *J. Neurosci.* 24, 1265–1271. doi: 10.1523/jneurosci.3823-03.2004

Russo, S. J., and Nestler, E. J. (2013). The brain reward circuitry in mood disorders. *Nat. Rev. Neurosci.* 14, 609–625. doi: 10.1038/nrn3381

Salamone, J. D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. *Neuron* 76, 470–485. doi: 10.1016/j.neuron.2012.10.021

Santamaría-García, H., Burgaleta, M., and Sebastián-Gallés, N. (2015). Neuroanatomical markers of social hierarchy recognition in humans: a combined ERP/MRI study. *J. Neurosci.* 35, 10843–10850. doi: 10.1523/JNEUROSCI.1457-14.2015

Santamaría-García, H., Pannunzi, M., Ayneto, A., Deco, G., and Sebastián-Gallés, N. (2013). "If you are good, I get better": the role of social hierarchy in perceptual decision-making. *Soc. Cogn. Affect. Neurosci.* 9, 1489–1497. doi: 10.1093/scan/nst133

Sapolsky, R. M. (2005). The influence of social hierarchy on primate health. *Science* 308, 648–652. doi: 10.1126/science.1106477

Sharp, T., Zetterström, T., and Ungerstedt, U. (1986). An *in vivo* study of dopamine release and metabolism in rat brain regions using intracerebral dialysis. *J. Neurochem.* 47, 113–122. doi: 10.1111/j.1471-4159.1986.tb02838.x

Soares-Da-Silva, P., and Garrett, M. (1990). Overflow of endogenous dopamine and 3,4-dihydroxyphenylacetic acid from tissues of the rat brain, elicited by electrical stimulation, depolarization by potassium and activation of carrier-mediated release. *Neuropharmacology* 29, 1151–1159. doi: 10.1016/0028-3908(90)90039-t

Sombers, L. A., Beyene, M., Carelli, R. M., and Wightman, R. M. (2009). Synaptic overflow of dopamine in the nucleus accumbens arises from neuronal activity in the ventral tegmental area. *J. Neurosci.* 29, 1735–1742. doi: 10.1523/jneurosci.5562-08.2009

Steinberg, E. E., Keiflin, R., Boivin, J. R., Witten, I. B., Deisseroth, K., and Janak, P. H. (2013). A causal link between prediction errors, dopamine neurons and learning. *Nat. Neurosci.* 16, 966–973. doi: 10.1038/nn.3413

Timmer, M., Cordero, M. I., Sevelinges, Y., and Sandi, C. (2011). Evidence for a role of oxytocin receptors in the long-term establishment of dominance hierarchies. *Neuropsychopharmacology* 36, 2349–2356. doi: 10.1038/npp.2011.125

Timmer, M., and Sandi, C. (2010). A role for glucocorticoids in the long-term establishment of a social hierarchy. *Psychoneuroendocrinology* 35, 1543–1552. doi: 10.1016/j.psyneuen.2010.05.011

Toledo-Rodriguez, M., and Sandi, C. (2007). Stress before puberty exerts a sex-and age-related impact on auditory and contextual fear conditioning in the rat. *Neural Plast.* 2007:71203. doi: 10.1155/2007/71203

Tye, K. M., Mirzabekov, J. J., Warden, M. R., Ferenczi, E. A., Tsai, H.-C., Finkelstein, J., et al. (2013). Dopamine neurons modulate neural encoding and expression of depression-related behaviour. *Nature* 493, 537–541. doi: 10.1038/nature11740

van der Kooij, M. A., Hollis, F., Lozano, L., Zalachoras, I., Abad, S., Zanoletti, O., et al. (2018). Diazepam actions in the VTA enhance social dominance and mitochondrial function in the nucleus accumbens by activation of dopamine D1 receptors. *Mol. Psychiatry* 23, 569–578. doi: 10.1038/mp.2017.135

van der Kooij, M. A., and Sandi, C. (2015). The genetics of social hierarchies. *Curr. Opin. Behav. Sci.* 2, 52–57. doi: 10.1016/j.cobeha.2014.09.001

van der Meer, M. A., and Redish, A. D. (2009). Covert expectation-of-reward in rat ventral striatum at decision points. *Front. Integr. Neurosci.* 3:1. doi: 10.3389/neuro.07.001.2009

Wang, F., Kessels, H. W., and Hu, H. (2014). The mouse that roared: neural mechanisms of social hierarchy. *Trends Neurosci.* 37, 674–682. doi: 10.1016/j.tins.2014.07.005

Wilkinson, R. G., and Pickett, K. E. (2006). Income inequality and population health: a review and explanation of the evidence. *Soc. Sci. Med.* 62, 1768–1784. doi: 10.1016/j.socscimed.2005.08.036

Wise, R. A. (2004). Dopamine, learning and motivation. *Nat. Rev. Neurosci.* 5, 483–494. doi: 10.1038/nrn1406

Witten, I. B., Steinberg, E. E., Lee, S. Y., Davidson, T. J., Zalocusky, K. A., Brodsky, M., et al. (2011). Recombinase-driver rat lines: tools, techniques and optogenetic application to dopamine-mediated reinforcement. *Neuron* 72, 721–733. doi: 10.1016/j.neuron.2011.10.028

Conflict of Interest Statement: 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: 06 June 2019
doi: 10.3389/fnbeh.2019.00119

A Visual Two-Choice Rule-Switch Task for Head-Fixed Mice

Szabolcs Biró, Bálint Lasztóczi and Thomas Klausberger**

Center for Brain Research, Division of Cognitive Neurobiology, Medical University of Vienna, Vienna, Austria

Cognitive flexibility is the innate ability of the brain to change mental processes and to modify behavioral responses according to an ever-changing environment. As our brain has a limited capacity to process the information of our surroundings in any given moment, it uses sets as a strategy to aid neural processing systems. With assessing the capability of shifting between task sets, it is possible to test cognitive flexibility and executive functions. The most widely used neuropsychological task for the evaluation of these functions in humans is the Wisconsin Card Sorting Test (WCST), which requires the subject to alter response strategies and use previously irrelevant information to solve a problem. The test has proven clinical relevance, as poor performance has been reported in multiple neuropsychiatric conditions. Although, similar tasks have been used in pre-clinical rodent research, many are limited because of their manual-based testing procedures and their hardware attenuates neuronal recordings. We developed a two-choice rule-switch task whereby head-fixed C57BL/6 mice had to choose correctly one of the two virtual objects presented to retrieve a small water reward. The animals learnt to discriminate the visual cues and they successfully switched their strategies according to the related rules. We show that reaching successful performance after the rule changes required more trials in this task and that animals took more time to execute decisions when the two rules were in conflict. We used optogenetics to inhibit temporarily the medial prefrontal cortex (mPFC) during reward delivery and consumption, which significantly increased the number of trials needed to perform the second rule successfully (i.e., succeed in switching between rules), compared to control experiments. Furthermore, by assessing two types of error animals made after the rule switch, we show that interfering with the positive feedback integration, but leaving the negative feedback processing intact, does not influence the initial disengagement from the first rule, but impedes the maintenance of the newly acquired response set. These findings support the role of prefrontal networks in mice for cognitive flexibility, which is impaired during numerous neuropsychiatric diseases, such as schizophrenia and depression.

Keywords: cognitive flexibility, rule-switching, prefrontal cortex, virtual reality, behavioral task, optogenetics, head-fixed

OPEN ACCESS

Edited by:
Bruno Poucet,
Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Verity J. Brown,
University of St Andrews, United Kingdom
Tommy Pattij,
VU University Medical Center, Netherlands

****Correspondence:***
Szabolcs Biró
sz.biro86@gmail.com
Thomas Klausberger
thomas.klausberger@meduniwien.ac.at

Received: *18 December 2018*
Accepted: *17 May 2019*
Published: *06 June 2019*

Citation:
Biró S, Lasztóczi B and Klausberger T (2019) A Visual Two-Choice Rule-Switch Task for Head-Fixed Mice. Front. Behav. Neurosci. 13:119. doi: 10.3389/fnbeh.2019.00119

INTRODUCTION

Cognitive flexibility is a crucial executive function which allows adaptive behavior by switching between different thoughts and actions, the complex rules of which are yet unknown. Deficit of this function has been observed in numerous neurological conditions, including schizophrenia, Alzheimer's and Parkinson's disease, autism spectrum disorder and unipolar depression (Downes et al., 1989; Freedman and Oscar-Berman, 1989; Hughes et al., 1994; Elliott et al., 1995; Merriam et al., 1999). Cognitive flexibility in humans has been measured with various behavioral methods, such as the Wisconsin Card Sorting Test (WCST; Berg, 1948) and the Cambridge Neuropsychological Test Automated Battery Intra-Extra Dimensional Set Shifting task (Robbins, 2000). These assessments measure rule acquisition and rule switching ability by a set of compound visual stimuli, with two or more superimposed perceptual dimensions. Subjects are required to categorize presented figures dependent upon their properties along these dimensions. The rule of discrimination itself is not explained to the participants; instead, feedback on the accuracy is provided after each response. Several trials after the initial rule acquisition, the sorting rule changes unbeknownst to the test subjects and the new rule has to be discerned. Many of the patients with the aforementioned conditions can resolve the initial rule for sorting or recognize the rule change, but because of perseveration of pre-potent responses, they have difficulties with adjusting their behavior once the relevance of categories changes.

Historically the WCST has been used to detect prefrontal damage in humans, signifying a critical role for prefrontal circuits in behavioral flexibility (Berg, 1948; Milner, 1963; Nelson, 1976; Lombardi et al., 1999). Human and non-human primate studies showed that the prefrontal cortex (PFC) coordinate multiple cognitive processes essential for shifting between rule-based strategies, including attentional set formation, rule encoding, and feedback integration amongst others (Monchi et al., 2001; Wallis et al., 2001; Lie et al., 2006; Mansouri et al., 2006; Reverberi et al., 2012). Although the anatomical homology of primate and rodent PFC is controversial (Preuss, 1995), a wealth of studies indicate that the rodent PFC might provide some cognitive capacities similar to primates. It has been demonstrated that activity patterns of neuronal populations in the medial PFC (mPFC) relate to abstract rules, behavioral responses, and conflicts of strategies during rule switching (Durstewitz et al., 2010; Bissonette and Roesch, 2015). In line with these observations, pharmacological inactivation or lesion of mPFC in rodents did not influence learning stimulus-response associations but hindered the application of new strategies upon rule contingency change (Ragozzino et al., 1999a,b; Birrell and Brown, 2000; Bissonette et al., 2008). These behavioral and functional similarities across species also proved rats and mice, to be compelling animal models in pre-clinical research of cognitive flexibility.

Even though components of such psychometric tests employed in human studies have been modified and adapted for rodent research, most tasks available to date still suffer some methodological limitations when it comes to fine dissection of neuronal circuits underlying cognitive flexibility (Bissonette et al., 2013). The most widely used set-shifting tasks made use of instinctive behavior and mimicking naturally occurring attentional sets, such as navigation, digging, taste and odor with great success (Birrell and Brown, 2000; Lagler et al., 2016; Malagon-Vina et al., 2018). On the other hand, they are manual based, requiring the experimenter to continuously interact with the test subject and the low trial number occasionally restricts statistical measures. A recent development of operant-based tasks, which combined automation with previously mentioned naturally occurring stimuli (odor, tactile, and visual), resolved this obstacle and it proved to be an effective tool in large scale pharmacological and genetic assessments (Scheggia et al., 2014). However, some difficulties still remain due to the chamber and the freely behaving design, which complicates neuronal recordings, in addition to the small and variable number of trials. These persisting limitations reveal an evident need for an automated task that allows a more sophisticated dissection of neuronal networks underlying cognitive flexibility by providing reliable measures and attenuating the difficulties of integrating cutting-edge recording techniques.

MATERIALS AND METHODS

Experimental Subjects

All procedures were carried out under a license approved by the Austrian Ministry of Science and animals were tested in accordance with the regulations of the Medical University of Vienna. The test subjects were in-house bred adult male C57BL/6 mice (25–30 g), between 2 and 3 months of age. Before any experiments were carried out, the animals were housed two to eight per cage in a climate-controlled (temperature: 21°C ± 2°C, humidity: 50% ± 20%) animal facility, maintaining a 12-h, non-reversed light-dark cycle, starting at 7 am with *ad libitum* access to food and water. Procedures and tests were conducted during the light phase.

Surgical Procedures

For the head-plate implantation, mice were anesthetised with isoflurane (4% induction, 2% maintenance) and their skull was fixed to a stereotactic frame, while the body temperature was stabilized with a heating pad. The skull was exposed, cleaned and sterilized with alcohol (70%) and iodine tincture, respectively. After the future craniotomy sites were marked [Bregma anterior-posterior (AP) 1.7 mm, medial-lateral (ML) ± 0.5 mm], the exposed skull was applied a coating of super glue to prevent bone infections. The stability of the head-plate was ensured with screws tightened into the nasal and the parietal bones, covered with acrylic cement (Refobacin®, Biomet). The exposed part of the skull was covered with silicon (Kwik-Sil, World Precision Instruments) to further attenuate the possibility of bone infection. Metamizol (Novalgin) was used as post-surgery analgesic. Following surgery, mice were housed individually. In addition, after the animals learned to perform the rule switch (conditions mentioned later), they underwent a craniotomy procedure in order to be able to collect electrophysiological data by inserting acute silicone

probes into the mPFC. These data were not considered for the purpose of the current study and will be employed in another publication.

Behavioral Training

After animals fully recovered from the implantation surgery, *ad libitum* water was taken away to start the water restriction. Mice were closely monitored to get ∼1 ml water each day to avoid losing more than 15% of their body weight. After 2–3 days of habituation, mice were introduced to a PhenoSys virtual reality (VR) system (**Figure 1A**). The head-plate was tightened to the head-fixation arm and the orientation of the reward tube was set before each session, such that the water droplets touch the mouth of mouse upon delivery. The animals were given ∼5 μl water every 7 s until satiety. After the animals learned to accept water droplets from the water delivery tube (2–3 days), they started the first level of the training process. In this stage, they learnt to interact with the VR through the JetBall and to discriminate figures upon their size. To achieve this, animals were trained twice daily for an hour (∼600 trials per training session) with 7 h of difference, to choose the bigger from the presented figures by rolling the ball to the corresponding side. At early stages of training, if an incorrect choice was made, the animal had to re-do the same trial. The "insist" on correcting errors helped the mice learning the paradigm, as well as it prevented unwanted satiety before any progress was achieved. On the other hand, mice had to be closely monitored not to use "insist" as a strategy (in case of error, choosing the other side) instead of learning the rules to acquire reward. These strategies were diminished with behavioral shaping (taking "insist" out partially or fully). Once the performance reached a 90% success rate over 40 trials (in about 1 week), the training schedule was changed to one training session per day in the afternoon, which lasted 30–50 min, depending on the performance of the animal, corresponding to approximately 300–500 trials. Mice were then similarly trained to discriminate figures upon their face pattern (i.e., to form the "Pattern" set). After they achieved the same criterion of performance choosing the patterned figure as well (approximately 1 week), they had to follow the "Big" rule on the next training session, again until their performance reached 90% over a 40-trial period. Alternating "Pattern" and "Big" rules in different training sessions continued until the animals could execute both rules with high proficiency in different sessions, on two consecutive days (∼8 weeks of training). Finally, the animals were introduced to the rule change within a session (i.e., rule-switch sessions). The rule-switch sessions were preceded by two training sessions on the initial rule, meaning, that the first rule of the rule-switch session was the same as the rule of the last two training sessions. The criterion of the successful rule acquisition was to achieve 13 correct trials in a 15-trial window (87% correct). The automatic rule switch was triggered when mice gave altogether 50 correct responses, where the last 14 were consecutively correct. As our behavioral paradigm was developed primarily as a tool to measure neuronal activity and the effects of neuronal manipulations on the behavior, the criterion for triggering the rule switch was designed to provide an extended period of good performance. Therefore, baseline data could be acquired in various conditions before the rule switch, which facilitates the detection of significant changes post-criterion.

Rule-Switching Paradigm and Behavioral Analysis

During a single trial of the task, first, the VR turns on and the animal is presented with the cues (**Figure 1B**). During the cue presentation, the VR is locked for 2 s, meaning the movement of the mouse is not registered by the system. Hence, animals have sufficient time for decision making, and it also avoids unintentional choices by steering inaccuracy. Once the VR unlocks, the animal is allowed to carry out decisions by steering the ball to either side. The ball movement drives the figure of the corresponding side towards the middle of the screen. When the VR movement reaches 30° on either side, it locks onto the chosen figure. At the same time, the animal is given feedback by the delivery or the lack of a water droplet, dependent upon whether it was a correct or incorrect response, respectively. After 2 s the VR turns off for another second, indicating the beginning of a new trial. Although the system with the current settings is not well suited to pinpoint the exact start of the decision execution, this time period is an adequate temporal measure of response. Also, it is worth noting that the length of this episode is determined completely by the response time of the animal, ranging from 100 ms to 3 s. Hence, trials with decision execution times greater than 3 s were considered grooming periods and were excluded from the analysis.

After the rule switch, trials can be divided into two main types by the presented cues (**Figure 1C**). In non-conflicting trials, giving a correct response following either of the rules results in a correct response (e.g., left: big patterned circle vs. right: small plain circle). On the other hand, in conflicting trials, the two rules oppose each other, thus a correct response according to the previously reinforced rule results in an incorrect response choice (e.g., left: small patterned vs. right: big plain). Trial contingency was programmed to have a 60% bias towards conflicting trials to help acquisition of the new rule and to achieve more powerful analysis. Errors after the rule switch were categorized as perseverative and regressive types in conflicting trials, while nonsense types during non-conflicting trials. Perseverative errors were choices, where animals pursue the subsequent rule following a negative feedback but prior to the first correct response. This indicates the persistent use of the initial response set, despite the evidence of the relevant category change. Errors were marked regressive after the first correct conflicting trial, as animals "regressed" to the no longer reinforced rule. Thus, regressive errors demonstrate the unsuccessful maintenance of the new cognitive rule, notwithstanding the positive feedback of correct trials. Finally, nonsense errors were responses which following neither of the rules resulted in reward, hence they were never-reinforced (small plain circle). Behavioral data analysis was performed using standard functions and custom-made scripts in MATLAB (MathWorks).

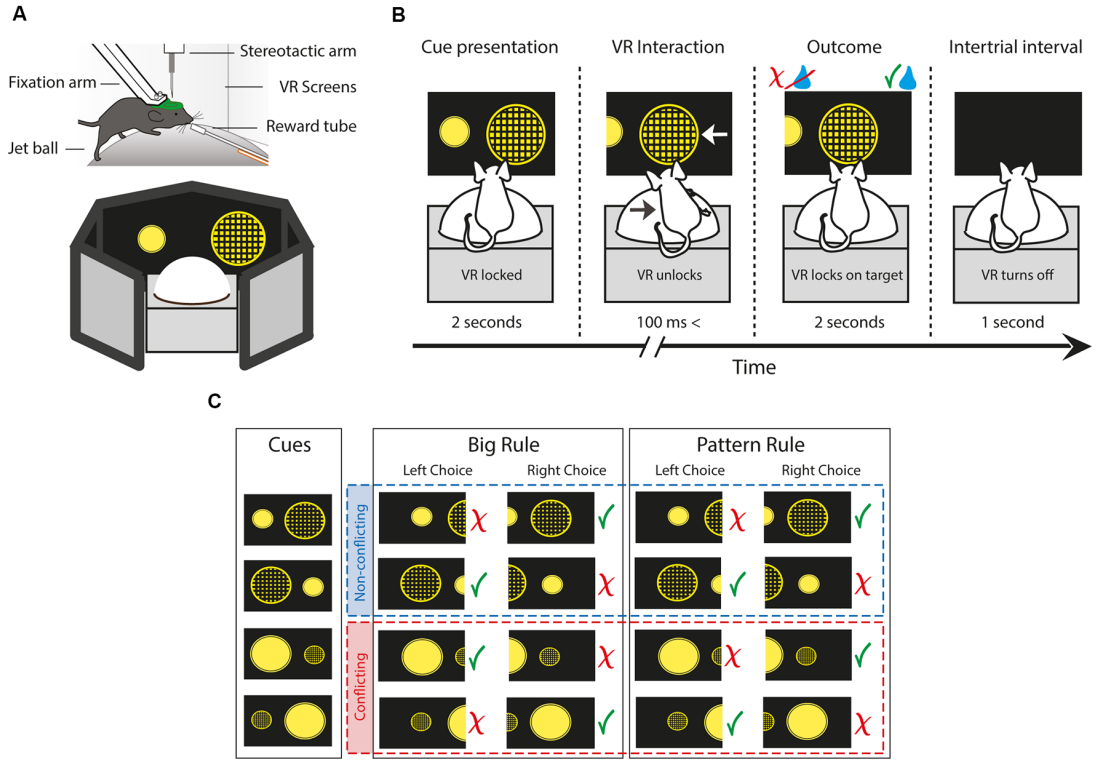


FIGURE 1 | The behavioral setup and paradigm of the two choice rule-switching task. **(A)** Schematic illustrating a head-fixed mouse running on a styrene ball supported by constant air flow. The virtual environment is presented on a 270° surround screen. **(B)** Each trial is divided into four parts. First, the animal is presented two figures. During the cue presentation, the movement of the animal is not conveyed to the system. When the virtual reality (VR) unlocks, the animal is allowed to carry out decisions by steering the ball to the side of the figure of choice, which moves towards the middle of the screen. The animal is given feedback by the delivery or the lack of a water reward. The beginning of a new trial is indicated by the blackout of the screen. **(C)** Diagram showing all cue variations, trial types and possible outcomes depending on the rule and choice. Green check marks indicate correct, red ex marks indicate incorrect responses. Red and blue dash-lined boxes group the non-conflicting and conflicting trials, respectively. The paradigm uses two perceptual dimensions: size and pattern. The animal has to turn the ball towards the side of the figure which is either bigger or has a pattern on it, contingent on the rule.

Optogenetic Procedures

Mice were anesthetized in the aforementioned way for the virus injection and optic fiber implantation. The AAV2/1-mDlx-channelrhodopsin (ChR2), an adeno-associated virus vector was bilaterally injected, that drives ChR2 expression through a mDlx enhancer, that restricts the expression of reporter genes to GABAergic cells. Specifics of the viral strategy for targeting and manipulating GABAergic interneurons were earlier described in detail (Dimidschstein et al., 2016). 0.5 µl virus was injected with the help of a pulled glass pipette into the prelimbic/infralimbic (PL/IL) area of the mPFC [Bregma AP 1.7 mm, ML, ± 0.3 mm, dorsal-ventral (DV) 1.5 mm] using a microsyringe pump. Two pieces of optic fiber (Ø200 µm, 0.22 NA, Thorlabs) were implanted transcranially above the PL area (1.7 mm AP, ± 0.4 mm ML, 1 mm DV) for bilateral stimulation. The position was secured by embedding the optic fibers in acrylic cement, firmly fixed to the head-plate. Behavioral experiments began 2–3 weeks after the virus injection.

After mice recovered from the optic fiber implantation surgery, they were trained for the rule-switching task the aforementioned way for 2–3 weeks, until the ChR2 protein was expressed in the target inhibitory cells. Experiments were scheduled in such a manner as two control and two optogenetic rule-switch sessions of each type ("Pattern to Big" and "Big to Pattern") would follow each other. Mice were connected to the laser through a fiber optic cable. The head-plate and the optic fiber implant were covered with an opaque head-piece to avoid the laser light to interfere with the vision of the animal, in both testing and control conditions for comparability reasons. During initial optogenetic experiments, blue light (473 nm) was shined with a stimulation protocol (7 ms ON, 3 ms OFF) yielding an illumination intensity of 10–15 mW measured at the tip of the implanted fiber (n = 2). Subsequent optimization of the stimulation protocol (5 ms ON, 15 ms OFF) resulted in 0.1–1 mW power output (n = 2), which sufficiently silenced the mPFC as well. The laser was driven by digital computer signals (TTL pulses) of the PhenoSys system controlled by a custom-written MATLAB script on the controlling computer. The laser was shined in every rewarded trial during reward consumption and the inter-trial interval.

Histology

After all behavioral and optogenetic experiments were finished, animals were anesthetized with urethane (3 g/kg) and intra-

cardially perfused with saline followed by a fixative solution (4% paraformaldehyde, 0.05% glutaraldehyde, 15% saturated picric acid in 0.1 M phosphate buffer, pH ∼7.4). The extracted brains were sectioned (coronal) with a vibratome (Leica VT 1000S, 70 μm thickness). Incubations and standard procedures used were described previously (Somogyi et al., 2004). ChR2 expression in the GABAergic cells was tested with double immunofluorescent reactions on individual free-floating sections with antibodies against ChR2 (mouse monoclonal; PROGEN Biotechnik GmbH; dilution: 1:10,000; for antibody specificity see Kleinlogel et al., 2011) and vesicular GABA transporter (guinea pig polyclonal; Frontier Institute Co., Ltd.; dilution: 1:500; for antibody specificity see Miyazaki et al., 2003; Fukudome et al., 2004) as detected with secondary antibodies conjugated to Alexa Fluor® 488 or Cy®5 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and imaged with immunofluorescent confocal microscopy [ZeissLSM 780; 63× oil immersion objective (NA 1.4)]. Positions of the optic fibers were assessed using a transmitted light microscope. One subject had to be removed from the optogenetic study for reasons of implant disposition.

All original data from this study will be made available upon reasonable request.

RESULTS

Behavioral Performance

Mice (n = 7) were trained to discriminate two visual cues presented in a virtual environment and, by following rules, make decisions dependent upon different perceptual dimensions of size or pattern. After a brief cue presentation, the animals had to turn the ball to left or to the right, corresponding to the side of the chosen figure. For example, if the rule was "Pattern," the target side was where the patterned figure was positioned. Upon a correct choice, animals collected water reward as a positive feedback, while in case of an incorrect choice, the negative feedback was the lack of reward. After the animals reached an extended period of stable good performance (50 correct responses, with the last 14 consecutively correct), the rule change was triggered. Following the previous example, if the starting rule was "Pattern," the second rule was switched to "Big" and the animal had to turn the ball to the side where the bigger figure was positioned. In order to succeed afterward, animals had to recognize the rule change, disengage from the first rule, infer and apply the new strategy by attending to other attributes of the same visual cues. The paradigm of the task is described in the "Materials and Methods" section in detail.

All mice trained to perform the set-shifting task managed to successfully discriminate between the visual cues and reached the criteria for successful performance during both the first and the second rule (**Figure 2A**). Altogether, 145 rule-switch session (out of 169) were considered successful, where 76 were of "Big-to-Pattern" and 69 were of "Pattern-to-Big" type. The analysis of the behavioral performance revealed that significantly more trials were needed to reach the criterion for the second rule, than for the first rule (t-test, means 28.3 vs. 144.4, SEM 1.4 and 14.8, $p < 0.001$, $t = -17.8$, $df = 288$, Cohen's $d = -2.09$, effect size = −0.72). To examine the effect of rule-shift and the visual cues on trial number needed to reach the criterion, a two-way ANOVA was conducted (**Figure 2B**). The simple main effects analysis showed that while switching from the first rule to the second rule increased the number of trials needed to reach the criterion ($F_{(1,24)} = 185.78$; $p < 0.001$), the rules *per se* (whether it was big or pattern) did not influence it ($F_{(1,24)} = 0.19$; $p = 0.668$), neither did the two factors interact ($F_{(1,24)} = 0.13$, $p = 0.7167$). Comparing trial length in different conditions (**Figure 2C**) revealed a significant difference between non-conflicting and conflicting trials after the rule switch (t-test, means 0.394. vs. 0.489, respectively; SEM 0.022 and 0.027, respectively; $p = 0.007$; $t = 2.7292$, $df = 288$, Cohen's $d = -0.32$, effect size = −0.16). This indicates that on trials where the two rules were in conflict, the animals took more time carrying out decisions. Altogether, these data confirm that animals learned to discriminate the presented cues, followed the appropriate rule, and they had difficulty switching between them when the relevant category changed.

As errors during conflicting trials after the rule switch provide essential feedback for cognitive rule adjustment, we categorized set-shifting errors as perseverative and regressive. During perseverative responses, subjects fail to shift to a new response set despite the negative feedback, and they execute choices following the previous rule, which does not apply anymore. Errors become regressive after subjects make the first conflicting correct choice, indicating that the newly-reinforced response set is identified, but then they are unable to maintain it, and instead they revert back to choices in accordance with the initial rule. Analyzing these two types of errors (**Figure 2D**) concluded that most of the errors were of regressive-types (t-test, means 7.75 vs. 55.35, SEM 0.62 and 2.92, $p < 0.001$, $t = -15.9$, $df = 266$, Cohen's $d = -1.95$, effect size = −0.7), which implies that mice had difficulty suppressing responses to the initial set. Additionally, animals made very few "non-sense" errors (i.e., choices that were never reinforced; **Figure 2E**; t-test, means 59.22 vs. 3.87, SEM 2.67 and 0.44, $p < 0.001$, $t = 20.5$, $df = 266$, Cohen's $d = 2.5$, effect size = 0.78), which suggests that other rule possibilities were less likely to be explored and that the mice acquired a high ball-handling precision during the training, making very few mistakes as a result of steering inaccuracy.

Optogenetic Experiments

To determine the behavioral effect of silencing the mPFC during positive-feedback epochs, we implemented an optogenetic system (Passecker et al., 2019), which achieves locally restricted inhibition of principal neurons through activation of GABAergic interneurons. For these experiments the same animals (n = 4), which underwent the earlier described behavioral tests, were bilaterally injected with an AAV2/1-mDlx-ChR2 virus (Dimidschstein et al., 2016), to selectively express the light-sensitive ChR2 channel in GABAergic cells (**Figure 3A**). Optic fibers were implanted above the PL area of the mPFC. After the animals recovered and their behavioral performance returned to a pre-surgery level, the task was performed in alternating sessions, with and without optogenetic stimulation, granting the

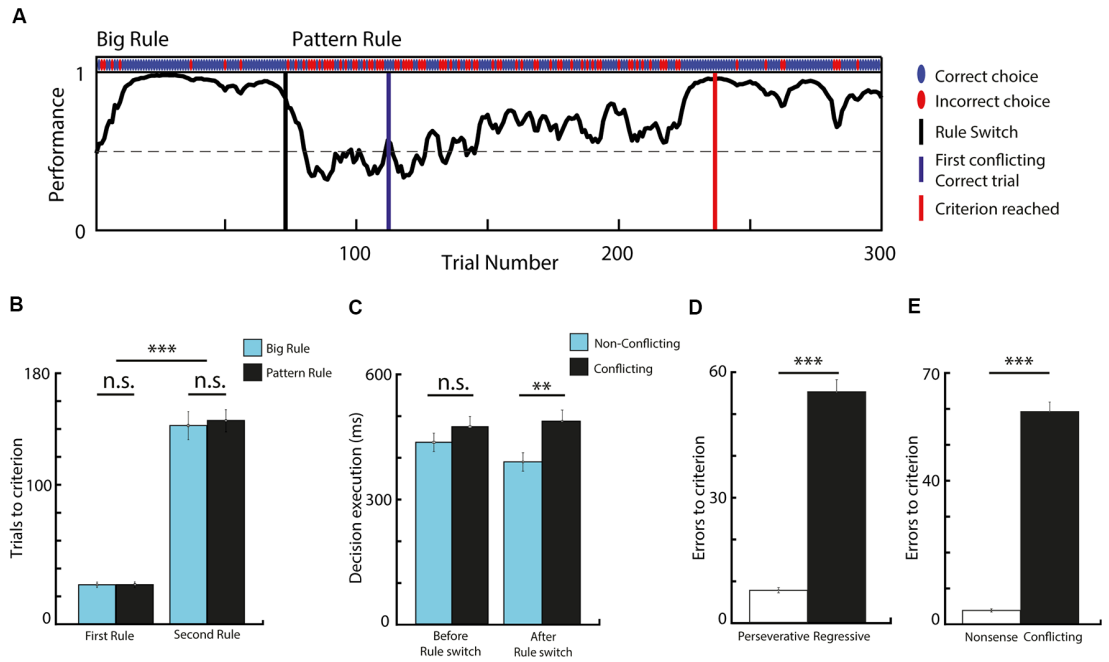


FIGURE 2 | Task performance and behavioral analysis. **(A)** Performance curve deduced from the binary data (correct vs. incorrect choices) *via* Markov-chain Monte–Carlo analysis of one behavioral session. **(B)** Comparing the number of trials needed to reach the criterion before and after rule switch, in respect to rule modality. The second rule required significantly more trials to reach criterion, while the rule-type had no effect. **(C)** Data showing decision execution time before and after rule switch, during conflicting and non-conflicting trials. Animals spent significantly more time making choices during conflicting trials compared to non-conflicting after the rule switch. **(D)** Bar graph comparing the number of errors before and after the first conflicting correct trial. Animals made significantly more regressive than perseverative errors. **(E)** Data showing that very small number of nonsense errors were made. $n = 7$ animals, $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. not significant; error bars show SEM.

advantage of comparing optogenetic and control experiments within the same animal. Inhibitory cells were activated by light application during reward delivery and the inter-trial interval in all rewarded trials, before and after rule switch (**Figure 3B**). As expected, performance on the initial rule was not affected by inhibition of the mPFC (**Figure 3C**). Mice took the same number of trials to reach the criterion, in both type of sessions (Wilcoxon rank sum, means 24.50 vs. 24.30, SEM 3.69 and 3.25, $p = 0.82$, $z = 0.23$). In contrast, reaching the criterion after a rule switch to the second rule took significantly more trials during light on, compared to control sessions (Wilcoxon rank sum, means 235.6 vs. 92.5, respectively; SEM 50.65 and 12.76, respectively; $p = 0.0111$, $z = 2.54$), which highlights an important role for the mPFC in reward integration during set-shifting, but not during single rule performance (**Figure 3D**).

To test whether optogenetic inactivation of mPFC had any effect on decision making and decision execution, we analyzed the lengths of VR interaction times of various trial conditions after the rule switch (**Figure 3E**). Similarly to the full dataset (**Figure 2C**), in control sessions, virus injected animals took more time to respond to the presented cues when the rules were in conflict (t-test, means 0.268 vs. 0.381, SEM 0.18 and 0.37, $p = 0.002$, Cohen's $d = -1.13$, effect size $= -0.49$), while this difference was not observed in light on sessions (t-test, means 0.283 vs. 0.326, SEM 0.41 and 0.48, $p = 0.123$, Cohen's $d = -0.28$, effect size $= -0.14$), suggesting that animals had difficulties suppressing impulsive responses when the normal activity of the mPFC is disturbed. However, decisive conclusions cannot be drawn as neither the conflicting (Wilcoxon rank sum, means 0.381 vs. 0.327, SEM 0.37 and 0.48, $p = 0.3734$, $z = -0.89$) nor the non-conflicting trial lengths (Wilcoxon rank sum, means 0.268 vs. 0.283, SEM 0.18 and 0.41, $p = 0.9737$, $z = -0.03$) differed in the two experimental settings when compared to each other. Lastly, assessing the number of perseverative and regressive errors (**Figure 3F**) showed that mice made markedly more regressive errors during optogenetic experiments compared to control sessions (Wilcoxon rank sum, means 78.30 vs. 32.25, respectively; SEM 18.28 and 6.00, respectively; $p = 0.038$, $z = -2.08$), while the number of perseverative errors did not differ in the two session types (Wilcoxon rank sum, means 6.10 vs. 6.92, respectively; SEM 1.87 and 1.77, respectively; $p = 0.8685$, $z = -0.17$). These results imply, that interfering with post-reward computations in the mPFC has no effect on the ability to alter cognitive rules and responses, though it hinders the maintenance of newly acquired response sets causing mice regressing more to no longer reinforced choices.

DISCUSSION

Technological developments of recent years have triggered an interest in implementing and updating mouse behavioral

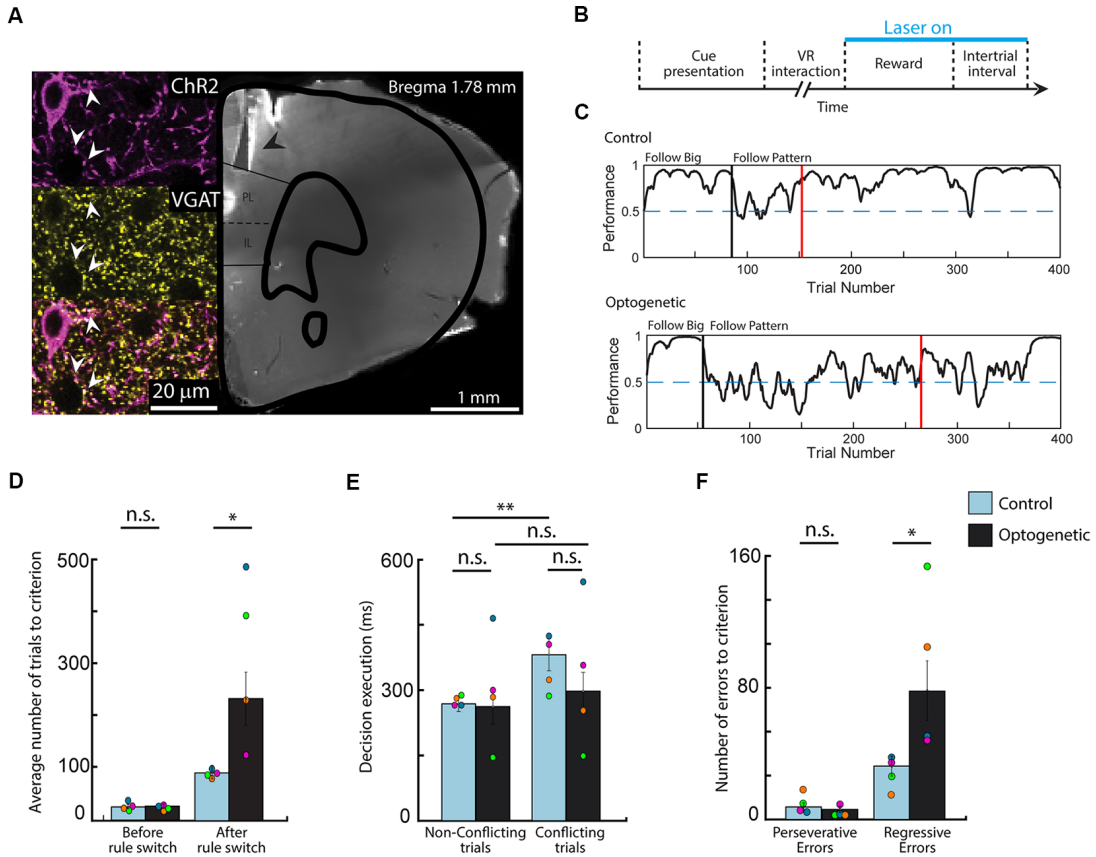


FIGURE 3 | Optogenetic silencing of the medial prefrontal cortex (mPFC) during positive feedback epochs induces symptoms of cognitive rigidity. **(A)** Photomicrograph and confocal scan of a brain slice indicating the location of the optic fibers (black arrow) and the immunolabeling of channelrhodopsin-2 (ChR2) expression in GABAergic cells in the prelimbic/infralimbic (PL/IL) area (white arrow). **(B)** Diagram of the optogenetic stimulation protocol. Light was shined in every rewarded trial, before and after rule switch (7 ms ON, 3 ms OFF; 10–15 mW; $n = 2$ and 5 ms ON, 15 ms OFF; 0.1–1 mW; $n = 2$). **(C)** Behavioral curves showing performance difference in rule switching during one control and one optogenetic session from the same animal. Performance was assessed using the binary data of correct and incorrect choices *via* Markov-chain Monte–Carlo analysis. Black vertical lines indicate the automated rule switch, while the red vertical lines mark the beginning of the 13 out of 15 correct trials. **(D)** Number of trials needed to reach the criterion on the first and on the second rule. **(E)** Comparing decision execution times after the rule switch shows that silencing the mPFC diminished the difference between conflicting and non-conflicting trial length in optogenetic experiments. **(F)** Data showing increased number of regressive errors but not perseverative errors during optogenetic sessions. $n = 4$ animals, $^{*}p < 0.05$; $^{**}p < 0.01$; n.s. not significant; error bars show SEM; Different colors mark the individual animals' averages.

tasks to more reliably measure cognitive functions (Cho et al., 2015; Havenith et al., 2018; Pinto et al., 2018). Their genetic flexibility, commercial availability, and their tolerance of head-fixation appointed them as an ideal animal model for experimental neuroscience (Trancikova et al., 2011; White, 2016; Stowers et al., 2017; Chen et al., 2019). In this study, we introduce a visual two-choice rule-switching task developed for head-fixed mice, which opens up new possibilities in preclinical research of cognitive flexibility. In this paradigm, mice learned to execute choices with high precision through an air-supported ball connected to a VR system to follow abstract rules contingent upon the size and the pattern of the presented cues. The trial length was designed to be short, which resulted in a large number of trials, allowing robust statistical measures and decreased variance. Our data shows that mice learned to differentiate the visual cues and they were able to switch between the appropriate strategies without having bias towards any of the task sets. Using bivalent visual cues effectively increased the number of trials needed to establish a good performance after the rule switch, which yielded several thousands of trials cumulatively. The performance decrement after rule switch also indicates that mice faced difficulty reconfiguring task-sets. This switch cost was also indicated by the increased decision making times on trials where rules were in conflict. These phenomena are well described in human rule switching (Wylie and Allport, 2000; Monsell, 2003; Schneider and Logan, 2007) and are in agreement with data from previous studies in mice showing that response time increases when difficult choices are made (Abraham et al., 2004; Young et al., 2010).

We also explored the behavioral effect of disrupting reward integration in the mPFC on switching between tasks by optogenetically silencing the area during post-reward epochs. Our optogenetic experiments had some limitations regarding the

low subject number, which introduced higher variance, and it is missing the viral control to test whether the laser light, *per se*, had any effect on the behavior. However, our results show no significant difference between the optogenetic and the control sessions during the initial rule acquisition, while the performance in sessions with optogenetic silencing of mPFC is clearly poorer after the rule switch. This finding suggests that the light itself did not influence the behavior, as it aligns well with previous research showing that disruption of the mPFC does not affect the initial rule performance in a task switch paradigm, it only hinders the acquisition of the second rule (Hampshire and Owen, 2006; Bissonette et al., 2008; Keeler and Robbins, 2011).

The mPFC is theorized to govern multiple cognitive processes, which work together to achieve a successful set shifting after rule change. These include initiation of new choices, inhibition of subsequent, ineffective responses, and promoting newly acquired, effective strategies. These processes can be monitored by delineating errors as perseverative and regressive types (Ragozzino, 2007; Gastambide et al., 2012). Human, non-human primate, and rodent studies suggest that mPFC is involved in the initial suppression of established response sets after rule contingencies change, thus promoting the selection of new choices, marked by an increase in perseverative errors (Dias et al., 1997; Ragozzino et al., 2003; Chudasama and Robbins, 2006; Ragozzino, 2007). In contrast, an increase in regressive errors indicates a failure to maintain the newly acquired response sets, which is dependent on the basal ganglia (Ragozzino et al., 2002; Ragozzino and Choi, 2004; Floresco et al., 2006; Palencia and Ragozzino, 2006). Thus, the cooperation of these functionally different brain areas facilitates cognitive flexibility by choosing an alternative response and promoting it over other possibilities. Contrary to earlier findings, however, we found that silencing the mPFC increased the number of regressive errors, instead of the perseverative errors. A possible explanation for these results may be the way perseverative errors were defined in the previous rodent studies. In these experiments, a window of multiple trials was applied and perseveration was defined when the majority of the trials in that block were incorrect. This definition of perseverative errors includes both perseverative and regressive errors, as defined here, and it does not quite capture the initial shift in response set, when the animal disengages from the primary rule for the first time. Furthermore, Oualian and Gisquet-Verrier who observed some perseverative behavior in their lesion studies as well, argue that in most of the aforementioned experiments animals chose to maintain the initial strategy because it still leads to reward for half of the trials (Delatour and Gisquet-Verrier, 1999, 2000; Oualian and Gisquet-Verrier, 2010). Following the previous rule after the rule change in our paradigm is a very inefficient strategy because of the small reward size, the length of the experiment, and the probability of encountering non-conflicting trials, which is only 40% after the rule change. Also, they hypothesized that the behavioral rigidity caused by the dysfunctional mPFC is due to the attenuated ability to resolve internal conflicts generated by the opposing previously learned strategy and the new rule. Therefore, our findings imply a more complex cooperation between the mPFC and the dorsomedial striatum in behavioral flexibility, suggesting that processing only negative feedback signals in the mPFC is enough to initiate a new response after rule contingencies change, but to successfully maintain it over the subsequent rule, positive feedback signals of the striatum have to be integrated in the mPFC.

As cognitive rigidity is prevalent in a large number of psychiatric disorders, continuous development of preclinical research tools is essential in order to dissect and understand the complex mechanisms, which bring about flexible behavior. The system we developed is well suited for neuronal recordings, as head-fixation augments stability and it has further advantages combined with other movement-sensitive techniques such as juxta-cellular recording and labeling (Lapray et al., 2012; Köszeghy et al., 2018). It is completely automatic, including sampling and synchronization of behavioral and physiological data, which immensely simplifies data collection and data analysis. Its parameters are flexibly programmable to test other components of cognitive flexibility, such as attentional set-shifting and reversal learning. As the reliable assessment of set-shifting using visual based tasks remains a challenge in mouse research (Floresco et al., 2008), we would like to test whether our system is capable of measuring visual attentional set shifts as a next step. By introducing a novel shape of different sizes and patterns, a total change paradigm can be established (Slamecka, 1968), creating a task which is analogous to the ones used in clinical practice (Sahakian and Owen, 1992). Finally, as one of the goals of the development was to create a task for mice, implementing genetically modified mouse models of neurocognitive diseases which produce cognitive rigidity as a symptom, could give us a more detailed insight into the pathophysiology of these conditions. Thus, this adaptability of the apparatus provides a multifaceted approach to tackle behavioral and neurobiological questions of cognitive flexibility that were rather troublesome with previous tasks.

In conclusion, in this study, we demonstrate a cognitive flexibility task that integrates recent technological advancements of neuroscience to overcome limitations of currently used tasks. The head-fixed design provides stability for various neuronal recording techniques while using mouse models widens the horizon of preclinical research of psychiatric diseases. The abstract task sets prevent mice to develop biases towards choices and also increase the difficulty of solving the task, which combined with short trial length not only increases the available time interval for measurements but also boosts the statistical power. Our initial finding suggests that selectively silencing the mPFC during correct trials concurrently with reward consumption does not affect the initial rule performance, but it induces signs of cognitive rigidity when new rule strategies are implemented. Furthermore, our data indicate that interfering with post reward computations in the mPFC but leaving the negative feedback periods intact, hinders the maintenance of the new response set after rule switching, but it does not disturb the ability to initially disengage from the first task, suggesting the importance of mPFC for cognitive flexibility.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Austrian Ministry of Science. The protocol was approved by the Austrian Ministry of Science.

AUTHOR CONTRIBUTIONS

SB, BL and TK contributed to the conception and design of the study. SB carried out the experiments and performed the analysis of the data. SB and TK wrote the manuscript.

FUNDING

This work was supported by grants W1205 (CCHD) and P27610 of the Austrian Science Fund.

ACKNOWLEDGMENTS

We thank Erzsébet Borok and Romana Hauer for the excellent technical assistance. Thomas Forro for the help with the VR. Hugo Malagon-Vina for the assistance with the data analysis. Michael Lagler and Johannes Passecker for their insights on the project.

REFERENCES

Abraham, N. M., Spors, H., Carleton, A., Margrie, T. W., Kuner, T., and Schaefer, A. T. (2004). Maintaining accuracy at the expense of speed: stimulus similarity defines odor discrimination time in mice. *Neuron* 44, 865–876. doi: 10.1016/j.neuron.2004.11.017

Berg, E. A. (1948). A simple objective technique for measuring flexibility in thinking. *J. Gen. Psychol.* 39, 15–22. doi: 10.1080/00221309.1948.9918159

Birrell, J. M., and Brown, V. J. (2000). Medial frontal cortex mediates perceptual attentional set shifting in the rat. *J. Neurosci.* 20, 4320–4324. doi: 10.1523/jneurosci.20-11-04320.2000

Bissonette, G. B., Martins, G. J., Franz, T. M., Harper, E. S., Schoenbaum, G., and Powell, E. M. (2008). Double dissociation of the effects of medial and orbital prefrontal cortical lesions on attentional and affective shifts in mice. *J. Neurosci.* 28, 11124–11130. doi: 10.1523/jneurosci.2820-08.2008

Bissonette, G. B., Powell, E. M., and Roesch, M. R. (2013). Neural structures underlying set-shifting: roles of medial prefrontal cortex and anterior cingulate cortex. *Behav. Brain Res.* 250, 91–101. doi: 10.1016/j.bbr.2013.04.037

Bissonette, G. B., and Roesch, M. R. (2015). Neural correlates of rules and conflict in medial prefrontal cortex during decision and feedback epochs. *Front. Behav. Neurosci.* 9:266. doi: 10.3389/fnbeh.2015.00266

Chen, I.-W., Ronzitti, E., Lee, B. R., Daigle, T. L., Dalkara, D., Zeng, H., et al. (2019). *In vivo* sub-millisecond two-photon optogenetics with temporally focused patterned light. *J. Neurosci.* 39, 3484–3497. doi: 10.1523/jneurosci.1785-18.2018

Cho, K. K. A., Hoch, R., Lee, A. T., Patel, T., Rubenstein, J. L. R., and Sohal, V. S. (2015). γ rhythms link prefrontal interneuron dysfunction with cognitive inflexibility in Dlx5/6(+/−) mice. *Neuron* 85, 1332–1343. doi: 10.1016/j.neuron.2015.02.019

Chudasama, Y., and Robbins, T. W. (2006). Functions of frontostriatal systems in cognition: comparative neuropsychopharmacological studies in rats, monkeys and humans. *Biol. Psychol.* 73, 19–38. doi: 10.1016/j.biopsycho.2006.01.005

Delatour, B., and Gisquet-Verrier, P. (1999). Lesions of the prelimbic-infralimbic cortices in rats do not disrupt response selection processes but induce delay-dependent deficits: evidence for a role in working memory? *Behav. Neurosci.* 113, 941–955. doi: 10.1037/0735-7044.113.5.941

Delatour, B., and Gisquet-Verrier, P. (2000). Functional role of rat prelimbic-infralimbic cortices in spatial memory: evidence for their involvement in attention and behavioural flexibility. *Behav. Brain Res.* 109, 113–128. doi: 10.1016/s0166-4328(99)00168-0

Dias, R., Robbins, T. W., and Roberts, A. C. (1997). Dissociable forms of inhibitory control within prefrontal cortex with an analog of the Wisconsin Card Sort Test: restriction to novel situations and independence from "on-line" processing. *J. Neurosci.* 17, 9285–9297. doi: 10.1523/jneurosci.17-23-09285.1997

Dimidschstein, J., Chen, Q., Tremblay, R., Rogers, S. L., Saldi, G. A., Guo, L., et al. (2016). A viral strategy for targeting and manipulating interneurons across vertebrate species. *Nat. Neurosci.* 19, 1743–1749. doi: 10.1038/nn.4430

Downes, J. J., Roberts, A. C., Sahakian, B. J., Evenden, J. L., Morris, R. G., and Robbins, T. W. (1989). Impaired extra-dimensional shift performance in medicated and unmedicated Parkinson's disease: evidence for a specific attentional dysfunction. *Neuropsychologia* 27, 1329–1343. doi: 10.1016/0028-3932(89)90128-0

Durstewitz, D., Vittoz, N. M., Floresco, S. B., and Seamans, J. K. (2010). Abrupt transitions between prefrontal neural ensemble states accompany behavioral transitions during rule learning. *Neuron* 66, 438–448. doi: 10.1016/j.neuron.2010.03.029

Elliott, R., McKenna, P. J., Robbins, T. W., and Sahakian, B. J. (1995). Neuropsychological evidence for frontostriatal dysfunction in schizophrenia. *Psychol. Med.* 25, 619–630. doi: 10.1017/s0033291700033523

Floresco, S. B., Block, A. E., and Tse, M. T. L. (2008). Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure. *Behav. Brain Res.* 190, 85–96. doi: 10.1016/j.bbr.2008.02.008

Floresco, S. B., Magyar, O., Ghods-Sharifi, S., Vexelman, C., and Tse, M. T. L. (2006). Multiple dopamine receptor subtypes in the medial prefrontal cortex of the rat regulate set-shifting. *Neuropsychopharmacology* 31, 297–309. doi: 10.1038/sj.npp.1300825

Freedman, M., and Oscar-Berman, M. (1989). Spatial and visual learning deficits in Alzheimer's and Parkinson's disease. *Brain Cogn.* 11, 114–126. doi: 10.1016/0278-2626(89)90009-2

Fukudome, Y., Ohno-Shosaku, T., Matsui, M., Omori, Y., Fukaya, M., Tsubokawa, H., et al. (2004). Two distinct classes of muscarinic action on hippocampal inhibitory synapses: M2-mediated direct suppression and M1/M 3-mediated indirect suppression through endocannabinoid signalling. *Eur. J. Neurosci.* 19, 2682–2692. doi: 10.1111/j.0953-816x.2004.03384.x

Gastambide, F., Cotel, M. C., Gilmour, G., O'Neill, M. J., Robbins, T. W., and Tricklebank, M. D. (2012). Selective remediation of reversal learning deficits in the neurodevelopmental MAM model of schizophrenia by a novel mGlu5 positive allosteric modulator. *Neuropsychopharmacology* 37, 1057–1066. doi: 10.1038/npp.2011.298

Hampshire, A., and Owen, A. M. (2006). Fractionating attentional control using event-related fMRI. *Cereb. Cortex* 16, 1679–1689. doi: 10.1093/cercor/bhj116

Havenith, M. N., Zijderveld, P. M., van Heukelum, S., Abghari, S., Glennon, J. C., and Tiesinga, P. (2018). The virtual-environment-foraging task enables rapid training and single-trial metrics of attention in head-fixed mice. *Sci. Rep.* 8:17371. doi: 10.1038/s41598-018-34966-8

Hughes, C., Russell, J., and Robbins, T. W. (1994). Evidence for executive dysfunction in autism. *Neuropsychologia* 32, 477–492. doi: 10.1016/0028-3932(94)90092-2

Keeler, J. F., and Robbins, T. W. (2011). Translating cognition from animals to humans. *Biochem. Pharmacol.* 81, 1356–1366. doi: 10.1016/j.bcp.2010.12.028

Kleinlogel, S., Terpitz, U., Legrum, B., Gökbuget, D., Boyden, E. S., Bamann, C., et al. (2011). A gene-fusion strategy for stoichiometric and co-localized expression of light-gated membrane proteins. *Nat. Methods* 8, 1083–1091. doi: 10.1038/nmeth.1766

Köszeghy, Á., Lasztóczi, B., Forro, T., and Klausberger, T. (2018). Spike-timing of orbitofrontal neurons is synchronized with breathing. *Front. Cell. Neurosci.* 12:105. doi: 10.3389/fncel.2018.00105

Lagler, M., Ozdemir, A. T., Lagoun, S., Malagon-Vina, H., Borhegyi, Z., Hauer, R., et al. (2016). Divisions of identified parvalbumin-expressing basket cells during working memory-guided decision making. *Neuron* 91, 1390–1401. doi: 10.1016/j.neuron.2016.08.010

Lapray, D., Lasztoczi, B., Lagler, M., Viney, T. J., Katona, L., Valenti, O., et al. (2012). Behavior-dependent specialization of identified hippocampal interneurons. *Nat. Neurosci.* 15, 1265–1271. doi: 10.1038/nn.3176

Lie, C. H., Specht, K., Marshall, J. C., and Fink, G. R. (2006). Using fMRI to decompose the neural processes underlying the wisconsin card sorting test. *Neuroimage* 30, 1038–1049. doi: 10.1016/j.neuroimage.2005.10.031

Lombardi, W. J., Andreason, P. J., Sirocco, K. Y., Rio, D. E., Gross, R. E., Umhau, J. C., et al. (1999). Wisconsin card sorting test performance following head injury: dorsolateral fronto-striatal circuit activity predicts perseveration. *J. Clin. Exp. Neuropsychol.* 21, 2–16. doi: 10.1076/jcen.21.1.2.940

Malagon-Vina, H., Ciocchi, S., Passecker, J., Dorffner, G., and Klausberger, T. (2018). Fluid network dynamics in the prefrontal cortex during multiple strategy switching. *Nat. Commun.* 9:309. doi: 10.1038/s41467-017-02764-x

Mansouri, F. A., Matsumoto, K., and Tanaka, K. (2006). Prefrontal cell activities related to monkeys' success and failure in adapting to rule changes in a wisconsin card sorting test analog. *J. Neurosci.* 26, 2745–2756. doi: 10.1523/jneurosci.5238-05.2006

Merriam, E. P., Thase, M. E., Haas, G. L., Keshavan, M. S., and Sweeney, J. A. (1999). Prefrontal cortical dysfunction in depression determined by Wisconsin Card Sorting Test performance. *Am. J. Psychiatry* 156, 780–782.

Milner, B. (1963). Effects of different brain lesions on card sorting: the role of the frontal lobes. *Arch. Neurol.* 9, 90–100. doi: 10.1001/archneur.1963.00460070100010

Miyazaki, T., Fukaya, M., Shimizu, H., and Watanabe, M. (2003). Subtype switching of vesicular glutamate transporters at parallel fibre-Purkinje cell synapses in developing mouse cerebellum. *Eur. J. Neurosci.* 17, 2563–2572. doi: 10.1046/j.1460-9568.2003.02698.x

Monchi, O., Petrides, M., Petre, V., Worsley, K., and Dagher, A. (2001). Wisconsin Card Sorting revisited: distinct neural circuits participating in different stages of the task identified by event-related functional magnetic resonance imaging. *J. Neurosci.* 21, 7733–7741. doi: 10.1523/JNEUROSCI.21-19-07733.2001

Monsell, S. (2003). Task switching. *Trends Cogn. Sci.* 7, 134–140. doi: 10.1016/S1364-6613(03)00028-7

Nelson, H. E. (1976). A modified card sorting test sensitive to frontal lobe defects. *Cortex* 12, 313–324. doi: 10.1016/s0010-9452(76)80035-4

Oualian, C., and Gisquet-Verrier, P. (2010). The differential involvement of the prelimbic and infralimbic cortices in response conflict affects behavioral flexibility in rats trained in a new automated strategy-switching task. *Learn. Mem.* 17, 654–668. doi: 10.1101/lm.1858010

Palencia, C. A., and Ragozzino, M. E. (2006). The effect of N-methyl-d-aspartate receptor blockade on acetylcholine efflux in the dorsomedial striatum during response reversal learning. *Neuroscience* 143, 671–678. doi: 10.1016/j.neuroscience.2006.08.024

Passecker, J., Mikus, N., Malagon-Vina, H., Anner, P., Dimidschstein, J., Fishell, G., et al. (2019). Activity of prefrontal neurons predict future choices during gambling. *Neuron* 101, 152.e7–164.e7. doi: 10.1016/j.neuron.2018.10.050

Pinto, L., Koay, S. A., Engelhard, B., Yoon, A. M., Deverett, B., Thiberge, S. Y., et al. (2018). An accumulation-of-evidence task using visual pulses for mice navigating in virtual reality. *Front. Behav. Neurosci.* 12:36. doi: 10.3389/fnbeh.2018.00036

Preuss, T. M. (1995). Do rats have prefrontal cortex? The Rose-Woolsey-Akert program reconsidered. *J. Cogn. Neurosci.* 7, 1–24. doi: 10.1162/jocn.1995.7.1.1

Ragozzino, M. E. (2007). The contribution of the medial prefrontal cortex, orbitofrontal cortex and dorsomedial striatum to behavioral flexibility. *Ann. N Y Acad. Sci.* 1121, 355–375. doi: 10.1196/annals.1401.013

Ragozzino, M. E., and Choi, D. (2004). Dynamic changes in acetylcholine output in the medial striatum during place reversal learning. *Learn. Mem.* 11, 70–77. doi: 10.1101/lm.65404

Ragozzino, M. E., Detrick, S., and Kesner, R. P. (1999a). Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. *J. Neurosci.* 19, 4585–4594. doi: 10.1523/JNEUROSCI.19-11-04585.1999

Ragozzino, M. E., Wilcox, C., Raso, M., and Kesner, R. P. (1999b). Involvement of rodent prefrontal cortex subregions in strategy switching. *Behav. Neurosci.* 113, 32–41. doi: 10.1037/0735-7044.113.1.32

Ragozzino, M. E., Kim, J., Hassert, D., Minniti, N., and Kiang, C. (2003). The contribution of the rat prelimbic-infralimbic areas to different forms of task switching. *Behav. Neurosci.* 117, 1054–1065. doi: 10.1037/0735-7044.117.5.1054

Ragozzino, M. E., Ragozzino, K. E., Mizumori, S. J. Y., and Kesner, R. P. (2002). Role of the dorsomedial striatum in behavioral flexibility for response and visual cue discrimination learning. *Behav. Neurosci.* 116, 105–115. doi: 10.1037//0735-7044.116.1.105

Reverberi, C., Görgen, K., and Haynes, J.-D. (2012). Compositionality of rule representations in human prefrontal cortex. *Cereb. Cortex* 22, 1237–1246. doi: 10.1093/cercor/bhr200

Robbins, T. W. (2000). Chemical neuromodulation of frontal-executive functions in humans and other animals. *Exp. Brain Res.* 133, 130–138. doi: 10.1007/978-3-642-59794-7_14

Sahakian, B. J., and Owen, A. M. (1992). Computerized assessment in neuropsychiatry using CANTAB: discussion paper. *J. R. Soc. Med.* 85, 399–402.

Scheggia, D., Bebensee, A., Weinberger, D. R., and Papaleo, F. (2014). The ultimate intra-/extra-dimensional attentional set-shifting task for mice. *Biol. Psychiatry* 75, 660–670. doi: 10.1016/j.biopsych.2013.05.021

Schneider, D. W., and Logan, G. D. (2007). Defining task-set reconfiguration: the case of reference point switching. *Psychon. Bull. Rev.* 14, 118–125. doi: 10.3758/bf03194038

Slamecka, N. J. (1968). A methodological analysis of shift paradigms in human discrimination learning. *Psychol. Bull.* 69, 423–438. doi: 10.1037/h0025762

Somogyi, J., Baude, A., Omori, Y., Shimizu, H., El Mestikawy, S., Fukaya, M., et al. (2004). GABAergic basket cells expressing cholecystokinin contain vesicular glutamate transporter type 3 (VGLUT3) in their synaptic terminals in hippocampus and isocortex of the rat. *Eur. J. Neurosci.* 19, 552–569. doi: 10.1111/j.0953-816x.2003.03091.x

Stowers, J. R., Hofbauer, M., Bastien, R., Griessner, J., Higgins, P., Farooqui, S., et al. (2017). Virtual reality for freely moving animals. *Nat. Methods* 14, 995–1002. doi: 10.1038/nmeth.4399

Trancikova, A., Ramonet, D., and Moore, D. J. (2011). "Genetic mouse models of neurodegenerative diseases. *Prog. Mol. Biol. Transl. Sci.* 100, 419–482. doi: 10.1016/B978-0-12-384878-9.00012-1

Wallis, J. D., Anderson, K. C., and Miller, E. K. (2001). Single neurons in prefrontal cortex encode abstract rules. *Nature* 411, 953–956. doi: 10.1038/35082081

White, B. H. (2016). What genetic model organisms offer the study of behavior and neural circuits. *J. Neurogenet.* 30, 54–61. doi: 10.1080/01677063.2016.1177049

Wylie, G., and Allport, A. (2000). Task switching and the measurement of "switch costs". *Psychol. Res.* 63, 212–233. doi: 10.1007/s004269900003

Young, J. W., Powell, S. B., Geyer, M. A., Jeste, D. V., and Risbrough, V. B. (2010). The mouse attentional-set-shifting task: a method for assaying successful cognitive aging? *Cogn. Affect. Behav. Neurosci.* 10, 243–251. doi: 10.3758/cabn.10.2.243

Conflict of Interest Statement: 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

BRIEF RESEARCH REPORT
published: 14 January 2020
doi: 10.3389/fnbeh.2019.00291

Increased Goal Tracking in Adolescent Rats Is Goal-Directed and Not Habit-Like

*Analise N. Rode, Bita Moghaddam† and Sara E. Morrison**

Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, United States

When a cue is paired with reward in a different location, some animals will approach the site of reward during the cue, a behavior called goal tracking, while other animals will approach and interact with the cue itself: a behavior called sign tracking. Sign tracking is thought to reflect a tendency to transfer incentive salience from the reward to the cue. Adolescence is a time of heightened sensitivity to rewards, including environmental cues that have been associated with rewards, which may account for increased impulsivity and vulnerability to drug abuse. Surprisingly, however, studies have shown that adolescents are actually less likely to interact with the cue (i.e., sign track) than adult animals. We reasoned that adolescents might show decreased sign tracking, accompanied by increased apparent goal tracking, because they tend to attribute incentive salience to a more reward-proximal "cue": the food magazine. On the other hand, adolescence is also a time of enhanced exploratory behavior, novelty-seeking, and behavioral flexibility. Therefore, adolescents might truly express more goal-directed reward-seeking and less inflexible habit-like approach to a reward-associated cue. Using a reward devaluation procedure to distinguish between these two hypotheses, we found that adolescents indeed exhibit more goal tracking, and less sign tracking, than a comparable group of adults. Moreover, adolescents' goal tracking behavior is highly sensitive to reward devaluation and therefore goal-directed and not habit-like.

Keywords: adolescent, rat, sign tracking, goal tracking, habit, devaluation, Pavlovian conditioning

OPEN ACCESS

Edited by:
Sean B. Ostlund, University of California, Irvine, United States

Reviewed by:
Donna J. Calu, University of Maryland, Baltimore, United States
Lindsey Robertson Hammerslag, University of Kentucky, United States

****Correspondence:***
Sara E. Morrison
sara.morrison@pitt.edu

†Present address:
Bita Moghaddam, Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR, United States

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

Received: *28 August 2019*
Accepted: *23 December 2019*
Published: *14 January 2020*

Citation:
Rode AN, Moghaddam B and Morrison SE (2020) Increased Goal Tracking in Adolescent Rats Is Goal-Directed and Not Habit-Like. Front. Behav. Neurosci. 13:291. doi: 10.3389/fnbeh.2019.00291

INTRODUCTION

Animals and humans vary widely in the degree to which they ascribe motivational value, or incentive salience, to reward-predictive cues. This variability can be measured using a Pavlovian conditioned approach (PCA) procedure in which a cue (e.g., extension of a lever) is followed by the delivery of a reward in a separate location. Under these circumstances, some animals will approach the location of reward delivery: a behavior known as goal tracking (Boakes, 1977). Other animals will approach and interact with the cue itself, a behavior known as sign tracking (Hearst and Jenkins, 1974). A growing body of evidence supports a relationship between sign tracking and certain maladaptive behaviors, including impulsive action (Lovic et al., 2011), initiation and maintenance of drug-taking (Flagel et al., 2009; Beckmann et al., 2011) and relapse after abstinence (Versaggi et al., 2016).

In both humans and non-human animals, adolescence is a time of enhanced sensitivity to rewards, including natural rewards such as sugar (Friemel et al., 2010) and drug rewards such as nicotine (Dannenhoffer and Spear, 2016) and alcohol (Doremus-Fitzwater and Spear, 2016). In many contexts, adolescents also exhibit greater behavioral and neural responsivity to cues that predict reward (Sturman et al., 2010; Burton et al., 2011; Sturman and Moghaddam, 2011). Finally, adolescents display enhanced impulsivity in tasks such as the 5-CSRTT (Burton and Fletcher, 2012). Given these factors, combined with heightened risk-taking (Gardner and Steinberg, 2005; Westbrook et al., 2018), it is unsurprising that adolescents are especially vulnerable to substance abuse (Chambers et al., 2003). Because sign tracking (ST) is also associated with factors contributing to drug abuse and addiction, it is reasonable to hypothesize that adolescents would be more prone to ST. However, studies have found the opposite: under normal circumstances, adolescents typically exhibit lower levels of sign tracking than adults (Anderson and Spear, 2011; Doremus-Fitzwater and Spear, 2011). Only under conditions of heightened stress, such as social isolation combined with food restriction, do adolescents develop equivalent or enhanced ST behavior relative to adults (Anderson et al., 2013; DeAngeli et al., 2017).

These observations give rise to two competing hypotheses. One possibility is that adolescents might display less apparent sign tracking than adults because they ascribe incentive salience to one or more alternative targets, such as the food magazine. This notion is supported by the finding that, among adult sign trackers, most apparent "goal tracking" behavior (which occurs alongside sign tracking in many animals) is insensitive to reward devaluation (Morrison et al., 2015). In other words, subjects that are predisposed to sign tracking in general may exhibit ST-like behavior directed toward the location of reward, not just the cue.

On the other hand, adolescence is a time of enhanced exploratory activity and novelty-seeking (Douglas et al., 2003), and adolescent rats often exhibit more cognitive and behavioral flexibility than adults (Simon et al., 2013; Westbrook et al., 2018). Indeed, some studies have shown that, despite their higher reward sensitivity, adolescents are less prone to habit formation than adults, including drug-seeking habits (Serlin and Torregrossa, 2015) and habits emerging from the pursuit of natural rewards (Naneix et al., 2012). Given these findings, we might expect to see more goal-directed behavior among adolescents (manifested as goal tracking) and less inflexible, habit-like conditioned approach towards a reward-associated cue (i.e., sign tracking).

To distinguish between these two hypotheses, we examined goal-directed vs. habit-like behavior in adolescents compared to adults in a Pavlovian setting. Adolescent and adult male rats were trained on a PCA task and subjected to a reward devaluation procedure to evaluate whether adolescents' lever- and magazine-oriented behavior is primarily goal-directed or habit-like.

MATERIALS AND METHODS

All procedures were performed in accordance with the standards of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Subjects

Subjects were 32 adolescent male Long-Evans rats obtained from Charles River Laboratory at 21 days of age. An adult comparison group consisted of 39 male Long-Evans rats obtained from Charles River Laboratory weighing 275–300 g upon arrival (approximately 9 weeks). All rats were allowed to acclimate to the housing colony for 7 days, and then habituated to gentle handling over the next 7 days. Among adolescents, training and experimental procedures took place between postnatal days 35 and 51. All animals were pair-housed on a 12 h light/dark cycle and all procedures took place during the dark period. Mild food restriction was initiated 2 days prior to the start of training, with adolescents receiving 10 g and adults 14 g of chow per day. Rats were weighed regularly to ensure that they did not fall below 85% of pre-restriction body weight (adults) or 85% of the weight of age-matched free-feeding controls (adolescents).

Apparatus and Training

Training and experiments took place in a standard operant chamber (Coulbourn Instruments) controlled by GraphicState 3.0 and equipped with a house light, pellet delivery system, food magazine recessed into the side wall, and a single retractable lever to one side of the magazine (counterbalanced among subjects). A white cue light was located above the lever. The magazine was equipped with an infrared photo-detector to record entries and exits.

Rats were trained using a PCA procedure similar to that used previously (Gillis and Morrison, 2019). Subjects were initially trained to retrieve sugar pellets (45 mg, Bio-Serv) from the magazine over two sessions consisting of 50 pellets delivered individually on a variable interval schedule averaging 60 s. Rats then received seven daily acquisition sessions on the PCA task, consisting of 25 trials separated by an interval selected from a truncated exponential distribution averaging 60 s. Trials began with the presentation of the cue, consisting of lever extension and a flashing cue light (5 Hz) for 8 s. Upon completion of the cue, the lever retracted, the cue light was extinguished, and a sugar pellet was delivered to the magazine.

Reward Devaluation and Testing

After 7 days of training on the PCA task, rats were subjected to devaluation of the sugar pellet reward *via* taste aversion conditioning. Rats were divided into behavior-matched groups based on the PCA index (see below) calculated for the last day of training. The two groups received either reward devaluation ("paired" group) or sham devaluation ("unpaired"). Rats in the paired group were given access to 50 sugar pellets over 10 min in an empty cage. Immediately afterward, both groups were injected with lithium chloride (LiCl; 0.6 M; 5 ml/kg i.p.). The next day, rats in the unpaired group were given similar access to sugar pellets. Immediately afterward, both groups were injected with vehicle (0.9% saline). Thus, both groups experienced the same exposure to sugar pellets and the same injections, but only the paired group experienced the sugar pellets in conjunction with lithium.

The day after vehicle injections, rats were given a test session in extinction. The test session was identical to the training sessions except that no rewards were delivered. On the same day, rats were given a consumption test consisting of 10 min of exposure to 50 sugar pellets in an empty cage.

Data Analysis

We quantified sign tracking and goal tracking by calculating a PCA index (Meyer et al., 2012) for each individual, which comprises the average of three ratios: (1) probability index, which compares the probability of lever deflection and magazine entry during the cue, calculated as ($P_{lever} - P_{magazine}$); (2) bias index, which compares the average number of lever deflections and magazine entries per cue presentation, calculated as (#lever − #receptacle/#lever + #receptacle); and (3) latency index, which compares the average latency from cue onset to lever deflection vs. magazine entry, calculated as ($lat_{magazine} - lat_{lever}$)/(cue length). When a behavior was not performed, the latency was defined as the cue length. Each of these indices ranges from −1.0 to +1.0, where more positive numbers indicate more sign tracking (relative to goal tracking) and more negative numbers indicate more goal tracking (relative to sign tracking).

In order to isolate the effects of devaluation from the effects of extinction, analyses were performed on data from the first 10 trials of both training and test sessions. As in some previous reports (e.g., Tunstall and Kearns, 2015), we operationally defined "sign trackers" as animals with an average PCA index greater than zero and "goal trackers" as animals with an average PCA index less than zero. Although in previous studies (e.g., Morrison et al., 2015) we have categorized animals using the mean or median PCA index of a specific group of animals, here we opted to use a fixed boundary in order to facilitate comparisons across groups (adults and adolescents).

All statistical comparisons were performed using a Wilcoxon signed-rank test (within-group comparisons) or a Wilcoxon rank-sum test (across-group comparisons). Where appropriate, alphas were corrected for multiple comparisons using the Holm-Sidak method.

RESULTS

We trained 32 adolescents and 39 adult rats in a PCA procedure similar to those we have used previously to study sign tracking and goal tracking behavior in adults (Morrison et al., 2015; Gillis and Morrison, 2019). In this task, sign tracking is represented by lever deflections and goal tracking by magazine entries during the 8 s lever/light cue, although neither behavior is required for delivery of the sugar pellet reward after cue termination.

Acquisition of Sign Tracking and Goal Tracking

We quantified the tendency of individuals towards sign-tracking vs. goal-tracking behavior by calculating a PCA index ranging from −1.0 (all GT, no ST) to +1.0 (all ST, no GT). The acquisition of sign tracking and goal tracking behaviors followed a similar time course among adolescent vs. adult sign trackers and goal trackers (**Figures 1A,B**). At the end of the 7 days of training, adolescent rats exhibited a slight but non-significant population bias towards goal tracking (**Figure 1C**; median PCA index not different from 0; $Z = -1.12$, $p = 0.26$) while adult rats exhibited a marked population bias towards sign tracking (**Figure 1D**; median PCA index >0, $Z = 3.18$, $p = 0.002$). The distribution of PCA index was significantly different between the two groups ($Z = -2.60$, $p = 0.01$), and this difference was driven by both fewer lever deflections ($Z = -2.00$, $p = 0.046$) and more magazine entries ($Z = 2.88$, $p = 0.004$) among adolescents compared to adults (**Figure 1E**). Thus, not only do adolescents perform fewer sign-tracking actions than adults, confirming prior findings (Anderson and Spear, 2011; Doremus-Fitzwater and Spear, 2011), they also perform more goal-tracking behavior.

Effects of Reward Devaluation

After 7 days of training on the PCA task, adolescent rats underwent reward devaluation *via* taste aversion conditioning: subjects in the "paired" group were given sugar pellets outside of the task environment, followed immediately by injection with LiCl solution to induce illness. Subjects in the "unpaired" group were also injected with LiCl; then, on a subsequent day, the unpaired group was allowed to eat sugar pellets, followed immediately by vehicle injection for all rats. Thus, all rats experienced the same exposure to sugar pellets and injections, but only the paired group experienced the temporal conjunction of sugar pellets and illness.

The next day, rats in both groups performed the PCA task in extinction (no rewards given) followed by a consumption test. As shown in **Figure 2A**, adolescent rats consumed many fewer sugar pellets following taste aversion conditioning (paired group; $Z = 3.30$, $p < 0.001$), but not following a sham devaluation procedure (unpaired group; $Z = -1.60$, $p = 0.11$). As we have previously shown in adults (Morrison et al., 2015), reward devaluation increased adolescents' ratio of sign tracking to goal tracking: adolescents in the paired group, but not the unpaired group, had a significantly higher PCA index following reward devaluation (**Figure 2B**; paired: $Z = -3.10$, $p = 0.002$; unpaired: $Z = -1.45$, $p = 0.15$). Moreover, in a direct comparison, the PCA index of adolescents in the paired group trended higher than those in the unpaired group during the test session ($Z = 1.60$, $p = 0.11$). Although we would expect some increase in the PCA index even in the unpaired group due to the effects of extinction, the increase was significantly greater in the paired group (**Figure 2C**; $Z = 1.98$, $p = 0.048$).

Following reward devaluation, the paired group showed significant increases in the probability of lever deflection relative to magazine entry (**Figure 2D**; $Z = -3.21$, $p = 0.001$), bias towards lever deflection relative to magazine entry during the cue (**Figure 2E**; $Z = -2.13$, $p = 0.03$), and latency to magazine entry relative to lever deflection (**Figure 2F**; $Z = -3.36$, $p < 0.001$), whereas the unpaired group showed no such changes (all comparisons, $p > 0.2$). Moreover, two of the three indices were significantly or trending higher for the paired group than for the unpaired group during the test session (latency index, $Z = 1.98$, $p = 0.048$; probability index, $Z = 1.70$, $p = 0.073$; bias index, $p > 0.2$). Finally, for all measures except bias index, the change

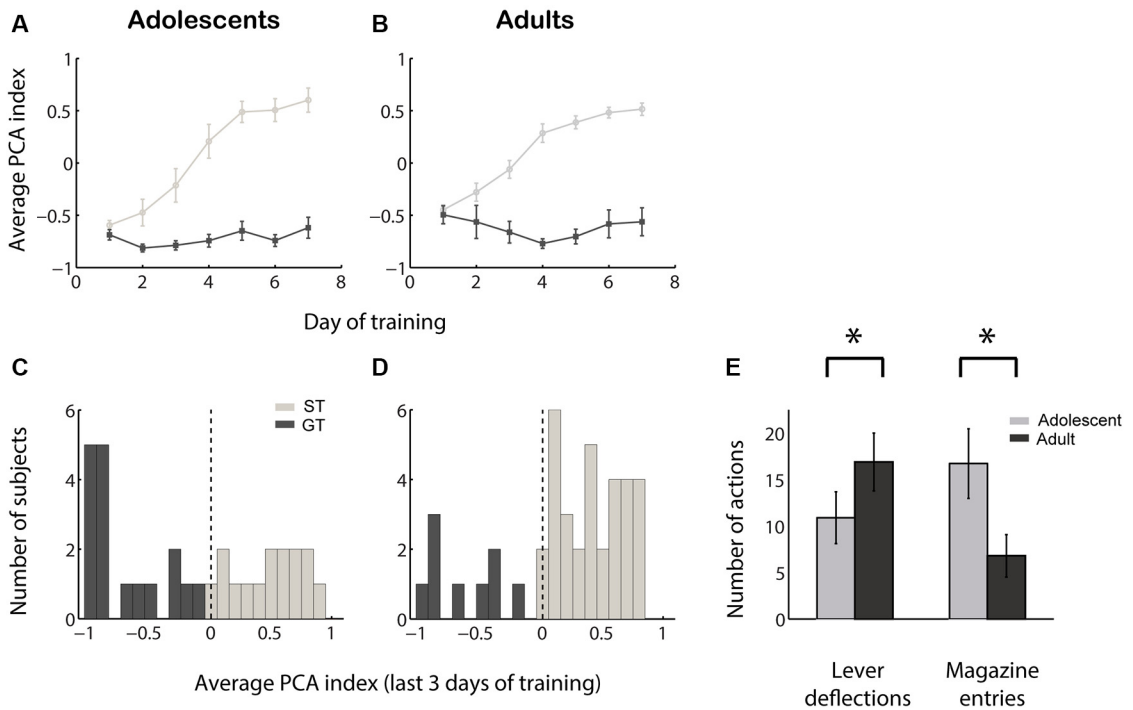


FIGURE 1 | Adolescents perform less sign tracking and more goal tracking than adults. **(A,B)** Average Pavlovian conditioned approach (PCA) index among sign trackers (light gray) and goal trackers (dark gray) over 7 days of training for adolescents **(A)** and adults **(B)**. Higher PCA index indicates more sign tracking relative to goal tracking. **(C,D)** Average PCA index for adolescents **(C)** and adults **(D)** over the last 3 days of the 7-day training period. Individuals categorized as sign trackers (ST) and goal trackers (GT) are indicated in light gray and dark gray, respectively. **(E)** Average raw counts of lever deflections and magazine entries during the cue over the last 3 days of training for adolescents (gray) and adults (black). Error bars, SEM. Asterisk, $p < 0.05$.

was significantly greater for the paired group than the unpaired group (data not shown; probability index, $Z = 2.34$, $p = 0.02$; latency index, $Z = 2.58$, $p = 0.01$). These changes were driven by both an increase in the average number of lever presses during the cue (**Figure 2G**; $Z = -2.20$, $p = 0.03$) and a robust decrease in the average number of magazine entries (**Figure 2H**; $Z = 3.20$, $p = 0.001$) among the paired group. Thus, reward devaluation resulted in an increase in the intensity of sign tracking behavior and a decrease in the intensity of goal-tracking behavior in adolescent rats.

For comparison, we performed an identical reward devaluation experiment using 39 adult rats (20 in the paired group, 19 unpaired). Similar to adolescents, adults consumed fewer sugar pellets following taste aversion conditioning (**Figure 3A**; $Z = 3.82$, $p < 0.001$), but not following sham devaluation ($p = 1$). Devaluation effects were similar but weaker in adults compared to adolescents, as might be expected given their greater degree of sign tracking relative to goal tracking: the PCA index of adults in the paired group trended higher following devaluation (**Figure 3B**; $Z = -1.79$, $p = 0.073$), whereas the PCA index of adults in the unpaired group did not ($Z = -1.49$, $p = 0.14$). This change was primarily driven by a decrease in magazine entries among adults in the paired group (**Figure 3D**; $p = 0.002$), which was larger than the small but significant decrease in magazine entries in the unpaired group ($p = 0.038$) likely caused by extinction effects. Neither group showed a significant change in the number of lever presses (**Figure 3C**; $p > 0.2$).

Individual Differences in Behavior and Devaluation Effects

We next asked whether, in adolescents as in adults, sensitivity to reward devaluation is a characteristic that varies with an individual's tendency to ascribe incentive salience to a cue. As shown in **Figure 1**, we divided subjects into sign trackers and goal trackers based on average PCA index: subjects with a PCA index >0 were categorized as sign trackers, and subjects with a PCA index <0 were categorized as goal trackers. We found that the population effects of reward devaluation (**Figure 2**) were almost entirely attributable to goal trackers. Only among goal trackers did PCA index show a significant increase following reward devaluation (**Figure 4A**; $Z = -2.43$, $p = 0.015$); the same was not true for sign trackers or following sham devaluation (all comparisons, $p > 0.1$). Furthermore, the PCA index of goal trackers in the paired group trended higher than goal trackers in the unpaired group during the test session ($p = 0.11$). Likewise, goal trackers, but not sign trackers, showed a significant increase in the probability of lever deflection relative to magazine entry (**Figure 4B**; $Z = -2.67$, $p = 0.008$) and a significant increase in latency to magazine entry relative to lever deflection (**Figure 4D**;

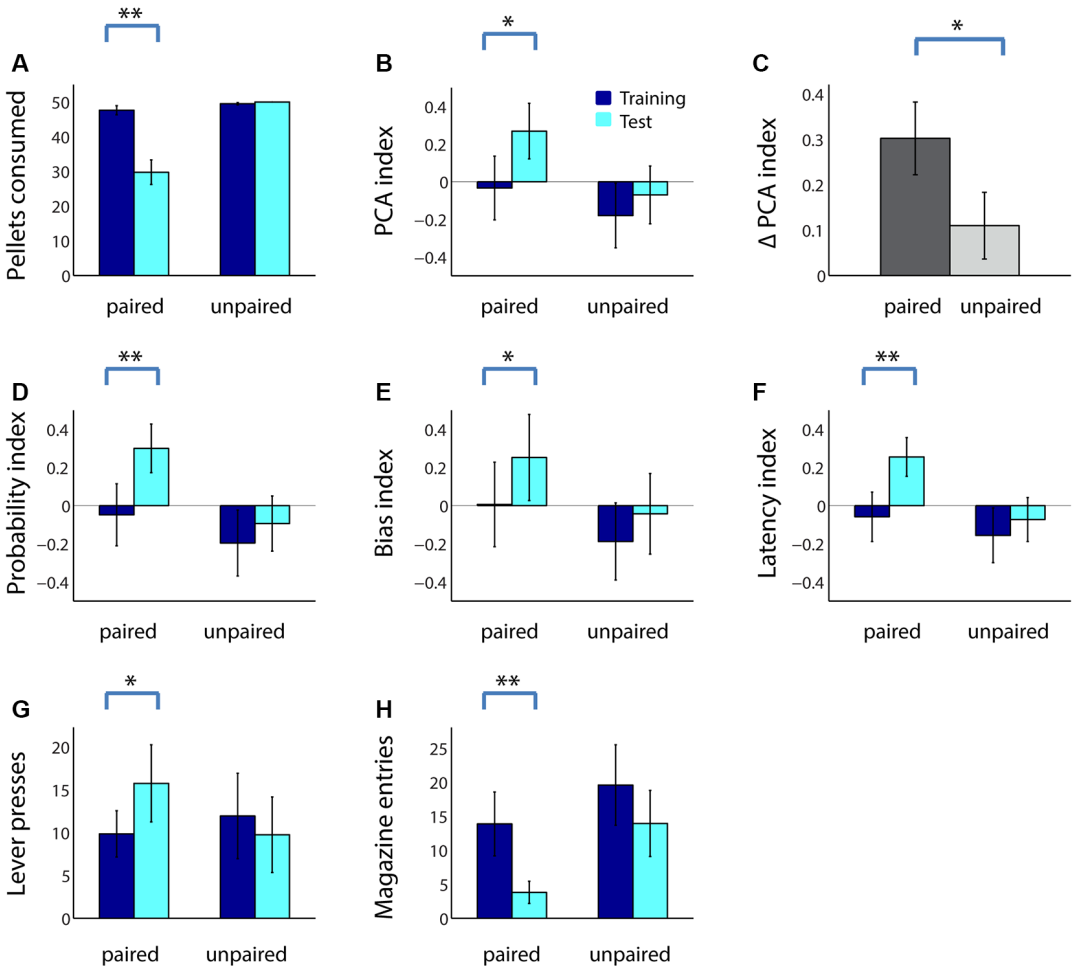


FIGURE 2 | Reward devaluation increases sign tracking and decreases goal tracking in adolescent rats. **(A)** The average number of sugar pellets consumed before (dark blue) and after (cyan) reward devaluation (paired group) or sham devaluation (unpaired group). **(B)** Average PCA index over the last 3 days of training dark (blue) and in the test session (cyan) for the paired and unpaired groups. Higher PCA index indicates more sign tracking relative to goal tracking. **(C)** Mean change in PCA index from training to test session for the paired (dark gray) and unpaired (light gray) groups. **(D–F)** Components of the PCA index. Average probability index **(D)**, bias index **(E)**, and latency index **(F)** over the last 3 days of training (dark blue) and in the test session (cyan) for the paired and unpaired groups. **(G,H)** Raw lever press count **(G)** and magazine entry count **(H)** over the last 3 days of training (dark blue) and in the test session (cyan) for the paired and unpaired groups. All panels, error bars indicate SEM. Double asterisk, $p < 0.001$; single asterisk, $p < 0.05$.

$Z = -2.67$, $p = 0.008$) following reward devaluation (although there was no significant change in bias index; **Figure 4C**). Direct comparisons revealed that both of these indices were higher in the paired group than the unpaired group during the test session (probability index, $p = 0.032$; latency index, $p = 0.024$).

Raw behavior counts showed that the changes in PCA index and its components were predominantly due to a robust devaluation-induced decrease in magazine entries during the cue among goal trackers (**Figure 4F**; $Z = 2.67$, $p = 0.008$) accompanied by a small but significant increase in lever presses (**Figure 4E**; $Z = -1.99$, $p = 0.046$). In contrast, there was no significant change in behavior among sign trackers or following sham devaluation (all comparisons, $p > 0.05$).

Finally, we investigated whether the effects of devaluation can be predicted by an individual's tendency towards sign-tracking or goal-tracking behavior on a subject-by-subject basis. Indeed, there was a significant negative correlation between an individual's pre-devaluation PCA index and subsequent change in the PCA index (**Figure 4G**; $r^2 = 0.24$, $p = 0.05$). A smaller negative relationship was seen in the unpaired group, likely due to the effects of extinction (**Figure 4H**; $r^2 = 0.20$, $p = 0.08$). Thus, adolescents' behavior is sensitive to reward devaluation to a degree commensurate with their baseline levels of goal tracking, whereas sign tracking is either unaffected or increased by reward devaluation.

DISCUSSION

In adult rats, sign-tracking behavior, compared with goal-tracking behavior, is relatively insensitive to reward devaluation, whether accomplished *via* pre-feeding (Patitucci et al., 2016; Conrad and Papini, 2018) or conditioned taste

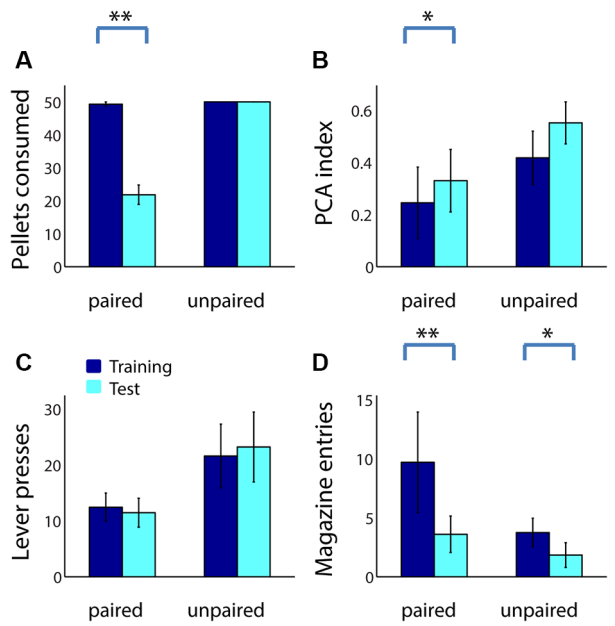


FIGURE 3 | Reward devaluation decreases goal tracking, but not sign tracking, in adult rats. **(A)** The average number of sugar pellets consumed before (dark blue) and after (cyan) reward devaluation (paired group) or sham devaluation (unpaired group). **(B)** Average PCA index over the last 3 days of training (dark blue) and in the test session (cyan) for the paired and unpaired groups. Higher PCA index indicates more sign tracking relative to goal tracking. **(C,D)** Raw lever press counts **(C)** and magazine entry counts **(D)** over the last 3 days of training (dark blue) and in the test session (cyan) for the paired and unpaired groups. All panels, error bars indicate SEM. Double asterisk, $p < 0.001$; single asterisk, $p < 0.05$.

aversion (CTA; Morrison et al., 2015; Smedley and Smith, 2018; although devaluation can affect the approach to the cue under some circumstances: see Cleland and Davey, 1982; Derman et al., 2018). Because resistance to reward devaluation is one of the defining features of habitual actions (Balleine and O'Doherty, 2010), these findings imply that sign tracking is a habit-like behavior, although it is distinct from a classically defined habit because it arises from Pavlovian rather than instrumental contingencies (Dayan and Berridge, 2014).

In the current study, we used reward devaluation to determine whether the Pavlovian conditioned behavior of adolescent rats is more goal-driven or habit-like. As in previous studies (Anderson and Spear, 2011; Doremus-Fitzwater and Spear, 2011), we found that adolescents exhibit less sign-tracking behavior (lever deflections) than a comparable group of adults under normal, low-stress circumstances. We extend these findings to show that adolescents also engage in more apparent goal-tracking behavior (magazine entries) than adults. There are two possible explanations for this observation: on one hand, the increase in magazine entries might reflect a tendency by adolescents to place incentive salience on a more reward-proximal cue: the food magazine itself. On the other hand, adolescents might simply engage in more goal-directed behavior, and less habit-like behavior, than adults.

Our results support the latter hypothesis: just as in adults, the magazine-oriented behavior of adolescents is markedly sensitive to reward devaluation and therefore fits the definition of goal-directed behavior. When we subjected adolescent rats to CTA, in which they learned to associate sugar pellets with LiCl-induced illness, they dramatically reduced their interactions with the food magazine during the cue. This finding challenges the idea that CTA might be relatively ineffective in adolescents (Hammerslag and Gulley, 2014) because they are less sensitive to the aversive consequences of rewarding stimuli (Doremus-Fitzwater and Spear, 2016). Perhaps more importantly, it shows that adolescents' magazine-directed behavior can fairly be termed "goal tracking," rather than sign tracking directed towards the food magazine.

The current findings provide evidence against the hypothesis that adolescents are less goal-oriented and more "stimulus-driven" than adults (Ernst et al., 2011; Hammerslag and Gulley, 2014), even though there is evidence that adolescents are more responsive to reward-associated cues in certain contexts. Adolescents are faster to acquire cued behaviors associated with drug rewards (Schramm-Sapyta et al., 2011), slower to extinguish such behaviors (Anker and Carroll, 2010; Meyer and Bucci, 2016), and exhibit stronger cue-induced reinstatement of drug-seeking (Brenhouse and Andersen, 2008). Nevertheless, adolescent rats are no more likely than adults to transfer incentive salience to a cue; on the contrary, based on their relatively low levels of sign tracking, it seems that adolescents are considerably less likely than adults to ascribe independent motivational value to cues. Adolescents' responsiveness to reward-associated cues might be better explained by their generally enhanced sensitivity to the rewarding properties of food and drugs (Friemel et al., 2010; Doremus-Fitzwater and Spear, 2016).

Indeed, there is a small but growing body of literature supporting the notion that adolescents can be more goal-driven than adults under the right circumstances. In addition to the finding that adolescents are less prone to sign tracking, studies have suggested that adolescents are less susceptible to habit formation (Serlin and Torregrossa, 2015) and that their cognition and behavior is more flexible compared to adults (Sturman and Moghaddam, 2012; Simon et al., 2013; Westbrook et al., 2018). This is consistent with adolescence as a time of enhanced exploratory behavior, novelty-seeking, and risk-taking (Douglas et al., 2003): adolescents are still learning the rules of their environment, including which stimuli and actions are most often followed by reward, and habit formation would be a hindrance to exploration and learning. Adults, on the other hand, can take advantage of the computational efficiency of engaging in cue-driven and/or habitual behavior.

The current findings complement the observation that adolescents perform more sign tracking, equivalent to or surpassing adult levels, when exposed to stressors such as social isolation combined with food restriction (Anderson et al., 2013; DeAngeli et al., 2017). Interestingly, food restriction by itself, as in the current study and that of Anderson et al. (2013), is inadequate to elevate adolescents' sign tracking above that of adults; it may be the case that the stresses of food restriction and social isolation are additive, or that social isolation

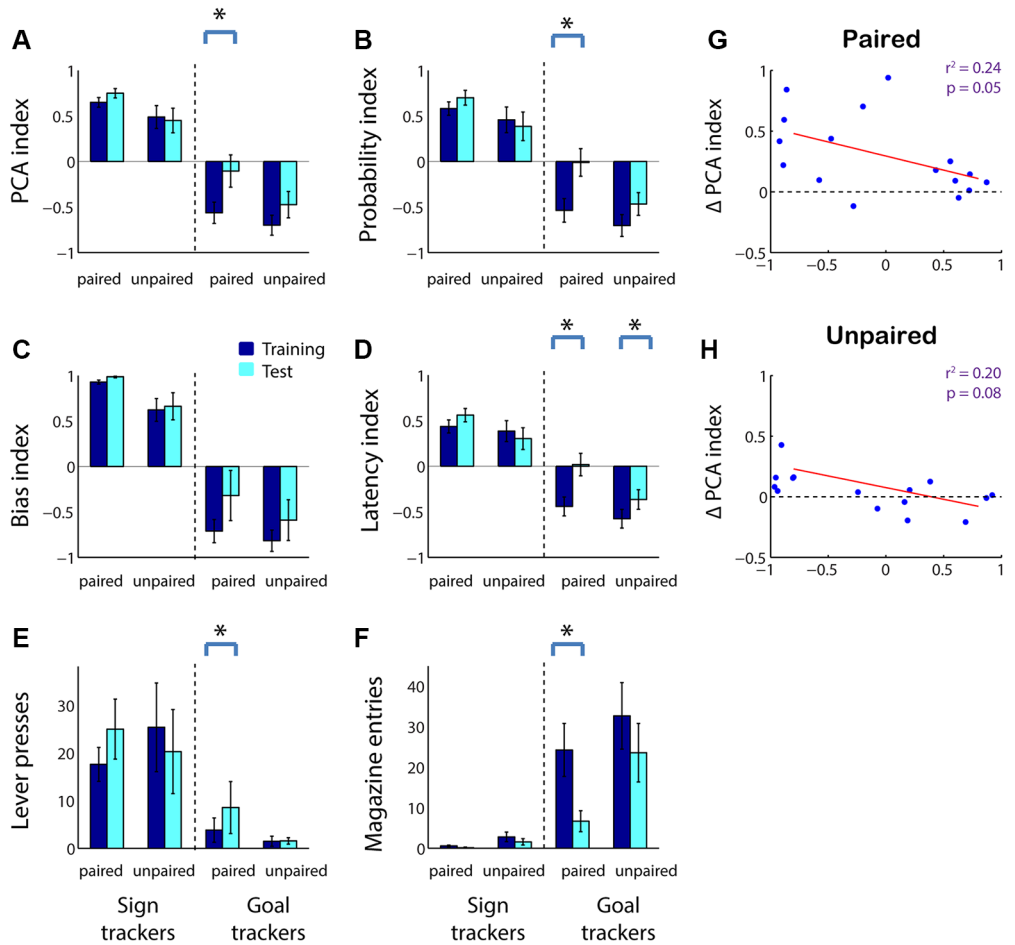


FIGURE 4 | In adolescents, reward devaluation primarily causes a reduction in goal tracking among goal tracker individuals. **(A–D)** Average PCA index **(A)**, probability index **(B)**, bias index **(C)**, and latency index **(D)** among sign trackers (left-hand side of panels) and goal trackers (right-hand side of panels) before and after reward devaluation (paired group) or sham devaluation (unpaired group). Higher PCA index indicates more sign tracking relative to goal tracking. Dark blue, average over the last 3 days of training. Cyan, test session (in extinction). **(E,F)** Raw lever press count **(E)** and magazine entry count **(F)** among sign trackers (left-hand side of panels) and goal trackers (right-hand side of panels) before and after reward devaluation (paired group) or sham devaluation (unpaired group). Dark blue, average over the last 3 days of training. Cyan, test session. All panels, error bars indicate SEM. Asterisk, $p < 0.05$. **(G,H)** Average PCA index over the last 3 days of training plotted against change in PCA index for the paired **(G)** and unpaired **(H)** groups. Regression lines in red.

has an especially stressful impact on adolescent individuals. Overall, these studies imply that adolescents are fully capable of transferring incentive salience to cues, consistent with their adult-like expression of Pavlovian-instrumental transfer (Naneix et al., 2012), but tend to do so only when under stress, possibly as an adaptation to limit exploration when resources or social support are scarce. Indeed, there is evidence that the mesolimbic dopamine system, which is thought to underlie sign tracking (Flagel et al., 2011), is specifically vulnerable to stress-induced abnormalities in adolescents (Buwalda et al., 2011).

A substantial body of literature supports the notion that sign tracking and goal tracking are the behavioral outputs of two parallel reinforcement learning processes—akin to model-free and model-based learning, respectively (Clark et al., 2012; Huys et al., 2014). Although there has been limited investigation of model-free vs. model-based learning in adolescents, especially in animal models, the predominant view is that model-free learning is present throughout the lifespan, whereas model-based learning emerges slowly and is not fully integrated into behavior until adulthood (Decker et al., 2016; Potter et al., 2017). Our finding that adolescent rats perform more goal tracking and less sign tracking—and that such goal tracking is truly goal-oriented behavior, based on its sensitivity to reward devaluation—complicates this view.

There are a number of possible reasons for these differing results, including species differences between the development of these systems in humans vs. rodents. Although there is evidence that humans, including children, display sign tracking- and goal tracking-like behavior in a Pavlovian conditioning context (Garofalo and di Pellegrino, 2015; Joyner et al., 2018), it is currently unknown whether human adolescents exhibit less sign tracking and more goal tracking than adults. Moreover, there

may be important developmental differences in the engagement of different learning systems during Pavlovian conditioning vs. instrumental tasks. In Pavlovian conditioning, the subject's actions have no impact on reward delivery; in contrast, in instrumental tasks, reward delivery is contingent upon the performance of a certain action or actions. There is a large body of literature demonstrating that Pavlovian and instrumental conditioning engage distinct, though overlapping, brain circuits, and that these circuits can interact in complex ways to generate behavior (Dayan and Berridge, 2014).

The distinction between Pavlovian and instrumental contexts may also be a factor in the influence of extinction on adolescent behavior. In the current study, the test session was performed in extinction in order to minimize new cue-outcome learning. Prior studies have shown that sign tracking, compared to goal tracking, is resistant to extinction behaviorally (Ahrens et al., 2016) and in the context of cue-related neural activity (Gillis and Morrison, 2019). Compared with our prior report in adults, in adolescents, we see a larger apparent effect of sham devaluation (e.g., see **Figure 4H**). However, when we minimized the effect of extinction by analyzing a smaller number of trials at the beginning of the test session (5 instead of 10), the near-significant effects disappeared in the unpaired (sham) group, but not the paired group (data not shown). The relatively large effect of extinction, separate from reinforcer devaluation, might be accounted for by the larger number of goal trackers among the adolescent population; but it raises the intriguing possibility that extinction has a stronger effect on goal tracking in adolescents compared with adults. This would be consistent with the evidence that adolescents are more behaviorally flexible and sensitive to context and/or changing contingencies (Simon et al., 2013; Serlin and Torregrossa, 2015). On the other hand, several studies have found that adolescents, compared with adults, are more likely to exhibit perseverative behavior during extinction of Pavlovian and instrumental tasks (Sturman et al., 2010; Andrzejewski et al., 2011; Meyer and Bucci, 2016). Further experiments will be needed to clarify the factors—e.g., Pavlovian vs. instrumental setting, stress levels, length of training—that lead to greater or lesser sensitivity to extinction in adolescents.

Overall, the current study provides new evidence that, depending on task context and environment, compared with adults, adolescent behavior is neither disproportionately cue-driven, nor habit-like, nor the result of model-free learning processes. Although there is substantial evidence that the neural circuits associated with model-free learning develop earlier than circuits associated with model-based learning (Naneix et al., 2012), it is clear that simple forms of goal-directed behavior are fully operational in adolescence (and perhaps earlier) and are favored over the formation of habit-like Pavlovian behavior. In the future, a fuller understanding of adolescent behavioral processes, and how they differ from those of adults, is needed in order to improve prevention and treatment of substance use disorders, for which adolescents are at greater risk.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Pittsburgh IACUC.

AUTHOR CONTRIBUTIONS

SM and BM designed the experiment. AR and SM carried out the experiment. SM analyzed the data and wrote the article with input from BM.

FUNDING

This work was supported by grants from the National Institutes of Health (NIH) to SM (DA045913) and BM (MH048404), a University of Pittsburgh Medical Center Competitive Medical Research Fund Award (SM), and a NARSAD Young Investigator Award (25185) from the Brain and Behavior Research Organization (SM).

ACKNOWLEDGMENTS

We would like to thank Mary Torregrossa for helpful discussions and Zachary Gillis, Kelly Glavin, Maya Phillips, and Cassandra Slover for help with additional data collection.

REFERENCES

Ahrens, A. M., Singer, B. F., Fitzpatrick, C. J., Morrow, J. D., and Robinson, T. E. (2016). Rats that sign-track are resistant to Pavlovian but not instrumental extinction. *Behav. Brain Res.* 296, 418–430. doi: 10.1016/j.bbr.2015.07.055

Anderson, R. I., Bush, P. C., and Spear, L. P. (2013). Environmental manipulations alter age differences in attribution of incentive salience to reward-paired cues. *Behav. Brain Res.* 257, 83–89. doi: 10.1016/j.bbr.2013.09.021

Anderson, R. I., and Spear, L. P. (2011). Autoshaping in adolescence enhances sign-tracking behavior in adulthood: impact on ethanol consumption. *Pharmacol. Biochem. Behav.* 98, 250–260. doi: 10.1016/j.pbb.2011.01.004

Andrzejewski, M. E., Schochet, T. L., Feit, E. C., Harris, R., Mckee, B. L., and Kelley, A. E. (2011). A comparison of adult and adolescent rat behavior in operant learning, extinction and behavioral inhibition paradigms. *Behav. Neurosci.* 125, 93–105. doi: 10.1037/a0022038

Anker, J. J., and Carroll, M. E. (2010). Reinstatement of cocaine seeking induced by drugs, cues, and stress in adolescent and adult rats. *Psychopharmacology* 208, 211–222. doi: 10.1007/s00213-009-1721-2

Balleine, B. W., and O'Doherty, J. P. (2010). Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. *Neuropsychopharmacology* 35, 48–69. doi: 10.1038/npp.2009.131

Beckmann, J. S., Marusich, J. A., Gipson, C. D., and Bardo, M. T. (2011). Novelty seeking, incentive salience and acquisition of cocaine self-administration in the rat. *Behav. Brain Res.* 216, 159–165. doi: 10.1016/j.bbr.2010.07.022

Boakes, R. A. (1977). "Performance on learning to associate a stimulus with positive reinforcement," in *Operant-Pavlovian Interactions*, eds H. Davis and H. M. B. Hurwitz (Hillsdale, NJ: Lawrence Erlbaum Associates), 67–97.

Brenhouse, H. C., and Andersen, S. L. (2008). Delayed extinction and stronger reinstatement of cocaine conditioned place preference in adolescent rats,

compared to adults. *Behav. Neurosci.* 122, 460–465. doi: 10.1037/0735-7044.122.2.460

Burton, C. L., and Fletcher, P. J. (2012). Age and sex differences in impulsive action in rats: the role of dopamine and glutamate. *Behav. Brain Res.* 230, 21–33. doi: 10.1016/j.bbr.2012.01.046

Burton, C. L., Noble, K., and Fletcher, P. J. (2011). Enhanced incentive motivation for sucrose-paired cues in adolescent rats: possible roles for dopamine and opioid systems. *Neuropsychopharmacology* 36, 1631–1643. doi: 10.1038/npp.2011.44

Buwalda, B., Geerdink, M., Vidal, J., and Koolhaas, J. M. (2011). Social behavior and social stress in adolescence: a focus on animal models. *Neurosci. Biobehav. Rev.* 35, 1713–1721. doi: 10.1016/j.neubiorev.2010.10.004

Chambers, R. A., Taylor, J. R., and Potenza, M. N. (2003). Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am. J. Psychiatry* 160, 1041–1052. doi: 10.1176/appi.ajp.160.6.1041

Clark, J. J., Hollon, N. G., and Phillips, P. E. (2012). Pavlovian valuation systems in learning and decision making. *Curr. Opin. Neurobiol.* 22, 1054–1061. doi: 10.1016/j.conb.2012.06.004

Cleland, G. G., and Davey, G. C. (1982). The effects of satiation and reinforcer devaluation on signal-centered behavior in the rat. *Learn. Motiv.* 13, 343–360. doi: 10.1016/0023-9690(82)90014-5

Conrad, S. E., and Papini, M. R. (2018). Reward shifts in forced-choice and free-choice autoshaping with rats. *J. Exp. Psychol. Anim. Learn. Cogn.* 44, 422–440. doi: 10.1037/xan0000187

Dannenhoffer, C. A., and Spear, L. P. (2016). Age differences in conditioned place preferences and taste aversions to nicotine. *Dev. Psychobiol.* 58, 660–666. doi: 10.1002/dev.21400

Dayan, P., and Berridge, K. C. (2014). Model-based and model-free Pavlovian reward learning: revaluation, revision, and revelation. *Cogn. Affect. Behav. Neurosci.* 14, 473–492. doi: 10.3758/s13415-014-0277-8

DeAngeli, N. E., Miller, S. B., Meyer, H. C., and Bucci, D. J. (2017). Increased sign-tracking behavior in adolescent rats. *Dev. Psychobiol.* 59, 840–847. doi: 10.1002/dev.21548

Decker, J. H., Otto, A. R., Daw, N. D., and Hartley, C. A. (2016). From creatures of habit to goal-directed learners: tracking the developmental emergence of model-based reinforcement learning. *Psychol. Sci.* 27, 848–858. doi: 10.1177/0956797616639301

Derman, R. C., Schneider, K., Juarez, S., and Delamater, A. R. (2018). Sign-tracking is an expectancy-mediated behavior that relies on prediction error mechanisms. *Learn. Mem.* 25, 550–563. doi: 10.1101/lm.047365.118

Doremus-Fitzwater, T. L., and Spear, L. P. (2011). Amphetamine-induced incentive sensitization of sign-tracking behavior in adolescent and adult female rats. *Behav. Neurosci.* 125, 661–667. doi: 10.1037/a0023763

Doremus-Fitzwater, T. L., and Spear, L. P. (2016). Reward-centricity and attenuated aversions: an adolescent phenotype emerging from studies in laboratory animals. *Neurosci. Biobehav. Rev.* 70, 121–134. doi: 10.1016/j.neubiorev.2016.08.015

Douglas, L. A., Varlinskaya, E. I., and Spear, L. P. (2003). Novel-object place conditioning in adolescent and adult male and female rats: effects of social isolation. *Physiol. Behav.* 80, 317–325. doi: 10.1016/j.physbeh.2003.08.003

Ernst, M., Daniele, T., and Frantz, K. (2011). New perspectives on adolescent motivated behavior: attention and conditioning. *Dev. Cogn. Neurosci.* 1, 377–389. doi: 10.1016/j.dcn.2011.07.013

Flagel, S. B., Akil, H., and Robinson, T. E. (2009). Individual differences in the attribution of incentive salience to reward-related cues: implications for addiction. *Neuropharmacology* 56, 139–148. doi: 10.1016/j.neuropharm.2008.06.027

Flagel, S. B., Clark, J. J., Robinson, T. E., Mayo, L., Czuj, A., Willuhn, I., et al. (2011). A selective role for dopamine in stimulus-reward learning. *Nature* 469, 53–57. doi: 10.1038/nature09588

Friemel, C. M., Spanagel, R., and Schneider, M. (2010). Reward sensitivity for a palatable food reward peaks during pubertal developmental in rats. *Front. Behav. Neurosci.* 4:39. doi: 10.3389/fnbeh.2010.00039

Gardner, M., and Steinberg, L. (2005). Peer influence on risk taking, risk preference, and risky decision making in adolescence and adulthood: an experimental study. *Dev. Psychol.* 41, 625–635. doi: 10.1037/0012-1649.41.4.625

Garofalo, S., and di Pellegrino, G. (2015). Individual differences in the influence of task-irrelevant Pavlovian cues on human behavior. *Front. Behav. Neurosci.* 9:163. doi: 10.3389/fnbeh.2015.00163

Gillis, Z. S., and Morrison, S. E. (2019). Sign tracking and goal tracking are characterized by distinct patterns of nucleus accumbens activity. *eNeuro* 6:ENEURO.0414-18.2019. doi: 10.1523/eneuro.0414-18.2019

Hammerslag, L. R., and Gulley, J. M. (2014). Age and sex differences in reward behavior in adolescent and adult rats. *Dev. Psychobiol.* 56, 611–621. doi: 10.1002/dev.21127

Hearst, E., and Jenkins, H. M. (1974). *Sign-Tracking: The Stimulus-Reinforcer Relation and Directed Action.* Austin, TX: Psychonomic Society.

Huys, Q. J., Tobler, P. N., Hasler, G., and Flagel, S. B. (2014). The role of learning-related dopamine signals in addiction vulnerability. *Prog. Brain Res.* 211, 31–77. doi: 10.1016/b978-0-444-63425-2.00003-9

Joyner, M. A., Gearhardt, A. N., and Flagel, S. B. (2018). A translational model to assess sign-tracking and goal-tracking behavior in children. *Neuropsychopharmacology* 43, 228–229. doi: 10.1038/npp.2017.196

Lovic, V., Saunders, B. T., Yager, L. M., and Robinson, T. E. (2011). Rats prone to attribute incentive salience to reward cues are also prone to impulsive action. *Behav. Brain Res.* 223, 255–261. doi: 10.1016/j.bbr.2011.04.006

Meyer, H. C., and Bucci, D. J. (2016). Age differences in appetitive Pavlovian conditioning and extinction in rats. *Physiol. Behav.* 167, 354–362. doi: 10.1016/j.physbeh.2016.10.004

Meyer, P. J., Lovic, V., Saunders, B. T., Yager, L. M., Flagel, S. B., Morrow, J. D., et al. (2012). Quantifying individual variation in the propensity to attribute incentive salience to reward cues. *PLoS One* 7:e38987. doi: 10.1371/journal.pone.0038987

Morrison, S. E., Bamkole, M. A., and Nicola, S. M. (2015). Sign tracking, but not goal tracking, is resistant to outcome devaluation. *Front. Neurosci.* 9:468. doi: 10.3389/fnins.2015.00468

Naneix, F., Marchand, A. R., Di Scala, G., Pape, J. R., and Coutureau, E. (2012). Parallel maturation of goal-directed behavior and dopaminergic systems during adolescence. *J. Neurosci.* 32, 16223–16232. doi: 10.1523/jneurosci.3080-12.2012

Patitucci, E., Nelson, A. J., Dwyer, D. M., and Honey, R. C. (2016). The origins of individual differences in how learning is expressed in rats: a general-process perspective. *J. Exp. Psychol. Anim. Learn. Cogn.* 42, 313–324. doi: 10.1037/xan0000116

Potter, T. C. S., Bryce, N. V., and Hartley, C. A. (2017). Cognitive components underpinning the development of model-based learning. *Dev. Cogn. Neurosci.* 25, 272–280. doi: 10.1016/j.dcn.2016.10.005

Schramm-Sapyta, N. L., Cauley, M. C., Stangl, D. K., Glowacz, S., Stepp, K. A., Levin, E. D., et al. (2011). Role of individual and developmental differences in voluntary cocaine intake in rats. *Psychopharmacology* 215, 493–504. doi: 10.1007/s00213-011-2216-5

Serlin, H., and Torregrossa, M. M. (2015). Adolescent rats are resistant to forming ethanol seeking habits. *Dev. Cogn. Neurosci.* 16, 183–190. doi: 10.1016/j.dcn.2014.12.002

Simon, N. W., Gregory, T. A., Wood, J., and Moghaddam, B. (2013). Differences in response initiation and behavioral flexibility between adolescent and adult rats. *Behav. Neurosci.* 127, 23–32. doi: 10.1037/a0031328

Smedley, E. B., and Smith, K. S. (2018). Evidence of structure and persistence in motivational attraction to serial Pavlovian cues. *Learn. Mem.* 25, 78–89. doi: 10.1101/lm.046599.117

Sturman, D. A., Mandell, D. R., and Moghaddam, B. (2010). Adolescents exhibit behavioral differences from adults during instrumental learning and extinction. *Behav. Neurosci.* 124, 16–25. doi: 10.1037/a0018463

Sturman, D. A., and Moghaddam, B. (2011). Reduced neuronal inhibition and coordination of adolescent prefrontal cortex during motivated behavior. *J. Neurosci.* 31, 1471–1478. doi: 10.1523/jneurosci.4210-10.2011

Sturman, D. A., and Moghaddam, B. (2012). Striatum processes reward differently in adolescents versus adults. *Proc. Natl. Acad. Sci. U S A* 109, 1719–1724. doi: 10.1073/pnas.1114137109

Tunstall, B. J., and Kearns, D. N. (2015). Sign-tracking predicts increased choice of cocaine over food in rats. *Behav. Brain Res.* 281, 222–228. doi: 10.1016/j.bbr.2014.12.034

Versaggi, C. L., King, C. P., and Meyer, P. J. (2016). The tendency to sign-track predicts cue-induced reinstatement during nicotine self-administration, and is enhanced by nicotine but not ethanol. *Psychopharmacology* 233, 2985–2997. doi: 10.1007/s00213-016-4341-7

Westbrook, S. R., Hankosky, E. R., Dwyer, M. R., and Gulley, J. M. (2018). Age and sex differences in behavioral flexibility, sensitivity to reward value, and risky decision-making. *Behav. Neurosci.* 132, 75–87. doi: 10.1037/bne0000235

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: 26 February 2020
doi: 10.3389/fnbeh.2020.00027

Neonatal 6-OHDA Lesion Model in Mouse Induces Cognitive Dysfunctions of Attention-Deficit/Hyperactivity Disorder (ADHD) During Young Age

Otmane Bouchatta[1,2,3], Houria Manouze[1], Saadia Ba-M'Hamed[1], Marc Landry[2,3†] and Mohamed Bennis[1†]*

[1] Laboratory of Pharmacology, Neurobiology and Behavior, Faculty of Sciences, Cadi Ayyad University, Marrakesh, Morocco, [2] University of Bordeaux, Bordeaux, France, [3] CNRS UMR 5297, Centre Paul Broca-Nouvelle Aquitaine, Interdisciplinary Institute of Neuroscience, Bordeaux, France

OPEN ACCESS

Edited by:
Sean B. Ostlund,
University of California, Irvine,
United States

Reviewed by:
Susan Ferguson,
University of Washington,
United States
Almira Vazdarjanova,
Augusta University, United States
Roberto Frau,
University of Cagliari, Italy

***Correspondence:**
Mohamed Bennis
mbennis@uca.ma

[†] These authors share senior authorship

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

Received: 08 August 2019
Accepted: 05 February 2020
Published: 26 February 2020

Citation:
Bouchatta O, Manouze H, Ba-M'Hamed S, Landry M and Bennis M (2020) Neonatal 6-OHDA Lesion Model in Mouse Induces Cognitive Dysfunctions of Attention-Deficit/Hyperactivity Disorder (ADHD) During Young Age. Front. Behav. Neurosci. 14:27. doi: 10.3389/fnbeh.2020.00027

Attention-deficit/hyperactivity disorder (ADHD) is a syndrome characterized by impaired attention, impulsivity and hyperactivity in children. These symptoms are often maintained in adults. During adolescence, prefrontal cortex develops connectivity with other brain regions to engage executive functions such as, latent inhibition, attention and inhibitory control. In our previous work, we demonstrated the validity of the neonatal 6-Hydroxydopamine (6-OHDA) mouse model, a classical neurodevelopmental model mimicking major symptoms of the human ADHD pathology. In order to evaluate pathological forms of executive functions and impulsive behavior in 6-OHDA mice during young age, we first tested latent inhibition (LI) after weaning, and then we evaluated the impulsive behavior using a cliff avoidance reaction test. Our results demonstrated that 6-OHDA mice showed disruption in latent inhibition, suggesting a deficit in selective attention, and displayed repetitive peering-down behavior, indicating a maladaptive impulsive behavior. Subsequently, to assess impulsivity and attention in young mice, we performed a modified 5-choice serial reaction time task test (5-CSRTT), optimizing the degree of food restriction for young animals and shortening the training duration. This test allowed us to demonstrate a deficit in inhibitory control and a loss of accuracy of 6-OHDA mice in the 5-CSRTT. In conclusion, we demonstrated that the 6-OHDA mouse model reproduces human symptoms of ADHD in childhood and early adulthood periods, as seen in human. Taken together, the 6-OHDA mouse model will be useful alongside other animal models to understand the neurobiological mechanisms underlying complex, heterogeneous neurological disorders.

Keywords: attention-deficit/hyperactivity disorder, 6-hydroxydopamine, executive functions, latent inhibition, attention, inhibitory control

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a developmental disorder identified particularly by hyperactivity, impulsivity and inattention (American Psychiatric Association, 2013) in children. It is well known that ADHD patients exhibit impairments across a range of cognitive abilities, such as learning performance (i.e., latent inhibition), executive functions, novelty-seeking and exploratory activity, and short-term memory (Lubow and Josman, 1993; Faraone and Biederman, 2005; Lubow et al., 2005; Arnsten, 2009; Lubow et al., 2014; Fried et al., 2016). In addition, teens with ADHD exhibit often emotional immaturity and tend to feel more comfortable interact with younger children (Stanford and Tannock, 2012). Their affective status is poorly controlled, they often display exaggerate negative or positive reactions that are unrelated to the situation, and become easily frustrated, irritable and angry (Barkley et al., 2010). ADHD is present in children and continues into adolescence and adulthood in up to half of diagnosed cases (Barkley and Murphy, 1998).

Various animal models have been developed for modeling the neurodevelopmental alterations that occur in ADHD. The most studied ADHD animal models are: spontaneously strained rat (SHR), coloboma mutant mouse, dopamine transporter knockout/down mouse (DAT-KO), and neonatal rat damaged by 6-hydroxydopamine. (i) The use of SHRs as a model of ADHD in the 1990s is linked to their hyperactivity (Wultz et al., 1990; Sagvolden et al., 1992). However, the hyperactivity in the animal is not systematically considered as a model of ADHD (Stanford and Tannock, 2012). Subsequent studies using behavioral tests showed inattention and impulsivity in this SHR model (Evenden and Meyerson, 1999; De Bruin et al., 2003; Jentsch, 2005; Bizot et al., 2007; Fox et al., 2008). (ii) Coloboma mice which have a mutation in the Snap25 sequence are hyperactive (Hess et al., 1996), exhibit also an impairment of latent inhibition, indicating inattention (Lubow and Josman, 1993; Bruno et al., 2007). In addition, this animal model displays also impulsive behavior as demonstrated by delayed reward paradigms which require subjects to choose between an immediately available small reward or a delayed greater reward (Bruno et al., 2007). (iii) In DAT-KO mice, and in addition to the hyperactivity showed by Giros et al. (1996), impulsivity and a decrease in learning performance and memory are described (Gainetdinov et al., 1999; Li et al., 2010). (iv) The neonatal 6-OHDA-lesioned rat model of ADHD has been developed since 1976 by Shaywitz et al. (1976) by selective chemical lesion of dopaminergic neurons in 5-day-old rats. At 2–3 weeks following the lesion, these rats exhibit hyperactivity comparable to that observed in childhood ADHD (Erinoff et al., 1979; Miller et al., 1981; Archer et al., 1988), but there are not impulsive (Arime et al., 2011). Furthermore, hyperactivity in this model has sometimes been associated with inattention (Oke and Adams, 1978; Archer et al., 1988). However, a comprehensive assessment of ADHD-like symptoms is still missing, and data in mouse remain largely unavailable. In our previous work (Bouchatta et al., 2018), we demonstrated the validity of the neonatal 6-OHDA-lesioned mouse model to mimic human ADHD syndrome. At a juvenile stage, they are hyperactive in a novel environment, and exhibit inattention and impulsive-like behavior in adulthood. In addition, we have also shown that this model presents also comorbid symptoms such as learning and memory deficits, antisocial and aggressive behaviors, and a high level of anxiety. However, the executive functions such as latent inhibition, attention and impulsivity have not been systematically investigated during young age in this 6-OHDA model.

Several operant tasks have been developed to assess and highlight the underlying mechanisms of the deficits exhibited by children with ADHD at a preclinical level (Carli et al., 1983; Robbins, 2002). The latent inhibition test is based on the fact that the pre-exposure of a normal animal to a stimulus without reinforcement, makes it indifferent, and delays subsequent conditioning to the same stimulus. This can be explained by an attentional filtration which decreases the attention to an usual stimulus (Matsuo et al., 2009). Impulsivity is characterized by uncontrolled behaviors that are premature, inappropriate and/or irrepressible (Eagle and Baunez, 2010). In animals, the cliff avoidance reaction (CAR) test refers to their innate avoidance reaction to a potential fall from a height. Impaired RCA indicates inadequate impulsive behaviors among adult rodents (Matsuoka et al., 2005; Kumakura et al., 2010; Kuroda et al., 2011) which indicates a deficient behavioral inhibition. The 5-choice serial reaction time task (5-CSRTT) has been used widely to evaluate both attention and impulsivity in adult rodents (Robbins, 2002). It was adapted from Leonard's five-choice serial reaction task originally designed to assess attentional processes in humans (Wilkinson, 1963). However, the 5-CSRTT cannot be simply extrapolated to adolescent animals for many reasons. First, the tasks usually take months to complete. However, adolescence covers only a few weeks in rodents (Barr et al., 2008), restraining the applicability of this operant task only to adult subjects. Second, the normal food restriction procedure in 5-CSRTT, which is applied to motivate animals to perform the task, could disrupt the normal growth of mice during adolescence and can affect impulsive behavior (Robbins, 2002). For these reasons, the 5-CSRTT needs to be adapted to reliably test impulsivity and inattention in young mice.

The aim of the present study is to evaluate executive functions in 6-OHDA mice between juvenile and young adult periods. We demonstrated a disruption in latent inhibition and impulsive CAR behavior in 6-OHDA juvenile mice. Moreover, we adapted the 5-CSRTT protocol to assess attention and impulsivity during the adolescence-like period in mice. We manipulated the inter-trial interval (ITI) and the stimulus duration (SD) to produce impulsive responding and engage the attention, respectively, upon a stable performance. Young 6-OHDA adult mice showed a significant decrease in accuracy, when attention was tested. Moreover, they also showed more premature responses than sham mice at ITI challenges, indicating a deficit in inhibitory control. In conclusion, our data suggest that young 6-OHDA mice exhibit a comprehensive set of behavioral deficits consistent with ADHD.

MATERIALS AND METHODS

Animals

We used 40 Swiss male mice, bred in the central animal facility of Cadi Ayyad University, Marrakech, Morocco, with water and food *ad libitum*. Pups were housed with their mothers in litters and kept under constant temperature conditions (22°C ± 2), under a 12h light/12 h dark cycle (with light on at 7 am). The study received approval of the Council Committee of the research laboratories of the Faculty of Sciences, Cadi Ayyad University. All procedures were conducted in conformity with the approved institutional protocols and within the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive (EU2010/63). All efforts were made to minimize any animal suffering.

Neonatal 6-OHDA Lesion at P5

Intracerebroventricular injection of 6-OHDA was performed at P5 in an adapted platform fixed to a stereotaxic instrument (David Kopf instrument, Tujunga, CA, United States) according to Bouchatta et al. (2018) protocol. Briefly, male pups were injected by desipramine hydrochloride (20 mg/kg, s.c.; Sigma-Aldrich, France) as norepinephrine uptake blocker. 30 min later, pups were anesthetized with hypothermia induced by placing pups on ice for 1 min, and then following precise parameters (0.6 mm lateral to the medial sagittal suture, 2 mm rostral to the lambda and 1.3 mm in depth from the skull), they received into one of the lateral ventricles 25 μg of 6-OHDA hydrobromide (Sigma-Aldrich) dissolved in 3 μl of ascorbic acid 0.1%, at 1.5 μl/min, whereas control mice received vehicle. Injections were performed manually using a 30G needle (Carpule, Bayer; Osaka, Japan) connected to a 25 μl Hamilton syringe. After the injection, the pups were warmed up at 37°C, and returned to their mothers until weaning.

Behavioral Test

All behavioral tests were performed for all animals (sham = 20; 6-OHDA = 20) between 8:00 and 12:00 a.m. to prevent any circadian related fluctuation with the performance of the animals. The behavioral tests were performed as follow: latent inhibition test [postnatal day (PND)21 and 22], cliff avoidance reaction test (PND 24) and the 5-CSRTT (from PND 26 to 70) (**Figure 1**). Before each test and in order to remove any trace of odor, the apparatus was cleaned with a 75% ethanol solution.

Latent Inhibition (LI) Test

The protocol was set as previously described (Matsuo et al., 2009). On the first day, each mouse was placed in a training apparatus. Experimental (sham and 6-OHDA) mice were separated into two groups: pre-exposed (P) group and non-pre-exposed (NP) group. The P group (n = 10) received 40 white noise tones (55 dB, 5 s duration, 25 s inter-stimulus interval), while the NP group (n = 10) received no stimulus during an equivalent period. After, tone-shock associations consisting of a 5 s tone co-terminating with a 2 s foot shock at 0.25 mA were delivered to both groups with a 25 s inter-stimulus interval. All mice were exposed to 3 tone-shock pairings. The mice were submitted to the tone (CS), and during the last second of the tone they received a footshock (US). At 5 min after the CS–US pairing, the CS–US pairing was carried out again. Mice were returned to the home cage 25 s later. On day 2, the mice were placed back in the conditioning chamber for 5 min and the freezing due to the contextual recall was recorded. On the same day, the mice were put in another box (35 × 35 × 40 cm) made of white opaque Plexiglas and after 180 s, a 180 s tone was delivered to measure cued freezing.

Cliff Avoidance Reaction (CAR) Test

CAR was evaluated using a round wooden platform (diameter 20 cm; thickness 2 cm), fixed on an iron rod 50 cm high (Yamashita et al., 2013). The test was initiated by gently placing the animal on the platform. The CAR was considered altered when the animal fell from the platform and the latency of the fall was recorded. The incidence of altered CAR was calculated as a percentage index for each group:% (CAR) {the number of intact CAR mice (which did not fall from the platforms)/total number of mice tested} × 100. After each fall, the mice were immediately returned to the platform, and the test was continued until 60 min had passed. In mice, which did not fall from the platforms, they were also tested for 60 min.

5-Choice Serial Reaction Time Task (5-CSRTT)

Apparatus

Mice were trained in computer-controlled operant chambers (24 × 20 × 15 cm) placed inside ventilated sound-attenuating compartment (Med Associates Inc., St. Albans, VT, United States) as described previously (Bouchatta et al., 2018).

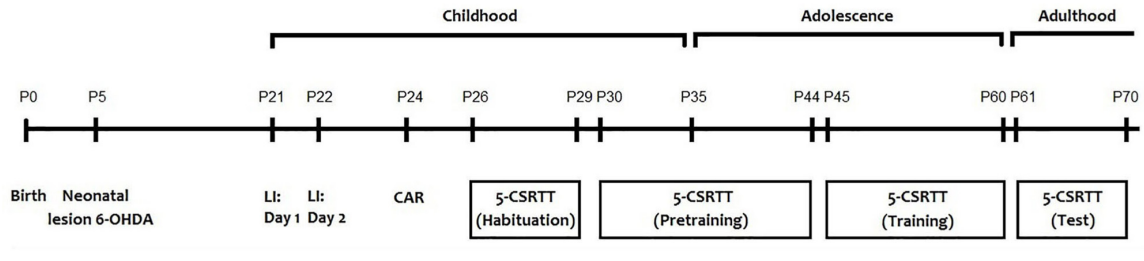


FIGURE 1 | Schematic representation of the experimental design.

Initial Handling and Feeding Protocol

Mice underwent 1-min of handling on PNDs 26, 27, 28, and 29 until they are completely habituated to being picked up (**Figure 1**). Twenty-four hours before the first training session on PND 30, available food was restricted to 1.0 g. During the 5-CSRTT training period, mice were given a diet as follows: providing 2.0 g food (3 weeks old), 2.5 g food (4 weeks old), 2.8 g food (5–7 weeks old), and 2.4 g food (8–9 weeks old). Eight hours before the training session, any remaining food was removed.

Methodological Approach

In a first training phase (one session), mice were placed in the chambers for 15 min with the house-light off. During this time, the pellets dispenser containing 15 food pellets was open in order to familiarize mice to eat the reinforcer in the magazine. In a second phase, the lighthouse was turned on, and mice were submitted to 2 training sessions (20 min per session) in which 20 food pellets were delivered in the magazine according to a variable time schedule (mean = 60 s). On the first session, the panel was blocked in order to maintain the food dispenser open. For all other following sessions, mice had to push away the panel in front of the food dispenser to receive the food pellet. During these two phases, each hole was covered by a metal cover. In a third phase, the house light was off, the central hole (hole 5) was illuminated, and accessible for the entire duration of the session (30 min). Each time the mouse introduced its nose into the illuminated hole (nose-poke), a food pellet was provided in the magazine. This training was maintained until the mice reached at least 50 nose-pokes during the session. Subsequently, mice were trained to react to a brief visual stimulus delivered randomly in one of the five spatial locations (holes 1, 3, 5, 7, 9), as previously described (Harati et al., 2011). This starts the inter-trial interval (ITI; 5 sec standard conditions). The food reinforcer is delivered when the subject nose pokes correctly within 5 s of extinguishing the light stimulus. The following trial is initiated upon exiting the food magazine. Once the mouse correctly responded to the illuminated hole, a reward pellet was delivered. Responses to non-illuminated holes had no consequence. If the subject's nose stings incorrectly or fails to respond within the limited 5-second timeout (considered as an omission), then the house light is turned on. If the animal pokes during the ITI, this is considered as a premature response, and the house light is illuminated. Subsequently, the subject must press the panel to start a new trial. When the basic performances are stabilized, we manipulate different tasks in order to modify the behavior of the mice. The light stimuli were presented in a pseudo-random manner (up to a maximum of 100 presentations). Initially, the stimulus duration was set to a long duration to facilitate learning (e.g., 32 s). During the subsequent sessions, the stimulus duration was progressively reduced (32, 16, 8, 4, 2, 1.8, 1.6, 1.4, 1.2, 1.0 s) until reaching the baseline value (0.8 s). The mice move to the following training level when they meet performance criteria (i.e., >70 trials, response latencies equivalent to or shorter than the stimulus duration, and $>80\%$ accuracy and $<20\%$ omissions; see **Figure 4**) during two consecutive sessions. The ITI duration and the length of the limited hold (the period after the extinction of the light stimulus, during which the subject can nose poke for a reward) were maintained unchanged during training (all 5 s).

Each session must be preceded by three consecutive days of stable basic performance respecting the criteria as indicated above. The altered duration of the ITI increases attentional load by disrupting the temporal predictability of the stimulus onset. Short ITI is in the range of 2–5 s, whereas long ITI is between 5 and 8 s. With an increase of the ITI duration, mice show increase in levels of premature responses that are independent of discriminative accuracy. In addition, increasing (from 0.8 to 2 s); or decreasing (from 0.8 to 0.2 s), the stimulus duration modulates attentional load.

The essential measures of performance are:

- The number of sessions at each training level defined by the stimulus duration levels.
- The accuracy of responding, defined as the number of correct commissions (correct responses/correct and incorrect responses).
- The total number of sessions to reach the baseline at 0.8 s stimulus duration.
- The number of premature nose pokes (the number of responses made during the ITI).
- The percentage of correct, incorrect, and omitted trials.
- The correct and incorrect reaction times (defined as the latency to respond in a hole after the stimulus light had been illuminated).

Statistical Analysis

Statistical analyses were conducted with SigmaPlot 11.0 software (SigmaStat, Systat Software Inc, San Jose, CA, United States). Homoscedasticity of all data sets were confirmed by using the Levene test, and thus parametric statistics were used in all cases. For the cliff avoidance test, the chi-square and Student's *t*-tests were used to compare between Sham and 6-OHDA groups. In addition, the three-way repeated measures ANOVA were used followed by a Tukey *post hoc* test to evaluate the difference between groups in the latent inhibition test. For each parameter of 5-CSRTT, the two-way repeated measures ANOVA followed by a Tukey *post hoc* test for multiple comparisons was performed. Results were presented as mean $\pm$ standard error of the mean (SEM), and significance was reported at $p < 0.05$.

RESULTS

To confirm that 6-OHDA mice display neurochemical features of ADHD and especially dopamine depletion, we examined TH-immunoreactivity (IR) in the striatum of sham and 6-OHDA adolescent mice.

We found a strong loss of TH-IR fibers in the striatum of 6-OHDA mice (**Supplementary Figure S1A**). Statistical analysis revealed that the 6-OHDA groups showed a significant decrease in the intensity of TH immunolabelling in comparison to sham ($p < 0.001$; **Supplementary Figure S1B**). In addition, TH-immunopositive area was significantly reduced in the striatum of 6-OHDA groups compared to sham ($p < 0.001$; **Supplementary Figure S1C**).

Abnormal Latent Inhibition in 6-OHDA Mice

The statistical analysis with the three-way ANOVA repeated measure demonstrated a significant effect of lesion [$F_{(1,396)} = 118.20$; $F_{(1,45)} = 13.21$; $p < 0.001$ and $F_{(1,54)} = 4.36$; $p < 0.05$; respectively], exposition to the tone [$F_{(1,396)} = 136.20$; $F_{(1,45)} = 49.57$ and $F_{(1,54)} = 33.05$; $p < 0.001$; respectively] and the time [$F_{(21,396)} = 60.96$; $F_{(4,45)} = 16.50$ and $F_{(5,54)} = 169.7$; $p < 0.001$; respectively] in the freezing percentage during conditioning (**Figure 2A**), the contextual recall (**Figure 2B**) and the cued tests (**Figure 2C**). In addition, the interaction Sham-lesion × P-NP × time had a significant effect in conditioning [$F_{(21,396)} = 3.51$], while in the contextual recall and the cued tests [$F_{(4,45)} = 0.14$; $F_{(5,54)} = 0.16$; $p > 0.05$; respectively] had no effect.

On one hand, the *post hoc* analysis indicated that sham mice pre-exposed (P) to the tone freeze less than non-pre-exposed (NP) animals during conditioning (from session 12: $q = 6.39$, $p < 0.05$ to session 19: $q = 9.59$, $p < 0.001$; **Figure 2A**) and the contextual recall test (session 2: $q = 5.20$, $p < 0.05$ and session 3: $q = 5.89$, $p < 0.01$; **Figure 2B**), indicating significant latent inhibition in sham mice. In contrast, the freezing behavior of pre-exposed 6-OHDA mice was not different from non-exposed 6-OHDA mice during conditioning, the context test, and the cued test ($p > 0.05$; **Figure 2C**), suggesting a deficit of 6-OHDA mice in latent inhibition and therefore poorly sustained attention. On the other hand, the *post hoc* analysis showed a significant decrease of the freezing behavior in the 6-OHDA NP group in comparison to the sham NP group during conditioning (from session 10: $q = 9.16$, $p < 0.001$ to session 21: $q = 8.77$; $p < 0.001$; **Figure 2A**) and the contextual recall test at session 5 ($q = 8.66$, $p < 0.001$; **Figure 2B**). However, there was no significant difference between sham and 6-OHDA groups during the cued test ($p > 0.05$; **Figure 2C**).

Impaired Cliff Avoidance Reaction in 6-OHDA Mice

A few minutes after the start of the test, sham mice bring their snouts close to the edge of the platform to examine it, avoiding falling. However, 6-OHDA mice repetitively investigate the edge of the platform and stay there longer. They tried to hang on the underside of the platform with their forelegs and often fell (**Figure 3A**; $t = 3.0$, $p < 0.01$). About 60% of 6-OHDA mice had impaired CAR during the 60 min test, while only 10% sham mice showed impaired CAR (**Figure 3B**; z = 2.34; $p < 0.05$, chi-square test). In addition, 6-OHDA mice fell from the platform within 20 min test period, but none of the sham mice fell (**Figure 3C**; $t = 10.74$; $p < 0.001$, Student's *t*-test).

Impaired Attention and Impulsivity in 6-OHDA Mice

The 5-CSRTT Training Effect on Body Weight

Our data indicated that there is no significant change in body weight between the experimental (sham and 6-OHDA) and control (free-fed) groups of the same age [**Figure 4A**; $F_{(2,18)} = 0.017$, $p > 0.05$].

Listed below are the results obtained for each of the commonly used tasks manipulations (**Figures 4–6**).

5-Choice Acquisition

We tested the different parameters of the 5-CSRTT acquisition in adolescent mouse. The stimulus duration (i.e., training level) is progressively shortened while the following parameters are assessed: number of sessions to maintain stable performance (**Figure 4B**), response accuracy (**Figure 4C**), the time needed for a correct reaction (**Figure 4D**), and percentage of omissions (**Figure 4E**). Two-way ANOVA repeated measures analysis was performed with lesion and training level as main factors. During the training, the results showed that the number of sessions, response accuracy, reaction time and omitted responses were affected by the lesion [$F_{(1,9)} = 192.70$; $F_{(1,9)} = 404.30$; $F_{(1,9)} = 450.10$ and $F_{(1,9)} = 354.20$; $p < 0.001$; respectively] and the training level [$F_{(9,81)} = 11.18$; $F_{(10,90)} = 6.01$; $F_{(10,90)} = 12.98$ and $F_{(10,90)} = 14.34$, $p < 0.001$; respectively]; while the interaction between the two factors was not affected [$F_{(9,81)} = 1.67$; $F_{(10,90)} = 0.09$; $F_{(10,90)} = 0.02$ and $F_{(10,90)} = 0.34$; $p > 0.05$; respectively]. In fact, the *post hoc* analysis showed a significant increase of the number of sessions in the 6-OHDA group in comparison to the sham group in all training levels (**Table 1**). Moreover, the reaction time and omitted responses were significantly increased in 6-OHDA mice as compared to the sham mice (**Table 1**). Meanwhile, the accuracy of responses was reduced significantly in the 6-OHDA group compared to the sham group (**Table 1**).

Long ITI

5-choice performance was assessed upon increasing ITI in young adult mouse. In all the parameters investigated [e.g., response accuracy (**Figure 5A**), reaction time (**Figure 5B**), omitted (**Figure 5C**), and premature responses (**Figure 5D**)], the two-way ANOVA repeated measures (lesion and ITI duration) revealed a significant effect of lesion [$F_{(1,9)} = 62.80$; $F_{(1,9)} = 77.96$; $F_{(1,9)} = 36.24$ and $F_{(1,9)} = 105.9$; $p < 0.001$; respectively] and ITI duration [$F_{(3,27)} = 7.43$; $F_{(3,27)} = 4.23$, $p < 0.05$; $F_{(3,27)} = 9.20$ and $F_{(3,27)} = 33.14$, $p < 0.001$; respectively] at the longest ITI duration, with no effect of the interaction of lesion × ITI duration [$F_{(3,27)} = 0.46$; $F_{(3,27)} = 0.08$; $F_{(3,27)} = 0.78$ and $F_{(3,27)} = 0.47$, $p > 0.05$; respectively). When the ITI was lengthened from 5 to 8 s, a significant increase in reaction time ($p < 0.05$), omitted ($p < 0.01$ and $p < 0.001$), and premature responses ($p < 0.001$) was observed in 6-OHDA mice (**Figures 5B–D**). Meanwhile, the 6-OHDA animals were significantly less accurate than sham animals from 5 ($p < 0.05$) to 8 s ($p < 0.01$) (**Figure 5A**). In addition, we did not observe difference on accuracy in sham mice when the ITI increased ($p > 0.05$); while at 8 s, the response accuracy was decreased significantly ($p < 0.05$) in 6-OHDA mice as compared to that observed at 5 s. The reaction time of both sham and 6-OHDA mice were significantly increased ($p < 0.05$) at 8 s as compared to 5 s. Indeed, the omitted and premature responses of the 6-OHDA group were increased significantly at 7 s ($p < 0.05$ and $p < 0.01$; respectively) and 8 s ($p < 0.01$ and $p < 0.001$; respectively) as compared to 5 s. However, those

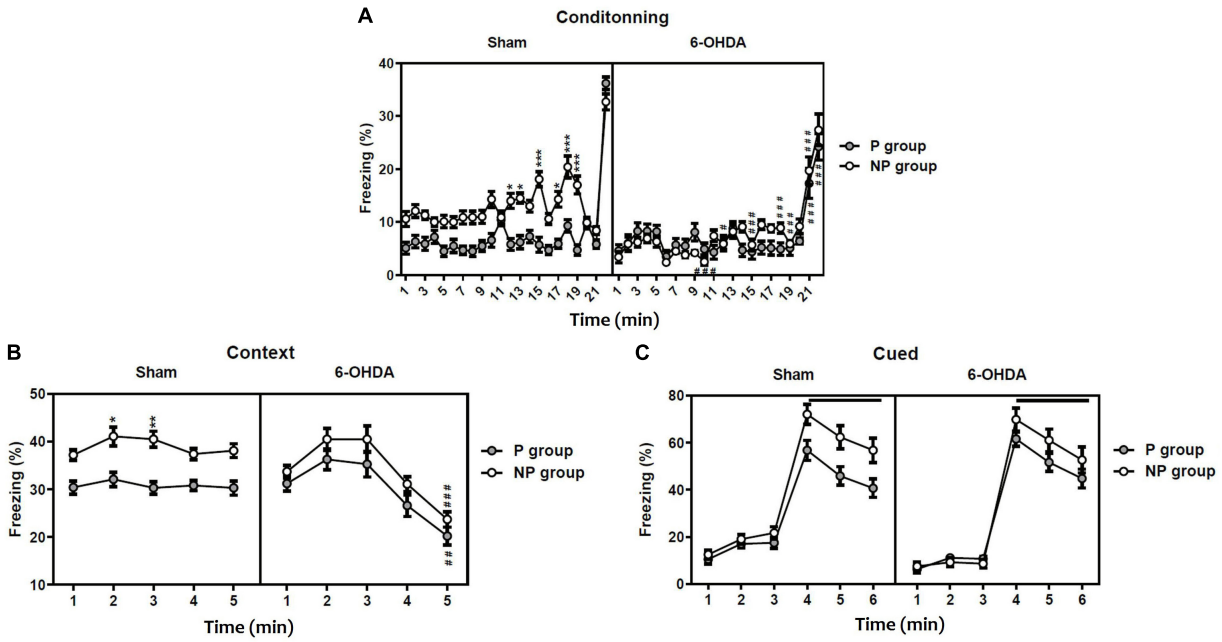


FIGURE 2 | Latent inhibition in sham and 6-OHDA mice. Percentage of freezing during conditioning **(A)**, contextual testing **(B)**, and cued testing **(C)**. Data is expressed as mean ± SEM, $n = 10$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with non-preexposed group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to 6-OHDA group (Three-way ANOVA followed by Tukey *post hoc* test). NP, non-pre-exposed; P, pre-exposed.

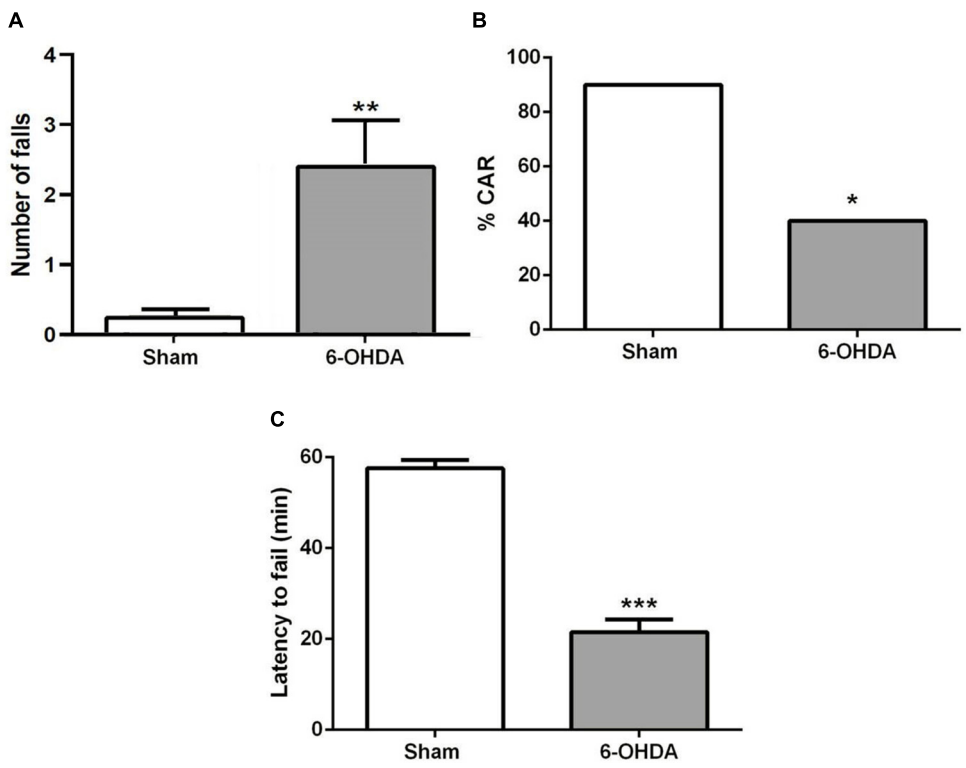


FIGURE 3 | (A) CAR in sham and 6-OHDA mice. Values represent the percentage of CAR. $^{*}p < 0.05$, compared with sham mice, $n = 10$ mice per group. **(B)** Latency from an initial placement on the platform until falling. **(C)** The latency to fail represented as mean ± SEM. $^{***}p < 0.001$ compared with sham mice, $n = 10$ mice per group. $^{**}p < 0.01$ compared with sham mice.

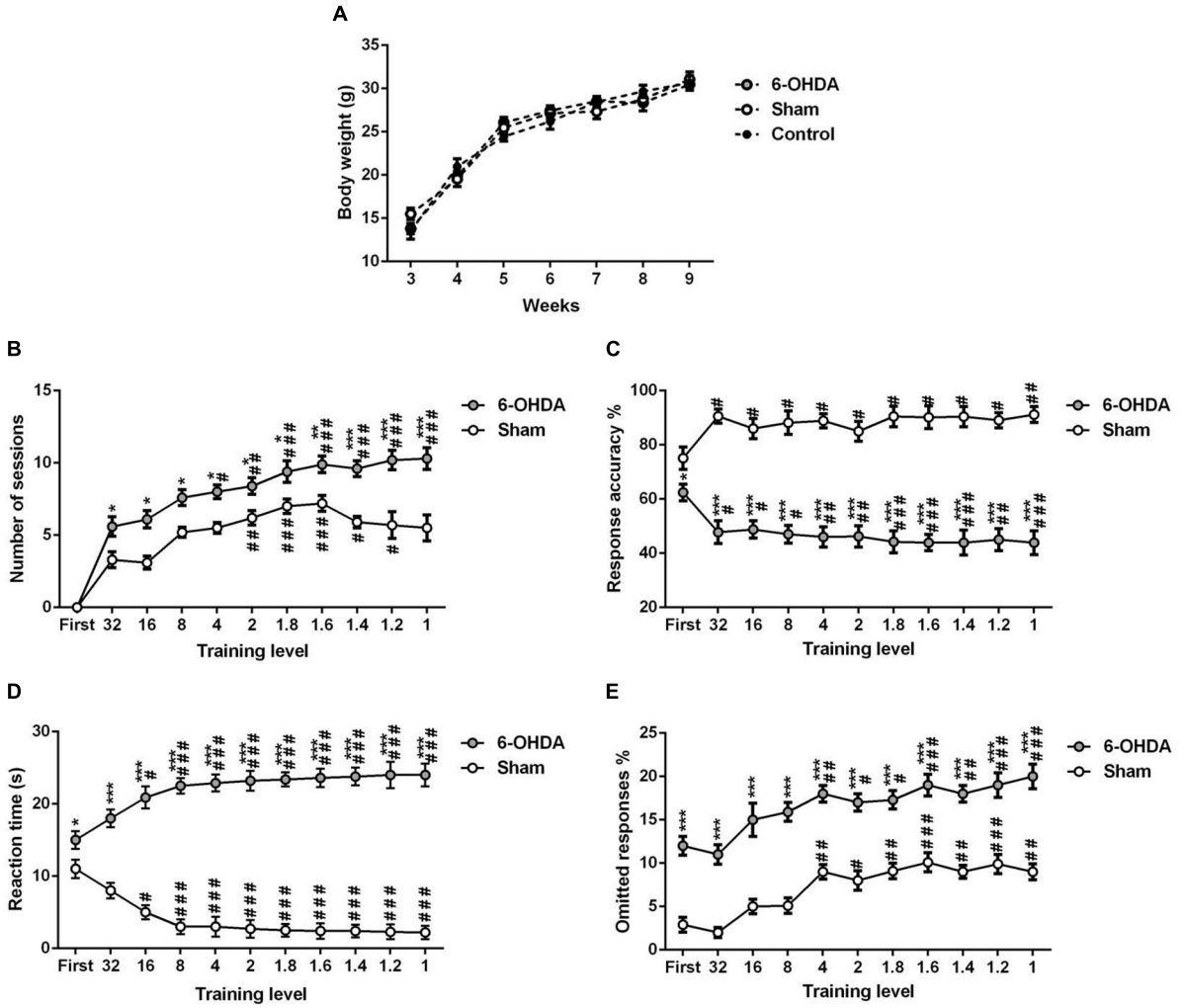


FIGURE 4 | 5-choice acquisition during adolescence period (ITI; 5 s standard conditions). Graphs show **(A)** body-weight of free-fed mice and food-restricted mice that underwent 5-CSRTT training, **(B)** number of sessions, **(C)** response accuracy, **(D)** correct reaction time, and **(E)** percentage of omitted responses at each training level during 5-choice acquisition in sham and 6-OHDA mice. Data is expressed as mean ± SEM, $n = 10$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. sham. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. 32 (Two-way ANOVA followed by Tukey *post hoc* test). ITI, intertrial interval.

parameters were increased significantly only at 8 s ($p < 0.01$ and $p < 0.001$; respectively) as compared to 5 s. in sham mice.

Reduced ITI

We measured the influence of decreasing the ITI in young adult mouse on the 5-choice performance [e.g., response accuracy (**Figure 6A**), response time (**Figure 6B**), and percentage of omission (**Figure 6C**)]. Two-way ANOVA repeated measures revealed that accuracy responses, reaction time and omitted responses were different between groups [$F_{(1,9)} = 285.80$, $F_{(1,9)} = 69.00$ and $F_{(1,9)} = 146.9$, $p < 0.001$; respectively] and varied with ITI duration [$F_{(3,27)} = 1.80$, $p < 0.05$; $F_{(3,27)} = 12.42$ and $F_{(3,27)} = 9.47$, $p < 0.001$; respectively]; while the interaction of group and ITI duration had no effect [$F_{(3,27)} = 0.51$; $F_{(3,27)} = 0.73$ and $F_{(3,27)} = 2.72$, $p > 0.05$; respectively]. The *post hoc* analysis showed a reduction of response accuracy in 6-OHDA group (5–4 s: $p < 0.01$ and 3–2 s: $p < 0.001$) at all ITI duration (5–2 s) as compared to the sham group. Meanwhile, from 5 until 2 s of ITI duration, the reaction time (5–4 s: $p < 0.05$ and 3–2 s: $p < 0.01$) and omitted responses (5–4 s: $p < 0.05$ and 3–2 s: $p < 0.001$) of the 6-OHDA group were higher when compared to the sham group (**Figure 6**). At 2 s ITI duration, the 6-OHDA mice were less accurate ($p < 0.05$), reacted more slowly ($p < 0.01$) and made more omission errors ($p < 0.001$) in comparison to the 5 s ITI duration. By contrast, only the reaction time at 2 s was increased ($p < 0.01$) in sham mice, while the accuracy and omitted responses at any ITI duration were not different from those of 5 s (**Figure 6**).

Reduced Stimulus Duration

The effect of shortening the stimulus duration was evaluated on the same parameters as above [e.g. response accuracy (**Figure 6D**), reaction time (**Figure 6E**), and omitted response

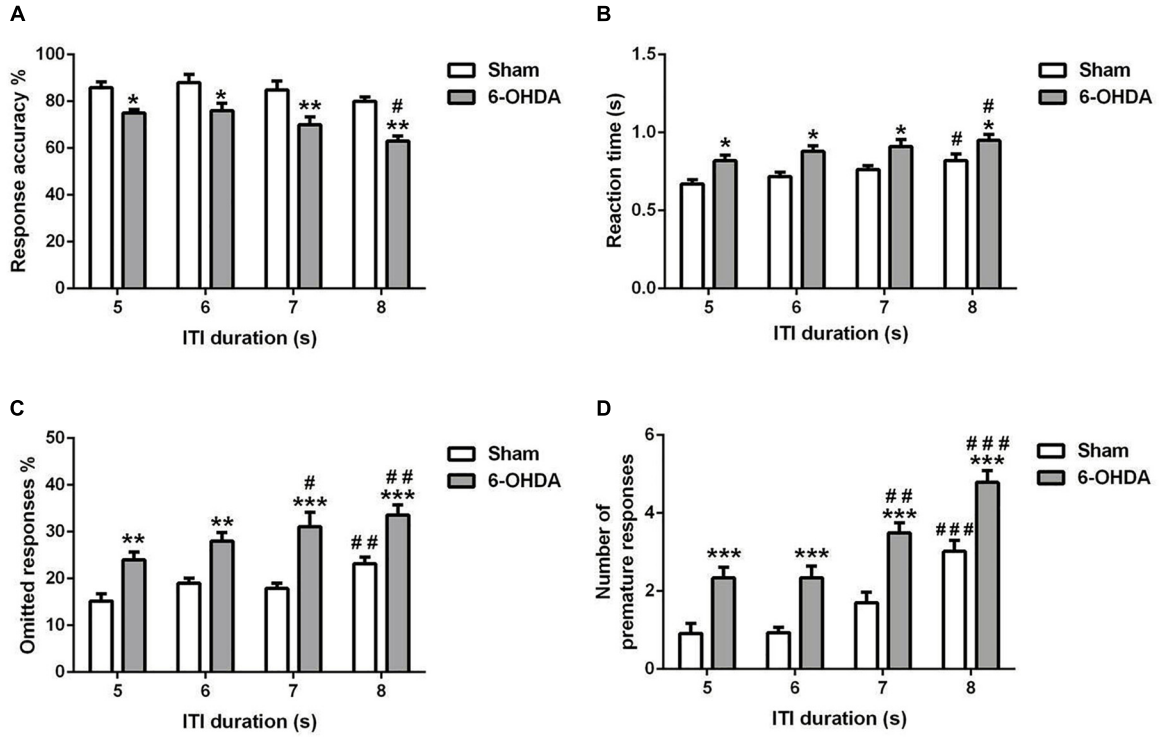


FIGURE 5 | Effects of increasing the ITI on the 5-choice performance during young adult period. Graphs show **(A)** response accuracy, **(B)** correct reaction time, **(C)** omission errors, and **(D)** premature responses at each of the ITI durations in sham and 6-OHDA mice. Data is expressed as mean ± SEM, $n = 10$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. ITI 5 s (Two-way ANOVA followed by Tukey *post hoc* test.

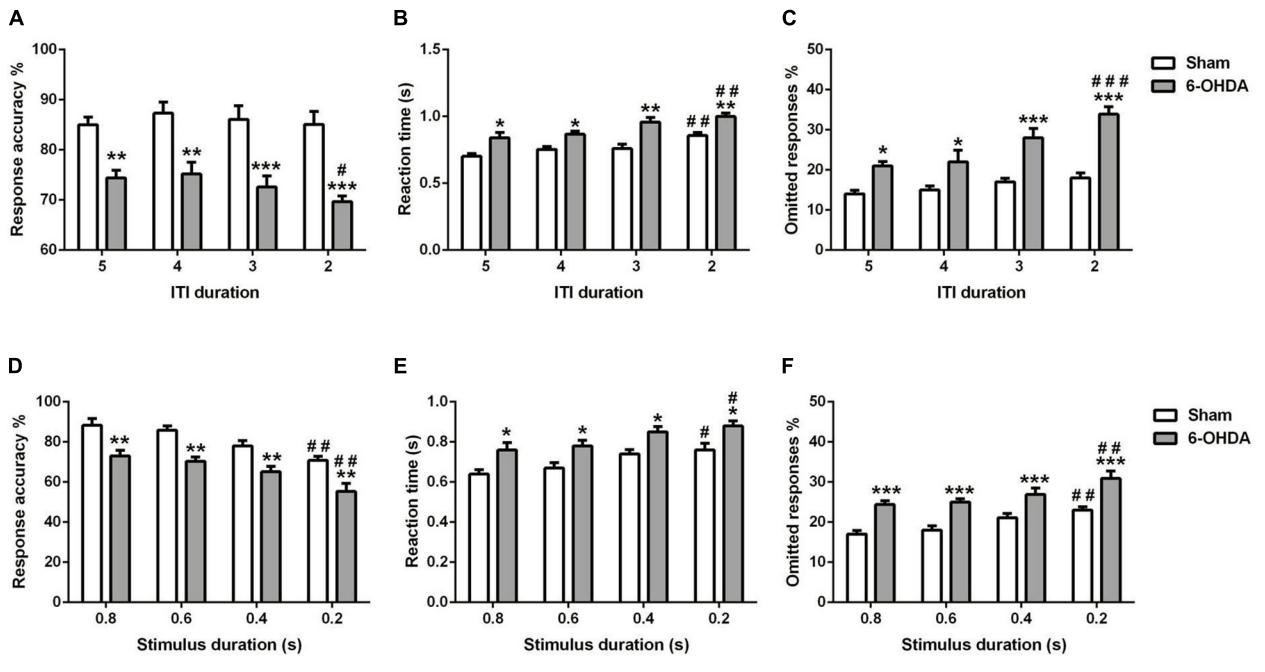


FIGURE 6 | Effects of decreasing the ITI **(A–C)** and SD **(D–F)** on 5-choice performance during young adult period. Graphs show **(A,C)** response accuracy, **(B,D)** correct reaction time, and **(C,F)** omission errors. Data is expressed as mean ± SEM, $n = 10$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. ITI 5 s or SD 0.8 s (Two-way ANOVA followed by Tukey *post hoc* test).

TABLE 1 | Statistical analysis of the mice groups effects (Sham/6-OHDA) and training level on number of sessions, response accuracy, reaction time, and omitted responses in 5-choice acquisition.

Parameters of the 5-CSRTT acquisition	Stimulus duration (seconds)	Groups		Two-way ANOVA with repeated measures	Tukey *post hoc*
		Sham	6-OHDA		6-OHDA vs. Sham
Number of sessions	First (60)	—	—		—
	32	3.30 ± 0.53 s	5.60 ± 0.67 s	Group effect:	$q = 3.12^{*}$
	16	3.10 ± 0.45 s	6.10 ± 0.60 s	$F_{(1,9)} = 192.7^{***}$	$q = 4.08^{*}$
	8	5.20 ± 0.35 s	7.60 ± 0.56 s	Level effect:	$q = 3.26^{*}$
	4	5.50 ± 0.37 s	8.00 ± 0.47 s	$F_{(9,81)} = 11.18^{***}$	$q = 3.40^{*}$
	2	6.20 ± 0.51 s	8.40 ± 0.58 s	Interaction:	$q = 2.99^{*}$
	1.8	7.00 ± 0.49 s	9.40 ± 0.74 s	$F_{(9,81)} = 1.67$	$q = 3.26^{*}$
	1.6	7.20 ± 0.55 s	9.90 ± 0.58 s		$q = 3.67^{**}$
	1.4	5.90 ± 0.40 s	9.60 ± 0.54 s		$q = 5.03^{***}$
	1.2	5.70 ± 0.91 s	10.20 ± 0.67 s		$q = 6.12^{***}$
	1	5.50 ± 0.90 s	10.30 ± 0.74 s		$q = 6.52^{***}$
Response accuracy	First (60)	75.10 ± 4.16 s	62.40 ± 3.00 s		$q = 3.17^{*}$
	32	90.60 ± 2.62 s	47.80 ± 4.25 s	Group effect:	$q = 10.70^{***}$
	16	86.00 ± 3.68 s	48.80 ± 3.20 s	$F_{(1,9)} = 404.3^{***}$	$q = 9.30^{***}$
	8	88.20 ± 4.32 s	47.00 ± 3.33 s	Level effect:	$q = 10.30^{***}$
	4	88.90 ± 2.61 s	46.00 ± 3.75 s	$F_{(10,90)} = 0.098$	$q = 10.73^{***}$
	2	85.00 ± 3.63 s	46.20 ± 3.90 s	Interaction:	$q = 9.70^{***}$
	1.8	90.50 ± 3.82 s	44.20 ± 4.09 s	$F_{(10,90)} = 6.00^{***}$	$q = 11.58^{***}$
	1.6	90.20 ± 4.23 s	43.90 ± 2.99 s		$q = 11.58^{***}$
	1.4	90.40 ± 3.69 s	43.90 ± 4.60 s		$q = 11.63^{***}$
	1.2	89.10 ± 2.77 s	45.00 ± 4.10 s		$q = 11.03^{***}$
	1	91.20 ± 2.87 s	43.90 ± 4.41 s		$q = 11.83^{***}$
Reaction time	First (60)	11.00 ± 1.25 s	15.00 ± 1.22 s		$q = 2.49^{*}$
	32	8.00 ± 1.08 s	18.00 ± 1.24 s	Group effect:	$q = 6.22^{***}$
	16	5.00 ± 0.97 s	20.90 ± 1.50 s	$F_{(1,9)} = 450.1^{***}$	$q = 9.90^{***}$
	8	3.00 ± 1.03 s	22.50 ± 1.07 s	Level effect:	$q = 12.14^{***}$
	4	3.00 ± 1.38 s	22.90 ± 1.18 s	$F_{(10,90)} = 0.016$	$q = 12.39^{***}$
	2	2.70 ± 1.21 s	23.20 ± 1.37 s	Interaction:	$q = 12.76^{***}$
	1.8	2.50 ± 0.88 s	23.40 ± 0.95 s	$F_{(10,90)} = 12.98^{***}$	$q = 13.01^{***}$
	1.6	2.40 ± 1.08 s	23.60 ± 1.29 s		$q = 13.20^{***}$
	1.4	2.40 ± 0.87 s	23.80 ± 1.20 s		$q = 13.32^{***}$
	1.2	2.30 ± 1.00 s	24.00 ± 1.81 s		$q = 13.51^{***}$
	1	2.20 ± 0.92 s	24.00 ± 1.59 s		$q = 13.57^{***}$
Omitted responses	First (60)	2.90 ± 0.86 s	12.00 ± 1.08 s		$q = 6.23^{***}$
	32	2.00 ± 0.59 s	11.00 ± 1.10 s	Group effect:	$q = 6.16^{***}$
	16	5.00 ± 0.81 s	15.00 ± 1.93 s	$F_{(1,9)} = 354.2^{***}$	$q = 6.84^{***}$
	8	5.10 ± 0.91 s	15.90 ± 1.07 s	Level effect:	$q = 7.39^{***}$
	4	9.00 ± 0.83 s	18.00 ± 0.96 s	$F_{(10,90)} = 14.34^{***}$	$q = 6.16^{***}$
	2	8.00 ± 1.11 s	17.00 ± 0.98 s	Interaction:	$q = 6.16^{***}$
	1.8	9.10 ± 0.91 s	17.30 ± 1.05 s	$F_{(10,90)} = 0.34$	$q = 5.61^{***}$
	1.6	10.10 ± 1.11 s	19.00 ± 1.23 s		$q = 6.09^{***}$
	1.4	9.00 ± 0.76 s	18.00 ± 0.96 s		$q = 6.16^{***}$
	1.2	9.90 ± 1.10 s	19.00 ± 1.40 s		$q = 6.16^{***}$
	1	9.00 ± 0.90 s	20.00 ± 1.41 s		$q = 7.53^{***}$

*The values reported are the mean ± SD (n = 10 per group). *p < 0.05 and ***p < 0.001 refers to the sham versus 6-OHDA group comparison.*

(**Figure 6F**)]. For all the parameters used, there is an effect of lesion [$F_{(1,9)} = 75.36$; $F_{(1,9)} = 67.06$; $F_{(1,9)} = 63.40$, $p < 0.001$; respectively] and stimulus duration [$F_{(3,27)} = 14.21$; $F_{(3,27)} = 9.28$; $F_{(3,27)} = 9.33$, $p < 0.001$; respectively]; with no effect of the interaction between those two factors [$F_{(3,27)} = 0.09$; $F_{(3,27)} = 0.02$; $F_{(3,27)} = 0.31$, $p > 0.05$; respectively]. The *post hoc* analysis showed that response accuracy was decreased significantly in both groups at 0.2 s of SD as compared to 0.8 s ($p < 0.01$; **Figure 6D**). Furthermore, the reaction time was increased at shortest SD (0.2 s) as compared to longest SD (0.8 s) in sham and 6-OHDA mice ($p < 0.05$; **Figure 6E**). However, the omitted response was increased when the 0.2 s of SD compared to 0.8 s in both of the groups ($p < 0.01$; **Figure 6F**).

DISCUSSION

In the present study, we demonstrated for the first time a disturbance in the latent inhibition and a poorly sustained attention in adolescent-like 6-OHDA mice, suggesting that not only adult, but also juvenile 6-OHDA mice show behavioral alterations associated with ADHD. Indeed, an alteration in the latent inhibition reflects a deficiency in selective attention, and a pathologically high tendency to replace or exchange the non-contingent association previously learned with the appropriate CS - US response (Hemsley, 1993). Even if no theory takes this phenomenon into account (Escobar et al., 2002), a disturbance in the latent inhibition is closely linked with the inattention/impulsivity distinctive of ADHD. In our study, 6-OHDA juvenile mice easily managed to associate the CS and US, as showed by the non-preexposed groups. This assumes that the impairment of the latent inhibition is probably not due to alteration in associative learning, but rather indicates deficit in selective attention.

In addition, we showed that 6-OHDA juvenile mice present highly impulsive behavior in the CAR test. However, optimizing the actions of animals requires serious control of their impulses. This control seems to be linked to distinct neuronal and neurochemical systems (Fineberg et al., 2010; Whelan et al., 2012). In addition, the CAR impairments seen in juvenile 6-OHDA mice may be contributed to repetitive exploratory behaviors due to persevering motor behavior.

In ADHD patients, both the attentional and impulse control deficits can be proved by the continuous performance task (CPT). In fact, because of their attention deficit, subjects with ADHD have slower and more variable reaction times and make more errors of omission (Epstein et al., 2003; Winstanley et al., 2006). These patients also exhibit reduced behavioral inhibition demonstrated by their high score of error commissions. In addition, high levels of impulsivity are determined in ADHD patients by numerous tests (Solanto, 1998; Winstanley et al., 2006). 5-CSRTT is a test usually used in rodents that can show behavioral inhibition aspect (Carli et al., 1983). In fact, the 5-CSRTT (Robbins, 2002) and 3-choice serial reaction time task (3-CSRTT) (Tsutsui-Kimura et al., 2009) has been usually used to study impulsivity in adult rats and mice. In addition, the main disadvantage of 5-CSRTT is that it lasts months to finished the mice training and reach stable performance levels. Another disadvantage of the 5-CSRTT is the mild food restriction used to motivate task performance (Asinof and Paine, 2014). In fact, this motivation to respond decreases as the session continues, since the subjects become full, and consequently their performance can be affected (Grottick and Higgins, 2002). However, pre-feeding subjects before the test is a way to determine whether satiety is involved in the effects of a particular manipulation (Grottick and Higgins, 2002; Bari et al., 2008; Nemeth et al., 2010). By cons, several methods have been recently developed to assess attention and impulsivity in mice (Remmelink et al., 2017; Bruinsma et al., 2019). The author's performed their method with particular equipment (The CombiCage and self-paced 5-CSRTT protocol) that allowed mice to learn fast. In their protocols, mice had 24-h/day continuous task access, during which they could earn unlimited food rewards based on tasks, and achieve task progress at their own pace. This free access to the task can induce an automation of the performance or usual responses; which could make the performance on the standard task unchangeable to particular manipulations.

In this study, we adapted the 5-CSRTT protocol in order to assess attention and inhibitory control during the adolescence-like period in mice. First, we controlled the food restriction in the youngest mice, to allow them to have a normal growth and to be sufficiently motivated to accomplish tasks. Thus, we have shown that this diet adopted during all the training phases allowed an almost normal development (**Figure 4**). In addition, the duration of training was similar to that of adult mice reported in our previous study (Bouchatta et al., 2018). These results prove that the diet applied to young mice induced sufficient motivation to acquire the tasks, without stunting. Second, we reduced the number of sessions needed to terminate the training. Third, we carried out training sessions without sanction, allowing many opportunities to discover a brief light stimulus during the accustomed training procedure. In our adapted 5-CSRTT protocol, the mice were conditioned to successfully respond to a 1 s stimulus after just 6 weeks (**Figure 4**), which is shorter than other procedures (Humby et al., 2005). Our data confirmed that sham and 6-OHDA mice learned the complex 5-CSRTT task. Both groups made more than 50% of correct responses at the first stage of training (**Figure 4**). These results agree with a similar protocol using a 3-choice serial reaction time task (Sasamori et al., 2018).

Attention is most often evaluated using the percentage of response omissions (Robbins, 2002; Amitai and Markou, 2011) as well as by the responses accuracy (Robbins, 2002; Bari et al., 2008; Amitai and Markou, 2011). In addition, it has been shown that this accuracy is not affected by locomotor ability, motivation or sedation (Asinof and Paine, 2014). For the first time, we demonstrated that sham and 6-OHDA adolescent-like mice exhibit different performance in the 5-CSRTT, especially when attentional demands are high.

This difference between sham and 6-OHDA lesioned mice observed throughout the session, would underlie the presence of a deficit of selective attention and difficulties in keeping sustained attention, similar to human situations. Various modifications of the parameters raise the attention demands of the 5-CSRTT, e.g. shortening the ITI or decreasing the duration of the stimulus (**Figure 6**), and indicate that young adult 6-OHDA mice exhibited a larger drop in precision when attention was tested. Response inhibition can be easily tested in the 5-CSRTT to assess impulsivity by increasing the extent of the ITI. Premature responses are those occurring during the ITI before the stimulus presentation, and lengthening the ITI leads to a consistent increase in the number of these premature responses. Premature responses are a form of impulsive behavior and represent failures in impulse control (Robbins, 2002; Bari et al., 2008) that reflects a lack of response inhibition (Evenden, 1999; Robbins, 2002). Taken together, we demonstrated that young adult 6-OHDA mice

showed a disturbance in the inhibitory control on the 5-CSRTT, as expressed by the increase of premature responses during the inter-trial interval task (**Figure 5**).

ADHD result from dopamine (DA) system dysfunction of certain cortical structures such as the prefrontal cortex, mainly the right-medial side (Sullivan and Brake, 2003), and subcortical areas, particularly the nucleus accumbens and the striatum (Russell et al., 2005). Neonatal 6-OHDA animal models showed a clear functional impairment of the dopaminergic system (Shaywitz et al., 1976; Luthman et al., 1989; Zhang et al., 2001; Moran-Gates et al., 2005). In addition, in rodents, the postnatal development of the nigrostriatal neuronal DAergic activity described during the first 2 weeks is more important for the final development of excitatory synapses in the corticostriatal pathway by reducing the glutamate release (Choi and Lovinger, 1997). Consequently, an increase in glutamatergic transmission would be obtained during a selective disturbance of this DAergic pathway during this critical period (Tang et al., 2001). It is therefore conceivable that the behavioral characteristics of ADHD could result from an alteration in dopaminergic modulation of neurotransmission in the cortico-striato-thalamo-cortical circuits.

CONCLUSION

The present study demonstrated defects in latent inhibition and poorly sustained attention, suggesting that 6-OHDA mice display supplementary behavioral impairments associate with ADHD. Moreover, 6-OHDA mice show inadequately impulsive behavior in the CAR test. We can then suppose that attentional deficits highlighted by 5-CSRTT could in part be due to this impulsive behavioral disturbance. However, we were able successfully to surmount the limitations of in effect, our modified 5-CSRTT protocol prevented growth disruptions and significantly reduced the training duration, allowing us to assess attention and impulsivity in mice adolescence. Therefore, it is now possible to assess parameters of neurodevelopmental disorders in rodent models in conditions that are close to the human situation. The 6-OHDA mouse model will be useful in understanding and supporting the basic neurobiological mechanisms of this heterogeneous, and complex disorder at different periods of neurodevelopment.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Council Committee of Research Laboratories of the Faculty of Sciences, Cadi Ayyad University. All procedures were conducted in accordance with the approved institutional protocols and within the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive (EU2010/63).

AUTHOR CONTRIBUTIONS

OB, SB-M, ML, and MB conceived the experiments. OB, HM, and SB-M performed the experiments. OB, HM, SB-M, ML, and MB analyzed the data. OB, SB-M, ML, and MB wrote the manuscript.

ACKNOWLEDGMENTS

We thank the Campus France PHC Toubkal 32508 PH, for their support for internships in the joint supervision of the thesis and the NEUREN PIRSES-GA-2012-318997 and Mediterranean Neuroscience Society for their help in internships and participation in conferences.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00027/full#supplementary-material

REFERENCES

American Psychiatric Association (2013). *Diagnostic and Statistical Manual of Mental Disorders: DSM-5*, 5th Edn. Washington, DC: American Psychiatric Publishing.

Amitai, N., and Markou, A. (2011). Comparative effects of different test day challenges on performance in the 5-choice serial reaction time task. *Behav. Neurosci.* 125, 764–774. doi: 10.1037/a0024722

Archer, T., Danysz, W., Fredriksson, A., Jonsson, G., Luthman, J., Sundstrom, E., et al. (1988). Neonatal 6-hydroxydopamine-induced dopamine depletions: motor activity and performance in maze learning. *Pharmacol. Biochem. Behav.* 31, 357–364. doi: 10.1016/0091-3057(88)90358-9

Arime, Y., Kubo, Y., and Sora, I. (2011). Animal models of attention-deficit/hyperactivity disorder. *Biol. Pharm. Bull.* 34, 1373–1376.

Arnsten, A. F. (2009). Toward a new understanding of attention-deficit hyperactivity disorder pathophysiology. *CNS Drugs* 23, 33–41. doi: 10.2165/00023210-200923000-00005

Asinof, S. K., and Paine, T. A. (2014). The 5-choice serial reaction time task: a task of attention and impulse control for rodents. *J. Vis. Exp.* 90:51574. doi: 10.3791/51574

Bari, A., Dalley, J. W., and Robbins, T. W. (2008). The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. *Nat. Protoc.* 3, 759–767. doi: 10.1038/nprot.2008.41

Barkley, R. A., and Murphy, K. R. (1998). *Attention-Deficit Hyperactivity Disorder: A Clinical Workbook*. New York, NY: Guilford Press.

Barkley, R. A., Murphy, K. R., and Fischer, M. (2010). *ADHD in Adults: What the Science Says*. New York, NY: Guilford Press.

Barr, R. S., Culhane, M. A., Jubelt, L. E., Mufti, R. S., Dyer, M. A., Weiss, A. P., et al. (2008). The effects of transdermal nicotine on cognition in nonsmokers

with schizophrenia and nonpsychiatric controls. *Neuropsychopharmacology* 33, 480–490. doi: 10.1038/sj.npp.1301423

Bizot, J. C., Chenault, N., Houze, B., Herpin, A., David, S., Pothion, S., et al. (2007). Methylphenidate reduces impulsive behaviour in juvenile Wistar rats, but not in adult Wistar, SHR and WKY rats. *Psychopharmacology* 193, 215–223. doi: 10.1007/s00213-007-0781-4

Bouchatta, O., Manouze, H., Bouali-Benazzouz, R., Kerekes, N., Ba-M'hamed, S., Fossat, P., et al. (2018). Neonatal 6-OHDA lesion model in mouse induces attention-deficit/hyperactivity disorder (ADHD)-like behaviour. *Sci. Rep.* 8:15349. doi: 10.1038/s41598-018-33778-0

Bruinsma, B., Terra, H., de Kloet, S. F., Luchicchi, A., Timmerman, A. J., Remmelink, E., et al. (2019). An automated home-cage-based 5-choice serial reaction time task for rapid assessment of attention and impulsivity in rats. *Psychopharmacology* 236, 2015–2026. doi: 10.1007/s00213-019-05189-0

Bruno, K. J., Freet, C. S., Twining, R. C., Egami, K., Grigson, P. S., and Hess, E. J. (2007). Abnormal latent inhibition and impulsivity in coloboma mice, a model of ADHD. *Neurobiol. Dis.* 25, 206–216. doi: 10.1016/j.nbd.2006.09.009

Carli, M., Robbins, T. W., Evenden, J. L., and Everitt, B. J. (1983). Effects of lesions to ascending noradrenergic neurons on performance of a 5-choice serial reaction task in rats; implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal. *Behav. Brain Res.* 9, 361–380. doi: 10.1016/0166-4328(83)90138-9

Choi, S., and Lovinger, D. M. (1997). Decreased probability of neurotransmitter release underlies striatal long-term depression and postnatal development of corticostriatal synapses. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2665–2670. doi: 10.1073/pnas.94.6.2665

De Bruin, N. M., Kiliaan, A. J., De Wilde, M. C., and Broersen, L. M. (2003). Combined uridine and choline administration improves cognitive deficits in spontaneously hypertensive rats. *Neurobiol. Learn. Mem.* 80, 63–79. doi: 10.1016/s1074-7427(03)00024-8

Eagle, D. M., and Baunez, C. (2010). Is there an inhibitory-response-control system in the rat? Evidence from anatomical and pharmacological studies of behavioral inhibition. *Neurosci. Biobehav. Rev.* 34, 50–72. doi: 10.1016/j.neubiorev.2009.07.003

Epstein, J. N., Erkanli, A., Conners, C. K., Klaric, J., Costello, J. E., and Angold, A. (2003). Relations between continuous performance test performance measures and ADHD behaviors. *J. Abnorm. Child Psychol.* 31, 543–554.

Erinoff, L., MacPhail, R. C., Heller, A., and Seiden, L. S. (1979). Age-dependent effects of 6-hydroxydopamine on locomotor activity in the rat. *Brain Res.* 164, 195–205. doi: 10.1016/0006-8993(79)90015-5

Escobar, M., Oberling, P., and Miller, R. R. (2002). Associative deficit accounts of disrupted latent inhibition and blocking in schizophrenia. *Neurosci. Biobehav. Rev.* 26, 203–216. doi: 10.1016/s0149-7634(01)00067-7

Evenden, J., and Meyerson, B. (1999). The behavior of spontaneously hypertensive and Wistar Kyoto rats under a paced fixed consecutive number schedule of reinforcement. *Pharmacol. Biochem. Behav.* 63, 71–82. doi: 10.1016/s0091-3057(98)00222-6

Evenden, J. L. (1999). Varieties of impulsivity. *Psychopharmacology* 146, 348–361. doi: 10.1007/pl00005481

Faraone, S. V., and Biederman, J. (2005). What is the prevalence of adult ADHD? Results of a population screen of 966 adults. *J. Atten. Disord.* 9, 384–391. doi: 10.1177/1087054705281478

Fineberg, N. A., Potenza, M. N., Chamberlain, S. R., Berlin, H. A., Menzies, L., Bechara, A., et al. (2010). Probing compulsive and impulsive behaviors, from animal models to endophenotypes: a narrative review. *Neuropsychopharmacology* 35, 591–604. doi: 10.1038/npp.2009.185

Fox, A. T., Hand, D. J., and Reilly, M. P. (2008). Impulsive choice in a rodent model of attention-deficit/hyperactivity disorder. *Behav. Brain Res.* 187, 146–152. doi: 10.1016/j.bbr.2007.09.008

Fried, R., Chan, J., Feinberg, L., Pope, A., Woodworth, K. Y., Faraone, S. V., et al. (2016). Clinical correlates of working memory deficits in youth with and without ADHD: a controlled study. *J. Clin. Exp. Neuropsychol.* 38, 487–496. doi: 10.1080/13803395.2015.1127896

Gainetdinov, R. R., Wetsel, W. C., Jones, S. R., Levin, E. D., Jaber, M., and Caron, M. G. (1999). Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* 283, 397–401. doi: 10.1126/science.283.5400.397

Giros, B., Jaber, M., Jones, S. R., Wightman, R. M., and Caron, M. G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* 379, 606–612. doi: 10.1038/379606a0

Grottick, A. J., and Higgins, G. A. (2002). Assessing a vigilance decrement in aged rats: effects of pre-feeding, task manipulation, and psychostimulants. *Psychopharmacology* 164, 33–41. doi: 10.1007/s00213-002-1174-3

Harati, H., Majchrzak, M., Cosquer, B., Galani, R., Kelche, C., Cassel, J. C., et al. (2011). Attention and memory in aged rats: impact of lifelong environmental enrichment. *Neurobiol. Aging* 32, 718–736. doi: 10.1016/j.neurobiolaging.2009.03.012

Hemsley, D. R. (1993). A simple (or simplistic?) cognitive model for schizophrenia. *Behav. Res. Ther.* 31, 633–645. doi: 10.1016/0005-7967(93)90116-c

Hess, E. J., Collins, K. A., and Wilson, M. C. (1996). Mouse model of hyperkinesis implicates SNAP-25 in behavioral regulation. *J. Neurosci.* 16, 3104–3111. doi: 10.1523/jneurosci.16-09-03104.1996

Humby, T., Wilkinson, L., and Dawson, G. (2005). Assaying aspects of attention and impulse control in mice using the 5-choice serial reaction time task. *Curr. Protoc. Neurosci.* 31, 8.5H.1–8.5H.15. doi: 10.1002/0471142301.ns0805hs31

Jentsch, J. D. (2005). Impaired visuospatial divided attention in the spontaneously hypertensive rat. *Behav. Brain Res.* 157, 323–330. doi: 10.1016/j.bbr.2004.07.011

Kumakura, K., Nomura, H., Toyoda, T., Hashikawa, K., Noguchi, T., Takeda, K., et al. (2010). Hyperactivity in novel environment with increased dopamine and impaired novelty preference in apoptosis signal-regulating kinase 1 (ASK1)-deficient mice. *Neurosci. Res.* 66, 313–320. doi: 10.1016/j.neures.2009.12.003

Kuroda, K., Yamada, S., Tanaka, M., Iizuka, M., Yano, H., Mori, D., et al. (2011). Behavioral alterations associated with targeted disruption of exons 2 and 3 of the Disc1 gene in the mouse. *Hum. Mol. Genet.* 20, 4666–4683. doi: 10.1093/hmg/ddr400

Li, B., Arime, Y., Hall, F. S., Uhl, G. R., and Sora, I. (2010). Impaired spatial working memory and decreased frontal cortex BDNF protein level in dopamine transporter knockout mice. *Eur. J. Pharmacol.* 628, 104–107. doi: 10.1016/j.ejphar.2009.11.036

Lubow, R. E., Braunstein-Bercovitz, H., Blumenthal, O., Kaplan, O., and Toren, P. (2005). Latent inhibition and asymmetrical visual–spatial attention in children with ADHD. *Child Neuropsychol.* 11, 445–457. doi: 10.1080/09297040590951578

Lubow, R. E., and Josman, Z. E. (1993). Latent inhibition deficits in hyperactive children. *J. Child Psychol. Psychiatry* 34, 959–973. doi: 10.1111/j.1469-7610.1993.tb01101.x

Lubow, R. E., Kaplan, O., and Manor, I. (2014). Latent inhibition in ADHD adults on and off medication: a preliminary study. *J. Atten. Disord.* 18, 625–631. doi: 10.1177/1087054712445783

Luthman, J., Fredriksson, A., Lewander, T., Jonsson, G., and Archer, T. (1989). Effects of d-amphetamine and methylphenidate on hyperactivity produced by neonatal 6-hydroxydopamine treatment. *Psychopharmacology* 99, 550–557. doi: 10.1007/bf00589907

Matsuo, N., Tanda, K., Nakanishi, K., Yamasaki, N., Toyama, K., Takao, K., et al. (2009). Comprehensive behavioral phenotyping of ryanodine receptor type3 (RyR3) knockout mice: decreased social contact duration in two social interaction tests. *Front. Behav. Neurosci.* 3:3. doi: 10.3389/neuro.08.003.2009

Matsuoka, Y., Furuyashiki, T., Yamada, K., Nagai, T., Bito, H., Tanaka, Y., et al. (2005). Prostaglandin E receptor EP1 controls impulsive behavior under stress. *Proc. Natl. Acad. Sci. U.S.A.* 102, 16066–16071. doi: 10.1073/pnas.0504908102

Miller, F. E., Heffner, T. G., Kotake, C., and Seiden, L. S. (1981). Magnitude and duration of hyperactivity following neonatal 6-hydroxydopamine is related to the extent of brain dopamine depletion. *Brain Res.* 229, 123–132. doi: 10.1016/0006-8993(81)90750-2

Moran-Gates, T., Zhang, K., Baldessarini, R. J., and Tarazi, F. I. (2005). Atomoxetine blocks motor hyperactivity in neonatal 6-hydroxydopamine-lesioned rats: implications for treatment of attentiondeficit hyperactivity disorder. *Int. J. Neuropsychopharmacol.* 8, 439–444. doi: 10.1017/s1461145705005249

Nemeth, C. L., Paine, T. A., Rittiner, J. E., Béguin, C., Carroll, F. I., Roth, B. L., et al. (2010). Role of kappa-opioid receptors in the effects of salvinorin A and ketamine on attention in rats. *Psychopharmacology* 210, 263–274. doi: 10.1007/s00213-010-1834-7

Oke, A. F., and Adams, R. N. (1978). Selective attention dysfunctions in adult rats neonatally treated with 6-hydoxydopamine. *Pharmacol. Biochem. Behav.* 9, 429–432. doi: 10.1016/0091-3057(78)90036-9

Remmelink, E., Chau, U., Smit, A. B., Verhage, M., and Loos, M. (2017). A one-week 5-choice serial reaction time task to measure impulsivity and attention in adult and adolescent mice. *Sci. Rep.* 7:42519. doi: 10.1038/srep42519

Robbins, T. W. (2002). The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. *Psychopharmacology* 163, 362–380. doi: 10.1007/s00213-002-1154-7

Russell, V. A., Sagvolden, T., and Johansen, E. B. (2005). Animal models of attention-deficit hyperactivity disorder. *Behav. Brain Funct.* 1:9. doi: 10.1186/1744-9081-1-9

Sagvolden, T., Metzger, M. A., Schiørbeck, H. K., Rugland, A. L., Spinnangr, I., and Sagvolden, G. (1992). The spontaneously hypertensive rat (SHR) as an animal model of childhood hyperactivity (ADHD): changed reactivity to reinforcers and to psychomotor stimulants. *Behav. Neural Biol.* 58, 103–112. doi: 10.1016/0163-1047(92)90315-u

Sasamori, H., Ohmura, Y., Kubo, T., Yoshida, T., and Yoshioka, M. (2018). Assessment of impulsivity in adolescent mice: a new training procedure for a 3-choice serial reaction time task. *Behav. Brain Res.* 343, 61–70. doi: 10.1016/j.bbr.2018.01.014

Shaywitz, B. A., Yager, R. D., and Klopper, J. H. (1976). Selective brain dopamine depletion in developing rats: an experimental model of minimal brain dysfunction. *Science* 191, 305–308. doi: 10.1126/science.942800

Solanto, M. V. (1998). Neuropsychopharmacological mechanisms of stimulant drug action in attention-deficit hyperactivity disorder: a review and integration. *Behav. Brain Res.* 94, 127–152. doi: 10.1016/s0166-4328(97)00175-7

Stanford, C., and Tannock, R. (eds). (2012). *Behavioral Neuroscience of Attention Deficit Hyperactivity Disorder and its Treatment*, Vol. 9. Berlin: Springer Science & Business Media.

Sullivan, R. M., and Brake, W. G. (2003). What the rodent prefrontal cortex can teach us about attention-deficit/hyperactivity disorder: the critical role of early developmental events on prefrontal function. *Behav. Brain Res.* 146, 43–55. doi: 10.1016/j.bbr.2003.09.015

Tang, K. C., Low, M. J., Grandy, D. K., and Lovinger, D. M. (2001). Dopamine-dependent synaptic plasticity in striatum during *in vivo* development. *Proc. Natl. Acad. Sci.* 98, 1255–1260. doi: 10.1073/pnas.031374698

Tsutsui-Kimura, I., Ohmura, Y., Izumi, T., Yamaguchi, T., Yoshida, T., and Yoshioka, M. (2009). The effects of serotonin and/or noradrenaline reuptake inhibitors on impulsive-like action assessed by the three-choice serial reaction time task: a simple and valid model of impulsive action using rats. *Behav. Pharmacol.* 20, 474–483. doi: 10.1097/FBP.0b013e3283305e65

Whelan, R., Conrod, P. J., Poline, J. B., Lourdusamy, A., Banaschewski, T., Barker, G. J., et al. (2012). Adolescent impulsivity phenotypes characterized by distinct brain networks. *Nat. Neurosci.* 15, 920–925. doi: 10.1038/nn.3092

Wilkinson, R. T. (1963). Interaction of noise with knowledge of results and sleep deprivation. *J. Exp. Psychol.* 66, 332–337. doi: 10.1037/h0044161

Winstanley, C. A., Eagle, D. M., and Robbins, T. W. (2006). Behavioral models of impulsivity in relation to ADHD: translation between clinical and preclinical studies. *Clin. Psychol. Rev.* 26, 379–395. doi: 10.1016/j.cpr.2006.01.001

Wultz, B., Sagvolden, T., Moser, E. I., and Moser, M. B. (1990). The spontaneously hypertensive rat as an animal model of attention-deficit hyperactivity disorder: effects of methylphenidate on exploratory behavior. *Behav. Neural Biol.* 53, 88–102. doi: 10.1016/0163-1047(90)90848-z

Yamashita, M., Sakakibara, Y., Hall, F. S., Numachi, Y., Yoshida, S., Kobayashi, H., et al. (2013). Impaired cliff avoidance reaction in dopamine transporter knockout mice. *Psychopharmacology* 227, 741–749. doi: 10.1007/s00213-013-3009-9

Zhang, K., Tarazi, F. I., and Baldessarini, R. J. (2001). Role of dopamine D(4) receptors in motor hyperactivity induced by neonatal 6-hydroxydopamine lesions in rats. *Neuropsychopharmacology* 25, 624–632. doi: 10.1016/s0893-133x(01)00262-7

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: 21 May 2020
doi: 10.3389/fnbeh.2020.00075

Ketamine Blocks Morphine-Induced Conditioned Place Preference and Anxiety-Like Behaviors in Mice

*Greer McKendrick[1,2], Hannah Garrett[2†], Holly E. Jones[2,3†], Dillon S. McDevitt[1,3], Sonakshi Sharma[2], Yuval Silberman[4] and Nicholas M. Graziane[5]**

[1]Neuroscience Graduate Program, Penn State College of Medicine, Hershey, PA, United States, [2]Department of Anesthesiology and Perioperative Medicine, Penn State College of Medicine, Hershey, PA, United States, [3]Summer Undergraduate Research Internship Program, Penn State College of Medicine, Hershey, PA, United States, [4]Department of Neural and Behavioral Sciences, Penn State College of Medicine, Pennsylvania State University, Hershey, PA, United States, [5]Departments of Anesthesiology and Perioperative Medicine and Pharmacology, Penn State College of Medicine, Hershey, PA, United States

OPEN ACCESS

Edited by:
Jee Hyun Kim,
Florey Institute of Neuroscience and Mental Health, Australia

Reviewed by:
Cristina Cadoni,
National Research Council (Cnr), Italy
Fabrizio Sanna,
University of Cagliari, Italy

***Correspondence:**
Nicholas M. Graziane
ngraziane@pennstatehealth.psu.edu

[†]These authors have contributed equally to this work

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

Received: 09 January 2020
Accepted: 23 April 2020
Published: 21 May 2020

Citation:
McKendrick G, Garrett H, Jones HE, McDevitt DS, Sharma S, Silberman Y and Graziane NM (2020) Ketamine Blocks Morphine-Induced Conditioned Place Preference and Anxiety-Like Behaviors in Mice. Front. Behav. Neurosci. 14:75. doi: 10.3389/fnbeh.2020.00075

Patients suffering from opioid use disorder often relapse during periods of abstinence, which is posited to be caused by negative affective states that drive motivated behaviors. Here, we explored whether conditioning mice with morphine in a conditioned place preference (CPP) training paradigm evoked anxiety-like behavior during morphine abstinence. To do this, mice were conditioned with morphine (10 mg/kg, i.p.) for 5 days. Twenty-four hours following conditioning, anxiety levels were tested by measuring time in the open arms of the elevated plus-maze. The next day, mice were placed in the three-compartment chamber to measure morphine-induced CPP. Our results show that following morphine conditioning, mice spent significantly less time in the open arm of the elevated plus-maze and expressed robust morphine CPP on CPP test day. Furthermore, we found that an acute treatment with (*R,S*)-ketamine (10 mg/kg, i.p.), a medication demonstrating promise for preventing anxiety-related phenotypes, 30 min before testing on post-conditioning day 1, increased time spent in the open arm of the elevated plus-maze in saline- and morphine-conditioned mice. Additionally, we found that the second injection of ketamine 30 min before CPP tests on post-conditioning day 2 prevented morphine-induced CPP, which lasted for up to 28 days post-conditioning. Furthermore, we found that conditioning mice with 10% (w/v) sucrose using an oral self-administration procedure did not evoke anxiety-like behavior, but elicited robust CPP, which was attenuated by ketamine treatment 30 min before CPP tests. Overall, our results suggest that the ketamine-induced block of morphine CPP may not be attributed solely to alleviating negative affective states, but potentially through impaired memory of morphine-context associations.

Keywords: negative affect, morphine, conditioned place preference, anxiety, opioid use disorder, ketamine, psychedelics

INTRODUCTION

The motivation to continually seek and obtain addictive substances during periods of abstinence or recovery is caused, in part, by the necessity to avoid aversive internal states (Solomon and Corbit, 1978). Evidence for this comes from patients with substance use disorders who self-report urges and intentions to take drugs to avoid drug-withdrawal symptoms (O'Brien, 1975; Baker et al., 2004; Wikler, 2013) or to cope with negative affect (Perkins and Grobe, 1992; Zinser et al., 1992; Wetter et al., 1994; Cooney et al., 1997; Conklin and Perkins, 2005; Fox et al., 2007). For example, abstinence from morphine, a highly addictive opioid, facilitates increases in anxiety (Gold et al., 1978, 1979), which is a potential factor in continued drug use (Martins et al., 2012).

To better understand the mechanisms mediating drug-craving and subsequent relapse, preclinical models have been developed whereby drug-seeking behaviors are monitored in drug-exposed rodents. In the conditioned place preference (CPP) paradigm, a drug is paired with a context during conditioning. This is followed by a test day whereby the time spent in the drug-paired context is measured. This behavioral paradigm is a form of Pavlovian learning whereby injection of a drug (i.e., unconditioned stimulus) elicits a hedonic feeling of pleasure (i.e., unconditioned response), which, when paired with a context (neutral stimulus), invokes incentive value to the context (i.e., now a conditioned stimulus), thus driving a behavioral response to "seek" the context (conditioned response). This is similar to sign-tracking behaviors (Huston et al., 2013), which refer to a behavior that is directed toward a stimulus as a result of that stimulus becoming associated with a reward (Huys et al., 2014). Therefore, CPP provides a valuable tool used to understand how drugs of abuse become associated with environmental contexts, which is implicated in context-induced drug craving and relapse (O'Brien and Ternes, 1986; O'Brien et al., 1992). We have found that 5 days of morphine (10 mg/kg) conditioning elicits robust morphine CPP (Graziane et al., 2016; McDevitt and Graziane, 2019). However, it is unclear whether this "drug context-seeking" behavior is mediated by negative affective states. Additionally, it is unclear whether a subanesthetic dose of ketamine, an anxiolytic agent (Engin et al., 2009), blocks morphine-induced CPP by mitigating morphine-induced negative affective states.

Here, we attempt to investigate whether morphine conditioning in our CPP paradigm generates negative affect during morphine abstinence. Additionally, we investigate whether an acute, subanesthetic dose of (*R,S*)-ketamine before testing is sufficient to disrupt morphine-induced anxiety and/or morphine-induced CPP behaviors. Lastly, it has been shown that an acute administration of (*R,S*)-ketamine is sufficient to block the expression of morphine CPP (Suzuki et al., 2000). Here, we investigate whether this ketamine-induced block of morphine CPP, in our behavioral training paradigm, is mediated by the impairment of drug-context associations or by the attenuation of morphine-induced negative affective states.

MATERIALS AND METHODS

Animals

All experiments were done following procedures approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee. Male C57BL/6J mice aged 5–8 weeks were purchased from Jackson Labs (stock #000664; Bar Harbor, ME, USA), singly-housed, and maintained on a regular 12 h light/dark cycle (lights on 07:00, lights off 19:00) with *ad libitum* food and water. Mice were singly housed for the following reasons. First, we have reliably developed morphine conditioned place preference (CPP) in singly-housed mice (Graziane et al., 2016; McDevitt and Graziane, 2019). Second, evidence suggests that socially isolated rodents are more vulnerable to developing drug-context associations (Whitaker et al., 2013). In humans, social isolation increases vulnerability to substance use disorders (Newcomb and Bentler, 1988; Sinha, 2008), which often are accompanied by the development of drug-context associations (O'Brien and Ternes, 1986; O'Brien et al., 1992; Xue et al., 2012). Therefore, our studies are designed to model this patient population.

Drugs

(−)-morphine sulfate pentahydrate was provided by the National Institute on Drug Abuse Drug Supply Program. Ketamine hydrochloride (racemic mixture of 50% *R*-ketamine and *S*-ketamine; Dechra Pharmaceuticals, Northwich, UK) was purchased from the Comparative Medicine Department at the Pennsylvania State University College of Medicine.

Non-contingent Conditioned Place Preference

Conditioned place preference (CPP) chambers (Med Associates) were located in the mouse housing room and consisted of three distinct compartments separated by manual guillotine-style doors. Each compartment had distinct contextual characteristics: the middle (neutral) compartment (7.2 cm × 12.7 cm × 12.7 cm) had gray walls and gray plastic floor, while the choice compartments (16.8 cm × 12.7 cm × 12.7 cm, each) had either white walls and stainless steel mesh floor or black walls and stainless steel grid floor. All compartments were illuminated with dim light during use. Immediately following use, the entire preference chamber was cleaned thoroughly with a scent-free soap solution. Mouse locations, activity counts, and time spent in each compartment were collected *via* automated data-collection software (Med Associates) *via* infrared photo beam strips lining each compartment. Morphine administration was verified with the Straub tail response and enhanced locomotor activity (Bilbey et al., 1960; Graziane et al., 2016; McDevitt and Graziane, 2019).

Habituation

Mice were placed in the center compartment with free access to all three compartments for 20 min once a day for 2 days. Time spent (seconds) in each compartment was recorded.

Conditioning

Twenty-four hours after habituation, mice received 5 days conditioning training. Morphine-paired compartments were

assigned based on the least preferred side (a biased approach; Tzschentke, 2007) calculated by averaging time spent in each compartment over the two habituation days. Similar to conditioning studies with alcohol (Gremel et al., 2006), we find that C57BL/6J mice will reliably develop morphine CPP using a biased approach. During conditioning, mice received an injection of saline and were placed into the most preferred compartment for 40 min. Six hours later, mice received an injection of saline (control group) or morphine (10 mg/kg, i.p.) and were placed into their least preferred compartment for 40 min (Koo et al., 2014; Graziane et al., 2016).

Post-conditioning

Forty-eight hours or 28 days after the last conditioning day, mice were placed in the 3-compartment chamber and allowed to move freely for 20 min. Our post-conditioning took place at a time point corresponding to 3 h before drug conditioning (e.g., morphine conditioning took place at 3 P.M., post-conditioning tests took place 2 or 28 days later at 12 P.M.). CPP scores were calculated as time spent in the drug-paired side minus the average time spent on the same side during preconditioning (Bohn et al., 2003). Activity counts are defined as any beam break within a current zone. This is inclusive of grooming, rearing, and lateral movements. Mice were treated with 0.9% saline (0.1 ml, i.p.) or with (*R,S*)-ketamine (10 mg/kg, i.p.) 30 min before the first CPP test. The dose of ketamine was selected based on preclinical data demonstrating that a 10 mg/kg dose of ketamine produces a maximal effect on morphine CPP (Suzuki et al., 2000) and produces plasma concentrations associated with subanesthetic ketamine doses capable of eliciting antidepressant effects in mice and humans (Zarate et al., 2012; Zanos et al., 2016).

Sucrose Oral Self-administration Conditioned Place Preference

Habituation

Mice were placed in the center compartment with free access to all three compartments for 20 min once a day for 2 days. Time spent (seconds) in each compartment was recorded.

Conditioning

Drinking bottles were created as described in Freet et al. (2013). Briefly, we modified 10 ml serological pipettes by tapering both ends, placing a stainless-steel sipper tube (Ancare; OT-300) in one end and a silicone stopper (Thermo Fisher Scientific; 09-704-1D) in the other. Bottles were inserted into plastic holders that were then placed directly into CPP chambers (for chamber description, see "Non-contingent Conditioned Place Preference" section), where they were positioned so that the sipper was ∼5 cm above the chamber floor. Pennsylvania State University Fabrication shop constructed plexiglass tops that were placed along the top of the 3-compartment apparatus and allowed for plastic bottle holders to be placed into chambers. Oral self-administration was recorded as the mL before and following all sessions. Similar to the i.p. CPP methodology, we utilized a biased approach in which the 10% sucrose (w/v) solution was placed in the least-preferred context. Twenty-four hours after habituation, mice underwent two 14 h overnight sessions (separated by 24 h), confined to the least preferred chamber on the first night (ON1) with access to water (control groups) or a 10% sucrose solution and confined to the most preferred side on the second night (ON2) with access to water. Mice then received 5 days of conditioning (C1–C5), where morning sessions consisted of 40 min in the most-preferred context with access to water. Six hours later, afternoon sessions consisted of 40 min in the least preferred context with access to water (control groups) or 10% sucrose solution.

Post-conditioning

Forty-eight hours or 21 days after the last conditioning day, mice were placed in the 3-compartment chamber and allowed to move freely for 20 min. Our post-conditioning took place at a time point corresponding to 3 h before drug conditioning (e.g., sucrose conditioning took place at 3 P.M., post-conditioning tests took place 2 or 21 days later at 12 P.M.). No bottles were present in the chambers on preference tests. CPP scores were calculated as time spent on the least preferred side on test day minus the average time spent on the same side during preconditioning (Bohn et al., 2003). Mice treated with (*R,S*)-ketamine (10 mg/kg, i.p.; water+ketamine and sucrose + ketamine groups) received injections 30 min before the first CPP test on post-conditioning day 2.

Elevated Plus Maze

The elevated plus-maze, a well-established method to measure anxiety in rodents, was implemented to measure anxiety-like behavior (Pellow et al., 1985; Handley and McBlane, 1993; Dawson and Tricklebank, 1995). The elevated-plus maze for mice (Stoelting, Item #60140) was raised approximately 50 cm from the ground. The floor of the elevated portion of the maze was gray. Two opposite arms (35 × 5 cm each) of the maze were enclosed by a 15 cm high wall and the remaining two arms were "open." A center space (5 cm^2) between these four arms was also not enclosed. The elevated portion of the apparatus was cleaned thoroughly with a scent-free soap solution after each trial. Behavioral tests were performed in the animal housing room under ambient light of the light cycle.

Twenty-four hours after the last conditioning day in the CPP apparatus, mice were placed in the center space facing the open arm and allowed to explore the apparatus for 5 min before being placed back into their home cage (Grisel et al., 2008). Each trial was video recorded using a GoPro camera (Hero7 white) and analyzed by researchers blinded to the treatment condition of the mice. Time in the open arm was measured when the body of the mouse cleared the center space. Mice were treated with 0.9% saline (0.1 ml, i.p.) or ketamine (10 mg/kg, i.p.) 30 min before the elevated plus-maze test.

Statistical Analysis

Statistical significance was assessed in GraphPad Prism software using a student's *t*-test, one- or two-way ANOVA with Bonferroni's correction for multiple comparisons as specified. *F* values for two-way ANOVA statistical comparisons represent interactions between variables unless stated otherwise. Two-tailed tests were performed for student's *t*-test. For

correlation analysis, Pearson's correlation coefficient, and subsequent linear regression, were determined. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Morphine Conditioning Elicits Anxiety-Like Behaviors During Morphine Abstinence

Repeated exposure to morphine increases levels of anxiety both in humans and in animal models of substance use disorders (Gold et al., 1978, 1979; Becker et al., 2017). Additionally, it is posited that relapse to opioids in abstinent patients is caused by negative affective states, thus driving drug-seeking behaviors (Solomon and Corbit, 1978; Koob and Le Moal, 2008; Evans and Cahill, 2016). In an attempt to provide evidence that morphine-induced CPP, using our training paradigm, is mediated, in part, by negative affective states, 24 h following the last morphine conditioning session (**Figure 1A**), we measured anxiety-like behavior using the elevated plus maze (EPM; Pellow et al., 1985). We found that morphine-treated mice, who showed robust locomotor sensitization by conditioning day 5 (**Figure 1B**), expressed a significant decrease in the percent time spent in the open arm of the EPM compared to saline-treated controls ($t_{(38)} = 3.35$, $p = 0.002$, student's t-test; **Figure 1C**). To correlate anxiety levels with CPP scores, mice underwent CPP tests 24 h following EPM tests (**Figure 1A**). We found that 5 days morphine conditioning elicited significant increases in place preference for the drug-paired compartment ($t_{(38)} = 5.61$, $p < 0.0001$, student's t-test; **Figure 1D**). However, we found no correlation between anxiety-like behaviors and CPP score in morphine-conditioned mice (Pearson's correlation coefficient = −0.162; simple linear regression: $F_{(1,15)} = 0.404$, $p = 0.53$, $R^2 = 0.03$) or in saline-conditioned, control mice (Pearson's correlation coefficient = −0.095; simple linear regression: $F_{(1,21)} = 0.191$, $p = 0.67$, $R^2 = 0.01$; **Figures 1E,F**). Overall, these results suggest that morphine conditioning in a CPP paradigm is sufficient to facilitate anxiety-like behaviors during short-term abstinence, but that the animal's anxiety-like behavior is not correlated with the amount of time spent in the morphine-paired compartment on CPP test day.

Ketamine Blocks Morphine-Induced Anxiety-Like Behaviors and Morphine CPP

Evidence suggests that (*R,S*)-ketamine, a noncompetitive NMDA receptor antagonist (Lodge et al., 1982; Kohrs and Durieux, 1998), is an effective treatment for anxiety and substance use disorders (Krupitsky et al., 2002; Ivan Ezquerra-Romano et al., 2018; Taylor et al., 2018). Because of this, we investigated whether an acute injection of (*R,S*)-ketamine (30 min before EPM and CPP testing) would be sufficient to block morphine-induced anxiety-like behaviors and/or morphine-induced CPP (**Figure 2A**). Following conditioning with morphine, which produced robust locomotor sensitization (**Figure 2B**), we found that the first (*R,S*)-ketamine injection before the EPM test on post-conditioning day 1 (PC1) significantly increased the percent time in the open arms of the EPM ($F_{(3,52)} = 22.2$, $p < 0.0001$, one-way ANOVA, Bonferroni *post hoc* test; **Figure 2C**). Additionally, we found that a second (*R,S*)-ketamine injection before CPP tests on post-conditioning day 2 (PC2) was sufficient to prevent morphine-induced CPP ($F_{(3,52)} = 14.04$, $p < 0.0001$, one-way ANOVA, Bonferroni *post hoc* test; **Figure 2D**), which was likely not attributed to ketamine-induced changes in locomotor activity ($F_{(3,52)} = 0.447$, $p = 0.72$, two-way repeated-measures ANOVA; **Figure 2E**).

Acute Ketamine Treatment Blocks the Long-Term Expression of Morphine CPP

We have previously shown that morphine-induced CPP, using the paradigm described in this study, is

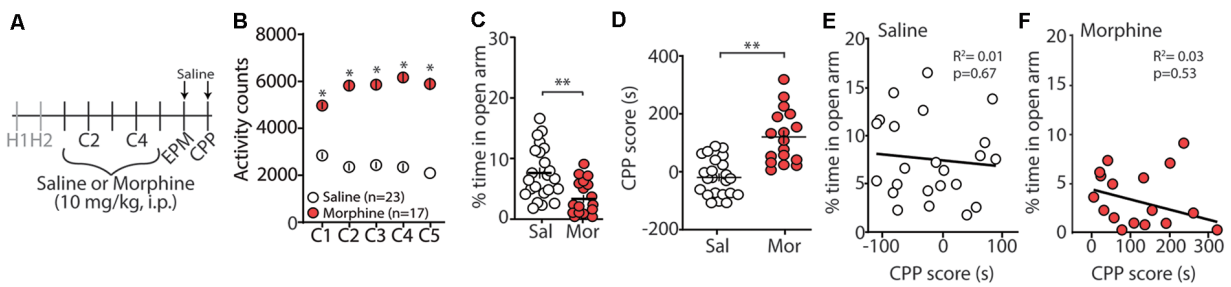


FIGURE 1 | Morphine conditioning in a conditioned place preference (CPP) paradigm elicits anxiety-like behaviors during 24 h abstinence. **(A)** Timeline and drug regimen of the behavioral procedure. Animals underwent 2 days of habituation (H), followed by 5 days of saline or morphine (10 mg/kg, i.p.) conditioning (C), before being subjected to tests measuring anxiety-like behaviors using an elevated plus maze (EPM) 24 h post-conditioning. Twenty-four hours post EPM tests, CPP tests were performed. Animals were injected with saline 30 min before EPM and CPP tests. **(B)** A summary showing that morphine conditioning over 5 days produces robust locomotor sensitization ($F_{(4,152)} = 17.1$, $p < 0.0001$, two-way repeated-measures ANOVA, Bonferroni *post hoc* test). **(C)** A summary showing that morphine (Mor)-conditioned mice spent significantly less time in the open arms of the elevated plus-maze compared to saline (Sal)-conditioned mice 24 h following the last conditioning day ($t_{(38)} = 3.35$, $p = 0.002$, student's t-test). **(D)** A summary showing that morphine conditioning produced reliable CPP ($t_{(38)} = 5.61$, $p < 0.0001$, student's t-test). **(E)** Correlation of the % time in the open arm of the elevated plus-maze and CPP score in saline- or **(F)** morphine-conditioned mice. $^*p < 0.05$, $^{**}p < 0.01$.

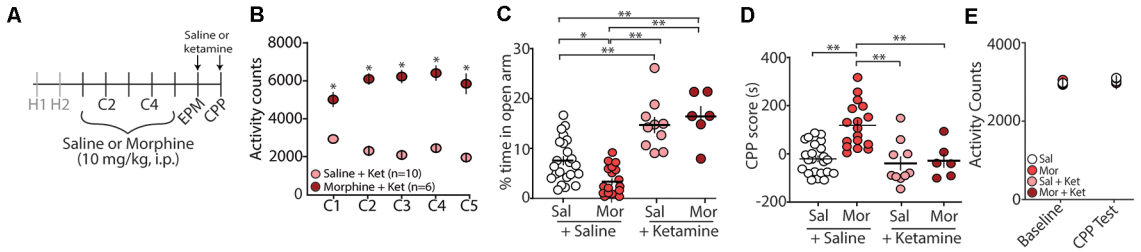


FIGURE 2 | Acute (*R,S*)-ketamine injection produces anxiolytic-like behaviors in mice 24 h after conditioning and blocks morphine-induced CPP. **(A)** Timeline and drug regimen of the behavioral procedure. Saline or (*R,S*)-ketamine (10 mg/kg, i.p.) was injected 30 min before EPM test with the second injection taking place 30 min before the first CPP test. **(B)** A summary showing that morphine conditioning over 5 days (C1–C5) produces robust locomotor sensitization ($F_{(4,56)} = 12.55$, $p < 0.0001$, two-way repeated-measures ANOVA, Bonferroni *post hoc* test). **(C)** A summary showing that (*R,S*)-ketamine significantly increased the time spent in the open arms of the elevated plus-maze in both saline (Sal)- and morphine (Mor)-conditioned mice ($F_{(3,52)} = 22.2$, $p < 0.0001$, one-way ANOVA, Bonferroni *post hoc* test; animals not receiving (*R,S*)-ketamine are the same data as shown in **Figure 1C**). **(D)** A summary showing that morphine produced reliable CPP at post-conditioning day 2, which was blocked by (*R,S*)-ketamine injected 30 min before testing ($F_{(3,52)} = 14.04$, $p < 0.0001$, one-way ANOVA, Bonferroni *post hoc* test; saline and morphine groups are the same animals as shown in **Figure 1D**). **(E)** A summary showing the activity counts in the CPP chamber during habituation (baseline) and the CPP test in saline (Sal)- or morphine (Mor)-conditioned mice treated with saline or (*R,S*)-ketamine 30 min before testing ($F_{(3,52)} = 0.447$, $p = 0.72$, two-way repeated-measures ANOVA). $^*p < 0.05$, $^{**}p < 0.01$.

sufficient to elicit long-lasting CPP for up to 28 days post-conditioning (Graziane et al., 2016). Because of this, we tested whether ketamine administration during early abstinence was sufficient to block the prolonged-expression of morphine-induced CPP (**Figure 3A**). We found that two injections of (*R,S*)-ketamine, one on post-conditioning day 1 (before elevated arm maze tests) and the second on post-conditioning day 2 (before CPP tests), were sufficient to prevent the prolonged-expression of morphine-induced CPP on PC28 (column factor: $F_{(3,38)} = 10.25$, $p < 0.0001$, two-way repeated-measures ANOVA, Bonferroni *post hoc* test; **Figure 3B**).

Acute Ketamine Treatment Prevents the Expression of Sucrose CPP

To further investigate whether the ketamine block of morphine CPP is through potential memory impairment and/or anxiolytic effects, we evaluated the effect of ketamine on the CPP of a natural reward (i.e., sucrose). We rationalized that if ketamine blocks morphine CPP by specifically alleviating negative affective states, without impairing memory of drug-context associations, then ketamine would be ineffective at blocking sucrose CPP, a natural reward, which does not evoke anxiety-like behaviors (**Figure 4C**). To test this, we conditioned mice over 7 days (**Figure 4A**) to orally self-administer water (controls) or sucrose in the least preferred compartment of the CPP chamber (see "Materials and Methods" section for conditioning paradigm). Mice conditioned with sucrose drank significantly more than mice conditioned with water over all conditioning days ($F_{(15,175)} = 462.1$, $p < 0.0001$, two-way repeated-measures ANOVA, Bonferroni *post hoc* test; **Figure 4B**). The water consumed in the most preferred chamber during conditioning days 1–5 did not differ between groups ($F_{(12,140)} = 0.596$, $p = 0.843$, two-way repeated-measures ANOVA; **Supplementary Figure S1**). On post-conditioning day 1 (PC1), anxiety-like behavior was measured using the EPM. We found that the percent time in the open arm of the EPM in sucrose-conditioned mice was not significantly different from mice conditioned with water ($t_{(17)} = 0.184$, $p = 0.856$, student's *t*-test; **Figure 4C**) suggesting that sucrose exposure did not elicit anxiety-like behaviors during short-term abstinence. Twenty-four hours later, on post-conditioning day 2 (PC2), water- and sucrose-conditioned mice underwent a CPP test 30 min after receiving an acute injection of (*R,S*)-ketamine (10 mg/kg, i.p.). Our data show that (*R,S*)-ketamine attenuated sucrose-induced CPP on PC2 ($F_{(3,35)} = 6.31$, $p = 0.0015$, one-way ANOVA, Bonferroni *post hoc* test; **Figure 4D**) and this ketamine-induced attenuation of sucrose CPP persisted to abstinence day 21 ($F_{(3,32)} = 5.51$, $p = 0.004$, one-way ANOVA, Bonferroni *post hoc* test; **Supplementary Figure S2**).

Lastly, we investigated whether the ketamine block of morphine-induced anxiety-like behavior and morphine-induced CPP was potentially attributed to ketamine-induced behavioral disinhibition, leading the animal to explore more. To do this, we monitored entrance counts and exploratory counts in the CPP chamber on test day. We found that there was no significant difference in the entrance or exploratory counts in the CPP chamber when comparisons were made between saline vs. ketamine injected mice undergoing the same treatment during conditioning (**Figures 4E,F**). These results suggest that the effects of ketamine on morphine-driven behaviors are unlikely mediated by behavioral disinhibition.

DISCUSSION

Our results show that the percent time spent in the open arms of the elevated plus-maze is decreased in animals conditioned with morphine. Additionally, we show that acute injection of (*R,S*)-ketamine 30 min before the EPM and CPP tests is sufficient to block morphine-induced anxiety-like behaviors and morphine-induced CPP (post-conditioning day 2 through post-conditioning day 28), as well as attenuates sucrose-induced

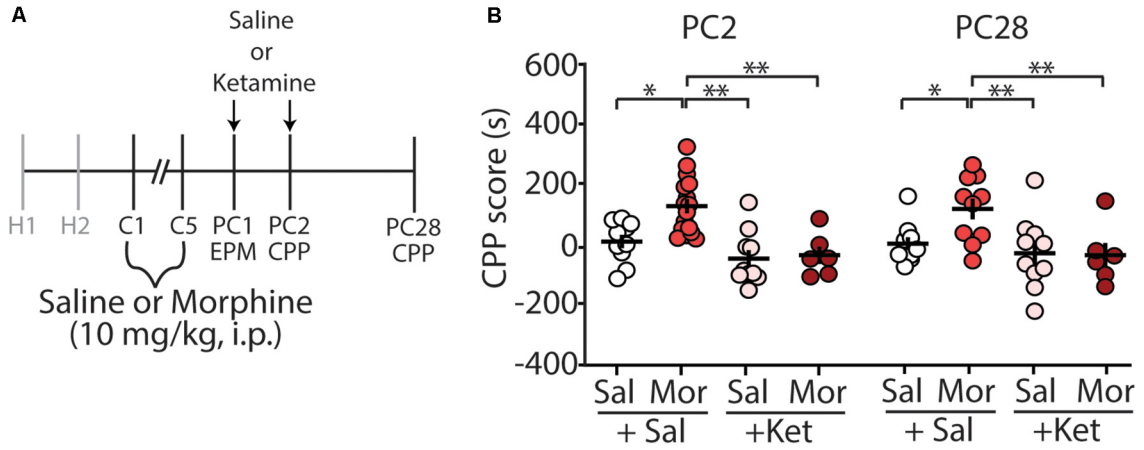


FIGURE 3 | (*R,S*)-ketamine administration during early abstinence is sufficient to prevent the prolonged retention of morphine-induced CPP at post-conditioning day 28. **(A)** Timeline and drug regimen of the behavioral procedure. (*R,S*)-ketamine (10 mg/kg, i.p.) was injected 30 min before the EPM test on post-conditioning day 1 (PC1) and again on the first CPP test on post-conditioning day 2 (PC2; i.e., each mouse received a ketamine injection before the EPM test and a second ketamine injection the next day before the CPP test). The second CPP test was run on PC28. **(B)** A summary showing that morphine produced reliable CPP 28 days post-conditioning, which was blocked by (*R,S*)-ketamine (column factor: $F_{(3,38)} = 10.25$, $p < 0.0001$, two-way repeated-measures ANOVA, Bonferroni *post hoc* test; PC2 data is the same data shown in **Figure 2D**). Abbreviation: EPM, elevated plus maze; CPP, conditioned place preference. $^{*}p < 0.05$, $^{**}p < 0.01$.

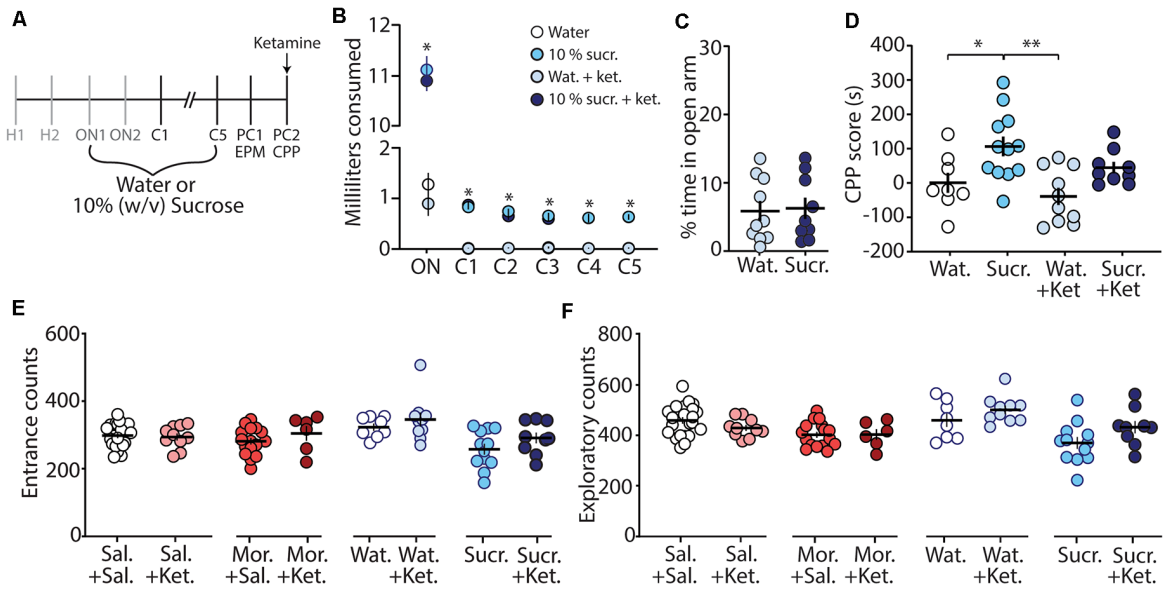


FIGURE 4 | Ketamine administration attenuates sucrose-induced conditioned place preference. **(A)** Timeline and sucrose regimen of the behavioral procedure. Following sucrose oral self-administration in the three-compartment apparatus, mice underwent EPM testing on post-conditioning day 1 (PC1). Twenty-four hours later, mice received no injection or (*R,S*)-ketamine (10 mg/kg, i.p.) 30 min before the conditioned place preference (CPP) test on post-conditioning day 2 (PC2). **(B)** A summary showing the milliliters of water or sucrose consumed for each training session in the least preferred chamber. Groups conditioned with sucrose (i.e., sucrose (sucr.) and sucrose + ketamine (sucr. + ket.) groups) drank significantly more than groups conditioned with water (i.e., water (Wat.) and water+ketamine (Wat. + Ket.) groups; $F_{(15,175)} = 462.1$, $p < 0.0001$, two-way repeated-measures ANOVA, Bonferroni *post hoc* test). **(C)** A summary showing that conditioning with sucrose had no effect on anxiety-like behaviors as both water- and sucrose-conditioned mice displayed similar % time in the open arm of the EPM ($t_{(17)} = 0.184$, $p = 0.856$, student's *t*-test). **(D)** A summary showing that oral self-administration of sucrose produced CPP at PC2, which was blocked by (*R,S*)-ketamine treatment ($F_{(3,35)} = 6.31$, $p = 0.0015$, one-way ANOVA, Bonferroni *post hoc* test). **(E)** A summary showing that ketamine injections 30 min before the CPP test did not impact entrance counts in the CPP apparatus (Sal. + Sal. vs. Sal. + Ket.: $t_{(31)} = 0.295$, $p = 0.770$; Mor. + Sal. vs. Mor. + Ket.: $t_{(21)} = 1.13$, $p = 0.272$; Wat. + Sal. vs. Wat. + Ket.: $t_{(16)} = 0.874$, $p = 0.395$; Sucr. + Sal. vs. Sucr. + Ket.: $t_{(19)} = 1.43$, $p = 0.168$, student's *t*-test). **(F)** A summary showing that ketamine injections 30 min before the CPP test did not impact exploratory counts in the CPP apparatus (Sal. + Sal. vs. Sal. + Ket.: $t_{(31)} = 1.42$, $p = 0.166$; Mor. + Sal. vs. Mor. + Ket.: $t_{(21)} = 0.045$, $p = 0.964$; Wat. + Sal. vs. Wat. + Ket.: $t_{(16)} = 1.26$, $p = 0.226$; Sucr. + Sal. vs. Sucr. + Ket.: $t_{(19)} = 1.80$, $p = 0.088$, student's *t*-test). $^{*}p < 0.05$, $^{**}p < 0.01$.

CPP (post-conditioning day 2 through post-conditioning day 21). We further find that ketamine, at least in the dose tested here, does not alter behavioral disinhibition in either morphine-CPP or sucrose-CPP mice. Together these findings indicate that ketamine may inhibit morphine CPP behaviors, at least in part, *via* reductions in withdrawal-induced anxiety-like behaviors. Our data do not, however, rule out the possibility that ketamine-induced effects on morphine CPP may also be mediated in part by impairing memory of morphine-context associations.

Anxiety-Like Behaviors During Morphine Abstinence

Morphine possesses anxiolytic-like properties during initial exposure (Kõks et al., 1999; Sasaki et al., 2002; Shin et al., 2003). However, during opioid abstinence, symptoms of anxiety (Gold et al., 1978, 1979; Li et al., 2009; Shi et al., 2009) or anxiety-like behaviors are observed (Cabral et al., 2009; Becker et al., 2017). Here, we show that 24 h following repeated morphine injections (once a day for 5 days), mice display anxiety-like behaviors in the elevated plus-maze (**Figure 1C**). These results are similar to previous studies showing escalating doses of morphine over 6 days induce anxiety-like behaviors in the marble burying task (Becker et al., 2017). Additionally, our observed morphine-induced anxiety-like behavior is timed with anxiogenic neurobiological responses that occur during acute opioid abstinence including, increases in norepinephrine release in the extended amygdala (Fuentealba et al., 2000; Aston-Jones and Harris, 2004), norepinephrine-induced modulation of the extended amygdala (Aston-Jones et al., 1999; Delfs et al., 2000; Smith and Aston-Jones, 2008), activation of the amygdalar corticotrophin-releasing factor (CRF) system (Heinrichs et al., 1995; Maj et al., 2003), and decreases in dopamine transmission (Diana et al., 1995). However, the observed morphine-induced anxiety-like behavior may be dependent upon morphine exposure as it has been shown that morphine does not elicit anxiety-like behaviors following three morphine injections (10 mg/kg) occurring every other day (Benturquia et al., 2007). This may be related to neurobiological mechanisms associated with different drug exposure regimens. We have previously shown that morphine exposure significantly increases the expression of silent synapses, excitatory glutamatergic synapses that express functional NMDA receptors, but lack functional α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Hanse et al., 2013), in the nucleus accumbens shell. We found that this increase in silent synapse expression is observed 24 h after the last of five morphine injections (once a day for 5 days), but not 24 h after the last of three morphine injections (once a day for 3 days; Graziane et al., 2016; Hearing et al., 2018; McDevitt and Graziane, 2018). Future experiments will be required to test whether this morphine-induced change in the nucleus accumbens shell regulates morphine-induced anxiety-like behaviors.

The observed anxiety-like behaviors following morphine conditioning in a three-chamber apparatus (**Figure 1F**) may suggest that animals seek the drug-paired chamber as a consequence of negative reinforcement to alleviate aversive affective states facilitated by opioid abstinence. Importantly, our injection regimen of morphine 10 mg/kg once a day for 5 consecutive days does not induce signs of somatic withdrawal in mice including jumping, wet dog shakes, teeth chattering, rearing, tremor, diarrhea, or mastication (Gallego et al., 2010). This coincides with the lack of observed somatic withdrawal symptoms following a more prolonged injection regimen of five daily morphine (10 mg/kg, i.p.) injections over 4 weeks (Robinson and Kolb, 1999). Although more studies are required, it is plausible that specific opioid dosing regimens may be implemented in a preclinical setting to separate opioid-induced negative affective states (e.g., anxiety) from confounds induced by somatic signs of opioid withdrawal, which are ineffective at reinstating opioid seeking or morphine CPP in opioid-dependent rodents (Shaham et al., 1996; Lu et al., 2005) as well as in humans (Miller et al., 1979). Separating opioid-induced negative affective states (e.g., anxiety) from confounds induced by somatic signs of opioid withdrawal is not a new idea and has been demonstrated previously with doses of naloxone (used to precipitate opioid withdrawal) that were sub-threshold for somatic signs of opioid withdrawal (Gracy et al., 2001).

Based on our results, it would be expected that facilitating a negative affective state during morphine abstinence would enhance the expression of morphine CPP. However, evidence suggests that this is not the case, as forced swim stress, which would be expected to elicit a strong negative affective state, immediately before CPP testing in morphine-conditioned animals has either no effect on morphine CPP (Attarzadeh-Yazdi et al., 2013) or significantly decreases morphine CPP (Haghparast et al., 2014). Additionally, corticosterone administration, which is expected to facilitate depression-like behaviors (Gregus et al., 2005), before CPP tests do not affect morphine CPP (Attarzadeh-Yazdi et al., 2013). These results are surprising especially considering the robust effect of stressful stimuli in reinstating morphine CPP in extinguished rodents (Ribeiro Do Couto et al., 2006; Wang et al., 2006; Karimi et al., 2014). Possibly, morphine CPP tested during abstinence (e.g., Attarzadeh-Yazdi et al., 2013) reaches a ceiling effect, making it unlikely that exposure to a stressor (e.g., forced swim) will enhance the CPP score (i.e., occlusion). It is also possible that the stressor elicits a decreased locomotor state potentially resulting in reduced morphine CPP (e.g., Haghparast et al., 2014).

Ketamine's Effects on Anxiety-Like Behaviors

Ketamine has recently been shown to be a potentially effective treatment for anxiety disorders (Glue et al., 2018; Shadli et al., 2018; Taylor et al., 2018). In humans, ketamine displays a biphasic dose-effect on anxiety, with low doses decreasing anxiety and higher doses increasing anxiety (Jansen, 1989; Krystal et al., 1994). Likewise, in rodents, ketamine induces anxiolytic-like behaviors (Engin et al., 2009; Zhang et al., 2015; Fraga et al., 2018) as well as anxiogenic-like phenotypes likely dependent upon the dose, the temporal relationship between ketamine injection and test onset, and rodent species (Silvestre et al., 1997; da Silva et al., 2010). Here, we

demonstrate that in C57BL/6J mice, acute injection of ketamine at 10 mg/kg, i.p. Thirty minutes before testing is sufficient to block morphine-induced anxiety-like behaviors during a 24 h abstinence period (**Figure 2C**). Additionally, we find that ketamine significantly increases the percent time in the open arm of the elevated plus-maze in mice conditioned with saline. This significant change observed in saline conditioned animals suggests that ketamine, at the dose and temporal relationship of ketamine injection and test onset, is sufficient to overcome baseline anxiety-like behaviors in animals exposed to a novel environment (i.e., EPM).

Despite the evidence suggesting that the antagonistic effects of ketamine on NMDA receptors in the bed nucleus of the stria terminalis attenuate negative affective states (Louderback et al., 2013), the mechanisms mediating the observed anxiolytic-like effects are unknown. In addition to acting as a non-competitive antagonist to NMDA receptors in the extended amygdala, evidence suggests that ketamine interacts with hyperpolarization-activated cyclic nucleotide-gated (HCN) channels as well as dopamine, serotonin, sigma, opioid, and cholinergic receptors (Scheller et al., 1996; Cai et al., 1997; Kubota et al., 1999; Lydic and Baghdoyan, 2002; Wang et al., 2012; Zanos et al., 2018). Additionally, ketamine metabolites are biologically active as antagonists to NMDA receptors (Ebert et al., 1997) and α7 nicotinic acetylcholine receptors (Moaddel et al., 2013), while also possessing agonistic activity for AMPA receptors (Zanos et al., 2016; Tyler et al., 2017). Because of the undiscriminating activity of ketamine and its metabolites, it has been difficult to pinpoint how ketamine influences anxiety states both in humans and in preclinical models.

Ketamine's Effects on Morphine-Induced Conditioned Place Preference

Using a paradigm known to induce robust CPP for up to 28 days post-conditioning (Graziane et al., 2016), we show that acute injection of ketamine 30 min before the CPP test on abstinence day 2 is sufficient to block morphine-induced CPP. These results are not likely caused by changes in locomotor activity as activity counts during habituation (baseline) were not significantly different from activity counts measured following ketamine administration (**Figure 2E**). Our results are in line with previous publications demonstrating that ketamine blocks morphine-induced CPP in mice (Suzuki et al., 2000). However, the effects on locomotor activity are conflicting. Whereas, our results and those from previous publications show that ketamine does not influence locomotor activity (Lindholm et al., 2012), others have found that locomotor activity is increased (Filibeck and Castellano, 1980) or decreased following ketamine administration (Akillioglu et al., 2012). These discrepancies are likely due to the temporal relationship between ketamine treatment and test onset. Here, we performed our tests 30 min following ketamine injection similar to previous studies (Lindholm et al., 2012), while tests performed 5 min or 15 min following ketamine administration appear to increase or decrease locomotor activity, respectively (Filibeck and Castellano, 1980; Akillioglu et al., 2012). The half-life of ketamine is ∼13–25 min in mice following i.p. administration (Maxwell et al., 2006; Zanos et al., 2016; Ganguly et al., 2018). Therefore, possibly the locomotor effects observed are due to ketamine action before metabolism, while the effects on negative affect are potentially attributed to ketamine metabolites including hydroxynorketamine (Li et al., 2015; Zanos et al., 2016). This hypothesis will need to be tested in future experiments. Moreover, our results are based on using a fixed dose of ketamine at 10 mg/kg, thus preventing dose-response observations. Future investigations are required to test how varying ketamine doses may influence morphine-induced CPP as well as morphine-induced anxiety-like behaviors.

Based on our findings that ketamine elicited anxiolytic-like behaviors following an acute injection, perhaps, the acute administration of ketamine was sufficient to prevent a negative affective state during 24 h morphine abstinence, thus facilitating the lack of motivation to seek a context paired with a drug reward (i.e., morphine-induced CPP). It is also plausible that the block of morphine-induced CPP by ketamine may be mediated by its effects on cognition and memory, thus blocking the recall of morphine-context associations (Ghoneim et al., 1985; Newcomer et al., 1999; Morgan et al., 2004; Malhotra et al., 1996; Pfenninger et al., 2002). Evidence suggests that ketamine-induced deficits in cognitive functioning and memory occur during the consolidation or, as shown in rodents, reconsolidation (Zhai et al., 2008) of information, rather than the retrieval of already learned associations (Honey et al., 2005). Furthermore, it has been shown in rodent models that the memory impairing effects of ketamine are not attributed to its effects on memory retrieval (Goulart et al., 2010). Therefore, acute injection of ketamine before CPP tests is not likely to influence already encoded morphine-context associations. However, we found that ketamine was effective at attenuating sucrose-induced CPP, despite the lack of anxiety-like behavior induced by sucrose conditioning (**Figures 4C,D**). Therefore, these data suggest that ketamine can interfere with memory associated with Pavlovian learning when administered before retrieval of already learned associations. We acknowledge that our data do not unequivocally demonstrate that the ketamine-induced block of morphine CPP is solely mediated by impairing already learned associations. Therefore, future studies are required to test whether blocking only morphine-induced negative affective states are sufficient to prevent morphine CPP.

Lastly, our data suggest that the effects of ketamine on morphine-induced anxiety-like behavior and morphine CPP are not likely a result of ketamine-induced behavioral disinhibition, which would be expected to increase exploratory behaviors. We found that ketamine did not affect entrance counts or exploratory behaviors in the CPP apparatus (**Figures 4E,F**).

Overall, our data suggest that ketamine may influence morphine CPP by altering negative affective states as well as by altering the memory of learned associations. However, this does not rule out that ketamine's effects on morphine-induced CPP may be mediated by other mechanisms of action as ketamine has proven effective for treating pain (Weisman, 1971; Laskowski et al., 2011; Jonkman et al., 2017), depression (Khorramzadeh and Lotfy, 1973; Sofia and Harakal, 1975), and inflammation (Roytblat et al., 1998; Beilin et al., 2007; Loix et al., 2011).

Ketamine as a Treatment Option for Substance Use Disorders

There is growing clinical and preclinical evidence that ketamine may be a potential treatment option for substance use disorders (Ivan Ezquerra-Romano et al., 2018; Jones et al., 2018). Through the use of Ketamine Assisted Psychotherapy (KAP; Ivan Ezquerra-Romano et al., 2018), alcohol-dependent patients (Krupitsky and Grinenko, 1997; Kolp et al., 2006), heroin-dependent patients (Krupitsky et al., 2002, 2007), and cocaine-dependent patients (Dakwar et al., 2017) showed greater rates of abstinence and reductions in drug craving. These results have been echoed in preclinical models of substance use disorders as acute ketamine injections significantly attenuate alcohol self-administration (Sabino et al., 2013) and prevent the reconsolidation of morphine-induced CPP (Zhai et al., 2008). Here, we discovered a novel and unexpected loss of long-term expression of morphine-induced CPP (PC28) in animals injected with (*R,S*)-ketamine at time points corresponding to 24 and 48 h post CPP conditioning. These results demonstrate the profound effect that (*R,S*)-ketamine has on reward-related behaviors and opens up many avenues including, investigating temporal effects of ketamine treatment at later time points following conditioning, the neurocircuit mechanisms mediating this prolonged ketamine effect on morphine-induced CPP, and the specificity for drug-context associations vs. other forms of memory. With the ever-increasing use of ketamine as an antidepressant in major depressive disorder (Berman et al., 2000; Diazgranados et al., 2010; Ibrahim et al., 2011; Zarate et al., 2012; Murrough et al., 2013b), applying its therapeutic use to patients suffering from substance use disorders holds potential value as an alternative treatment option.

Limitations to the Use of Ketamine as a Treatment Option for Substance Use Disorders

Despite its therapeutic value, ketamine has undesirable side effects including drowsiness, confusion, dizziness, and dissociative psychiatric side effects (Zarate et al., 2006; Diazgranados et al., 2010; Ibrahim et al., 2011; Murrough et al., 2013a). Additionally, evidence suggests that ketamine impairs cognition and memory (Harris et al., 1975; Ghoneim et al., 1985; Malhotra et al., 1996; Newcomer et al., 1999; Pfenninger et al., 2002; Morgan et al., 2004; Honey et al., 2005; Mathew et al., 2010; Driesen et al., 2013) and may cause urological effects (Middela and Pearce, 2011). A limitation of ketamine use as a treatment option for substance use disorders is its abuse potential (Liu et al., 2016). However, controlled studies in patients addressing the abuse potential of low-dose ketamine are lacking and if the long-lasting ketamine effects shown here in mice translate to human patients, the abuse liability can be mitigated by monthly physician-administered injections.

CONCLUSION

Here, we found that morphine conditioning in a three-compartment apparatus that elicits robust CPP was sufficient to evoke anxiety-like behaviors in mice. Additionally, we provided evidence that acute ketamine pretreatment produces anxiolytic-like behaviors and blocks morphine-induced CPP for a prolonged period, suggesting that ketamine is a potential option for attenuating negative reinforcement as well as learned associations that are implicated in substance use disorders.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

GM, HG, HJ, DM, SS, YS, and NG designed the experiments, performed the analyses, and wrote the manuscript. HJ, GM, HG, DM, and SS performed behavioral training and testing.

FUNDING

The study was supported by the Brain and Behavioral Research Foundation NARSAD Young Investigator Award (27364NG), the Pennsylvania State Junior Faculty Scholar Award (NG), the Pennsylvania Department of Health using Tobacco CURE Funds (NG), and the Pennsylvania State Research Allocation Project Grant (NG). Morphine was provided by the Drug Supply Program of NIDA NIH.

ACKNOWLEDGMENTS

We thank Dr. Diane McCloskey for edits and comments on this project as well as Dr. Patrick Randall and Dr. Zheng-Ming Ding for their comments on the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00075/full#supplementary-material.

FIGURE S1 | A summary showing that there is no significant difference in the amount of water consumed in the most preferred side among all groups ($F_{(12,140)} = 0.596$, $p = 0.843$, two-way repeated-measures ANOVA).

FIGURE S2 | (*R,S*)-ketamine administration during early abstinence blocks the prolonged retention of sucrose-induced CPP at post conditioning day 21. **(A)** Time line and drug regimen of the behavioral procedure. (*R,S*)-ketamine (10 mg/kg, i.p.) was injected 30 min prior to the first CPP test on post conditioning day 2 (PC2). **(B)** Summary showing that oral self-administration of sucrose produced CPP for the sucrose-paired context 21 days after conditioning. This prolonged expression of sucrose-induced CPP was blocked by (*R,S*)-ketamine when injected 30 min prior to testing on PC2 ($F_{(3,32)} = 5.51$, $p = 0.004$, one-way ANOVA, Bonferroni *post hoc* test). $^{*}p < 0.05$, $^{**}p < 0.01$.

REFERENCES

Akillioglu, K., Melik, E. B., Melik, E., and Boga, A. (2012). Effect of ketamine on exploratory behaviour in BALB/C and C57BL/6 mice. *Pharmacol. Biochem. Behav.* 100, 513–517. doi: 10.1016/j.pbb.2011.10.014

Aston-Jones, G., and Harris, G. C. (2004). Brain substrates for increased drug seeking during protracted withdrawal. *Neuropharmacology* 47, 167–179. doi: 10.1016/j.neuropharm.2004.06.020

Aston-Jones, G., Delfs, J. M., Druhan, J., and Zhu, Y. (1999). The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. *Ann. N Y Acad. Sci.* 877, 486–498. doi: 10.1111/j.1749-6632.1999.tb09284.x

Attarzadeh-Yazdi, G., Karimi, S., Azizi, P., Yazdi-Ravandi, S., Hesam, S., and Haghparast, A. (2013). Inhibitory effects of forced swim stress and corticosterone on the acquisition but not expression of morphine-induced conditioned place preference: involvement of glucocorticoid receptor in the basolateral amygdala. *Behav. Brain Res.* 252, 339–346. doi: 10.1016/j.bbr.2013.06.018

Baker, T. B., Piper, M. E., McCarthy, D. E., Majeskie, M. R., and Fiore, M. C. (2004). Addiction motivation reformulated: an affective processing model of negative reinforcement. *Psychol. Rev.* 111, 33–51. doi: 10.1037/0033-295x.111.1.33

Becker, J. A. J., Kieffer, B. L., and Le Merrer, J. (2017). Differential behavioral and molecular alterations upon protracted abstinence from cocaine versus morphine, nicotine, THC and alcohol. *Addict. Biol.* 22, 1205–1217. doi: 10.1111/adb.12405

Beilin, B., Rusabrov, Y., Shapira, Y., Roytblat, L., Greemberg, L., Yardeni, I. Z., et al. (2007). Low-dose ketamine affects immune responses in humans during the early postoperative period. *Br. J. Anaesth.* 99, 522–527. doi: 10.1093/bja/aem218

Benturquia, N., Le Guen, S., Canestrelli, C., Lagente, V., Apiou, G., Roques, B. P., et al. (2007). Specific blockade of morphine- and cocaine-induced reinforcing effects in conditioned place preference by nitrous oxide in mice. *Neuroscience* 149, 477–486. doi: 10.1016/j.neuroscience.2007.08.003

Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S., et al. (2000). Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry* 47, 351–354. doi: 10.1016/s0006-3223(99)00230-9

Bilbey, D. L., Salem, H., and Grossman, M. H. (1960). The anatomical basis of the straub phenomenon. *Br. J. Pharmacol. Chemother.* 15, 540–543. doi: 10.1111/j.1476-5381.1960.tb00277.x

Bohn, L. M., Gainetdinov, R. R., Sotnikova, T. D., Medvedev, I. O., Lefkowitz, R. J., Dykstra, L. A., et al. (2003). Enhanced rewarding properties of morphine, but not cocaine, in β(arrestin)-2 knock-out mice. *J. Neurosci.* 23, 10265–10273. doi: 10.1523/JNEUROSCI.23-32-10265.2003

Cabral, A., Ruggiero, R. N., Nobre, M. J., Brañdao, M. L., and Castilho, V. M. (2009). GABA and opioid mechanisms of the central amygdala underlie the withdrawal-potentiated startle from acute morphine. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 334–344. doi: 10.1016/j.pnpbp.2008.12.012

Cai, Y.-C., Ma, L., Fan, G.-H., Zhao, J., Jiang, L.-Z., and Pei, G. (1997). Activation of N-methyl-d-aspartate receptor attenuates acute responsiveness of δ-opioid receptors. *Mol. Pharmacol.* 51, 583–587. doi: 10.1124/mol.51.4.583

Conklin, C. A., and Perkins, K. A. (2005). Subjective and reinforcing effects of smoking during negative mood induction. *J. Abnorm. Psychol.* 114, 153–164. doi: 10.1037/0021-843x.114.1.153

Cooney, N. L., Litt, M. D., Morse, P. A., Bauer, L. O., and Gaupp, L. (1997). Alcohol cue reactivity, negative-mood reactivity and relapse in treated alcoholic men. *J. Abnorm. Psychol.* 106, 243–250. doi: 10.1037/0021-843x.106.2.243

da Silva, F. C. C., do Carmo De Oliveira Cito, M., da Silva, M. I. G., Moura, B. A., De Aquino Neto, M. R., Feitosa, M. L., et al. (2010). Behavioral alterations and pro-oxidant effect of a single ketamine administration to mice. *Brain Res. Bull.* 83, 9–15. doi: 10.1016/j.brainresbull.2010.05.011

Dakwar, E., Hart, C. L., Levin, F. R., Nunes, E. V., and Foltin, R. W. (2017). Cocaine self-administration disrupted by the N-methyl-D-aspartate receptor antagonist ketamine: a randomized, crossover trial. *Mol. Psychiatry* 22, 76–81. doi: 10.1038/mp.2016.39

Dawson, G. R., and Tricklebank, M. D. (1995). Use of the elevated plus maze in the search for novel anxiolytic agents. *Trends Pharmacol. Sci.* 16, 33–36. doi: 10.1016/s0165-6147(00)88973-7

Delfs, J. M., Zhu, Y., Druhan, J. P., and Aston-Jones, G. (2000). Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature* 403, 430–434. doi: 10.1038/35000212

Diana, M., Pistis, M., Muntoni, A., and Gessa, G. (1995). Profound decrease of mesolimbic dopaminergic neuronal activity in morphine withdrawn rats. *J. Pharmacol. Exp. Ther.* 272, 781–785. doi: 10.1097/00008877-199505001-00100

Diazgranados, N., Ibrahim, L., Brutsche, N. E., Newberg, A., Kronstein, P., Khalife, S., et al. (2010). A randomized add-on trial of an N-methyl-D-aspartate antagonist in treatment-resistant bipolar depression. *Arch. Gen. Psychiatry* 67, 793–802. doi: 10.1001/archgenpsychiatry.2010.90

Driesen, N. R., McCarthy, G., Bhagwagar, Z., Bloch, M. H., Calhoun, V. D., D'Souza, D. C., et al. (2013). The impact of NMDA receptor blockade on human working memory-related prefrontal function and connectivity. *Neuropsychopharmacology* 38, 2613–2622. doi: 10.1038/npp.2013.170

Ebert, B., Mikkelsen, S., Thorkildsen, C., and Borgbjerg, F. M. (1997). Norketamine, the main metabolite of ketamine, is a non-competitive NMDA receptor antagonist in the rat cortex and spinal cord. *Eur. J. Pharmacol.* 333, 99–104. doi: 10.1016/s0014-2999(97)01116-3

Engin, E., Treit, D., and Dickson, C. T. (2009). Anxiolytic- and antidepressant-like properties of ketamine in behavioral and neurophysiological animal models. *Neuroscience* 161, 359–369. doi: 10.1016/j.neuroscience.2009.03.038

Evans, C. J., and Cahill, C. M. (2016). Neurobiology of opioid dependence in creating addiction vulnerability. *F1000Res.* 5:1748. doi: 10.12688/f1000research.8369.1

Filibeck, U., and Castellano, C. (1980). Strain dependent effects of ketamine on locomotor activity and antinociception in mice. *Pharmacol. Biochem. Behav.* 13, 443–447. doi: 10.1016/0091-3057(80)90252-x

Fox, H. C., Bergquist, K. L., Hong, K. I., and Sinha, R. (2007). Stress-induced and alcohol cue-induced craving in recently abstinent alcohol-dependent individuals. *Alcohol. Clin. Exp. Res.* 31, 395–403. doi: 10.1111/j.1530-0277.2006.00320.x

Fraga, D. B., Olescowicz, G., Moretti, M., Siteneski, A., Tavares, M. K., Azevedo, D., et al. (2018). Anxiolytic effects of ascorbic acid and ketamine in mice. *J. Psychiatr. Res.* 100, 16–23. doi: 10.1016/j.jpsychires.2018.02.006

Freet, C. S., Wheeler, R. A., Leuenberger, E., Mosblech, N. A., and Grigson, P. S. (2013). Fischer rats are more sensitive than Lewis rats to the suppressive effects of morphine and the aversive κ-opioid agonist spiradoline. *Behav. Neurosci.* 127, 763–770. doi: 10.1037/a0033943

Fuentealba, J. A., Forray, M. I., and Gysling, K. (2000). Chronic morphine treatment and withdrawal increase extracellular levels of norepinephrine in the rat bed nucleus of the stria terminalis. *J. Neurochem.* 75, 741–748. doi: 10.1046/j.1471-4159.2000.0750741.x

Gallego, X., Murtra, P., Zamalloa, T., Canals, J. M., Pineda, J., Amador-Arjona, A., et al. (2010). Increased opioid dependence in a mouse model of panic disorder. *Front. Behav. Neurosci.* 3:60. doi: 10.3389/neuro.08.060.2009

Ganguly, S., Panetta, J. C., Roberts, J. K., and Schuetz, E. G. (2018). Ketamine pharmacokinetics and pharmacodynamics are altered by P-glycoprotein and breast cancer resistance protein efflux transporters in mice. *Drug Metab. Dispos.* 46, 1014–1022. doi: 10.1124/dmd.117.078360

Ghoneim, M. M., Hinrichs, J. V., Mewaldt, S. P., and Petersen, R. C. (1985). Ketamine: behavioral effects of subanesthetic doses. *J. Clin. Psychopharmacol.* 5, 70–77. doi: 10.1097/00004714-198504000-00003

Glue, P., Neehoff, S. M., Medlicott, N. J., Gray, A., Kibby, G., and McNaughton, N. (2018). Safety and efficacy of maintenance ketamine treatment in patients with treatment-refractory generalised anxiety and social anxiety disorders. *J. Psychopharmacol.* 32, 663–667. doi: 10.1177/0269881118762073

Gold, M. S., Redmond, D. E. Jr., and Kleber, H. D. (1978). Clonidine blocks acute opiate-withdrawal symptoms. *Lancet* 2, 599–602. doi: 10.1016/s0140-6736(78)92823-4

Gold, M. S., Redmond, D. E. Jr., and Kleber, H. D. (1979). Noradrenergic hyperactivity in opiate withdrawal supported by clonidine reversal of opiate withdrawal. *Am. J. Psychiatry* 136, 100–102. doi: 10.1176/ajp.136.1.100

Goulart, B. K., de Lima, M. N. M., de Farias, C. B., Reolon, G. K., Almeida, V. R., Quevedo, J., et al. (2010). Ketamine impairs recognition memory consolidation and prevents learning-induced increase in hippocampal brain-derived neurotrophic factor levels. *Neuroscience* 167, 969–973. doi: 10.1016/j.neuroscience.2010.03.032

Gracy, K. N., Dankiewicz, L. A., and Koob, G. F. (2001). Opiate withdrawal-induced fos immunoreactivity in the rat extended amygdala parallels the development of conditioned place aversion. *Neuropsychopharmacology* 24, 152–160. doi: 10.1016/s0893-133x(00)00186-x

Graziane, N. M., Sun, S., Wright, W. J., Jang, D., Liu, Z., Huang, Y. H., et al. (2016). Opposing mechanisms mediate morphine- and cocaine-induced generation of silent synapses. *Nat. Neurosci.* 19, 915–925. doi: 10.1038/nn.4313

Gregus, A., Wintink, A. J., Davis, A. C., and Kalynchuk, L. E. (2005). Effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats. *Behav. Brain Res.* 156, 105–114. doi: 10.1016/j.bbr.2004.05.013

Gremel, C. M., Gabriel, K. I., and Cunningham, C. L. (2006). Topiramate does not affect the acquisition or expression of ethanol conditioned place preference in DBA/2J or C57BL/6J mice. *Alcohol. Clin. Exp. Res.* 30, 783–790. doi: 10.1111/j.1530-0277.2006.00091.x

Grisel, J. E., Bartels, J. L., Allen, S. A., and Turgeon, V. L. (2008). Influence of β-endorphin on anxious behavior in mice: interaction with EtOH. *Psychopharmacology* 200, 105–115. doi: 10.1007/s00213-008-1161-4

Haghparast, A., Fatahi, Z., Alamdary, S. Z., Reisi, Z., and Khodagholi, F. (2014). Changes in the levels of p-ERK, p-CREB, and c-fos in rat mesocorticolimbic dopaminergic system after morphine-induced conditioned place preference: the role of acute and subchronic stress. *Cell. Mol. Neurobiol.* 34, 277–288. doi: 10.1007/s10571-013-0011-z

Handley, S. L., and McBlane, J. W. (1993). An assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs. *J. Pharmacol. Toxicol. Methods* 29, 129–138. doi: 10.1016/1056-8719(93)90063-k

Hanse, E., Seth, H., and Riebe, I. (2013). AMPA-silent synapses in brain development and pathology. *Nat. Rev. Neurosci.* 14, 839–850. doi: 10.1038/nrn3642

Harris, J. A., Biersner, R. J., Edwards, D., and Bailey, L. W. (1975). Attention, learning, and personality during ketamine emergence: a pilot study. *Anesth. Analg.* 54, 169–172. doi: 10.1213/00000539-197503000-00001

Hearing, M., Graziane, N., Dong, Y., and Thomas, M. J. (2018). Opioid and psychostimulant plasticity: targeting overlap in nucleus accumbens glutamate signaling. *Trends Pharmacol. Sci.* 39, 276–294. doi: 10.1016/j.tips.2017.12.004

Heinrichs, S. C., Menzaghi, F., Schulteis, G., Koob, G. F., and Stinus, L. (1995). Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal. *Behav. Pharmacol.* 6, 74–80. doi: 10.1097/00008877-199501000-00011

Honey, G. D., Honey, R. A., Sharar, S. R., Turner, D. C., Pomarol-Clotet, E., Kumaran, D., et al. (2005). Impairment of specific episodic memory processes by sub-psychotic doses of ketamine: the effects of levels of processing at encoding and of the subsequent retrieval task. *Psychopharmacology* 181, 445–457. doi: 10.1007/s00213-005-0001-z

Huston, J. P., Silva, M. A., Topic, B., and Müller, C. P. (2013). What's conditioned in conditioned place preference? *Trends Pharmacol. Sci.* 34, 162–166. doi: 10.1016/j.tips.2013.01.004

Huys, Q. J. M., Tobler, P. N., Hasler, G., and Flagel, S. B. (2014). "Chapter 3—the role of learning-related dopamine signals in addiction vulnerability," in *Progress in Brain Research*, eds M. Diana, G. Di Chiara and P. Spano (Elsevier), 31–77.

Ibrahim, L., Diazgranados, N., Luckenbaugh, D. A., Machado-Vieira, R., Baumann, J., Mallinger, A. G., et al. (2011). Rapid decrease in depressive symptoms with an N-methyl-d-aspartate antagonist in ECT-resistant major depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1155–1159. doi: 10.1016/j.pnpbp.2011.03.019

Ivan Ezquerra-Romano, I., Lawn, W., Krupitsky, E., and Morgan, C. J. A. (2018). Ketamine for the treatment of addiction: evidence and potential mechanisms. *Neuropharmacology* 142, 72–82. doi: 10.1016/j.neuropharm.2018.01.017

Jansen, K. (1989). Near death experience and the NMDA receptor. *BMJ* 298, 1708–1708. doi: 10.1136/bmj.298.6689.1708-b

Jones, J. L., Mateus, C. F., Malcolm, R. J., Brady, K. T., and Back, S. E. (2018). Efficacy of ketamine in the treatment of substance use disorders: a systematic review. *Front. Psychiatry* 9, 277–277. doi: 10.3389/fpsyt.2018.00277

Jonkman, K., Dahan, A., van de Donk, T., Aarts, L., Niesters, M., and Van Velzen, M. (2017). Ketamine for pain. *F1000Res.* 6:F1000. doi: 10.12688/f1000research.11372.1

Karimi, S., Attarzadeh-Yazdi, G., Yazdi-Ravandi, S., Hesam, S., Azizi, P., Razavi, Y., et al. (2014). Forced swim stress but not exogenous corticosterone could induce the reinstatement of extinguished morphine conditioned place preference in rats: involvement of glucocorticoid receptors in the basolateral amygdala. *Behav. Brain Res.* 264, 43–50. doi: 10.1016/j.bbr.2014.01.045

Khorramzadeh, E., and Lotfy, A. O. (1973). The use of ketamine in psychiatry. *Psychosomatics* 14, 344–346. doi: 10.1016/s0033-3182(73)71306-2

Kohrs, R., and Durieux, M. E. (1998). Ketamine: teaching an old drug new tricks. *Anesth. Analg.* 87, 1186–1193. doi: 10.1097/00000539-199811000-00039

Kõks, S., Soosaar, A., Võikar, V., Bourin, M., and Vasar, E. (1999). BOC-CCK-4, CCK(B)receptor agonist, antagonizes anxiolytic-like action of morphine in elevated plus-maze. *Neuropeptides* 33, 63–69. doi: 10.1054/npep.1999.0015

Kolp, E., Friedman, H. L., Young, M. S., and Krupitsky, E. (2006). Ketamine enhanced psychotherapy: preliminary clinical observations on its effectiveness in treating alcoholism. *Hum. Psychol.* 34, 399–422. doi: 10.1207/s15473333thp3404_7

Koo, J. W., Lobo, M. K., Chaudhury, D., Labonté, B., Friedman, A., Heller, E., et al. (2014). Loss of BDNF signaling in D1R-expressing NAc neurons enhances morphine reward by reducing GABA inhibition. *Neuropsychopharmacology* 39, 2646–2653. doi: 10.1038/npp.2014.118

Koob, G. F., and Le Moal, M. (2008). Review. Neurobiological mechanisms for opponent motivational processes in addiction. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 3113–3123. doi: 10.1098/rstb.2008.0094

Krupitsky, E. M., Burakov, A. M., Dunaevsky, I. V., Romanova, T. N., Slavina, T. Y., and Grinenko, A. Y. (2007). Single versus repeated sessions of ketamine-assisted psychotherapy for people with heroin dependence. *J. Psychoactive Drugs* 39, 13–19. doi: 10.1080/02791072.2007.10399860

Krupitsky, E., Burakov, A., Romanova, T., Dunaevsky, I., Strassman, R., and Grinenko, A. (2002). Ketamine psychotherapy for heroin addiction: immediate effects and two-year follow-up. *J. Subst. Abuse Treat.* 23, 273–283. doi: 10.1016/s0740-5472(02)00275-1

Krupitsky, E. M., and Grinenko, A. Y. (1997). Ketamine psychedelic therapy (KPT): a review of the results of ten years of research. *J. Psychoactive Drugs* 29, 165–183. doi: 10.1080/02791072.1997.10400185

Krystal, J. H., Karper, L. P., Seibyl, J. P., Freeman, G. K., Delaney, R., Bremner, J. D., et al. (1994). Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Arch. Gen. Psychiatry* 51, 199–214. doi: 10.1001/archpsyc.1994.03950030035004

Kubota, T., Hirota, K., Yoshida, H., Takahashi, S., Anzawa, N., Ohkawa, H., et al. (1999). Effects of sedatives on noradrenaline release from the medial prefrontal cortex in rats. *Psychopharmacology* 146, 335–338. doi: 10.1007/s002130051125

Laskowski, K., Stirling, A., McKay, W. P., and Lim, H. J. (2011). A systematic review of intravenous ketamine for postoperative analgesia. *Can. J. Anaesth.* 58, 911–923. doi: 10.1007/s12630-011-9560-0

Li, X., Martinez-Lozano Sinues, P., Dallmann, R., Bregy, L., Hollmen, M., Proulx, S., et al. (2015). Drug pharmacokinetics determined by real-time analysis of mouse breath. *Angew. Chem. Int. Ed. Engl.* 54, 7815–7818. doi: 10.1002/anie.201503312

Li, S. X., Shi, J., Epstein, D. H., Wang, X., Zhang, X. L., Bao, Y. P., et al. (2009). Circadian alteration in neurobiology during 30 days of abstinence in heroin users. *Biol. Psychiatry* 65, 905–912. doi: 10.1016/j.biopsych.2008.11.025

Lindholm, J. S. O., Autio, H., Vesa, L., Antila, H., Lindemann, L., Hoener, M. C., et al. (2012). The antidepressant-like effects of glutamatergic drugs ketamine and AMPA receptor potentiator LY 451646 are preserved in BDNF+/− heterozygous null mice. *Neuropharmacology* 62, 391–397. doi: 10.1016/j.neuropharm.2011.08.015

Liu, Y., Lin, D., Wu, B., and Zhou, W. (2016). Ketamine abuse potential and use disorder. *Brain Res. Bull.* 126, 68–73. doi: 10.1016/j.brainresbull.2016.05.016

Lodge, D., Anis, N. A., and Burton, N. R. (1982). Effects of optical isomers of ketamine on excitation of cat and rat spinal neurones by amino acids and acetylcholine. *Neurosci. Lett.* 29, 281–286. doi: 10.1016/0304-3940(82)90330-5

Loix, S., De Kock, M., and Henin, P. (2011). The anti-inflammatory effects of ketamine: state of the art. *Acta Anaesthesiol. Belg.* 62, 47–58.

Louderback, K. M., Wills, T. A., Muglia, L. J., and Winder, D. G. (2013). Knockdown of BNST GluN2B-containing NMDA receptors mimics the actions of ketamine on novelty-induced hypophagia. *Transl. Psychiatry* 3:e331. doi: 10.1038/tp.2013.103

Lu, L., Chen, H., Su, W., Ge, X., Yue, W., Su, F., et al. (2005). Role of withdrawal in reinstatement of morphine-conditioned place preference. *Psychopharmacology* 181, 90–100. doi: 10.1007/s00213-005-2207-5

Lydic, R., and Baghdoyan, H. A. (2002). Ketamine and MK-801 decrease acetylcholine release in the pontine reticular formation, slow breathing, and disrupt sleep. *Sleep* 25, 617–622. doi: 10.1093/sleep/25.6.615

Maj, M., Turchan, J., Śmiaowska, M., and Przewocka, B. (2003). Morphine and cocaine influence on CRF biosynthesis in the rat central nucleus of amygdala. *Neuropeptides* 37, 105–110. doi: 10.1016/s0143-4179(03)00021-0

Malhotra, A. K., Pinals, D. A., Weingartner, H., Sirocco, K., Missar, C. D., Pickar, D., et al. (1996). NMDA receptor function and human cognition: the effects of ketamine in healthy volunteers. *Neuropsychopharmacology* 14, 301–307. doi: 10.1016/0893-133x(95)00137-3

Martins, S. S., Fenton, M. C., Keyes, K. M., Blanco, C., Zhu, H., and Storr, C. L. (2012). Mood and anxiety disorders and their association with non-medical prescription opioid use and prescription opioid-use disorder: longitudinal evidence from the National Epidemiologic Study on Alcohol and Related Conditions. *Psychol. Med.* 42, 1261–1272. doi: 10.1017/s0033291711002145

Mathew, S. J., Murrough, J. W., aan het Rot, M., Collins, K. A., Reich, D. L., and Charney, D. S. (2010). Riluzole for relapse prevention following intravenous ketamine in treatment-resistant depression: a pilot randomized, placebo-controlled continuation trial. *Int. J. Neuropsychopharmacol.* 13, 71–82. doi: 10.1017/s1461145709000169

Maxwell, C. R., Ehrlichman, R. S., Liang, Y., Trief, D., Kanes, S. J., Karp, J., et al. (2006). Ketamine produces lasting disruptions in encoding of sensory stimuli. *J. Pharmacol. Exp. Ther.* 316, 315–324. doi: 10.1124/jpet.105.091199

McDevitt, D. S., and Graziane, N. M. (2018). Neuronal mechanisms mediating pathological reward-related behaviors: A focus on silent synapses in the nucleus accumbens. *Pharmacol. Res.* 136, 90–96. doi: 10.1016/j.phrs.2018.08.025

McDevitt, D. S., and Graziane, N. M. (2019). Timing of morphine administration differentially alters paraventricular thalamic neuron activity. *eNeuro* 6:ENEURO.0377–0319.2019. doi: 10.1523/eneuro.0377-19.2019

Middela, S., and Pearce, I. (2011). Ketamine-induced vesicopathy: a literature review. *Int. J. Clin. Pract.* 65, 27–30. doi: 10.1111/j.1742-1241.2010.02502.x

Miller, D. B., Dougherty, J. A., and Wikler, A. (1979). Interoceptive conditioning through repeated suppression of morphine-abstinence. II. Relapse-testing. *Pavlov. J. Biol. Sci.* 14, 170–176. doi: 10.1007/BF03001978

Moaddel, R., Abdrakhmanova, G., Kozak, J., Jozwiak, K., Toll, L., Jimenez, L., et al. (2013). Sub-anesthetic concentrations of (R,S)-ketamine metabolites inhibit acetylcholine-evoked currents in α7 nicotinic acetylcholine receptors. *Eur. J. Pharmacol.* 698, 228–234. doi: 10.1016/j.ejphar.2012.11.023

Morgan, C. J., Mofeez, A., Brandner, B., Bromley, L., and Curran, H. V. (2004). Acute effects of ketamine on memory systems and psychotic symptoms in healthy volunteers. *Neuropsychopharmacology* 29, 208–218. doi: 10.1038/sj.npp.1300342

Murrough, J. W., Iosifescu, D. V., Chang, L. C., Al Jurdi, R. K., Green, C. E., Perez, A. M., et al. (2013a). Antidepressant efficacy of ketamine in treatment-resistant major depression: a two-site randomized controlled trial. *Am. J. Psychiatry* 170, 1134–1142. doi: 10.1176/appi.ajp.2013.13030392

Murrough, J. W., Perez, A. M., Pillemer, S., Stern, J., Parides, M. K., aan het Rot, M., et al. (2013b). Rapid and longer-term antidepressant effects of repeated ketamine infusions in treatment-resistant major depression. *Biol. Psychiatry* 74, 250–256. doi: 10.1016/j.biopsych.2012.06.022

Newcomb, M. D., and Bentler, P. M. (1988). Impact of adolescent drug use and social support on problems of young adults: a longitudinal study. *J. Abnorm. Psychol.* 97, 64–75. doi: 10.1037//0021-843x.97.1.64

Newcomer, J. W., Farber, N. B., Jevtovic-Todorovic, V., Selke, G., Melson, A. K., Hershey, T., et al. (1999). Ketamine-induced NMDA receptor hypofunction as a model of memory impairment and psychosis. *Neuropsychopharmacology* 20, 106–118. doi: 10.1016/s0893-133x(98)00067-0

O'Brien, C. P. (1975). Experimental analysis of conditioning factors in human narcotic addiction. *Pharmacol. Rev.* 27, 533–543.

O'Brien, C. P., Childress, A. R., McLellan, A. T., and Ehrman, R. (1992). Classical conditioning in drug-dependent humans. *Ann. N Y Acad. Sci.* 654, 400–415. doi: 10.1111/j.1749-6632.1992.tb25984.x

O'Brien, E. R., and Ternes, J. W. (1986). "Classical conditioning in human opioid dependence," in *Behavioral Analysis of Drug Dependence*, ed. S. I. Goldberg (Orlando, FL: Academic), 329–356.

Pellow, S., Chopin, P., File, S. E., and Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14, 149–167. doi: 10.1016/0165-0270(85)90031-7

Perkins, K. A., and Grobe, J. E. (1992). Increased desire to smoke during acute stress. *Br. J. Addict.* 87, 1037–1040. doi: 10.1111/j.1360-0443.1992.tb03121.x

Pfenninger, E. G., Durieux, M. E., and Himmelseher, S. (2002). Cognitive impairment after small-dose ketamine isomers in comparison to equianalgesic racemic ketamine in human volunteers. *Anesthesiology* 96, 357–366. doi: 10.1097/00000542-200202000-00022

Ribeiro Do Couto, B., Aguilar, M. A., Manzanedo, C., Rodríguez-Arias, M., Armario, A., and Miñarro, J. (2006). Social stress is as effective as physical stress in reinstating morphine-induced place preference in mice. *Psychopharmacology* 185, 459–470. doi: 10.1007/s00213-006-0345-z

Robinson, T. E., and Kolb, B. (1999). Morphine alters the structure of neurons in the nucleus accumbens and neocortex of rats. *Synapse* 33, 160–162. doi: 10.1002/(sici)1098-2396(199908)33:2<160::aid-syn6>3.0.co;2-s

Roytblat, L., Talmor, D., Rachinsky, M., Greemberg, L., Pekar, A., Appelbaum, A., et al. (1998). Ketamine attenuates the interleukin-6 response after cardiopulmonary bypass. *Anesth. Analg.* 87, 266–271. doi: 10.1097/00000539-199808000-00006

Sabino, V., Narayan, A. R., Zeric, T., Steardo, L., and Cottone, P. (2013). mTOR activation is required for the anti-alcohol effect of ketamine, but not memantine, in alcohol-preferring rats. *Behav. Brain Res.* 247, 9–16. doi: 10.1016/j.bbr.2013.02.030

Sasaki, K., Fan, L. W., Tien, L. T., Ma, T., Loh, H. H., and Ho, I. K. (2002). The interaction of morphine and γ-aminobutyric acid (GABA)ergic systems in anxiolytic behavior: using mu-opioid receptor knockout mice. *Brain Res. Bull.* 57, 689–694. doi: 10.1016/s0361-9230(01)00785-7

Scheller, M., Bufler, J., Hertle, I., Schneck, H. J., Franke, C., and Kochs, E. (1996). Ketamine blocks currents through mammalian nicotinic acetylcholine receptor channels by interaction with both the open and the closed state. *Anesth. Analg.* 83, 830–836. doi: 10.1097/00000539-199610000-00031

Shadli, S. M., Kawe, T., Martin, D., Mcnaughton, N., Neehoff, S., and Glue, P. (2018). Ketamine effects on EEG during therapy of treatment-resistant generalized anxiety and social anxiety. *Int. J. Neuropsychopharmacol.* 21, 717–724. doi: 10.1093/ijnp/pyy032

Shaham, Y., Rajabi, H., and Stewart, J. (1996). Relapse to heroin-seeking in rats under opioid maintenance: the effects of stress, heroin priming, and withdrawal. *J. Neurosci.* 16, 1957–1963. doi: 10.1523/jneurosci.16-05-01957.1996

Shi, J., Li, S. X., Zhang, X. L., Wang, X., Le Foll, B., Zhang, X. Y., et al. (2009). Time-dependent neuroendocrine alterations and drug craving during the first month of abstinence in heroin addicts. *Am. J. Drug Alcohol Abuse* 35, 267–272. doi: 10.1080/00952990902933878

Shin, I. C., Kim, H. C., Swanson, J., Hong, J. T., and Oh, K. W. (2003). Anxiolytic effects of acute morphine can be modulated by nitric oxide systems. *Pharmacology* 68, 183–189. doi: 10.1159/000070457

Silvestre, J. S., Nadal, R., Pallares, M., and Ferre, N. (1997). Acute effects of ketamine in the holeboard, the elevated-plus maze and the social interaction test in Wistar rats. *Depress Anxiety* 5, 29–33. doi: 10.1002/(sici)1520-6394(1997)5:1<29::aid-da5>3.0.co;2-0

Sinha, R. (2008). Chronic stress, drug use, and vulnerability to addiction. *Ann. N Y Acad. Sci.* 1141, 105–130. doi: 10.1196/annals.1441.030

Smith, R. J., and Aston-Jones, G. (2008). Noradrenergic transmission in the extended amygdala: role in increased drug-seeking and relapse during protracted drug abstinence. *Brain Struct. Funct.* 213, 43–61. doi: 10.1007/s00429-008-0191-3

Sofia, R. D., and Harakal, J. J. (1975). Evaluation of ketamine HCl for anti-depressant activity. *Arch. Int. Pharmacodyn. Ther.* 214, 68–74.

Solomon, R. L., and Corbit, J. D. (1978). An opponent-process theory of motivation. *Am. Econ. Rev.* 68, 12–24.

Suzuki, T., Kato, H., Aoki, T., Tsuda, M., Narita, M., and Misawa, M. (2000). Effects of the non-competitive NMDA receptor antagonist ketamine on morphine-induced place preference in mice. *Life Sci.* 67, 383–389. doi: 10.1016/s0024-3205(00)00639-1

Taylor, J. H., Landeros-Weisenberger, A., Coughlin, C., Mulqueen, J., Johnson, J. A., Gabriel, D., et al. (2018). Ketamine for social anxiety disorder: a randomized, placebo-controlled crossover trial. *Neuropsychopharmacology* 43, 325–333. doi: 10.1038/npp.2017.194

Tyler, M. W., Yourish, H. B., Ionescu, D. F., and Haggarty, S. J. (2017). Classics in chemical neuroscience: ketamine. *ACS Chem. Neurosci.* 8, 1122–1134. doi: 10.1021/acschemneuro.7b00074

Tzschentke, T. M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.* 12, 227–462. doi: 10.1111/j.1369-1600.2007.00070.x

Wang, J., Fang, Q., Liu, Z., and Lu, L. (2006). Region-specific effects of brain corticotropin-releasing factor receptor type 1 blockade on footshock-stress- or drug-priming-induced reinstatement of morphine conditioned place preference in rats. *Psychopharmacology* 185, 19–28. doi: 10.1007/s00213-005-0262-6

Wang, M., Wong, A. H., and Liu, F. (2012). Interactions between NMDA and dopamine receptors: a potential therapeutic target. *Brain Res.* 1476, 154–163. doi: 10.1016/j.brainres.2012.03.029

Weisman, H. (1971). Anesthesia for pediatric ophthalmology. *Ann. Ophthalmol.* 3, 229–232.

Wetter, D. W., Smith, S. S., Kenford, S. L., Jorenby, D. E., Fiore, M. C., Hurt, R. D., et al. (1994). Smoking outcome expectancies: factor structure, predictive validity and discriminant validity. *J. Abnorm. Psychol.* 103, 801–811. doi: 10.1037/0021-843x.103.4.801

Whitaker, L. R., Degoulet, M., and Morikawa, H. (2013). Social deprivation enhances VTA synaptic plasticity and drug-induced contextual learning. *Neuron* 77, 335–345. doi: 10.1016/j.neuron.2012.11.022

Wikler, A. (2013). *Opioid Dependence: Mechanisms and Treatment.* New York, NY: Springer.

Xue, Y. X., Luo, Y. X., Wu, P., Shi, H. S., Xue, L. F., Chen, C., et al. (2012). A memory retrieval-extinction procedure to prevent drug craving and relapse. *Science* 336, 241–245. doi: 10.1126/science.1215070

Zanos, P., Moaddel, R., Morris, P. J., Georgiou, P., Fischell, J., Elmer, G. I., et al. (2016). NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature* 533, 481–486. doi: 10.1038/nature 17998

Zanos, P., Moaddel, R., Morris, P. J., Riggs, L. M., Highland, J. N., Georgiou, P., et al. (2018). Ketamine and ketamine metabolite pharmacology: insights into therapeutic mechanisms. *Pharmacol. Rev.* 70, 621–660. doi: 10.1124/pr.117.015198

Zarate, C. A. Jr., Brutsche, N., Laje, G., Luckenbaugh, D. A., Venkata, S. L., Ramamoorthy, A., et al. (2012). Relationship of ketamine's plasma metabolites with response, diagnosis and side effects in major depression. *Biol. Psychiatry* 72, 331–338. doi: 10.1016/j.biopsych.2012.03.004

Zarate, C. A. Jr., Singh, J. B., Carlson, P. J., Brutsche, N. E., Ameli, R., Luckenbaugh, D. A., et al. (2006). A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch. Gen. Psychiatry* 63, 856–864. doi: 10.1001/archpsyc.63.8.856

Zhai, H., Wu, P., Chen, S., Li, F., Liu, Y., and Lu, L. (2008). Effects of scopolamine and ketamine on reconsolidation of morphine conditioned place preference in rats. *Behav. Pharmacol.* 19, 211–216. doi: 10.1097/fbp.0b013e3282fe88a0

Zhang, L.-M., Zhou, W.-W., Ji, Y.-J., Li, Y., Zhao, N., Chen, H.-X., et al. (2015). Anxiolytic effects of ketamine in animal models of posttraumatic stress disorder. *Psychopharmacology* 232, 663–672. doi: 10.1007/s00213-014-3697-9

Zinser, M. C., Baker, T. B., Sherman, J. E., and Cannon, D. S. (1992). Relation between self-reported affect and drug urges and cravings in continuing and withdrawing smokers. *J. Abnorm. Psychol.* 101, 617–629. doi: 10.1037/0021-843x.101.4.617

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: 31 July 2020
doi: 10.3389/fnbeh.2020.00122

N-Acetylcysteine and Acetylsalicylic Acid Inhibit Alcohol Consumption by Different Mechanisms: Combined Protection

María Elena Quintanilla[1]† Fernando Ezquer[2†], Paola Morales[1,3], Marcelo Ezquer[2] Belen Olivares[4], Daniela Santapau[2], Mario Herrera-Marschitz[1] and Yedy Israel[1]*

[1]Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile, [2]Centro de Medicina Regenerativa, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago, Chile, [3]Department of Neuroscience, Faculty of Medicine, University of Chile, Santiago, Chile, [4]Centro de Química Médica, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago, Chile

OPEN ACCESS

Edited by:
Marcello Solinas,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Jerome Jeanblanc,
INSERM U1247 Groupe de Recherche sur l'Alcool et les Pharmacodépendances (GRAP), France
Anna Brancato,
University of Palermo, Italy

***Correspondence:**
María Elena Quintanilla
equintanilla@med.uchile.cl

†These authors have contributed equally to this work

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

Received: 19 April 2020
Accepted: 23 June 2020
Published: 31 July 2020

Citation:
Quintanilla ME, Ezquer F, Morales P, Ezquer M, Olivares B, Santapau D, Herrera-Marschitz M and Israel Y (2020) N-Acetylcysteine and Acetylsalicylic Acid Inhibit Alcohol Consumption by Different Mechanisms: Combined Protection. Front. Behav. Neurosci. 14:122. doi: 10.3389/fnbeh.2020.00122

Chronic ethanol intake results in brain oxidative stress and neuroinflammation, which have been postulated to perpetuate alcohol intake and to induce alcohol relapse. The present study assessed the mechanisms involved in the inhibition of: (i) oxidative stress; (ii) neuroinflammation; and (iii) ethanol intake that follow the administration of the antioxidant N-acetylcysteine (NAC) and the anti-inflammatory acetylsalicylic acid (ASA) to animals that had consumed ethanol chronically. At doses used clinically, NAC [40 mg/kg per day orally (p.o.)] and ASA (15 mg/kg per day p.o.) significantly inhibited chronic alcohol intake and relapse intake in alcohol-preferring rats. The coadministration of both drugs reduced ethanol intake by 65% to 70%. N-acetylcysteine administration: (a) induced the Nrf2-ARE system, lowering the hippocampal oxidative stress assessed as the ratio of oxidized glutathione (GSSG)/reduced glutathione (GSH); (b) reduced the neuroinflammation assessed by astrocyte and microglial activation by immunofluorescence; and (c) inhibited chronic and relapse ethanol intake. These effects were blocked by sulfasalazine, an inhibitor of the xCT transporter, which incorporates cystine (precursor of GSH) and extrudes extracellular glutamate, an agonist of the inhibitory mGlu2/3 receptor, which lowers the synaptic glutamatergic tone. The inhibitor of mGlu2/3 receptor (LY341495) blocked the NAC-induced inhibition of both relapse ethanol intake and neuroinflammation without affecting the GSSG/GSH ratio. Unlike N-acetylcysteine, ASA inhibited chronic alcohol intake and relapse *via* lipoxin A4, a strong anti-inflammatory metabolite of arachidonic acid generated following the ASA acetylation of cyclooxygenases. Accordingly, the lipoxin A4 receptor inhibitor, WRW4, blocked the ASA-induced reduction of ethanol intake. Overall, *via* different mechanisms, NAC and ASA administered in clinically relevant doses combine their effects inhibiting ethanol intake.

Keywords: N-acetylcysteine, aspirin, ethanol, relapse, rats

INTRODUCTION

The proposal put forward by Quertemont et al. (2005) that acetaldehyde generated in the metabolism of ethanol by brain catalase would play a role in eliciting the reinforcing effects of ethanol has been supported by a number of studies. Reports from Canada, Spain, United States, Italy, and Chile have shown that: (i) the administration of inhibitors of catalase or acetaldehyde trapping agents inhibits alcohol self-administration in animals (Aragon and Amit, 1992; Koechling and Amit, 1994; Tampier et al., 1995; Orrico et al., 2017; Peana et al., 2015); (ii) rats self-administer acetaldehyde into the brain ventral tegmental area (VTA), at concentrations that are three orders of magnitude lower than those required for ethanol to induce its self-administration (Rodd et al., 2005); and (iii) a VTA-directed gene transduction aimed at either inhibiting catalase synthesis or overexpressing the low K_m aldehyde dehydrogenase 2 (ALDH2) virtually blocks the self-administration of ethanol (Karahanian et al., 2011, 2015). Additionally, (iv) it has been shown that salsolinol, the adduct formed between acetaldehyde and dopamine, likely mediates the early reinforcing effects of acetaldehyde (Quintanilla et al., 2014; Melis et al., 2015; Quintanilla et al., 2016a; Rodd et al., 2003; Rojkovicova et al., 2008). The reader is referred to a comprehensive review on the actions of acetaldehyde (Correa et al., 2012).

The logical extension that inhibiting the molecular mechanisms that initiate ethanol intake would also inhibit chronic alcohol intake proved to be wrong; several studies showed that ethanol intake by rats that had learned to chronically self-administer ethanol was not inhibited by either: (a) anticatalase inhibitors (Peana et al., 2015); (b) acetaldehyde trapping agents (Peana et al., 2015; Orrico et al., 2017); or (c) anticatalase gene transduction or Aldh2 gene overexpression (Quintanilla et al., 2012; Karahanian et al., 2015).

From the above reports, mechanism(s) different from those that initiate the self-administration of ethanol must account for this "autopilot" continuation of alcohol intake. For several addictive drugs, relapse self-administration has been reported to be inhibited by the antioxidant drug N-acetylcysteine (NAC; Duailibi et al., 2017; Garcia-Keller et al., 2019). For alcohol, studies by Quintanilla et al. (2016a, 2018), Lebourgeois et al. (2018, 2019), and Israel et al. (2019) showed that the administration of NAC markedly inhibits both chronic alcohol intake and relapse intake. Treatments with NAC were shown to significantly inhibit both oxidative stress and neuroinflammation (Quintanilla et al., 2018; Israel et al., 2019).

The above studies dovetail with studies from the laboratories of Crews, Guerri, and Harris, who showed that chronic alcohol intake or its administration leads to neuroinflammation (Alfonso-Loeches et al., 2010; Mayfield et al., 2013; Crews and Vetreno, 2016). Neuroinflammation and oxidative stress highly interrelate and self-perpetuate each other (Schreck et al., 1992; Schulze-Osthoff et al., 1992; Canty et al., 1999; Kastl et al., 2014; Israel et al., 2019). Studies by Qin et al. (2007) showed that the administration of a single systemic dose of lipopolysaccharide (LPS) results in brain inflammation that remains for many months. Studies by Blednov et al. (2011) showed that the administration of LPS to mice increased the preference for increasing concentrations of alcohol solutions, an effect that was long-lasting. Noteworthy, oral alcohol intake (yielding acetaldehyde generated in the gut) leads to an increased gut leakiness, which allows bacterial LPS to enter into the portal circulation (Ferrier et al., 2006). Noteworthy, in detoxified alcoholics, a strong relationship is seen between plasma LPS and proinflammatory cytokine levels and alcohol craving (Leclercq et al., 2017).

While NAC has been used for decades as a strong antioxidant, the actual mechanism of its antioxidant effect is not fully clear. Recent studies showed that NAC amide (NACA), an analog of NAC, attenuated the oxidative stress in rats following traumatic brain injury *via* activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)–antioxidant response element (ARE) signal pathway (Zhou et al., 2018). *In vitro*, an overexpression of the Nrf2 system has been shown to up-regulate the activity of the promoter of the cystine/glutamate antiporter xCT in several types of cells (Habib et al., 2015; Shih et al., 2003). Thus, NAC *via* Nrf2 could reduce the glutamatergic tone both by activation of the xCT cystine-glutamate antiporter and increasing cystine as substrate for the antiporter, thus activating the inhibitory presynaptic metabotropic mGlu2/3 receptor.

Recent studies in alcohol-preferring rats showed that both neuroinflammation and chronic alcohol intake/relapse are inhibited by administration of acetylsalicylic acid (ASA; Israel et al., 2019). These effects of ASA were accompanied by increases in the levels of the glutamate transporter GLT-1 (see also Romera et al., 2007; Sobrado et al., 2009). Increases in GLT-1 levels and a reduction of chronic ethanol intake following the administration of β-lactam antibiotics have also been reported by Sari and associates in rats selected as alcohol drinkers (Rao et al., 2015; Sari et al., 2016). Recent and previous studies also indicate that the β-lactam ceftriaxone displays anti-inflammatory effects (Amin et al., 2012; Ochoa-Aguilar et al., 2018).

A number of studies have shown that ASA, even at low anticlotting doses, acutely acetylates cyclooxygenase-1 (Cox-1) in platelets, and chronically, it also acetylates Cox-2 leading to the generation of a powerful anti-inflammatory agent; namely, 15-R epi-lipoxin A4 (lipoxin A4 or ATL), a metabolite of arachidonic acid (Romano et al., 2015; Serhan and Levy, 2018). Whether the inhibitory effect of ASA on ethanol intake is due to the generation of lipoxin A4 is not known.

In operant self-administration studies, the presentation of a stimulus previously associated with alcohol self-administration leads to marked increases in glutamate release in nucleus accumbens (Gass et al., 2011). An increased release of glutamate is postulated to play an important role in addictive drug relapse and craving (see Scofield et al., 2016). Thus, it is hypothesized that increases in the GLT-1 transporter, as reported for ASA (Israel et al., 2019), added to an increase in an xCT-mediated presynaptic mGlu2/3 inhibitory tone induced by NAC, should provide greater inhibitory effect on alcohol intake when these drugs are administered together.

In the present studies, we investigated whether a reduction of ethanol intake induced by NAC follows the activation of the hippocampal Nrf2-ARE antioxidant signaling pathway, which

could be prevented by inhibition of the xCT cystine-glutamate transporter and by inhibition of the glutamate presynaptic metabotropic mGlu2/3 receptor. Further, we evaluated whether blocking the lipoxin A4 receptor blunts the inhibition of ethanol intake and alcohol relapse intake exerted by ASA.

MATERIALS AND METHODS

Animals

Adult female Wistar-derived rats, selectively bred for over 90 generations as alcohol consumers (University of Chile Bibulous; UChB; Quintanilla et al., 2006; Israel et al., 2017), were used in the experiments. Animals were maintained on a 12-h light–dark cycle (lights off at 7:00 PM) and regularly fed a soy protein, peanut-meal rodent diet (Cisternas, Santiago, Chile). Female rats were used because females maintain stable body weights (within 10%) over time, which is of value in long-term studies. Furthermore, Guerri and associates (Pascual et al., 2017) showed that female mice were more susceptible than males to develop an ethanol-induced neuroinflammation. Experimental procedures were approved by the Ethics Committee for Experiments with Laboratory Animals at the Medical Faculty of the University of Chile (protocol CBA# 0994 FMUCH) and by the Chilean Council for Science and Technology Research.

Drugs

Alcohol solutions were prepared from absolute graded ethanol (Merck, Darmstadt, Germany) diluted to 10% or 20% alcohol solutions (vol/vol) in tap water. N-acetylcysteine (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in saline, adjusted with NaOH to pH 7.2, and administered in volume of 5.0 ml/kg per day. Acetylsalicylic acid (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in water and adjusted with NaOH to pH 7.2. To study the effect of the combination of NAC + ASA on chronic ethanol intake (Experiment 1), NAC was administered i.p. for 2 days at a loading dose of 70 mg/kg per day (Israel et al., 2019), each dose being one-half of the loading dose (140 mg/kg) administered clinically in the treatment of acetaminophen hepatotoxicity (Fisher and Curry, 2019), followed by a lower maintenance dose of 40 mg/kg per day (by oral gavage) administered for 11 days. The maintenance dose of NAC chosen (40 mg/kg per day) was similar to that used in humans for cocaine treatment studies (Nocito Echevarria et al., 2017). Acetylsalicylic acid was administered at a dose of 15 mg/kg per day, by oral gavage (Israel et al., 2019), for 13 days. This dose is considerably lower than that used as chronic treatment for arthritis (Colebatch et al., 2012). Doses of ASA of 15–30 mg/kg have been 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 given in a volume of 5 ml/kg per day by oral gavage, for 11 days. In the three subsequent experiments, aimed at identifying the underlying mechanisms involved in the effect of NAC and ASA on alcohol relapse (**Figures 3**, **7**, **8**), we used a shorter treatment period (2 or 4 days) and a higher concentration of NAC (100 mg/kg per daily) and ASA (15 mg/kg per day, **Figure 7**; and 30 mg/kg per day, **Figure 8**) both administered p.o. Other drugs were administered parenterally to achieve a better bioavailability at the time of administration of NAC or ASA. Their schedules of administration are described for each experiment. The mGlu2/3 receptor inhibitor LY341495 disodium salt (Tocris Cookson Limited, Bristol, UK) was dissolved in 66 mM phosphate-saline buffer (pH 8.0) and administered intraperitoneally (i.p.) at a dose of 1 mg/kg per day (delivered as 5.0 ml/kg), as previously reported (Fukumoto et al., 2014). The xCT transporter inhibitor sulfasalazine (SZ; Sigma-Aldrich; cat. no. SO883) stock solution was prepared dissolving sulfasalazine (SZ) in dimethyl sulfoxide (80 mg/ml), as previously reported (Bernabucci et al., 2012). Fresh sulfasalazine was prepared every day by dilution of the stock solution in isotonic saline and administered i.p. at a dose of 8 mg/kg per day delivered as 5 ml/kg.

Lipoxin A4 (stock ethanol solution 25 μg/250 μl; Cayman Chemical, Ann Arbor, MI, USA), an ASA-triggered endogenous lipid mediator with potent anti-inflammatory properties (Medeiros et al., 2013), which acts as an agonist of the formyl peptide receptor 2 (FPR2/ALX), a lipoxin receptor, was prepared by dilution of the stock ethanol solution in isotonic saline and administered at a dose of 7 μg/kg delivered as 1 ml/kg intravenously (i.v.), as recommended (Wu et al., 2013). WRW4 (Tocris Bioscience Reagent, Bristol, UK; cat. no. 2262), an antagonist of the FPR2/ALX receptor, was dissolved in isotonic saline and administered at a dose of 0.3 mg/kg per day, delivered as 1 ml/kg, into the tail vein.

Brain Tissue Samples

We selected the whole left hippocampus for oxidized glutathione (GSSG)/reduced glutathione (GSH) and reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR) studies. Right hippocampus was used for immunohistochemical studies to evaluate astrocyte and microglial reactivity focusing on the stratum radiatum of CA1 region according to our previous studies (Ezquer et al., 2019; Israel et al., 2019) and according to Gómez et al. (2018).

Quantification of mRNA Levels of Nrf2 Regulated Genes in Hippocampus

Twenty-four hours after the oral administration of NAC, ASA, NAC + ASA, or saline, for 11 consecutive days, chronically ethanol-consuming rats were anesthetized with chloral hydrate (280 mg/kg, i.p.) and euthanized to obtain hippocampal samples, as indicated above. Total RNA was isolated using Trizol (Invitrogen, Grand Island, NY, USA). One microgram of total RNA was used to perform reverse transcription with MmlV RT (Invitrogen) and oligo dT primers. Real-time PCR reactions were performed to amplify the Nrf2 activated genes heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (Nqo1), peroxiredoxin (Prdx), and γ-glutamyl-cysteine synthetase (GCLC) using a Light-Cycler 1.5 thermocycler (Roche, Indianapolis, IN, USA). To ensure that amplicons were generated from mRNA and not from genomic DNA, controls without RT during the reverse transcription reaction were included. Relative quantification was performed using the ΔΔCT method. The

mRNA level for each target gene was normalized against the mRNA level shown by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. The primers used for qPCR amplifications were designed by the authors: HO-1 forward: 5′-CTATCGTGCTCGCATGAAC-3′; HO-1 reverse: 5′-CAGCTCCTCAAACAGCTCAA-3′; Nqo1 forward: 5′-CTCGCCTCATGCGTTTTTG-3′; Nqo1 reverse: 5′-CCCCTAATCTGACCTCGTTCAT-3′; GCLC forward: 5′-CTGAGGCAAGATACCTTTATGACC-3′; GCLC reverse: 5′-GTAGCTATCTATTGAGTCATACCGAGAC-3′; Prdx forward: 5′-GGAGGATTGGGACCCATGAAC-3′; Prdx reverse: 5′-AGAGCGGCCAACAGGAAGATC-3′; GAPDH forward: 5′-GACATGCCGCCTGGAGAAAC-3′; GAPDH reverse: 5′-AGCCCAGGATGCCCTTTAGT-3′.

Glutathione Determination

Following continuous chronic ethanol intake for 48 days, animals were alcohol deprived for 14 days and treated on the last 4 days of deprivation with either LY341495/NAC, SZ/NAC, LY341495/vehicle, SZ/vehicle, or vehicle/vehicle and then offered ethanol access for two consecutive days to evaluate alcohol relapse intake. Water was also available. Immediately, after the second day of reaccess to ethanol (after the alcohol reading at day 63, the animals were anesthetized with chloral hydrate (280 mg/kg, i.p.) and euthanized to obtain hippocampal samples. Brain oxidative stress was determined as the GSSG/GSH ratio in hippocampus as previously described (Ezquer et al., 2019). Glutathione reductase (cat. no. G3664), NADPH (cat. no. N1630), and DTNB (5,50-dithiobis-2-nitrobenzoic acid; cat. no. D-8130) were used for the determination of glutathione, purchased from Sigma-Aldrich.

Determination of Astrocyte and Microglia Immunoreactivity

Following continuous chronic ethanol intake for 48 days, animals were alcohol deprived for 14 days and treated for the last 4 days of deprivation with either LY341495/NAC, SZ/NAC, LY341495/vehicle, SZ/vehicle, or vehicle/vehicle and then offered ethanol access for two consecutive days. Water was always available. Immediately, after the second day of reaccess to ethanol, the animals were anesthetized with chloral hydrate (280 mg/kg, i.p.) and euthanized to obtain hippocampal samples. Immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) and the microglial marker ionized-calcium–binding adaptor molecule 1 (Iba-1) was evaluated in coronal cryosections of hippocampus (30 μm thick) as previously reported (Ezquer et al., 2019). Nuclei were counterstained with DAPI. Microphotographs were taken from the stratum radiatum of 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 primary astrocytic processes and density of Iba-1–positive microglial cells were assessed using FIJI image analysis software (http://fiji.sc/Fiji) as previously reported (Ezquer et al., 2019).

Experiment 1: Effect of Oral Administration of NAC, ASA, or Their Combination on Chronic Alcohol Intake and mRNA Expression of Nrf2-ARE Regulated Genes in the Hippocampus

Twenty-four female UChB rats, weighing 190–210 g were single housed in cages at the age of 60 days and maintained on a 12-h light-dark cycle (lights off at 7:00 PM). Subsequently, rats received continuous concurrent free-choice access to 10% (vol/vol) ethanol solution and water for 77 days. On day 78, rats were allowed to concurrent three-bottle choice access of 10% and 20% (vol/vol) ethanol solutions and water for 24 additional days. On day 88, rats were divided in four groups: (n = 6 per group), namely, (1) vehicle group: rats were given saline on the first 2 days by the i.p. route and water by oral gavage for the following 11 days; (2) NAC group: On the first 2 days, NAC was administered by the i.p. route (70 mg/kg per day), as a loading dose, and subsequently by oral gavage (40 mg/kg per day) for the following 11 days; (3) ASA group: rats were given saline on the first 2 days by the i.p. route and thereafter administered ASA (15 mg/kg per day) by oral gavage, for the following 11 days; and (4) NAC + ASA (NAC + ASA) group: On the first 2 days, NAC was administered by the i.p. route (70 mg/kg per day), and subsequently, rats were daily administered the combined NAC (40 mg/kg per day) plus ASA (15 mg/kg per day) dose by oral gavage, for 11 days. Ethanol intake was recorded daily and expressed as grams of ethanol consumed/kg body weight for the complete 11-day period. Preliminary studies for this group of animals have been reported (Israel et al., 2019). Twenty-four hours after the last NAC, ASA, or NAC + ASA administration and saline controls, the animals were anesthetized with chloral hydrate (280 mg/kg, i.p.) to obtain hippocampal samples for quantification of mRNA levels of Nrf2-ARE regulated genes.

Experiment 2. Effect of Oral Administration of NAC on Relapse-Like Ethanol Drinking: Role of Glutamate mGlu2/3 Receptor and of the xCT-Cystine/Glutamate Exchanger

Thirty-six female UChB rats, weighing 190–210 g, were single housed in cages at the age of 60 days and maintained on a 12-h light–dark cycle (lights off at 7:00 PM). Subsequently, rats received continuous concurrent free-choice access in the home cage to 10% (vol/vol) ethanol solution and water for 36 day. On day 37, rats were allowed to concurrent three-bottle choice access to 10% and 20% (vol/vol) ethanol solutions and water for 11 additional days. On day 48, after induction of chronic ethanol intake, animals were deprived of ethanol for 14 days and thereafter allowed reaccess to ethanol solutions (10% and 20%) and water, for 2 days. Water was always available through all the experiment. While the chronic ethanol ingestion period of the present experiment was shorter (47 days) than that of experiment 1, the rats showed a marked relapse intake to ethanol after two weeks of deprivation (alcohol reaccess on day 62), likely due to neuroinflammation and oxidative stress that self-perpetuate each other after ethanol is discontinued (see Israel

et al., 2019). It is further noted that NAC does not inhibit alcohol intake if rats are allowed to drink ethanol for a short time (up to 18 days), whereas an inhibitory effect was clearly observed after 60 days of chronic intake (Quintanilla et al., 2016b).

On the last 4 days of the 14-day ethanol deprivation period, rats were divided into five groups (n = 6 rats/group): (1) LY341495/NAC group: rats were given LY341495, an inhibitor of the mGlu2/3 receptor, at a dose of 1 mg/kg per day (i.p.), 15 min before NAC administration (100 mg/kg per day by oral gavage (Fukumoto et al., 2014) for 4 days; (2) vehicle/NAC group: rats were given vehicle (isotonic saline) in a single dose (5 ml/kg; i.p.), 15 min before of NAC administration (100 mg/kg per day, by oral gavage); (3) SZ/NAC group: rats were given SZ, an inhibitor of xCT-cystine/glutamate exchanger, at a dose of 8 mg/kg per day (i.p.) 15 min before NAC administration (100 mg/kg per day, by oral gavage; Bernabucci et al., 2012); (4) LY341495/vehicle group: rats were given LY341495 at a dose of 1 mg/kg per day (i.p.), 15 min before vehicle administration (water 5 ml/kg per day, by oral gavage); (5) SZ/vehicle group: rats were given SZ (8 mg/kg per day i.p.) 15 min before vehicle administration (water 5 ml/kg per day, by oral gavage); or (6) vehicle/vehicle group: rats were given a single dose of isotonic saline *via* i.p. 15 min before a dose of vehicle (water by oral gavage). Once the experiment had ended, after recording the ethanol intakes at the second day (day 63) of alcohol relapse, the animals were anesthetized with chloral hydrate (280 mg/kg, i.p.) and euthanized to obtain hippocampal samples for determination of GSSG and GSH and astrocyte and microglia immunoreactivity.

It is important to note that drug administration in this study was always conducted in the daylight part of the circadian cycle. The relapse intake after ethanol reaccess [alcohol deprivation effect (ADE)] was started at 1:30 PM to 3:30 PM to dissociate ethanol intake from food consumption (lights on at 7 AM and off at 7 PM).

Experiment 3. Effect of ASA or Lipoxin A4 Administration on Chronic Ethanol Intake: Role of the Formyl Peptide Receptor 2/Lipoxin A4 (FPR2/ALX)

Twenty female UChB rats, weighing 190–210 g, were single housed in cages at the age of 60 days and maintained on a 12-h light–dark cycle (lights off at 7:00 PM). Subsequently, rats received continuous concurrent free-choice access in the home cage to 10% (vol/vol) ethanol solution and water for 87 consecutive days. On day 88, rats were allowed to concurrent three-bottle choice access to 10% and 20% (vol/vol) ethanol solutions and water for 19 additional days. On day 102, rats were divided into five groups (five rats/group) that received for three consecutive days: (1) vehicle/ASA group: rats were given vehicle into the tail vein (i.v.) 5 min before ASA administration (15 mg/kg per day) given by oral gavage; (2) lipoxin (LXA)/vehicle group: rats were given vehicle (i.v.) 5 min before LXA administration (7.0 μg/kg per day) into the tail vein (Wu et al., 2013), an ASA-triggered endogenous lipid mediator with potent anti-inflammatory properties agonist of the FPR2/ALX receptor (Medeiros et al., 2013; Hughes et al., 2017); (3) WRW4/ASA group: rats were given WRW4, a FPR2/ALX receptor antagonist (0.3 mg/kg per day, i.v.; Sordi et al., 2013) 5 min before ASA administration (15 mg/kg per day, by oral gavage); (4) WRW4/vehicle group: rats were given WRW4 (0.3 mg/kg per day i.v.) 5 min before vehicle administration (water 5 ml/kg per day, by oral gavage); and (5) vehicle/vehicle group: rats were given vehicle into the tail vein (i.v.) 5 min before vehicle administration (water 5 ml/kg per day, by oral gavage). Ethanol intake was recorded daily.

Experiment 4. Effect of the Administration of ASA on the Relapse-Like Ethanol Drinking: Role of the Formyl Peptide Receptor 2/Lipoxin A4 (FPR2/ALX)

Twenty female UChB rats, weighing 190–210 g, were single housed in cages at the age of 60 days and maintained on a 12-h light–dark cycle (lights off at 7:00 PM). Subsequently, rats received continuous concurrent free-choice access in the home cage to 10% (vol/vol) ethanol solution and water for 56 consecutive days. On day 57, rats were allowed three-bottle choice access to 10% and 20% (vol/vol) ethanol solutions and water for 17 additional days. On day 74, after induction of chronic ethanol intake, animals were deprived of ethanol for 14 days and thereafter allowed reaccess to ethanol solutions (10% and 20%), for 2 days. Water was always available throughout the experiment. During the last 3 days of ethanol deprivation, rats were divided into the following four groups (5 rats/group): (1) vehicle/ASA group: rats were given vehicle (isotonic saline; 1 ml/kg per day i.v.) 5 min before ASA administration (30 mg/kg per day, by oral gavage); (2) WRW4/ASA group: rats were given WRW4 (0.3 mg/kg per day i.v.), an antagonist of FPR2/ALX receptor (Sordi et al., 2013) 5 min before ASA administration (30 mg/kg per day, by oral gavage); (3) WRW4/vehicle group: rats were given WRW4 (0.3 mg/kg per day i.v.) 5 min before water administration (5 ml/kg per day, by oral gavage); (4) vehicle/vehicle group: rats were given vehicle (isotonic saline, 1 ml/kg per day, i.p.) 5 min before water administration (5 ml/kg per day by oral gavage).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). 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 (**Figures 1**, **2**, **4**, **5**, **6**) or two-way (**Figures 3**, **7**, **8**) analysis of variance (ANOVA) was used followed by a Tukey or Fisher *post hoc* test. When only two groups were compared, statistical significance was determined by Student's *t*-test. A level of $P < 0.05$ was considered for statistical significance.

To facilitate text reading, full statistical ANOVAs are presented in the legends to Figures.

RESULTS

Figure 1A shows that chronic ethanol intake of control animals (vehicle-treated; 16.0 ± 0.3 g/kg per day; mean ± SEM, n = 6)

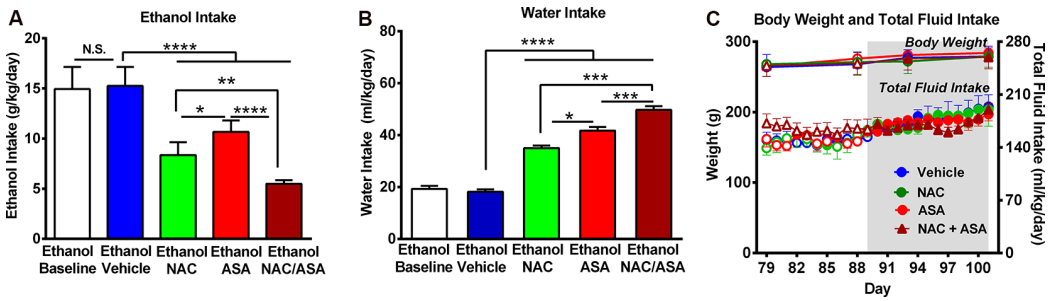


FIGURE 1 | Administration of N-acetylcysteine (NAC; 40 mg/kg per day p.o.) + acetylsalicylic acid (ASA; 15 mg/kg per day p.o.) marked reduced ethanol intake in rats that had chronically consumed ethanol. **(A)** Significant inhibition of chronic alcohol intake following the oral administration of NAC or ASA vs. vehicle group [one-way analysis of variance (ANOVA): $F_{4, 25} = 46.53$, $^{****}P < 0.0001$; Tukey *post hoc*: vehicle-treated vs. NAC-treated and vs. ASA-treated groups, $^{****}P < 0.0001$, $n = 6$ rats per group]. Inhibition of ethanol intake was further increased by the coadministration of NAC + ASA when compared with either NAC or ASA group ($^{**}P < 0.01$, NAC + ASA–treated vs. NAC- treated group; $^{****}P < 0.0001$, NAC + ASA–treated vs. ASA-treated group). Bars represent mean ± SEM of 11 consecutive days of ethanol intake, during which rats received by oral gavage NAC, ASA, NAC + ASA, or vehicle (from day 91 to day 101 of ethanol intake). Chronic ethanol intake of all groups (10-day baseline) prior to vehicle administration (mean ± SEM, 15.0 ± 0.9 g ethanol/kg per day) was not different from that of rats that received vehicle (mean ± SEM, 15.3 ± 0.8 g ethanol/kg per day; A: white bar vs. blue bar). Additionally, we found no significant differences between the baseline ethanol intakes of the four groups prior to drug/vehicle treatment [mean ± SEM, 15.0 ± 0.80 (ethanol vehicle); 14.0 ± 1.4 (ethanol NAC); 14.0 ± 0.2 (ethanol ASA); and 14.0 ± 0.7 (ethanol NAC + ASA) g ethanol/kg per day, $n = 6$/group; ANOVA: $F_{3, 20} = 0.2968$, P: N.S.] **(B)** NAC, ASA, NAC + ASA markedly increased water intake compared with the control (ethanol vehicle) and baseline group (ANOVA: $F_{4, 25} = 132.5$, $P < 0.0001$). **(C)** The administration of NAC, ASA, or the combination of NAC and ASA did not affect total fluid intake (ANOVA: $F_{3, 48} = 1.406$, P: N.S.) or body weight- (ANOVA: $F_{3, 12} = 0.416$, P: N.S) compared with vehicle administration. $^{*}P < 0.05$ means significant difference between Ethanol NAC and Ethanol ASA groups. $^{***}P < 0.001$ means significant differences between Ethanol NAC/ASA with Ethanol NAC and Ethanol ASA groups. N.S., not statistically significant.

was inhibited by 45% by NAC administration (8.9 ± 0.3 g/kg per day; mean ± SEM; $n = 6$) and inhibited by 33% by ASA (administration (10.7 ± 0.5 g/kg per day; mean ± SEM, $n = 6$). Ethanol intake of animals receiving both NAC and ASA (NAC + ASA; 5.5 ± 0.1 g/kg per day; mean ± SEM, $n = 6$) was decreased by 66% vs. that shown by vehicle-treated animals ($P < 0.001$) and lower than that shown by rats receiving either NAC or ASA alone ($P < 0.001$). In **Figure 1A**, chronic ethanol intake (10 days–baseline) prior to vehicle administration was not different [not statistically significant (N.S.)] from that of rats receiving vehicle (white bar vs. blue bar). **Figure 1B** shows that NAC, ASA, and NAC + ASA administration markedly increased water intake compared to that shown by the control (ethanol vehicle) and baseline groups. **Figure 1C** shows that the administration of NAC, ASA, or the combination of NAC and ASA did not affect total fluid intake or body weight compared to vehicle administration.

Figure 2 shows the administration of NAC (40 mg/kg per day) markedly increased the expression in the hippocampus of a number of genes known to respond to Nrf2 activation of the ARE (Kansanen et al., 2013): (A) HO-1; (B) NAD(P)H:quinone oxidoreductase 1 (Nqo1); (C) GCLC; and (D) Prdx. Acetylsalicylic acid administration (15 mg/kg per day) did not raise the expression of HO-1, Nqo1, GCLC, or Prdx.

A separate experiment was conducted to determine the mechanism(s) by which NAC inhibits alcohol relapse intake (ADE), addressing the role played on ethanol intake by the xCT cystine-glutamate transporter and by the glutamate mGlu2/3 inhibitory receptor on alcohol relapse intake. In this experiment, rats were allowed to ingest alcohol for 48 days and subsequently alcohol-deprived for 14 days. After the deprivation period, alcohol reaccess (10% and 20% vol/vol) was allowed on days 62 and 63. Water was always available throughout the experiment. **Figure 3** shows the complete loss of the NAC-induced inhibition of alcohol relapse intake afforded by the i.p. administration of both the inhibitor of the xCT transporter SZ (8 mg/kg per day i.p; Bernabucci et al., 2012) or the mGlu2/3 receptor inhibitor LY341495 (1 mg/kg per day i.p.; Fukumoto et al., 2014). These inhibitors were administered 15 min prior to NAC (100 mg/kg per day) administration, given by oral gavage, on the last 4 days of ethanol deprivation (days 58, 59, 60, and 61) 24 h prior to ethanol reaccess. On days 62 and 63, alcohol reaccess was allowed. Upon ethanol reaccess, the intake by the control group (e.g., vehicle/vehicle group) was on the first day of ethanol reaccess (13.3 ± 0.8 g/kg per day, $n = 5$; day 62) significantly higher ($P < 0.05$) than the intake prior to the 14-day deprivation period (10.5 ± 0.9 g/kg per day, $n = 5$). The administration of NAC inhibited ethanol intake by 70% to 75% ($P < 0.01$) both on days 62 and 63, an effect that was fully abolished ($P < 0.001$) by the administration of the xCT inhibitor SZ (8 mg/kg per day) or by the metabotropic Glu2/3 receptor antagonist LY341495 (1 mg g/kg per day).

As indicated above, an *in vitro* Nrf2 gene overexpression leads to increases in xCT gene expression (Shih et al., 2003; Habib et al., 2015). In the present study, NAC (100 mg/kg per day, administered on the last 4 days of the 14-day ethanol deprivation period) led to a small but significant increase (24%) in xCT mRNA level (1.1 ± 0.05 × CT mRNA/GADPH mRNA level; mean ± SEM, $n = 6$) compared to vehicle-treated rats (0.90 ± 0.04 × CT mRNA/GADPH mRNA level; mean ± SEM, $n = 6$; Student's t-test: 2.437; $P < 0.03$).

It has been reported that upon alcohol relapse intake following deprivation (ADE), UChB rats display a binge-like drinking behavior within the first hour of alcohol reaccess (Israel et al.,

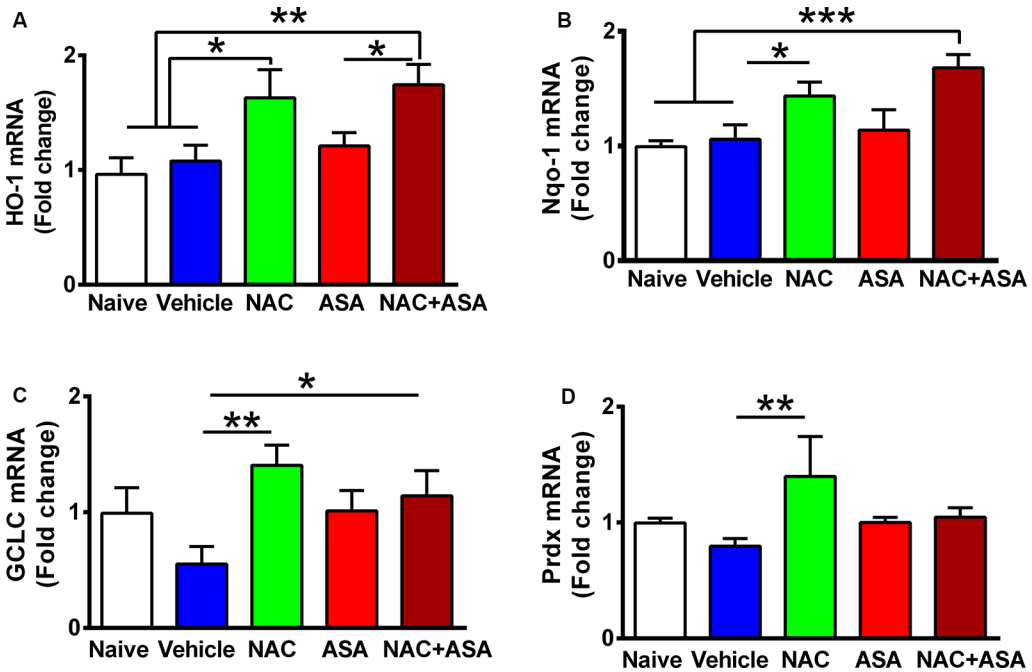


FIGURE 2 | Oral administration of N-acetylcysteine (NAC; 40 mg/kg per day), but not acetylsalicylic acid (ASA; 15 mg/kg per day), increased the expression of Nrf2-ARE regulated genes in the hippocampus of chronically alcohol-consuming rats. Reduction of chronic ethanol intake induced by the oral administration of NAC (40 mg/kg per day) shown in **Figure 1** was associated with a significant increase in the hippocampal level of Nrf2-ARE regulated genes compared to chronic ethanol drinking rats receiving vehicle or compared to naive rats that had ingested only water. A one-way analysis of variance (ANOVA) performed on ethanol intake data revealed significant effect of treatment on **(A)** heme oxygenase 1 (HO-1) mRNA levels (ANOVA $F_{(4, 24)} = 6.417$, $^{**}P < 0.01$); Tukey *post hoc* test revealed that rats treated with NAC or NAC + ASA showed in the hippocampus a greater mRNA expression of HO-1 vs. vehicle group ($^{*}P < 0.05$ vehicle-treated vs. NAC- and $^{**}P < 0.01$ vs. NAC + ASA-treated groups). **(B)** Quinone oxidoreductase 1 (Nqo1) levels. ANOVA $F_{\text{treatment}(4, 26)} = 5.332$, $^{**}P < 0.01$. *Post hoc* test revealed that rats treated with NAC or NAC + ASA showed greater mRNA expression of Nqo1 vs. vehicle group ($^{*}P < 0.05$ vehicle-treated vs. NAC-treated group, $^{***}P < 0.001$ vehicle-treated and naive vs. NAC + ASA-treated group). **(C)** γ-Glutamyl-cysteine synthetase (GCLC) mRNA levels. ANOVA $F_{\text{treatment}(4, 26)} = 2.852$, $^{**}P < 0.01$. *Post hoc* test revealed rats treated with NAC or NAC + ASA showed greater mRNA expression of GCLC vs. vehicle group ($^{**}P < 0.01$ vehicle-treated vs. NAC-treated group, $^{*}P < 0.05$ vehicle-treated vs. NAC + ASA–treated group) and **(D)** peroxiredoxin (Prdx) mRNA levels. ANOVA $F_{\text{treatment}(4, 26)} = 2.912$. *Post hoc* test revealed rats treated with NAC showed greater mRNA expression of Prdx vs. vehicle group ($^{**}P < 0.01$ vehicle-treated vs. NAC-treated group). Data of each target gene were normalized by the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. Data are presented as mean ± SEM, $n = 6$ rats per experimental group.

2019; Quintanilla et al., 2019). It is noted that **Figure 3** shows the daily (24-h) intake that follows the deprivation and ethanol reaccess. **Figure 4** shows the relapse of ethanol drinking for the above animal set evaluated during the first 60 min of ethanol reaccess. In the first hour following ethanol reaccess, rats consumed 1.58 ± 0.09 g ethanol/kg, $n = 6$, intake that was inhibited by 75% to 80% ($P < 0.01$) by NAC (0.35 ± 0.19 g ethanol/kg, $n = 6$) administration. Both the xCT transporter inhibitor SZ and the mGlu2/3 receptor inhibitor LY341495 administered 15 min prior to the oral administration of NAC (100 mg/kg) fully blocked the inhibitory effect of NAC on ethanol intake ($P < 0.001$). Noteworthy, by itself, the mGlu2/3 receptor inhibitor LY341495 or the xCT transporter inhibitor SZ did not inhibit the binge-like relapse ethanol intake.

The mechanism of the strong antioxidant effect of NAC has not been fully elucidated. Conceivably, NAC can: (a) directly increase intracellular cysteine for the synthesis of intracellular glutathione; (b) generate glutathione following the Nrf2-mediated increase in γ-glutamyl-cysteine synthetase, a rate-limiting enzyme in the synthesis of glutathione (Lu, 2013); and/or (c) generate glutathione following an xCT-mediated transport of extracellular cystine into the cells. **Figure 5** shows that the marked 2-fold to 3-fold increase in the hippocampal GSSG/GSH ratio induced by chronic alcohol intake ($P < 0.01$) was fully reversed by the administration of NAC (100 mg/kg per day per o.s. for 3 days; $P < 0.01$). Sulfasalazine administration fully reversed ($P < 0.01$) the effect of NAC on the GSSG/GSH ratio, supporting the view that the xCT-mediated influx of cystine into the cells is required to provide cysteine for the synthesis of glutathione. The mGlu2/3 receptor inhibitor LY341495 did moderately, but not significantly reduce the inhibitory effect of NAC on GSSG/GSH levels.

As indicated previously, a number of studies have shown that chronic alcohol treatment leads to neuroinflammation. **Figure 6** shows that chronic ethanol intake even if followed by an alcohol deprivation period and relapse leads to significant increase in astrocyte activation evidenced by an increase in the length and thickness of astrocyte processes (GFAP immunoreactivity) and by an increase in microglial density (Iba-1 immunoreactivity). Unexpectedly, the restoration of

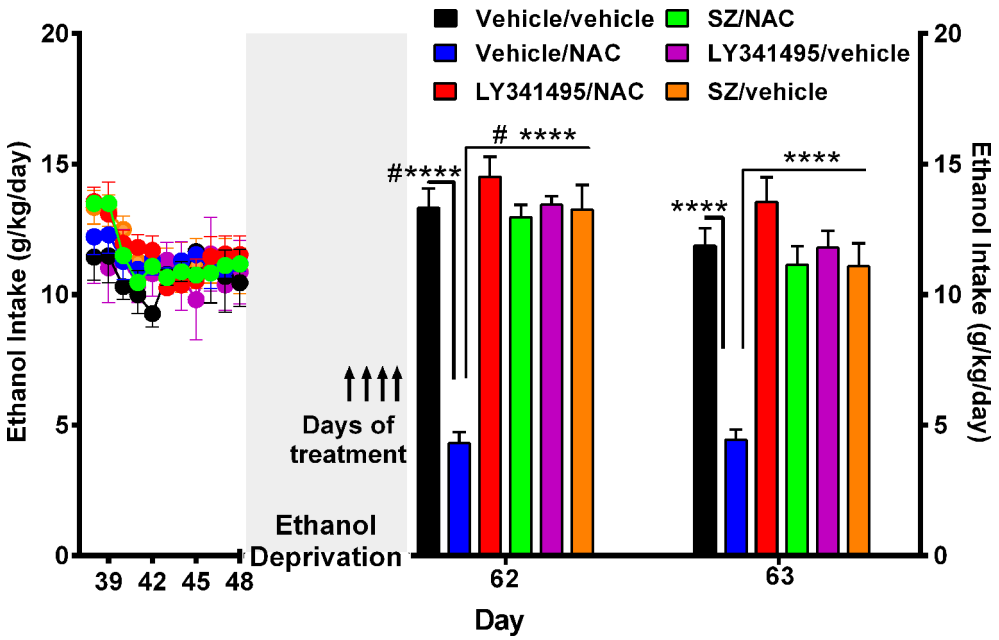


FIGURE 3 | N-acetylcysteine (NAC)–induced inhibition of alcohol relapse-like drinking is prevented by blockade of the glutamate mGlu2/3 receptors (LY3441495) and the xCT-cystine-glutamate exchanger (sulfasalazine, SZ). Following 48 days of chronic ethanol intake and a subsequent ethanol deprivation for 2 weeks, rats received their respective treatment for only the last 4 days of deprivation (arrows in Figure). Given the short administration period, the dose of NAC was increased to 100 mg/kg per day. Ethanol reaccess was allowed 24 h after the last dose of SZ or LY3441495. Following the 2 weeks of alcohol deprivation and ethanol reaccess, the high ethanol intake of the vehicle/vehicle group (black bars) on days 62 and 63 demonstrate the alcohol deprivation relapse. Two-way analysis of variance (treatment × day) performed on ethanol intake data obtained the first (day 62) and second (day 63) days of ethanol reaccess post deprivation revealed significant effect of treatment ($F_{treatment(5,46)} = 31.0$, $^{****}P < 0.0001$), day ($F_{day(1,46)} = 21.73$, $^{****}P < 0.0001$), and significant treatment × day interaction ($F_{interaction(5,46)} = 20.65$, $^{****}P < 0.0001$). Tukey *post hoc* analysis indicated that NAC (vehicle/NAC) induced a significant reduction of alcohol relapse drinking ($^{****}P < 0.0001$ vehicle/NAC group compared to vehicle/vehicle group), whereas pretreatment with LY341495, an antagonist of the mGlu2/3 receptors, or sulfasalazine (SZ), an inhibitor of the xCT-cystine/glutamate exchanger, fully blocked the NAC-induced reduction of alcohol relapse ($^{****}P < 0.0001$ LY341495/NAC group vs. vehicle/NAC group; and $^{****}P < 0.0001$ SZ/NAC group vs. vehicle/NAC group). However, sulfasalazine at the dose of 8 mg/kg per day administered without NAC (SZ/vehicle group) did not *per se* inhibit ethanol intake the first or second reaccess day; nor did it show a significant anti-inflammatory effect (*vide infra* **Figure 6**) vs. the vehicle/vehicle group (Tukey *post hoc* SZ/vehicle vs. vehicle/vehicle group: N.S.). Similarly, LY341495 at a dose of 1 mg/kg per day administered without NAC (LY341495/vehicle group) did not *per se* inhibit ethanol intake the first or second reaccess day; nor did it show a significant anti-inflammatory effect (*vide infra* **Figure 6**) vs. the vehicle/vehicle group (Tukey *post hoc* LY341495/vehicle vs. vehicle/vehicle group: N.S.). In addition, sulfsalazine or LY341495 treatments did not influence total fluid intake. Furthermore, 24 h after the administration of sulfasalazine or LY341495, rats were given reaccess to ethanol to recorder their consumption. Data are presented as mean ± SEM, $n = 6$ rats per experimental group. $^{\#}P < 0.05$ means significant difference with the same group before deprivation. N.S., not statistically significant.

the normal levels of astrocyte activation and microglial density afforded by NAC administration was fully blocked by both SZ and LY341495, suggesting that the glutamatergic tone plays a role in the generation and/or perpetuation of neuroinflammation.

As indicated earlier, ASA has been shown to induce remarkable anti-inflammatory effects *via* the generation of lipoxin A4 from arachidonic acid (also referred to ASA-triggered lipoxin or ATL-lipoxin). **Figure 7** shows that the administration of both ASA and lipoxin A4 inhibited chronic alcohol intake, effects that were fully blocked ($P < 0.001$) by administration of WRW4, the antagonist of the lipoxin A4 receptor (FPR2).

Figure 8 shows that the administration of the antagonist of the lipoxin A4 receptor FPR2/ALX (WRW4) also blocked the inhibitory effect of ASA on relapse alcohol intake. In these studies, rats were offered free-choice access to 10% (vol/vol) ethanol solution and water for 56 consecutive days. On day 57, rats were allowed three-bottle choice access to 10% and 20% (vol/vol) ethanol solutions and water for 17 additional days. On day 74, animals were deprived of ethanol for 14 days and thereafter were allowed reaccess to ethanol solutions (10% and 20%) for 2 days. Water was always available throughout the experiment. During the last 3 days of ethanol deprivation, on days 85, 86, and 87, rats were divided in four groups: (1) vehicle/ASA group: received vehicle (isotonic saline; 1 ml/kg per day i.v. 5 min prior to ASA administration at 30 mg/kg per day, by oral gavage); (2) WRW4/ASA group: received WRW4 (0.3 mg/kg per day i.v. 5 min prior to ASA administration at 30 mg/kg per day, by oral gavage); (3) WRW4/vehicle group received WRW4 (0.3 mg/kg per day i.v. 5 min prior to vehicle (water) administration by oral gavage; and (4) vehicle/vehicle group received vehicle [isotonic saline i.v. 5 min before vehicle (water) administration by oral gavage]. On days 88 and 89 (24 h after discontinuation of all treatments), reaccess to ethanol (10% and 20% vol/vol solutions) was allowed. Water was always available. As can be seen, a 50% to 55% of the ASA-induced

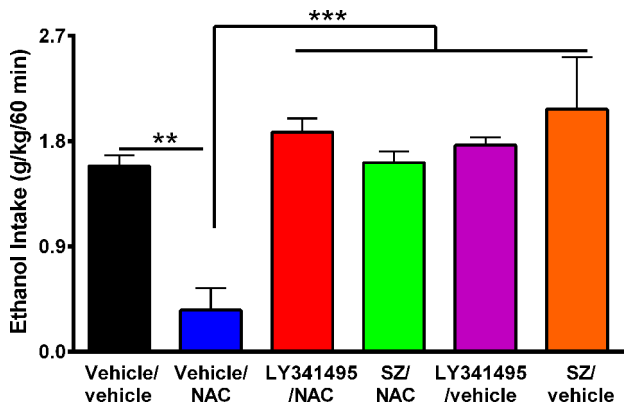


FIGURE 4 | The N-acetylcysteine (NAC)–induced inhibition of binge-like ethanol intake in the first 60 min of ethanol reaccess was fully blocked by inhibition of the glutamate mGlu2/3 receptor or the xCT-cystine/glutamate exchanger. Data showed ethanol intake of all groups during the first 60 min of ethanol reaccess. Analysis of variance of data indicates significant effect of treatments vs. vehicle/vehicle group ($F_{(5,23)} = 11.56$, $^{***}P < 0.001$). *Post hoc* analysis indicated that NAC (100 mg/kg per day for 4 days) induced a significant reduction of the alcohol relapse-like drinking (vehicle/NAC group compared to vehicle/vehicle group $^{**}P < 0.01$), whereas pretreatment with LY341495, an antagonist of mGlu2/3 receptors, or sulfasalazine (SZ), an inhibitor of xCT-cystine/glutamate exchanger fully prevented the NAC-induced reduction of alcohol relapse (LY341495/NAC group vs. vehicle/NAC group $^{***}P < 0.001$ and SZ/NAC group vs. vehicle/NAC group $^{***}P < 0.001$). Data are presented as mean ± SEM, $n = 6$ rats per experimental group.

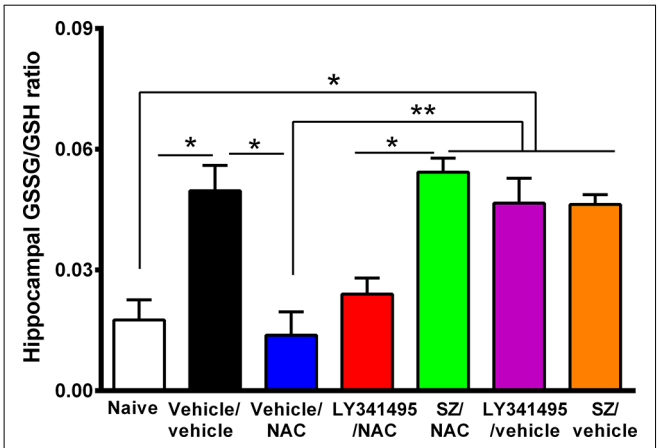


FIGURE 5 | Marked increase in oxidative stress in the hippocampus of animals that had consumed ethanol chronically and were deprived for 14 days followed by 2 days of ethanol reaccess was reversed by the administration of N-acetylcysteine (NAC; 100 mg/kg for 4 days), an effect that was prevented by an inhibitor of xCT-cystine/glutamate exchanger. Oxidative stress determined as the GSSG/GSH ratio is markedly increased by chronic and post deprivation ethanol intake shown in **Figure 4** [one-way analysis of variance (ANOVA): $F_{(6,28)} = 10$, $P < 0.0001$; Tukey *post hoc*: naive (water drinking) compared to ethanol vehicle/vehicle group, $^{*}P < 0.05$]. In addition, NAC fully normalized the ethanol-induced increase in GSSG/GSH ratio (*post hoc*: vehicle/NAC-treated compared to vehicle/vehicle group $^{*}P < 0.05$). Sulfasalazine (SZ), an inhibitor of xCT-cystine/glutamate exchanger, prevented the NAC-induced normalization of GSSG/GSH ratio (*post hoc*: vehicle/NAC-treated compared to SZ/NAC group $^{**}P < 0.01$) but not an antagonist of mGlu2/3 receptor (LY341495/NAC vs. vehicle/vehicle group $P > 0.05$ N.S.). Data are presented as mean ± SEM, $n = 6$ rats per experimental group. N.S., not statistically significant.

reduction of ethanol relapse was blocked ($P < 0.05$) by the administration of WRW4, an antagonist of FPR2/ALX receptor.

DISCUSSION

The present study shows that different mechanisms are involved in the inhibition of chronic alcohol intake/relapse drinking induced by NAC and ASA, which allows the combined administration of low doses of both drugs to achieve a larger inhibition of chronic alcohol intake/alcohol relapse, of possible relevance for the treatment of alcohol use disorders (AUDs).

As shown in the present studies, alcohol-induced neuroinflammation and oxidative stress remain for very long periods after chronic ethanol intake is discontinued, which along with the presentation of long-lasting conditioned cues, lead to relapse, characteristic of the chronicity of AUD (alcohol withdrawal removed in *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*). Acetylsalicylic acid and NAC would be most valuably in a clinical setting to prevent relapse (patient being abstinent) rather than to reduce the alcohol intake of a patient actively consuming alcohol, also avoiding any untoward effects of the combination of ethanol and ASA.

Often, in therapeutic approaches using NAC, a loading dose is recommended, followed by lower maintenance doses (Fisher and Curry, 2019). The loading dose of 70 mg/kg administered in the present study is one-half of the Food and Drug Administration–recommended loading dose of NAC for the treatment of acetaminophen (paracetamol) liver injury. After the 2-day administration, blood levels of NAC (half-life 5.6 h: Sansone and Sansone, 2011) would be still below those achieved after a single clinical loading dose of 140 mg/kg. In translational terms, the administration of a loading dose by a health professional would allow determining whether drug hypersensitivity (present in 2%–3% of the general population) is seen. It may also deter AUD individuals from self-administration of over-the-counter sold NAC (available in many countries, including the United States, Canada, France, Italy, and Germany).

The maintenance dose of NAC of 40 mg/kg per day administered along with ASA (15 mg/kg per day) for 11 days reduced chronic ethanol intake by two-thirds. When NAC was administered at dose of 100 mg/kg per day (*per os*) for only 4 days (to match the schedule of administration of LY341495 or SZ) during the alcohol deprivation period, a marked inhibition (75% to >80%) was observed on: (i) alcohol relapse; (ii) oxidative stress; and (iii) neuroinflammation. The effect of NAC on alcohol relapse and neuroinflammation was fully reversed by the xCT inhibitor SZ (8 mg/kg per day). These results support the suggestion (Lebourgeois et al., 2019) that NAC might be effective in reducing ethanol intake by activation of the xCT -mediated glutamate transporter. The mGlu2/3 receptor inhibitor LY341495 had only a marginal effect on reversing the GSSG/GSH ratio, indicating that lowering of oxidative stress induced by NAC is not primarily associated with the glutamatergic tone.

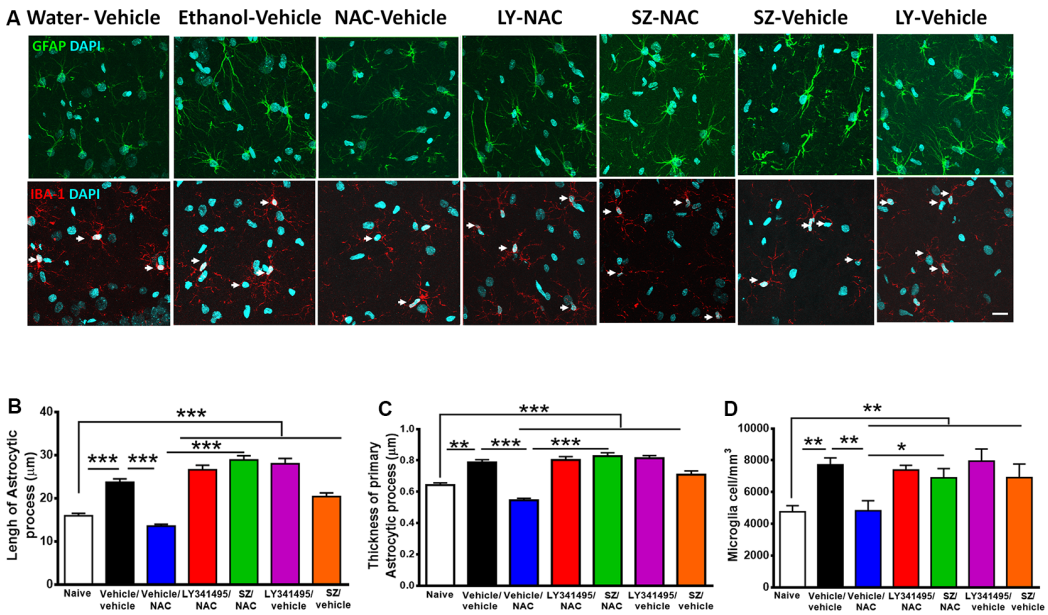


FIGURE 6 | Alcohol-induced neuroinflammation is inhibited by N-acetylcysteine (NAC; 100 mg/kg per day): effect blocked by inhibitors of xCT-transporter and mGlu2/3 receptor. (**A**, Top) Astrocyte immunofluorescence (GFAP immunoreactivity, green; DAPI, blue; A, bottom: microglia immunofluorescence (IBA-1 immunoreactivity, red; DAPI, blue, depicted by white arrows). Scale Bar 25 µm. Rats ingested alcohol chronically for 48 days followed by 14 days of ethanol deprivation and further 2 days of ethanol reaccess. Alcohol-treated rats treated with vehicle (vehicle/vehicle group) led to an increase in length (analysis of variance: $F_{6, 1531} = 55.61$, $^{***}P < 0.001$; Tukey *post hoc*: naive compared to vehicle/vehicle-treated group $^{***}P < 0.001$) and thickness ($F_{6, 733} = 35.77$, $^{***}P < 0.001$; Tukey *post hoc*: naive compared to vehicle/vehicle-treated group $^{**}P < 0.01$) of astrocyte processes **(B,C)** and to an increase in microglial density ($F_{6, 44} = 4.543$; $^{**}P < 0.01$; Tukey *post hoc*: naive compared to vehicle/vehicle-treated group $^{*}P < 0.05$; **D**) compared to (naive) rats drinking water. The administration of NAC (vehicle/NAC) fully normalized the ethanol-induced increase in astrocyte length (Tukey *post hoc*: vehicle/vehicle-treated compared to vehicle/NAC-treated group $^{***}P < 0.001$) and thickness (*post hoc*: vehicle/vehicle-treated compared to vehicle/NAC-treated group $^{***}P < 0.001$). N-acetylcysteine also blocked the ethanol-induced increase in microglial density (**D**; Tukey *post hoc*: vehicle/vehicle-treated compared to vehicle/NAC-treated group $^{*}P < 0.05$). Pretreatment prior to ethanol reaccess with LY341495, an antagonist of glutamate mGlu2/3 receptors and SZ, an inhibitor of xCT-cystine/glutamate exchanger, prevented the NAC-induced normalization of length (vehicle/NAC-treated compared to LY341495Y/NAC-treated group $^{***}P < 0.001$ and vs. SZ/NAC-treated group $^{***}P < 0.001$) and thickness (vehicle/NAC-treated compared to LY341495Y/NAC-treated group $^{***}P < 0.001$; and compared to SZ/NAC-treated group $^{***}P < 0.001$) of astrocytic process. Pretreatment with LY341495 or sulfasalazine also blocked the effect of NAC on microglial density (vehicle/NAC-treated compared to LY341495Y/NAC-treated treated group $^{*}P < 0.05$, and compared to SZ/NAC-treated group $^{*}P < 0.05$). Data are presented as mean ± SEM, $n = 6$ rats per experimental group.

A note on the specificity of SZ is in order. At doses of 25, 50, and 100 mg/kg per day, SZ was reported to induce analgesic effects in an animal model of inflammatory pain, while in the same study a dose of 8 mg/kg per day showed no effects (Bernabucci et al., 2012). Such a dose (8 mg/kg per day) was chosen in this study. It was recently reported that SZ administered at 30 mg/kg per day was minimally effective on the treatment of inflammatory bowel disease in rats, whereas a marked effect required the coadministration of anti-inflammatory mesenchymal stem cells (Yousefi-Ahmadipour et al., 2019). In a recently developed model of inflammatory bowel disease in rat, a dose of SZ of 300 mg/kg per day was used to show therapeutic effects (Ghattamaneni et al., 2019). In an established model of arthritis in rats, SZ was administered chronically at a dose of 80 mg/kg per day (Fener et al., 1990). In the present study at the dose of 8 mg/kg per day, an anti-inflammatory effect *per se* was not observed by SZ.

The results shown by the present study strongly suggest that NAC administration inhibited ethanol-induced oxidative stress (GSSG/GSH ratio) activating the Nrf2-ARE system, which is in line with a report that an analog of NAC, NACA, attenuated the oxidative stress induced by a traumatic brain injury in rats *via* the same antioxidant system (Zhou et al., 2018). The Nrf2 system activates the transcription of a number of antioxidant enzymes, including HO, NAD(P)H, quinone oxidoreductase 1, Prdx, and γ-glutamyl-cysteine synthetase, the latter a rate-limiting enzyme in the synthesis of glutathione. An additional rate-limiting factor in the synthesis of glutathione is the availability of intracellular cysteine (Lu, 2013). The Nrf2 system additionally up-regulates the activity of the promoter of the xCT cystine-glutamate transporter gene (Shih et al., 2003; Habib et al., 2015), a transporter that allows the influx of cystine into cells by exchanging it with glutamate (Baker et al., 2002). In the present study, the expression of the xCT was marginally but significantly increased by NAC administration.

Whether an activation of xCT transporter function due to an increased substrate availability and/or an increased xCT expression plays a role in the NAC-induced reduction of hippocampal GSSG/GSH ratio, the administration of the xCT inhibitor SZ was expected to reduce or block such an effect

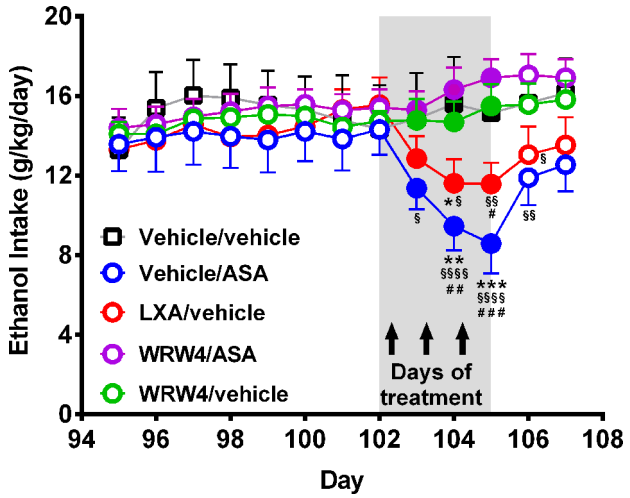


FIGURE 7 | Oral administration of acetylsalicylic acid (ASA) or intravenous administration of lipoxin A4 inhibited chronic ethanol intake an effect that was fully blocked by the administration of WRW4, an antagonist of the lipoxin FPR2/ALX receptor. Rats chronically ingesting ethanol for 102 days were treated for three consecutive days with either: vehicle, ASA (15 mg/kg, p.o.), lipoxin A4 (LXA; 7 μg/kg, i.v.), or WRW4 (0.3 mg/kg administered i.v. 5 min prior to ASA or vehicle). Acetylsalicylic acid or LXA A4 treatment reduced chronic ethanol intake compared to the vehicle group [two-way analysis of variance indicates significant effect of treatment ($F_{treatment(4, 108)} = 14.23$, $^{***}P < 0.001$) but not of day ($F_{day(5, 108)} = 1.262$, $P = 0.2856$)]. Tukey *post hoc* test revealed that the administration of three daily doses of ASA reduced ethanol intake: vs. the vehicle group ($^{**}P < 0.01$, the second and $^{***}P < 0.001$ the third day of treatment), vs. the WRW4/ASA group ($^{§}P < 0.05$ the first, $^{§§§}P < 0.001$ the second, $^{§§§§}P < 0.0001$ the third day of treatment, and $^{§§}P < 0.01$ the first posttreatment day) and vs. the WRW4/vehicle group ($^{\#\#}P < 0.01$, the second and $^{\#\#\#}P < 0.01$ the third day of treatment). In the same way, three daily doses of lipoxin A4 reduced ethanol intake: vs. vehicle group ($^{*}P < 0.05$, the second day of treatment), vs. the WRW4/ASA group ($^{§}P < 0.05$ the second, $^{§§}P < 0.01$ the third day of treatment, and $^{§§}P < 0.01$ the first posttreatment day) and vs. the WRW4/vehicle group ($^{\#}P < 0.05$, the third day of treatment). Data are presented as mean ± SEM, $n = 6$ rats per experimental group.

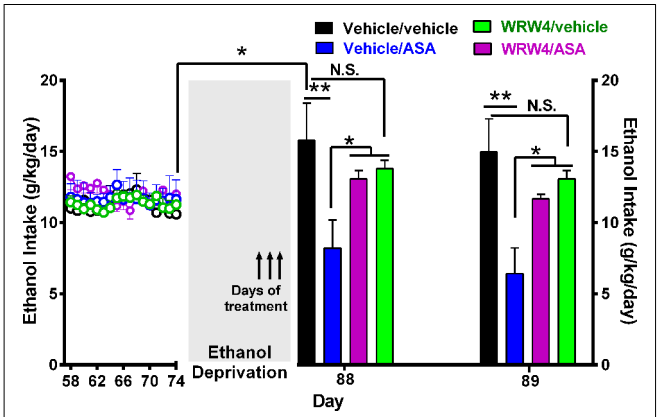


FIGURE 8 | Oral administration of acetylsalicylic acid (ASA, 30 mg/kg per day) inhibited the alcohol postdeprivation relapse drinking, an effect blocked by the administration of WRW4, an antagonist of the lipoxin FPR2/ALX receptor. Following 74 days of ethanol intake, on the last 3 days of a 14-day ethanol deprivation, animals were treated as indicated by arrows. Bars show daily ethanol intake after the 2-week ethanol deprivation followed by 2 days of ethanol reaccess. The high ethanol intake of the control group (vehicle/vehicle group; black bar) on the first day (day 88) of alcohol reaccess (16.0 ± 2.0; mean ± SEM, $n = 5$) vs. its intake prior to deprivation (11.2 ± 0.34; mean ± SEM, $n = 5$) demonstrates the alcohol deprivation relapse [$^{*}P < 0.05$ vehicle/vehicle group on the first day of ethanol re-exposure (black bar) compared to the mean of the last 11 baseline days of ethanol intake of the same group (empty black circles)]. Two-way analysis of variance of (treatment × day) performed on ethanol intake data obtained the first (day 88) and second (day 89) day of ethanol reaccess postdeprivation revealed significant effect of treatment ($F_{treatment(3, 26)} = 9.315$), but not of day. *Post hoc* Fisher analysis indicated that ASA (vehicle/ASA group) administration 24 h before ethanol reaccess induced a significant ($^{**}P < 0.01$) inhibition of ethanol intake vs. the vehicle/vehicle group the first and second day of ethanol reaccess, whereas pretreatment with WRW4, an antagonist of the lipoxin receptor (FPR2), blocked the ASA-induced reduction of alcohol relapse the first and second day of ethanol reaccess ($^{*}P < 0.05$ vehicle/ASA group vs. WRW4/ASA and WRW4/vehicle group). Data are presented as mean ± SEM, $n = 6$ rats per experimental group. N.S., not statistically significant.

of NAC. As indicated, this was indeed observed; SZ fully blocked the effect of NAC in lowering the GSSG/GSH ratio. This finding, added to the observation that SZ also fully blocked the NAC-induced inhibition of relapse-like ethanol intake, suggests that NAC acts *via* the xCT transporter extruding glutamate into the extracellular space (Baker et al., 2002) reaching levels known to stimulate presynaptic mGlu2/3 autoreceptors, negatively modulating synaptic glutamate release (Schoepp et al., 1999). This effect of NAC is of additional value for reducing ethanol intake because mGlu2/3 receptor has been reported to be depressed in animals chronically exposed to alcohol (Meinhardt et al., 2013; Ding et al., 2016). An increased mGlu2/3 receptor action by NAC is further supported by the finding in the present study that pretreatment of animals with the mGluR2/3 receptor antagonist, LY341495, fully blocked the NAC-induced inhibition of alcohol relapse.

Operant ethanol self-administration studies by Gass et al. (2011) showed that the presentation of cues previously associated with alcohol self-administration led to marked increases in glutamate release in nucleus accumbens. An increased synaptic release of glutamate appears to play an important role in addictive drug craving and relapse for most drugs of abuse (Scofield et al., 2016). In the present ADE study, alcohol reaccess provides conditioned cues such as ethanol odor, reported in heavy alcohol users to activate nucleus accumbens (Kareken et al., 2004) and taste (Remedios et al., 2014). An mGlu2/3 receptor-mediated inhibition of the glutamate tone by NAC is expected to improve the effect when combined with an increased synaptic GLT-1 transport, as previously reported for ASA (Israel et al., 2019). N-acetylcysteine is expected to have a synergic effect on the inhibition of alcohol intake induced by β-lactam antibiotics such as ceftriaxone and ampicillin, which increase GLT-1 activity (Rao et al., 2015; Sari et al., 2016). Such a synergic effect with NAC may be of value in the use of central nervous system–directed β-lactam antibiotics in clinical studies.

The doses of NAC and ASA used to reduce alcohol intake in the present study are within the range used for clinical studies. N-acetylcysteine at doses among 2,400–3,600 mg/day have been used for the treatment of cocaine, nicotine, alcohol, and cannabis relapse in humans (Duailibi et al., 2017; Squeglia et al., 2018). The

15 or 30 mg/kg per day dose of ASA used does not induce gastric irritation in the rat (Wallace et al., 2004). The average daily dose of ASA used chronically in the treatment of rheumatoid arthritis is 2,600 mg/day (Fries et al., 1993). Thus, in humans, doses of ASA could be markedly increased vs. those used in this study. The combination of NAC (1,000 mg/day) plus ASA (1,000 mg/day) has been administered with positive results in the treatment of bipolar depression (Bauer et al., 2019).

A possible depressant effect in the animals in the present study, in which the rats were single housed without environmental enrichment, is unlikely as a cause of neuroinflammation, because as shown in **Figure 6**, control animals housed individually and receiving water as the only fluid do not show astrocyte or microglial abnormalities. Nevertheless, the existence of nonhistological abnormalities, or stress often present in AUD, cannot be discarded and may have a role in the inhibition of neuroinflammation exerted by the combination of NAC and ASA (see Bauer et al., 2019).

A methodological aspect of interest relates to the minimal length of chronic alcohol intake required, by a model of spontaneous alcohol intake, to observe a marked effect of NAC. It is important to use a model where NAC (at the doses used) has a clear inhibitory effect on ethanol intake, such that a possible association with neuroinflammation or oxidative stress can be investigated. We have reported that NAC does not inhibit alcohol intake if rats are allowed ethanol intake for a short period (20 days; Quintanilla et al., 2016b), time at which an ethanol intake plateau had not been reached. However, in the same study, an inhibitory effect of NAC on ethanol intake was clearly observed following 60 days of chronic alcohol intake, time at which intake was constant. In the present study, chronic ethanol intake for 47 days (36 days of 10% ethanol access plus 11 days of 10% and 20% ethanol access), followed by a 14-day deprivation period, showed a marked relapse intake, which was inhibited by NAC (day 62). An inhibitory effect of NAC at such time is in line with a self-perpetuating neuroinflammation even after ethanol deprivation. The present work with UChB rats shows the shortest time (voluntary intake plus deprivation) that has even been reported for rats showing neuroinflammation. In previous studies, the authors have shown alcohol-induced neuroinflammation after longer times of ethanol access (>91 days). Studies in mice (Alfonso-Loeches et al., 2010) that had ingested 10% alcohol for 150 days showed significant neuroinflammation both in cerebral cortex and hippocampus. Whether shorter times will also show an ethanol-induced neuroinflammation is not known.

As indicated earlier, ASA acutely acetylates Cox-1 and chronically also Cox-2, leading to the generation of the powerful anti-inflammatory 15- R epi-Lipoxin A4 (or ASA triggered lipoxin ATL), a metabolite of arachidonic acid (Romano et al., 2015; Serhan and Levy, 2018). The present study shows that the administration of lipoxin A4 partially inhibited chronic alcohol intake. Furthermore, the inhibitory effect of ASA on alcohol relapse intake was significantly reduced by the administration of WRW4, an antagonist of the lipoxin receptor FPR2/ALX, suggesting that the generation of lipoxin is the main mechanism behind the reduction of chronic alcohol intake induced by ASA. To our knowledge, this is the first report of the inhibitory effect of lipoxin A4, a macrophage recruitment inhibitor (Serhan and Levy, 2018) on alcohol intake.

Overall, the present study shows that NAC and ASA inhibit alcohol intake by different mechanisms, resulting in a combined effect that could allow the chronic use of both drugs at clinically relevant doses.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by Medical Faculty of the University of Chile (Protocol CBA# 0994 FMUCH) and by the Chilean Council for Science and Technology Research (CONICYT).

AUTHOR CONTRIBUTIONS

MQ and FE: conception and design, collection of data, data analysis, manuscript writing, and final approval of manuscript. PM: design, collection of data, data analysis, and final approval of manuscript. ME: conception and design, collection of data, data analysis, financial support, manuscript writing, and final approval of manuscript. BO and DS: collection of data, data analysis, and final approval of manuscript. MH-M: conception and final approval of manuscript. YI: conception and design, financial support, manuscript draft and final approval of manuscript.

FUNDING

These studies were supported by FONDECYT grants #1180042, 1190562 and 1200287.

ACKNOWLEDGMENTS

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

REFERENCES

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A. M., Sanchez-Vera, I., and Guerri, C. (2010). Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. *J. Neurosci.* 30, 8285–8295. doi: 10.1523/JNEUROSCI.0976-10.2010

Amin, B., Hajhashemi, V., Hosseinzadeh, H., and Abnous, K. (2012). Antinociceptive evaluation of ceftriaxone and minocycline alone and in combination in a neuropathic pain model in rat. *Neuroscience* 224, 15–25. doi: 10.1016/j.neuroscience.2012.07.058

Aragon, C. M., and Amit, Z. (1992). The effect of 3-amino-1,2,4-triazole on voluntary ethanol consumption: evidence for brain catalase involvement in

the mechanism of action. *Neuropharmacology* 31, 709–712. doi: 10.1016/0028-3908(92)90150-n

Baker, D. A., Xi, Z. X., Shen, H., Swanson, C. J., and Kalivas, P. W. (2002). The origin and neuronal function of *in vivo* nonsynaptic glutamate. *J. Neurosci.* 22, 9134–9141. doi: 10.1523/JNEUROSCI.22-20-09134.2002

Bauer, I. E., Green, C., Colpo, G. D., Teixeira, A. L., Selvaraj, S., Durkin, K., et al. (2019). A double-blind, randomized, placebo-controlled study of aspirin and N-acetylcysteine as adjunctive treatments for bipolar depression. *J. Clin. Psychiatry* 80:18m12200. doi: 10.4088/jcp.18m12200

Bernabucci, M., Notartomaso, S., Zappulla, C., Fazio, F., Cannella, M., Motolese, M., et al. (2012). N-Acetyl-cysteine causes analgesia by reinforcing the endogenous activation of type-2 metabotropic glutamate receptors. *Mol. Pain* 8:77. doi: 10.1186/1744-8069-8-77

Blednov, Y. A., Benavidez, J. M., Geil, C., Perra, S., Morikawa, H., and Harris, R. A. (2011). Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain Behav. Immun.* 25, S92–S105. doi: 10.1016/j.bbi.2011.01.008

Canty, T. G. Jr., Boyle, E. M. Jr., Farr, A., Morgan, E. N., Verrier, E. D., and Pohlman, T. H. (1999). Oxidative stress induces NF-κB nuclear translocation without degradation of IκBα. *Circulation* 100, II361–II364. doi: 10.1161/01.cir.100.suppl_2.ii-361

Colebatch, A. N., Marks, J. L., van der Heijde, D. M., and Edwards, C. J. (2012). Safety of nonsteroidal antiinflammatory drugs and/or paracetamol in people receiving methotrexate for inflammatory arthritis: a Cochrane systematic review. *J. Rheumatol. Suppl.* 90, 62–73. doi: 10.3899/jrheum.120345

Correa, M., Salamone, J. D., Segovia, K. N., Pardo, M., Longoni, R., Spina, L., et al. (2012). Piecing together the puzzle of acetaldehyde as a neuroactive agent. *Neurosci. Biobehav. Rev.* 36, 404–430. doi: 10.1016/j.neubiorev.2011.07.009

Crews, F. T., and Vetreno, R. P. (2016). Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology* 233, 1543–1557. doi: 10.1007/s00213-015-3906-1

Ding, Z. M., Ingraham, C. M., Rodd, Z. A., and McBride, W. J. (2016). Alcohol drinking increases the dopamine-stimulating effects of ethanol and reduces D2 auto-receptor and group II metabotropic glutamate receptor function within the posterior ventral tegmental area of alcohol preferring (P) rats. *Neuropharmacology* 109, 41–48. doi: 10.1016/j.neuropharm.2016.05.023

Duailibi, M. S., Cordeiro, Q., Brietzke, E., Ribeiro, M., LaRowe, S., Berk, M., et al. (2017). N-acetylcysteine in the treatment of craving in substance use disorders: systematic review and meta-analysis. *Am. J. Addict.* 26, 660–666. doi: 10.1111/ajad.12620

Ezquer, F., Quintanilla, M. E., Morales, P., Ezquer, M., Lespay-Rebolledo, C., Herrera-Marschitz, M., et al. (2019). Activated mesenchymal stem cell administration inhibits chronic alcohol drinking and suppresses relapse-like drinking in high-alcohol drinker rats. *Addict. Biol.* 24, 17–27. doi: 10.1111/adb.12572

Fener, P., Bannwarth, B., Gillet, P., Netter, P., Regent, D., Pourel, J., et al. (1990). Influence of sulfasalazine on established collagen arthritis in rats. *Clin. Exp. Rheumatol.* 8, 167–170.

Ferrier, L., Bérard, F., Debrauwer, L., Chabo, C., Langella, P., Bueno, L., et al. (2006). Impairment of the intestinal barrier by ethanol involves enteric microflora and mast cell activation in rodents. *Am. J. Pathol.* 168, 1148–1154. doi: 10.2353/ajpath.2006.050617

Fisher, E. S., and Curry, S. C. (2019). Evaluation and treatment of acetaminophen toxicity. *Adv. Pharmacol.* 85, 263–283. doi: 10.1016/bs.apha.2018.12.004

Fries, J. F., Ramey, D. R., Singh, G., Morfeld, D., Bloch, D. A., and Raynauld, J. P. (1993). A reevaluation of aspirin therapy in rheumatoid arthritis. *Arch. Intern. Med.* 153, 2465–2471. doi: 10.1001/archinte.153.21.2465

Fukumoto, K., Iijima, M., and Chaki, S. (2014). Serotonin-1A receptor stimulation mediates effects of a metabotropic glutamate 2/3 receptor antagonist, 2S-2-amino-2–(1S,2S-2-carboxycycloprop-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495) and an N-methyl-D-aspartate receptor antagonist, ketamine, in the novelty-suppressed feeding test. *Psychopharmacology* 231, 2291–2298. doi: 10.1007/s00213-013-3378-0

Garcia-Keller, C., Smiley, C., Monforton, C., Melton, S., Kalivas, P. W., and Gass, J. (2019). N-Acetylcysteine treatment during acute stress prevents stress-induced augmentation of addictive drug use and relapse. *Addict. Biol.* 7:e12798. doi: 10.1111/adb.12798

Gass, J. T., Sinclair, C. M., Cleva, R. M., Widholm, J. J., and Olive, M. F. (2011). Alcohol-seeking behavior is associated with increased glutamate transmission in basolateral amygdala and nucleus accumbens as measured by glutamate-oxidase-coated biosensors. *Addict. Biol.* 16, 215–228. doi: 10.1111/j.1369-1600.2010.00262.x

Ghattamaneni, N. K. R., Panchal, S. K., and Brown, L. (2019). An improved rat model for chronic inflammatory bowel disease. *Pharmacol. Rep.* 71, 149–155. doi: 10.1016/j.pharep.2018.10.006

Gómez, G. I., Falcon, R. V., Maturana, C. J., Labra, V. C., Salgado, N., Rojas, C. A., et al. (2018). Heavy alcohol exposure activates astroglial hemichannels and pannexons in the hippocampus of adolescent rats: effects on neuroinflammation and astrocyte arborization. *Front. Cell. Neurosci.* 12:472. doi: 10.3389/fncel.2018.00472

Habib, E., Linher-Melville, K., Lin, H. X., and Singh, G. (2015). Expression of xCT and activity of system x_c^- are regulated by NRF2 in human breast cancer cells in response to oxidative stress. *Redox Biol.* 5, 33–42. doi: 10.1016/j.redox.2015.03.003

Hughes, E. L., Becker, F., Flower, R. J., Buckingham, J. C., and Gavins, F. N. E. (2017). Mast cells mediate early neutrophil recruitment and exhibit anti-inflammatory properties *via* the formyl peptide receptor 2/lipoxin A_4 receptor. *Br. J. Pharmacol.* 174, 2393–2408. doi: 10.1111/bph.13847

Israel, Y., Karahanian, E., Ezquer, F., Morales, P., Ezquer, M., Rivera-Meza, M., et al. (2017). Acquisition, maintenance and relapse-like alcohol drinking: lessons from the uchb rat line. *Front. Behav. Neurosci.* 11:57. doi: 10.3389/fnbeh.2017.00057

Israel, Y., Quintanilla, M. E., Ezquer, F., Morales, P., Santapau, D., Berrios-Carcamo, P., et al. (2019). Aspirin and N-acetylcysteine co-administration markedly inhibit chronic ethanol intake and block relapse binge drinking: role of neuroinflammation-oxidative stress self-perpetuation. *Addict. Biol.* 15:e12853. doi: 10.1111/adb.12853

Kansanen, E., Kuosmanen, S. M., Leinonen, H., and Levonen, A. L. (2013). The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. *Redox Biol.* 1, 45–49. doi: 10.1016/j.redox.2012.10.001

Karahanian, E., Quintanilla, M. E., Tampier, L., Rivera-Meza, M., Bustamante, D., Gonzalez-Lira, V., et al. (2011). Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. *Alcohol. Clin. Exp. Res.* 35, 606–612. doi: 10.1111/j.1530-0277.2011.01439.x

Karahanian, E., Rivera-Meza, M., Tampier, L., Quintanilla, M. E., Herrera-Marschitz, M., and Israel, Y. (2015). Long-term inhibition of ethanol intake by the administration of an aldehyde dehydrogenase-2 (ALDH2)-coding lentiviral vector into the ventral tegmental area of rats. *Addict. Biol.* 20, 336–344. doi: 10.1111/adb.12130

Kareken, D. A., Claus, E. D., Sabri, M., Dzemidzic, M., Kosobud, A. E., Radnovich, A. J., et al. (2004). Alcohol-related olfactory cues activate the nucleus accumbens and ventral tegmental area in high-risk drinkers: preliminary findings. *Alcohol. Clin. Exp. Res.* 28, 550–557. doi: 10.1097/01.alc.0000122764.60626.af

Kastl, L., Sauer, S. W., Ruppert, T., Beissbarth, T., Becker, M. S., Suss, D., et al. (2014). TNF-α mediates mitochondrial uncoupling and enhances ROS-dependent cell migration *via* NF-κB activation in liver cells. *FEBS Lett.* 588, 175–183. doi: 10.1016/j.febslet.2013.11.033

Koechling, U. M., and Amit, Z. (1994). Effects of 3-amino-1,2,4-triazole on brain catalase in the mediation of ethanol consumption in mice. *Alcohol* 11, 235–239. doi: 10.1016/0741-8329(94)90036-1

Lebourgeois, S., González-Marin, M. C., Antol, J., Naassila, M., and Vilpoux, C. (2019). Evaluation of N-acetylcysteine on ethanol self-administration in ethanol-dependent rats. *Neuropharmacology* 150, 112–120. doi: 10.1016/j.neuropharm.2019.03.010

Lebourgeois, S., González-Marin, M. C., Jeanblanc, J., Naassila, M., and Vilpoux, C. (2018). Effect of N-acetylcysteine on motivation, seeking and relapse to ethanol self-administration. *Addict. Biol.* 23, 643–652. doi: 10.1111/adb.12521

Leclercq, S., de Timary, P., Delzenne, N. M., and Stärkel, P. (2017). The link between inflammation, bugs, the intestine and the brain in alcohol dependence. *Transl. Psychiatry* 7:e1048. doi: 10.1038/tp.2017.15

Lu, S. C. (2013). Glutathione synthesis. *Biochim. Biophys. Acta* 1830, 3143–3153. doi: 10.1016/j.bbagen.2012.09.008

Mayfield, J., Ferguson, L., and Harris, R. A. (2013). Neuroimmune signaling: a key component of alcohol abuse. *Curr. Opin. Neurobiol.* 23, 513–520. doi: 10.1016/j.conb.2013.01.024

Medeiros, R., Kitazawa, M., Passos, G. F., Baglietto-Vargas, D., Cheng, D., Cribbs, D. H., et al. (2013). Aspirin-triggered lipoxin A4 stimulates alternative activation of microglia and reduces Alzheimer disease-like pathology in mice. *Am. J. Pathol.* 182, 1780–1789. doi: 10.1016/j.ajpath.2013.01.051

Meinhardt, M. W., Hansson, A. C., Perreau-Lenz, S., Bauder-Wenz, C., Stahlin, O., Heilig, M., et al. (2013). Rescue of infralimbic mGluR2 deficit restores control over drug-seeking behavior in alcohol dependence. *J. Neurosci.* 33, 2794–2806. doi: 10.1523/JNEUROSCI.4062-12.2013

Melis, M., Carboni, E., Caboni, P., and Acquas, E. (2015). Key role of salsolinol in ethanol actions on dopamine neuronal activity of the posterior ventral tegmental area. *Addict. Biol.* 20, 182–193. doi: 10.1111/adb.12097

Nocito Echevarria, M. A., Andrade Reis, T., Ruffo Capatti, G., Siciliano Soares, V., da Silveira, D. X., and Fidalgo, T. M. (2017). N-acetylcysteine for treating cocaine addiction—a systematic review. *Psychiatry Res.* 251, 197–203. doi: 10.1016/j.psychres.2017.02.024

Ochoa-Aguilar, A., Ventura-Martinez, R., Sotomayor-Sobrino, M. A., Jaimez, R., Coffeen, U., Jimenez-Gonzalez, A., et al. (2018). Ceftriaxone and clavulanic acid induce antiallodynia and anti-inflammatory effects in rats using the carrageenan model. *J. Pain Res.* 11, 977–985. doi: 10.2147/jpr.s154661

Orrico, A., Marti-Prats, L., Cano-Cebrian, M. J., Granero, L., Polache, A., and Zornoza, T. (2017). Pre-clinical studies with D-penicillamine as a novel pharmacological strategy to treat alcoholism: updated evidences. *Front. Behav. Neurosci.* 11:37. doi: 10.3389/fnbeh.2017.00037

Pascual, M., Montesinos, M., Marcos, M., Torres, J.-L., Costa-Alba, F., Lasom, F.-J., et al. (2017). Gender differences in the inflammatory cytokine and chemokine profiles induced by binge ethanol drinking in adolescence. *Addict. Biol.* 22, 1829–1841. doi: 10.1111/adb.12461

Peana, A. T., Porcheddu, V., Bennardini, F., Carta, A., Rosas, M., and Acquas, E. (2015). Role of ethanol-derived acetaldehyde in operant oral self-administration of ethanol in rats. *Psychopharmacology* 232, 4269–4276. doi: 10.1007/s00213-015-4049-0

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., et al. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453–462. doi: 10.1002/glia.20467

Quertemont, E., Eriksson, C. J., Zimatkin, S. M., Pronko, P. S., Diana, M., Pisano, M., et al. (2005). Is ethanol a pro-drug? Acetaldehyde contribution to brain ethanol effects. *Alcohol. Clin. Exp. Res.* 29, 1514–1521. doi: 10.1097/01.alc.0000175015.51329.45

Quintanilla, M. E., Ezquer, F., Morales, P., Santapau, D., Berrios-Carcamo, P., Ezquer, M., et al. (2019). Intranasal mesenchymal stem cell secretome administration markedly inhibits alcohol and nicotine self-administration and blocks relapse-intake: mechanism and translational options. *Stem Cell Res. Ther.* 10:205. doi: 10.1186/s13287-019-1304-z

Quintanilla, M. E., Israel, Y., Sapag, A., and Tampier, L. (2006). The UChA and UChB rat lines: metabolic and genetic differences influencing ethanol intake. *Addict. Biol.* 11, 310–323. doi: 10.1111/j.1369-1600.2006.00030.x

Quintanilla, M. E., Morales, P., Ezquer, F., Ezquer, M., Herrera-Marschitz, M., and Israel, Y. (2018). Commonality of ethanol and nicotine reinforcement and relapse in wistar-derived UChB rats: inhibition by N-acetylcysteine. *Alcohol. Clin. Exp. Res.* 42, 1988–1999. doi: 10.1111/acer.13842

Quintanilla, M. E., Rivera-Meza, M., Berrios-Carcamo, P., Cassels, B. K., Herrera-Marschitz, M., and Israel, Y. (2016a). (R)-Salsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake. *Addict. Biol.* 21, 1063–1071. doi: 10.1111/adb.12268

Quintanilla, M. E., Rivera-Meza, M., Berrios-Carcamo, P., Salinas-Luypaert, C., Herrera-Marschitz, M., and Israel, Y. (2016b). Beyond the "First Hit": marked inhibition by N-acetyl cysteine of chronic ethanol intake but not of early ethanol intake. Parallel effects on ethanol-induced saccharin motivation. *Alcohol. Clin. Exp. Res.* 40, 1044–1051. doi: 10.1111/acer.13031

Quintanilla, M. E., Rivera-Meza, M., Berrios-Cárcamo, P. A., Bustamante, D., Buscaglia, M., Morales, P., et al. (2014). Salsolinol, free of isosalsolinol, exerts ethanol-like motivational/sensitization effects leading to increases in ethanol intake. *Alcohol* 48, 551–559. doi: 10.1016/j.alcohol.2014.07.003

Quintanilla, M. E., Tampier, L., Karahanian, E., Rivera-Meza, M., Herrera-Marschitz, M., and Israel, Y. (2012). Reward and relapse: complete gene-induced dissociation in an animal model of alcohol dependence. *Alcohol. Clin. Exp. Res.* 36, 517–522. doi: 10.1111/j.1530-0277.2011.01606.x

Rao, P. S., Goodwani, S., Bell, R. L., Wei, Y., Boddu, S. H., and Sari, Y. (2015). Effects of ampicillin, cefazolin and cefoperazone treatments on GLT-1 expressions in the mesocorticolimbic system and ethanol intake in alcohol-preferring rats. *Neuroscience* 295, 164–174. doi: 10.1016/j.neuroscience.2015.03.038

Remedios, J., Woods, C., Tardif, C., Janak, P. H., and Chaudhri, N. (2014). Pavlovian-conditioned alcohol-seeking behavior in rats is invigorated by the interaction between discrete and contextual alcohol cues: implications for relapse. *Brain Behav.* 4, 278–289. doi: 10.1002/brb3.216

Rodd, Z. A., Bell, R. L., Zhang, Y., Goldstein, A., Zaffaroni, A., McBride, W. J., et al. (2003). Salsolinol produces reinforcing effects in the nucleus accumbens shell of alcohol-preferring (P) rats. *Alcohol. Clin. Exp. Res.* 27, 440–449. doi: 10.1097/01.alc.0000056612.89957.b4

Rodd, Z. A., Bell, R. L., Zhang, Y., Murphy, J. M., Goldstein, A., Zaffaroni, A., et al. (2005). Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring (P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* 30, 330–338. doi: 10.1038/sj.npp.1300561

Rojkovicova, T., Mechref, Y., Starkey, J. A., Wu, G., Bell, R. L., McBride, W. J., et al. (2008). Quantitative chiral analysis of salsolinol in different brain regions of rats genetically predisposed to alcoholism. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 863, 206–214. doi: 10.1016/j.jchromb.2008.01.016

Romano, M., Cianci, E., Simiele, F., and Recchiuti, A. (2015). Lipoxins and aspirin-triggered lipoxins in resolution of inflammation. *Eur. J. Pharmacol.* 760, 49–63. doi: 10.1016/j.ejphar.2015.03.083

Romera, C., Hurtado, O., Mallolas, J., Pereira, M. P., Morales, J. R., Romera, A., et al. (2007). Ischemic preconditioning reveals that GLT1/EAAT2 glutamate transporter is a novel PPARγ target gene involved in neuroprotection. *J. Cereb. Blood Flow Metab.* 27, 1327–1338. doi: 10.1038/sj.jcbfm.9600438

Sansone, R. A., and Sansone, L. A. (2011). Getting a knack for NAC: N-acetylcysteine. *Innov. Clin. Neurosci.* 8, 10–14.

Sari, Y., Toalston, J. E., Rao, P. S. S., and Bell, R. L. (2016). Effects of ceftriaxone on ethanol, nicotine or sucrose intake by alcohol-preferring (P) rats and its association with GLT-1 expression. *Neuroscience* 326, 117–125. doi: 10.1016/j.neuroscience.2016.04.004

Schoepp, D. D., Jane, D. E., and Monn, J. A. (1999). Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* 38, 1431–1476. doi: 10.1016/s0028-3908(99)00092-1

Schreck, R., Albermann, K., and Baeuerle, P. A. (1992). Nuclear factor κB: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic. Res. Commun.* 17, 221–237. doi: 10.3109/10715769209079515

Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. A., and Fiers, W. (1992). Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J. Biol. Chem.* 267, 5317–5323.

Scofield, M. D., Heinsbroek, J. A., Gipson, C. D., Kupchik, Y. M., Spencer, S., Smith, A. C., et al. (2016). The nucleus accumbens: mechanisms of addiction across drug classes reflect the importance of glutamate homeostasis. *Pharmacol. Rev.* 68, 816–871. doi: 10.1124/pr.116.012484

Serhan, C. N., and Levy, B. D. (2018). Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators. *J. Clin. Invest.* 128, 2657–2669. doi: 10.1172/jci97943

Shih, A. Y., Johnson, D. A., Wong, G., Kraft, A. D., Jiang, L., Erb, H., et al. (2003). Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J. Neurosci.* 23, 3394–3406. doi: 10.1523/JNEUROSCI.23-08-03394.2003

Sobrado, M., Pereira, M. P., Ballesteros, I., Hurtado, O., Fernandez-Lopez, D., Pradillo, J. M., et al. (2009). Synthesis of lipoxin A4 by 5-lipoxygenase mediates PPARγ-dependent, neuroprotective effects of rosiglitazone in experimental stroke. *J. Neurosci.* 29, 3875–3884. doi: 10.1523/JNEUROSCI.5529-08.2009

Sordi, R., Menezes-de-Lima, O. Jr., Horewicz, V., Scheschowitsch, K., Santos, L. F., and Assreuy, J. (2013). Dual role of lipoxin A4 in pneumosepsis pathogenesis. *Int. Immunopharmacol.* 17, 283–292. doi: 10.1016/j.intimp.2013.06.010

Squeglia, L. M., Tomko, R. L., Baker, N. L., McClure, E. A., Book, G. A., and Gray, K. M. (2018). The effect of N-acetylcysteine on alcohol use during a cannabis cessation trial. *Drug Alcohol Depend.* 185, 17–22. doi: 10.1016/j.drugalcdep.2017.12.005

Tampier, L., Quintanilla, M. E., and Mardones, J. (1995). Effects of aminotriazole on ethanol, water, and food intake and on brain catalase in UChA and UChB rats. *Alcohol* 12, 341–344. doi: 10.1016/0741-8329(95)00014-i

Wallace, J. L., Zamuner, S. R., McKnight, W., Dicay, M., Mencarelli, A., del Soldato, P., et al. (2004). Aspirin, but not NO-releasing aspirin (NCX-4016), interacts with selective COX-2 inhibitors to aggravate gastric damage and inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 286, G76–G81. doi: 10.1152/ajpgi.00295.2003

Wu, B., Walker, J. A., Temmermand, D., Mian, K., Spur, B., Rodriguez, A., et al. (2013). Lipoxin A_4 promotes more complete inflammation resolution in sepsis compared to stable lipoxin A_4 analog. *Prostaglandins Leukot. Essent. Fatty Acids* 89, 47–53. doi: 10.1016/j.plefa.2013.04.005

Yousefi-Ahmadipour, A., Rashidian, A., Mirzaei, M. R., Farsinejad, A., PourMohammadi-Nejad, F., Ghazi-Khansari, M., et al. (2019). Combination therapy of mesenchymal stromal cells and sulfasalazine attenuates trinitrobenzene sulfonic acid induced colitis in the rat: the S1P pathway. *J. Cell. Physiol.* 234, 11078–11091. doi: 10.1002/jcp.27944

Zhou, Y., Wang, H. D., Zhou, X. M., Fang, J., Zhu, L., and Ding, K. (2018). N-acetylcysteine amide provides neuroprotection *via* Nrf2-ARE pathway in a mouse model of traumatic brain injury. *Drug Des. Dev. Ther.* 12, 4117–4127. doi: 10.2147/dddt.s179227

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership