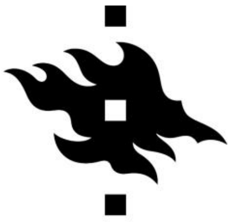

The effect of lysergic acid diethylamide on brain BDNF levels and Pavlovian extinction learning in mice

Master’s Programme in Neuroscience
Cell and Systems Physiology study track

Author:
Liisa Heleena Ilkka

Supervisors:
Esa Korpi
Lauri Elsilä

Helsinki
2023

Abstract

Faculty: Faculty of Biological and Environmental Sciences
Degree programme: Master's Programme in Neuroscience
Study track: Cell and Systems Physiology
Author: Liisa Heleena Ilkka
Title: The effect of lysergic acid diethylamide on brain BDNF levels and Pavlovian extinction learning in mice
Level: Master's Thesis
Month and year: May, 2023
Number of pages: 36
Keywords: LSD, psychedelics, addiction, extinction learning, BDNF
Supervisor or supervisors: Esa Korpi, Lauri Elsilä
Where deposited: HELDA – Digital Repository of the University of Helsinki
Additional information:
Abstract:

After decades of lull, the use of psychedelics as therapeutical agents has regained both scientific and public attention. The so-called classical psychedelics are currently studied in the treatment of multiple psychiatric conditions, including addiction. The current understanding implies that psychedelics mediate their subjective effects through serotonergic 5-HT_{2A} receptor binding. The activation of the receptor also leads to increases in brain-derived neurotrophic factor (BDNF) expression, a critical part of neuroplasticity mechanisms. At a behavioral level, facilitated neuroplasticity can be observed for example as quicker learning. Because addiction often involves associative learning between the pleasure produced by the drug use and the environmental cues, learning away from these associations could help to prevent relapses and enhance recovery. In this study we aimed to assess LSD's effect on BDNF levels in amygdalar and cortical regions, and their connection to extinction learning in fear and conditioned place preference paradigms. To evaluate time window for enhanced neuroplasticity, we chose two time points for BDNF level measurement, 24 and 48 h after the LSD injections. In addition, we chose both male and female mice for behavioral experiments to study possible differences between the sexes. We did not observe statistically significant differences between the treatment groups in BDNF levels or behavioral experiments after the single LSD injections. Despite that, this study provides perspectives for improving the experimental setups, as well as helps to evaluate still unanswered questions around the connection between psychedelics and neuroplasticity.

Tiivistelmä

Tiedekunta: Bio- ja ympäristötieteellinen tiedekunta

Koulutusohjelma: Neurotieteen maisteriohjelma

Opintosuunta: Solu- ja systeemifysiologia

Tekijä: Liisa Heleena Ilkka

Työn nimi: Lysergihapon dietyyliamidin vaikutus aivojen BDNF tasoihin ja Pavlovilaiseen sammutusoppimiseen hiirillä

Työn laji: Maisterin tutkielma

Aika: Toukokuu, 2023

Sivumäärä: 36

Avainsanat: LSD, psykedeelit, addiktio, sammutusoppiminen, BDNF

Säilytyspaikka: HELDA – Helsingin yliopiston digitaalinen arkisto

Muita tietoja:

Tiivistelmä:

Psykedeelien terapeuttinen käyttö on herättänyt runsaasti huomiota ja keskustelua usean hiljaisen vuosikymmenen jälkeen. Niin kutsuttuja klassisia psykedeelejä tutkitaan tällä hetkellä monien psykiatristen häiriöiden, esimerkiksi riippuvuuksien, hoidossa. Nykykäsityksen mukaan klassiset psykedeelit välittävät pääasiallisen psykoaktiiviset vaikutuksensa serotoniinin 5-HT_{2A} -reseptoriin sitoutumalla. Reseptorin aktivaatio saa myös aikaan lisääntyneen aivoperäisen hermokasvutekijän (BDNF) erittymisen, mikä on merkittävä osa luonnollisen neuroplastisuuden mekanismeja. Käyttäytymisen tasolla voimistunut neuroplastisuus saattaa näyttäytyä esimerkiksi tehostuneena oppimisena. Koska riippuvuuksien taustalla on usein ehdollistuminen päihteiden aiheuttaman mielihyvän ja ympäristön vihjeiden välillä, voi näistä poisoppiminen ehkäistä retkahduksia ja edistää toipumista. Tässä tutkimuksessa pyrimme selvittämään LSD:n vaikutusta BDNF-proteiinipitoisuuksiin mantelitumakkeen ja aivokuoren alueilla sekä näiden yhteyttä sammutusoppimiseen hiirillä pelko- ja paikkaehdollistumismalleissa. Voimistuneen plastisuuden aikaikkunaa hahmottaaksemme valitsimme BDNF-tasojen tarkastelemiseen kaksi aikapistettä, 24 ja 48 tuntia LSD-injektioiden jälkeen. Lisäksi valitsimme käyttäytymiskokeisiin sekä uros- että naarashiiriä mahdollisten sukupuolierojen kartoittamiseksi. Emme havainneet muutoksia BDNF-tasoissa tai käyttäytymismalleissa yksittäisten LSD annostusten jälkeen. Tästä huolimatta tutkimus tarjoaa näkökulmia koeasetelmien parantamiseksi sekä auttaa kartoittamaan vielä vastaamatta olevia kysymyksiä psykedeelien ja neuroplastisuuden yhteydestä

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Abbreviations

$5\text{-}HT_{2A}$	Serotonin 2A receptor
ANOVA	Analysis of variance
BDNF	Brain- derived neurotrophic factor
BSA	Bovine serum albumin
DMT	*N,N*-Dimethyltryptamine
DOI	2,5-Dimethoxy-4-iodoamphetamine
ELISA	Enzyme linked immunosorbent assay
LSD	Lysergic acid diethylamide
mTOR	Mammalian target of rapamycin
TCB-2	(4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide
TrkB	Tropomyosin receptor kinase B

1 Introduction

Addiction is often summarized as a chronic relapsing disorder characterized by compulsive drug seeking and use despite the harms (DSM-5; American Psychiatric Association, 2013). An estimated 38.6 million people suffer from drug use disorder worldwide (United nations office on drugs and crime & division for treaty affairs, 2022). According to European drug report 2022, Finland has the highest number of drug-related deaths among people under 25 years of age in Europe (European drug report: trends and developments, 2022). Despite the large economic and social burden, pharmacological treatment options for addictions are limited. The search for solutions has re-opened the research on psychedelics in the treatment of substance use disorders.

From a neurobiological perspective, addictive drugs target the brain's reward system and the dopaminergic tracts (Koob and Volkow, 2016). When used repeatedly, drugs of abuse can cause changes in the reward system, leading to pathological reward- and habit- driven drug- seeking behavior. Incentive salience theory of addiction is used to explain motivation behind drug use (Robinson and Berridge, 1993). The theory separates terms "liking" and "wanting" in the development of drug abuse. When the addiction progress, the craving of drug increases ("wanting") although the experienced pleasure ("liking") decreases. The "wanting" is reinforced through associative learning, where previously neutral environmental cues become associated with the pleasure produced by the drug use. This conditioned reinforcement is enabled by neuroadaptations in the mesocorticolimbic dopamine system (Koob and Volkow, 2016; Robinson and Berridge, 1993). The changes can lead to intense cravings induced by the cue alone, making person with addiction vulnerable to relapses even after long periods drug-free. Because cravings have a major role in relapses, reducing the cue- drug memories through extinction learning might be a possible target of pharmacological treatment (Kaplan et al., 2011; Torregrossa and Taylor, 2013). Because increasing serotonin signaling seems to facilitate extinction learning, potentially by promoting neuroplasticity, targeting the serotonergic system with pharmacological agents has been proposed for one possible treatment option (Klöbl et al., 2022; Zhang et al., 2013). In addition to selective serotonin reuptake inhibitors, psychedelics have gained interest as a way to augment these endogenous plasticity mechanisms.

The word psychedelics refers to a group of substances with an ability to produce altered states of consciousness and perception. These alterations often contain changes in sensory processing, perception and a variety of other psychological sensations, such as feelings of universal unity and loss of the sense of self (Liechti, 2017). So-called classical psychedelics include lysergic acid diethylamide (LSD), dimethyltryptamine (DMT), mescaline and psilocybin. Due to their effect on

serotonergic system, these compounds can also be classified as serotonergic psychedelics. The agonistic action of the serotonergic psychedelics on the specific serotonin receptor subtype 5-HT_{2A} has been shown to mediate the hallucinogenic properties of psychedelics (Vollenweider et al., 1998). In the Western world, LSD is probably one of the most known psychedelics. It is a semi-synthetic substance, first isolated and synthesized by Albert Hoffman in 1938 (Hofmann, 1979). After the discovery of LSD's strong psychological effects, it gained interest in psychiatric research together with other serotonergic psychedelics. The earlier trials focusing on problematic alcohol use were summarized in a meta-analysis published in 2012 (Krebs and Johansen, 2012). According to six randomized trials, a single LSD administration reduced alcohol consumption in short-term (2-3 months) and medium-term (6 months) observations. A more recent open-label trial indicates similar results (Bogenschutz et al., 2015). The study combined two psilocybin administrations with motivation enhancement therapy. Ten participants with alcohol use disorder were followed for 36 weeks. Heavy drinking days decreased 26% and overall drinking days 27.2%. Although the study lacks control groups and has a small sample size, the results were encouraging. Following up these findings, a larger double-blinded study published in 2022 reports similar results (Bogenschutz et al., 2022). Out of 95 participants 49 received psilocybin combined to a psychotherapy regimen. The study found significant reduction in heavy drinking days in both groups. While the comparison to an active placebo decreased the observed effect size, the psilocybin group reported 13.9% less heavy drinking days compared to the control group encouraging further study on the topic.

One popular hypothesis explaining the therapeutic effects of psychedelics is based on neuroplasticity (Calder and Hasler, 2022). The term is often used to describe multiple different changes in brain's structure and functioning in response to changes in environment or experiences. On the molecular level, increased neuroplasticity contains alterations in intracellular signaling, including changes in gene transcription, protein synthesis and signaling pathways (de Vos et al., 2021). It is argued that LSD and other serotonergic psychedelics can promote plasticity in 5-HT_{2A}, mammalian target of rapamycin (mTOR)- and tropomyosin receptor kinase B (TrkB) -dependent manner (Ly et al., 2018).

Acute plasticity effects of LSD are seen within the first hours of administration. In rats, LSD injection induced upregulation of plasticity related genes after 1.5 hours (Nichols and Sanders-Bush, 2002). The genes detected are involved in diverse cellular functioning and mostly belong to immediate early genes. There are also studies proposing that psychedelics increase the levels of neurotrophic factors, such as brain-derived neurotrophic factor BDNF, in limbic brain areas (Desouza et al., 2021; Gewirtz et al., 2002; Vaidya et al., 1997). In rodents, changes in *Bdnf* mRNA levels are detectable after two hours of psychedelic administration (Desouza et al., 2021; Gewirtz et al., 2002), whereas a study done in healthy subjects show enhanced levels of BDNF protein in plasma samples as early as four hours after the administration (Becker et al., 2022). The increase seen in BDNF levels after

psychedelics is likely caused by 5-HT_{2A} mediated regulation of BDNF mRNA expression (Vaidya et al., 1997). LSD's primary binding targets are thought to be the 5-HT_{2A} receptors densely expressed on the glutamatergic pyramidal cells in the deep cortical layers V and VI (de Vos et al., 2021). Some studies suggest that blocking the 5-HT_{2A} receptors with ketanserin inhibits LSDs psychological effects, but also its ability to induce neurogenesis and spinogenesis (Becker et al., 2022; Ly et al., 2018, but see also: Shao et al., 2021). Binding of LSD to the receptor triggers multiple intracellular pathways leading to increased glutamate bursts. Glutamate is linked to synaptic plasticity mainly through its effect on AMPA receptors (Diering and Huganir, 2018; Gulyaeva, 2017). Stimulation of AMPA results in further glutamate and BDNF release. BDNF binds to its receptor TrkB which leads to the activation of the mTOR signaling pathway. mTOR in turn stimulates BDNF synthesis, creating a positive feedback loop enabling long-lasting neural plasticity.

Our hypothesis links the BDNF expression to associative and extinction learning, both known to be part of addiction behaviors (Di Chiara, 1999; Hogarth et al., 2013). In animal models, extinction learning is usually studied as an extinction of conditioned fear or appetitive conditioning. Fear conditioning studies suggests that BDNF is essential for successful extinction (Chhatwal et al., 2006; Paredes et al., 2022; Peters et al., 2010). Chhatwal et al. (2006) demonstrated that BDNF release and the following TrkB signaling in the basolateral amygdala is necessary for the normal consolidation of an extinction memory. It has also been demonstrated that 5-HT_{2A} has a role in consolidation and extinction of fear memory (Zhang et al., 2013). The 5-HT_{2A} agonist TCB-2 was able to ease the acquisition of extinction of a fear memory when administrated before the extinction training. Given the psychedelics' ability to bind to the 5-HT_{2A} and stimulate BDNF release, we hypothesize that introducing psychedelics to the extinction learning settings accelerates extinction learning through enhanced consolidation of the forming extinction memory. If proven efficient, LSD's effect on extinction learning provides a new insight into the use of psychedelics in psychiatry and may carry clinically significant information about addictions and their treatment.

2 Aims of the study

The aim of this project was to study how LSD affects extinction of Pavlovian conditioning, and whether a correlation of these changes can be traced to molecular level. The workflow and experimental design are presented in Figure 1. We hypothesized that LSD increases BDNF expression and enhances extinction learning through endogenous neuroplastic mechanisms. To test our hypothesis, we aimed to measure BDNF- protein concentrations after a single LSD injection. We chose to collect samples from specific brain areas known to be important in the extinction of Pavlovian conditioning, namely prefrontal cortical areas like the prelimbic cortex and infralimbic cortex, and amygdalar areas, especially the basolateral amygdala (Bouton et al., 2021). To study the protein concentration level changes, we used enzyme linked immunosorbent assay (ELISA). By collecting samples from two time points, 24 and 48 hours after the LSD injection, we aimed to establish how the tissue BDNF levels change over time with previous collaborative work showing elevations in cortical samples at the 24 h timepoint (Moliner et al., 2020). With behavioral methods, we aimed to test if LSD affects the extinction of Pavlovian conditioning. Collaborative work in the lab has also previously demonstrated that LSD has an accelerating effect on the extinction of contextual fear conditioning (Moliner et al., 2020). In our study we aimed to extend the results to both sexes and to see if the same holds true for appetitive Pavlovian conditioning with conditioned place preference. If proven efficient, this study would increase the understanding on how psychedelics mediate their possible therapeutical potential and help guide the way towards meaningful clinical applications in the treatment of addictions.

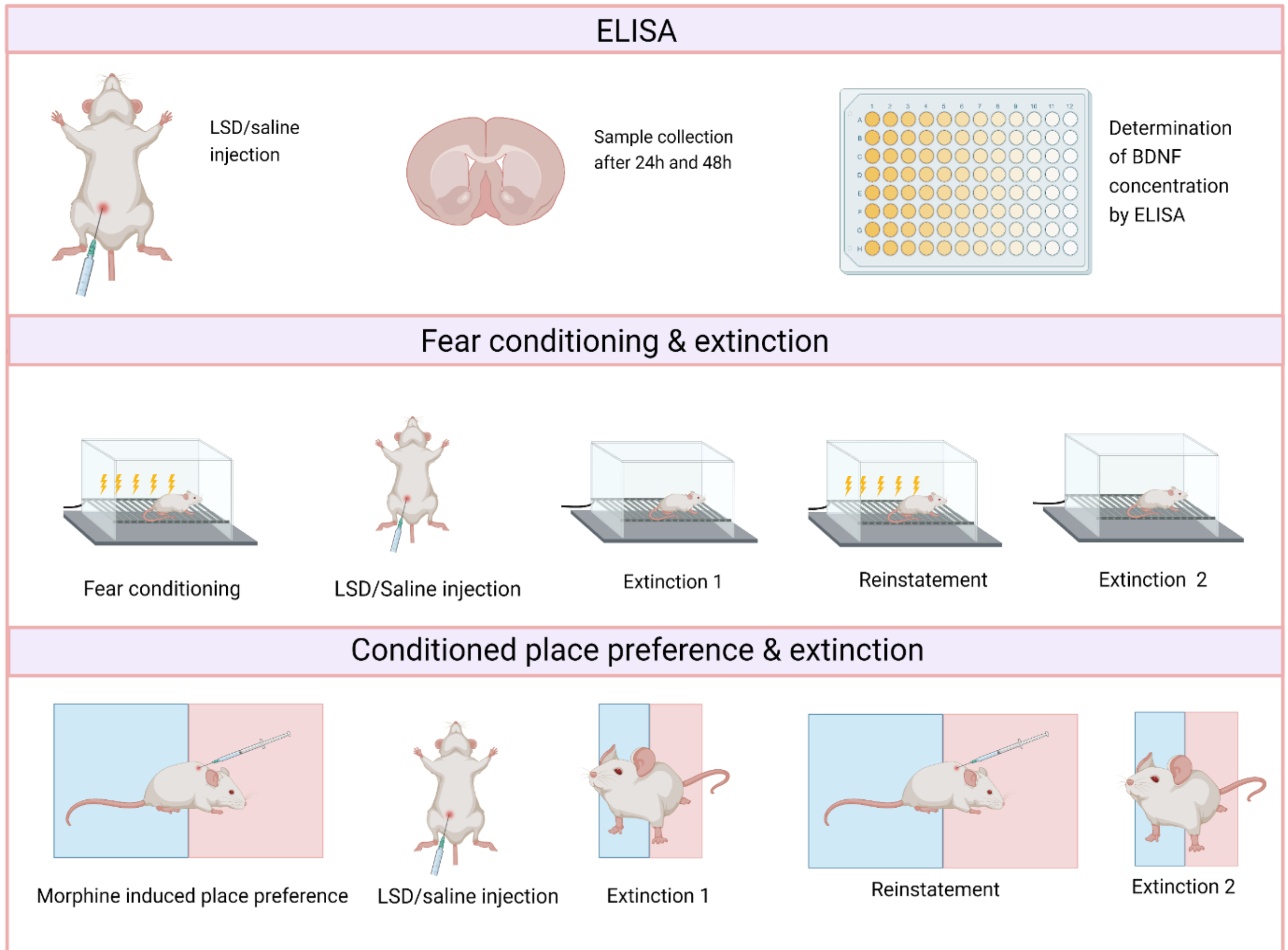

Figure 1. The experimental design. The aim of the project was to study if the single LSD injection influences brain BDNF protein levels and extinction learning. The BDNF levels were measured with ELISA in two different time point, 24 h and 48 h after the injections. Extinction learning was studied in two different behavioral experiments, fear conditioning and conditioned place preference. Image created in BioRender.com (2023).

3 Materials and Methods

3.1 Animals and handling

42 female and 42 male C57BL/6JCrl mice (Charles River Laboratories, Massachusetts, USA) weighing between 17.5 – 30.4 grams (average: 23 grams) were used in the study. The mice were 8 weeks old when arriving to the animal facility of the University of Helsinki (Biomedicum). The mice were housed in groups of three or four per cage, under standard clean room settings at 21 ˚C room temperature. The mice used in the tissue sample collection for the ELISA experiment (36 mice total, 18 females, 18 males) were housed in a 12-hour light/dark cycle with lights on at 6:00 am and off at 6:00 pm, and the mice used in behavioral studies housed in a reversed light-dark cycle with lights off at 9:00 am and on at 9:00 pm. Individually ventilated GM500 cages (Tecniplast, Buguggiate, Italy) housing the animals contained wooden chip bedding and nesting material. Plastic in-cage nests, handling tubes and wooden blocks were provided for enrichment. The mice had free access to ordinary rodent chow (Teklad™, Inotiv, West Lafayette, IN, USA) and water throughout the study.

All procedures were approved by the Finnish Project Authorisation Board (project license number: ESAVI/1218/2021) and followed the institutional and national guidelines. The number of used animals was limited as small as possible without losing the statistical power. We made a conscious choice to use both male and female animals in our experiments to avoid sex bias (Beery and Zucker, 2011).

We initiated a handling procedure for the mice undergoing behavioral tests a day after their arrival. We performed a five-day habituation period, where the mice were gradually trained to handling procedures. The period started with holding a hand still in the cage and slowly moving the hand around. Starting from day 2, we began to touch the mice lightly and lifting them away from the home cage with an open palm. Handling habituation lasted 5-8 minutes per cage daily.

The mice used for the tissue collection for ELISA were habituated with a shortened, two-day method, during which they were lifted away from the cage with plastic tube and given intraperitoneal injections of saline. Because no behavioral test was performed to these mice, we did not consider the five-day taming protocol necessary.

3.2 Pharmacological treatments

We prepared both the LSD (Sigma Aldrich, St. Louis, MO, USA) and the morphine (Yliopiston apteekki, Helsinki, Finland) solution by diluting to sterile saline. Sterile saline was also used as a control group vehicle in all settings, and people giving the injections and analyzing the data were blinded for the treatment group allocations until the initial data analysis was finished. All injection volumes were 10 ml/kg.

The given LSD doses were 0.1 mg/kg, and all the injections were given intraperitoneally.

The given morphine doses were 20 mg/kg, calculated as hydrochloride salt form. We chose to give morphine injections subcutaneously, as it has been shown to reach the peak level in brain faster compared to intraperitoneal injection (Cerletti et al., 1980). The used doses were chosen based on the literature and earlier, similar studies done in our group, the LSD dose being head twitch response-inducing and previously extinction-facilitating, and morphine dose readily place preference-inducing (Kiiskinen et al., 2019; Moliner et al., 2020).

3.3 Tissue collection and ELISA

3.3.1 Brain tissue collection for biochemical analysis

Tissue samples were collected from total of 24 mice (12 female, 12 male). The mice were 10 weeks old at the beginning of the experiment. We divided the mice into groups in a pseudo- randomized way, so that each treatment group was balanced between sexes and time points. We injected the mice intraperitoneally with 0.1 mg/kg LSD or similar volume of saline as a negative control.

To study how the brain BDNF expression levels change within 48 h after LSD administration, we chose two different time points for sample collection, 24 h and 48 h after a single LSD injection. We terminated the mice individually with CO_2 and decapitation. Immediately after, we dissected the brain and briefly washed them in 1 x phosphate- buffered saline. We sectioned the brain with a coronal brain matrix (1 mm slice intervals; Zivic Instruments, Pittsburgh, PA, USA) and further microdissected the amygdalar areas with a scalpel on ice and collected the samples to 1.5 ml Eppendorf tubes (Fig. 2). The tissue samples were snap frozen on dry ice and stored in -80 ˚C until further experiments.

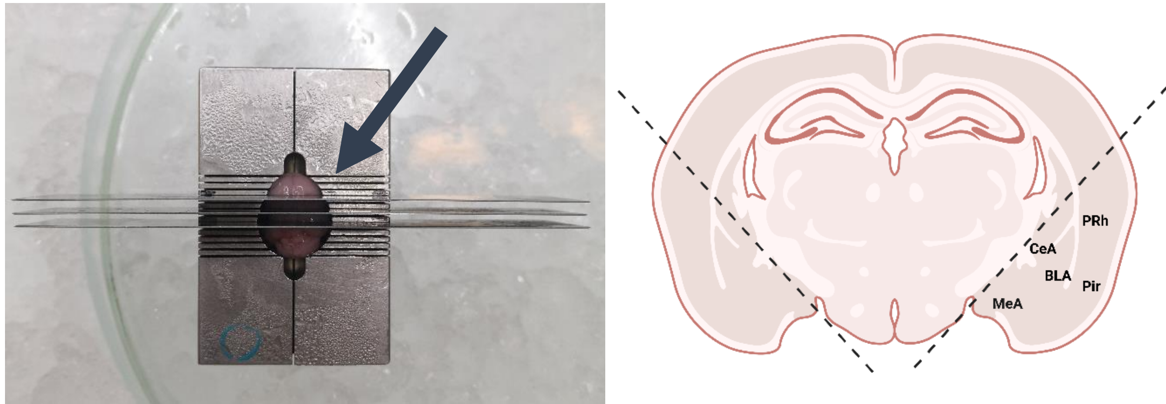

Figure 2. Collected brain areas. The brain was cut with three blades, placed on slots 4, 6 and 8. The first cut started approximately 3 mm from the anterior site. Each cut produced a 2 mm slice. The cortex sample covered the entire anterior part of the first cut (arrow on the left), therefore including, in addition to the prefrontal cortical areas of interest, parts of motor and associative cortices as well as parts of the limbic system. The amygdala samples were cut from both sides as described above. The collected amygdalar area contained basolateral amygdala (BLA), central nucleus of the amygdala (CeA) and medial amygdala (MeA), but also perirhinal cortex (PRh) and piriform cortex (Pir). Image on the right created in BioRender.com (2023).

3.3.2 Brain tissue lysate preparation and total protein quantification

We homogenized the samples by adding NP++ lysis buffer (Table 1) directly in the tubes where the samples were stored. We sonicated the tissue samples with a tip-sonicator (QSonica Q125, Newtown, CT, USA) for three rounds of five pulses with 40% amplitude. We centrifuged the samples in +4 °C for 15 minutes, 15 000 rcf and collected the supernatants. For the protein quantification, we used the Lowry assay-based DC (detergent compatible) protein assay (Bio-Rad, Hercules, CA, USA) reagents and performed the process according to the manufacturer's protocol (LIT448, section 5.2. “Microplate Assay Protocol”). To keep in line with the bovine serum albumin (BSA) standards (0, 0.1, 0.2, 0.5, 0.75, 1.0, 1.5 and 2.0 µg/µl) we prepared a 1:20 dilution of the samples (1 µl sample and 19 µl of distilled water). The absorbance was read at 750 nm using the Varioskan Flash- plate reader (Thermo Scientific, Waltham, MA, USA) and the sample protein levels were calculated by fitting the absorbance values to the regression line based on the standards.

3.3.3 ELISA

We determined BDNF concentrations from both amygdala and cortex samples following a slightly modified version of a previously described protocol (Karpova et al., 2010) for ELISA using BDNF antibodies received from F. Hoffmann-La Roche AG (Basel, Switzerland; a kind gift from Dr. Eero Castrén). Based on the protein quantification results, we diluted the samples in Hanks buffer (Table 1) to a total volume of 195 µl with 150 µg of protein. To release the epitope, the samples underwent

an acidic treatment. We acidified the samples at room temperature to pH 3 with 6 µl 1 M HCl and neutralized to pH 7 with 6 µl 1 M NaOH after 15 minutes.

We added 170 µl of the treated samples in duplicates to a Maxisorb Nunc ELISA plate (Thermo Scientific) previously coated with primary BDNF antibody (#1B, Roche; 1:1000 in carbonate buffer, overnight, 4 °C) and blocked with blocking buffer (Table 1). We added 30 µl of the secondary peroxidase (POD)- conjugated BDNF antibody (#9D, Roche; 1:1000 in 6.66% BSA and 0.66% Triton X-100 in Hanks buffer) to the plate. The plate was incubated overnight (4 °C) and washed four times with 300 µl phosphate buffered saline with 0.1% Tween® 20 (PBST). For the colorimetric reaction, we added 200 µl of BM Blue POD substrate (3,3',5,5'-tetramethylbenzidine liquid substrate, MP Biomedicals, Irvine, CA, USA) and stopped the reaction with 50 µl of 1M H_2SO_4 after 20 minutes. Absorbance was measured with the plate reader (Varioskan Flash, Thermo Scientific) at 490 nm. the sample BDNF levels were calculated by fitting the absorbance values of the means of the duplicates to the regression line based on the standards. The BDNF values used in the statistical analysis were the calculated amounts of total pg of BDNF in a mg of total protein in the original samples.

Table 1. Table of the buffers used and their ingredients; BSA - bovine serum albumin; HEPES- 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid; NP-40 - polyethylene glycol nonylphenyl ether.

NP++ lysis buffer (for 50ml)	1 ml 100 mM Na_3VO_4 2 ml complete inhibitors mix 47 ml (ad 50 ml) NP - - buffer
NP -- (for 250ml)	1340 ml 3 M Tris-HCl, pH 8.0 5480 ml 5 M NaCl 2 ml NP-40 19.2 ml 0.5 M NaF 20 ml glycerol 202 (ad 250 ml) H_2O
Hanks buffer	125 mM NaCl 5 mM KCl 1.2 mM NaH_2PO_4 1 mM $CaCl_2$ 1.2 mM $MgCl_2$ 1 mM $ZnCl_2$ 10 mM Glucose 25 mM HEPES 0.25% BSA PH 7.4 adjusted with NaOH

Blocking buffer	Hanks buffer 2% BSA 0.1% Triton x-100

3.4 Fear conditioning & extinction

We used 24 mice (12 male, 12 female) in the contextual fear conditioning experiment, all 10 weeks old when the experiment started. The mice were placed in transparent plexiglass conditioning chambers with metal grid floors (Med Associates Inc. Fairfax, VT, USA). We induced fear acquisition by exposing the mice for the context of the conditioning chamber for 8 minutes, during which the mice received 5 foot-shocks (2 s, 0.6 mA, interval: 1 shock per 80 s) delivered through the metal grid floor. Freezing behavior was recorded with automated Video freeze software system (Med Associates Inc.). Minimum freeze duration was set to 50 frames and motion threshold to 18 au (an artificial unit based on motion index calculated by the software). Bedding material was added to the chamber to increase the familiarity. After each mouse, we wiped the metal grids with dry paper and removed any remains of urine or feces. Between the sexes, we thoroughly washed the grids with tap water and changed the beddings to prevent olfactory cues. Measured parameters included the average motion, number of freeze episodes, time freeze and percentage freezing. The percentage freezing was used for the statistical comparison between the treatment groups.

As the pharmacological intervention, the mice received either saline or LSD injections (0.1 mg/kg, i.p) 24 h after the fear conditioning. The mice were randomly assigned into two groups counterbalanced by the cages and the sexes.

The first extinction period started 48 h after the fear conditioning and lasted three days. The mice were placed in the same context as on the fear conditioning day for 8 minutes, but without the foot-shocks. Freezing behavior was recorded as described above.

After 17 days of the extinction, we reinstated the extinguished fear memory by exposing the mice to shocks in the same setting as on the conditioning day. The second extinction phase started a day after the reinstatement and contained two sessions with one day in between. We performed the extinction as described for the first extinction period above.

3.5 Conditioned place preference

In total 24 mice were used in conditioned place preference experiment (12 females, 12 males). The mice were 9 weeks old when the experiment started. Eight polycarbonate boxes (45×22.5×15 cm; Tecniplast) and transparent lids with ventilation holes were used in the conditioned place preference experiment. We used two different floor materials for the conditioning: blue acrylic floor with a grid-like shape and pink polyethylene floor with a wave-like shape. To avoid lingering olfactory cues, we used different floor pieces between the two sexes. After each trial, we thoroughly washed the used floors and boxes with tap water.

We conducted the experiment with **a habituation session**. We injected the mice subcutaneously with saline and placed them in the boxes covered 1:1 with both floor materials. The mice were allowed to explore the arena freely for 15 minutes. Movement was recorded with video-based tracking software EthoVision® XT10 (Noldus, Wageningen, Netherlands).

The conditioning phase started the day after the habituation and lasted for 8 days total. Each day contained 4 sessions with 4 or 8 mice per session, and each mouse underwent one trial per day. We divided the mice in a balanced manner into two conditioning groups. The pink- conditioning group received subcutaneously 20 mg/kg morphine on the pink floor material and the blue- conditioning group received the morphine on the blue floor material. The mice received an identical volume of sterile saline on the opposing floor materials. During the 8 conditioning days, the mice received total of 4 injections of morphine and 4 injections of saline on alternating days with the starting floors and injections counterbalanced throughout. We placed the mice to the arena with morphine- or saline-paired floor material directly after the injections for 30 minutes.

After the conditioning phase, we **tested** for the morphine- induced place preference. We performed the test with similar settings as the habituation. All mice received an injection of saline and were placed in the arena with both floor materials for 15 minutes. The conditioned place preference would be interpreted successful if the mouse spends significantly more time in the morphine-paired floor material compared to the saline-paired material.

The experiment was designed to continue with an **extinction period**, where mice were randomly assigned to receive either a saline or LSD injection (0.1 mg/kg, i.p.). Extinction phase was supposed to start 24 h after the injections mimicking the design in fear conditioning experiment. During the following 11 days mice would have been placed in the arena with both floor materials for 30 minutes. All mice would have received only saline injections. Extinction can be declared successful after there is no significant difference between the time spent in conditioned floor material versus unconditioned

floor material. Since the conditioning phase did not produce reliable reward-related association, as discussed more in detail below in Results, the extinction phase was never carried out.

3.6 Head Twitch Response

Head twitch response is used as a behavioral model for the hallucinogenic effects caused by serotonergic psychedelics (Halberstadt and Geyer, 2013). Here, the response was measured as a control experiment. We injected 12 male mice (the same that underwent the fear conditioning experiment) intraperitoneally with either 1.0 mg/kg LSD or saline. Imidiatelly after the injections, the mice were placed the mice in 5 L glass beakers and video recorded (Nikon D3400 digital single-lens reflex camera, 60 frames per second) for 10 minutes. Two beakers placed next to each other, one mouse in each, were recorded at the same time. A white plastic plate was placed under the beakers to add contrast. After the recording, we placed the mice back into their home cages. We cleaned the beakers after each mouse with water and 70% ethanol. The number of head twitches were scored visually from the videos.

3.7 Statistical analysis

We used GraphPad Prism 8.1.0 for statistical analysis and graphics. We tested the normality of all data by Shapiro- Wilk test. Statistical significance was set at $p < 0.05$. Unpaired t-test with Welch's corrections was used to observe differences between the two treatment groups in ELISA (LSD vs saline), conditioned place preference (time on pink vs time on blue) and head twitch response (LSD vs saline) experiment. For fear conditioning, repeated measures two-way analysis of variance (ANOVA) was used to measure differences in freezing percentages caused by treatment (LSD vs saline) and time.

4 Results

4.1 BDNF protein levels

To determine whether a single dose of LSD has an effect on the BDNF protein concentrations, we analyzed the data received from the ELISA. We compared the mean concentrations of the treatment groups with the unpaired t-test. Results are presented in the Figure 3.

In this study design, we detected no significant differences between the vehicle and LSD groups in either cortex or amygdala samples. The average BDNF concentrations in 24 h cortex samples were 23.17 pg/mg for the LSD treated mice and 29.97 pg/mg for the saline treated mice (Fig.3A; t(7.087)= 1.14, p= 0.29). In the 48 h time group, the average concentration was 44.52 pg/mg for the LSD group and 34.40 pg/mg for the saline group (Fig. 3B; t(9.96)= 1.10, p= 0.30). In the amygdala samples, the average concentrations after 24 h of the administrations were 45.92 pg/mg for the LSD group and 35.87 pg/mg for the saline group (Fig. 3C: t(7.13)= 0.88, p= 0.41). The overall BDNF concentrations in cortex samples ranged between 5.85 pg/mg to 57.69 pg/mg and in amygdala samples between 15.07 pg/mg to 73.89 pg/mg.

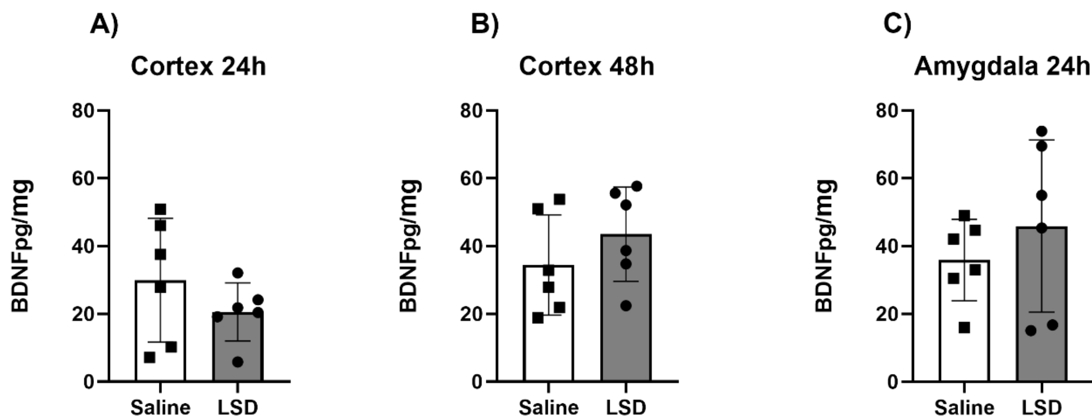

Figure 3. The BDNF concentrations (pg/mg) in different brain areas and time points after injections. Unpaired t-tests performed for between-group mean comparisons showed no statistically significant differences between the treatments. The data is presented as mean ± standard deviation with individual datapoints as dots.

When comparing the differences between the two timepoints within the theoretical treatment groups, unpaired t-test showed that the average BDNF concentrations in the LSD treated cortex samples

were significantly higher at 48 h than at 24 h timepoint (Fig. 4B; t(8.31)= 3.44, p= 0.0083). The saline treated cortex samples average BDNF concentration stayed in a similar range in both timepoints (Fig. 4A; t(9.58)= 0.46, p= 0.65).

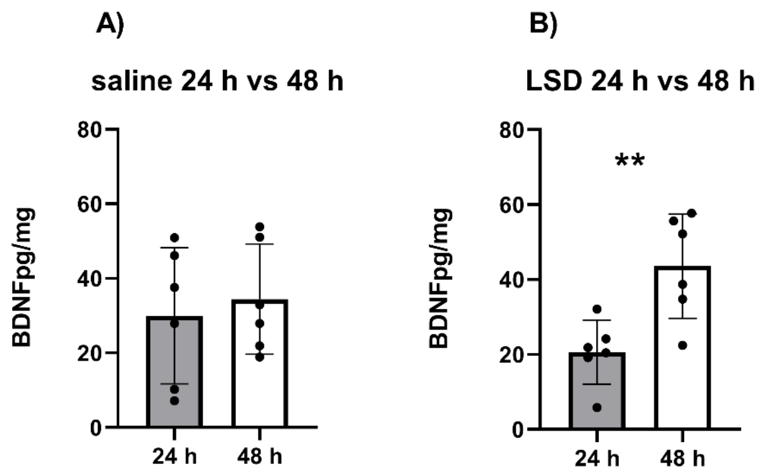

Figure 4. The change in BDNF concentrations of the cortex samples during the 24 hours. Unpaired t-test was performed to detect the differences between time points. Samples from saline treated animals showed similar concentration after 24h and 48h of injections (5A). For the LSD treated animals, the BDNF concentration of samples was significantly higher after 48h of injections (5B). The data is presented as mean ± standard deviation with individual datapoints as dots.

4.2 Fear conditioning and extinction

We evaluated the contextual fear conditioning by comparing the freezing percentages, i.e. the time mice spent freezing in relation to the total duration of the trial. The line graph of the freezing percentages for the whole experiment is presented in the Figure 6. When observing the experiment as a whole, there was no statistically significant difference in the freezing percentages between the treatment groups (F(7,15)= 0.40, p= 0.90). Overall, our fear conditioning and extinction study design showed no statistically significant differences in freezing behavior between LSD and saline treated mice.

The three-day extinction paradigm was sufficient to extinguish the fear before the reinstatement (Fig. 5). There was no significant difference between the freezing percentages of treatment groups during the first extinction period (Fig. 5; from conditioning to ext 1.3; F(3,66)= 0.60, p= 0.61). Both groups show decrease in the freezing percentages after the second three-day extinction, but neither of the groups reached the baseline during the experiment.

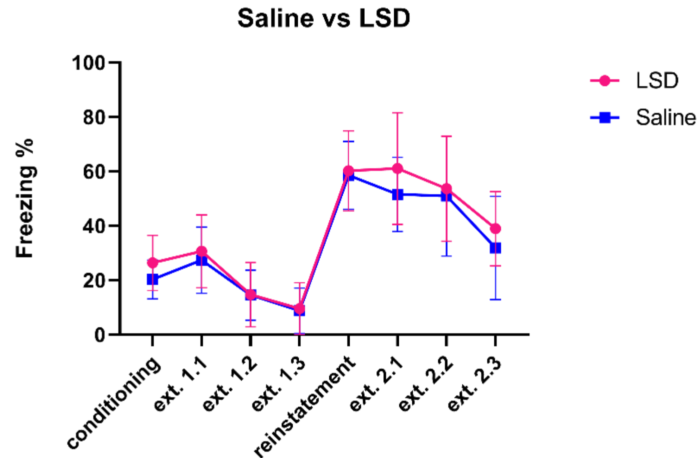

Figure 5. Two-way ANOVA revealed no statistically significant differences between the treatment groups in the present contextual fear conditioning design. Dots represent the mean of the freezing percentage for each day and error bars the standard deviation.

The line graphs separated between the sexes are shown in the Figure 6. The conditioning and the following three extinction days showed no difference between treatment groups in either of sexes (Fig. 6; from conditioning to ext. 1.3; females; $F(3,30)= 0.089$, $p= 0.97$, males; $F(3,30)= 0.99$, $p= 0.41$). Both sexes showed an increase in freezing behavior when the unconditioned stimulus was restored. LSD had no significant effect on the second extinction in either sex (Fig. 6; from reinstatement to ext 2.3; females; $F(3,30)= 0.51$, $p= 0.68$, males; $F(3,30)= 1.79$, $p= 0.17$).

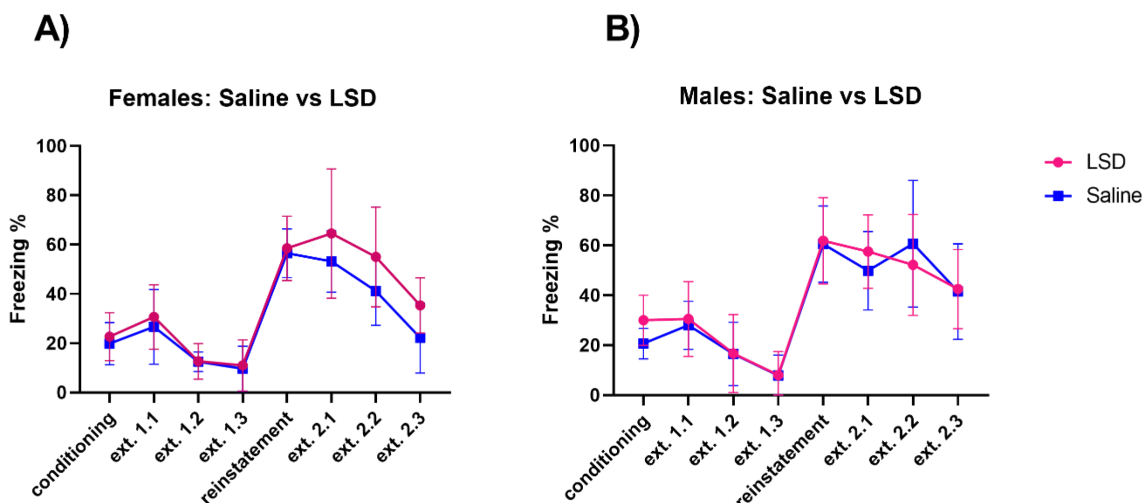

Figure 6. Two-way ANOVA showed no significant difference between treatment groups in either of sexes. The dots represent the mean of freezing percentages for each day. The error bars show the standard deviation.

When comparing the sexes in the treatment groups, two-way ANOVA revealed a significant Time × Sex interaction in the saline treated group (Fig. 7A; $F(7,70)= 2.17$, $p= 0.04$). The cause for this can be seen in the Figure 7, where in the day 2 of the second extinction paradigm (ext. 2.2) the males showed an increase in the average freezing percentages whereas the females average freezing percentage continues to drop. LSD group showed no notable differences between the sexes in any phase of the study (Fig. 7B; $F(7,70)= 0.58$, $p= 0.76$).

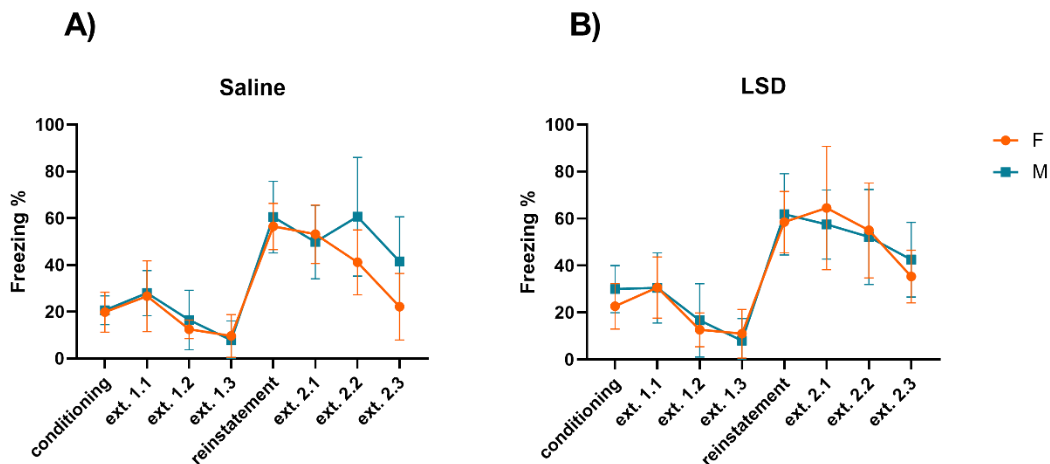

Figure 7. The freezing percentages separated between treatment groups comparing the sexes with two-way ANOVA. The saline group **A)** showed significant time x sex interaction on the second day of extinction after reinstatement. The LSD group **B)** showed similar freezing percentages in both sexes throughout the study. The dots represent the mean of the freezing percentage for each day and the error bars show the standard deviation. F (In orange) = female; M (in blue) = male.

4.3 Conditioned place preference

To test the formation of morphine induced place preference, we compared the time the mice spent in their morphine-paired floor material versus the time spent in their saline-paired floor material during the 15 minutes test period. We used unpaired t- test to determine the difference in the average time spent in the morphine-paired floor material before and after the conditioning phase.

We found no significant increase in the preference of the floor materials after morphine conditioning in either of the treatment groups (Fig. 8). This indicates that the place preference was not formed, and we terminated the experiment.

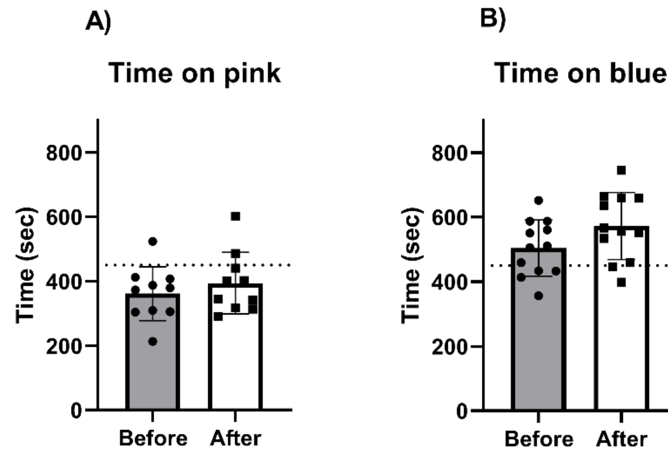

Figure 8. Time on the pink **(A)** and blue floor material **(B)** before and after the conditioning. The subjects on the graph **A)** represent the pink conditioning group and the subjects on the graph **B)** represent the blue conditioning group. No significant differences in the preference were detected in either of the conditioning group when comparing the times spent on the paired floor material before and after the morphine conditioning. Data shows the means in columns and standard deviation as the error bars. The individual values of each mouse represented as dots.

By analyzing the data from habituation day, we discovered that the blue floor material was initially significantly more preferred (Fig. 9). The preference was seen when comparing all the mice together (Fig. 9A; t(46.00)= 6.71, df= 46.00, p< 0.0001), and when analyzing the sexes separately, we noticed that the initial preference for the blue floor material was statistically significant amongst the females (Fig. 9B; t(22.00)= 6.81, p< 0.0001), but not in the males (Fig. 10C; t(18.00)= 2.08, p= 0.052).

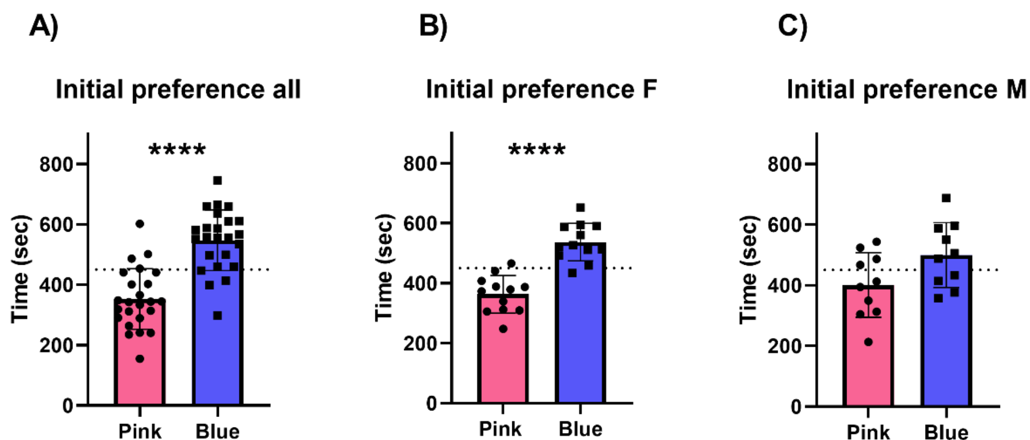

Figure 9. The initials preferenes described as the time spent on floor materials during the habituation day. Unpaired t-test showed statistically significant difference (p< 0.0001) in initial preference analysed for all subjects , where the blue floor material was more prefered **(A)**. When analyzed seperately, females **(B)** show statistically significant differences in initial preference but males don't **(C)**. Data show the means where the ndividual values of each mouse is represented as dots and error bars are the standard deviation. . F = female; M = male.

To evaluate weather the morphine administration and effect was successful, we analyzed the difference in the average velocities between morphine and saline treatments. In addition to Straub tail, increased locomotor activity is described to be a typical reaction to morphine (Gupta et al., 1988; Patti et al., 2005). The data is shown in the Figure 11. Two-way ANOVA showed a statistically significant difference in the mean velocity between the morphine and saline injections ($F(1,46)= 305.3$, $p< 0.0001$). The overall mean velocity after saline injections was 0.032 m/s and after morphine injectinos 0.123 m/s.

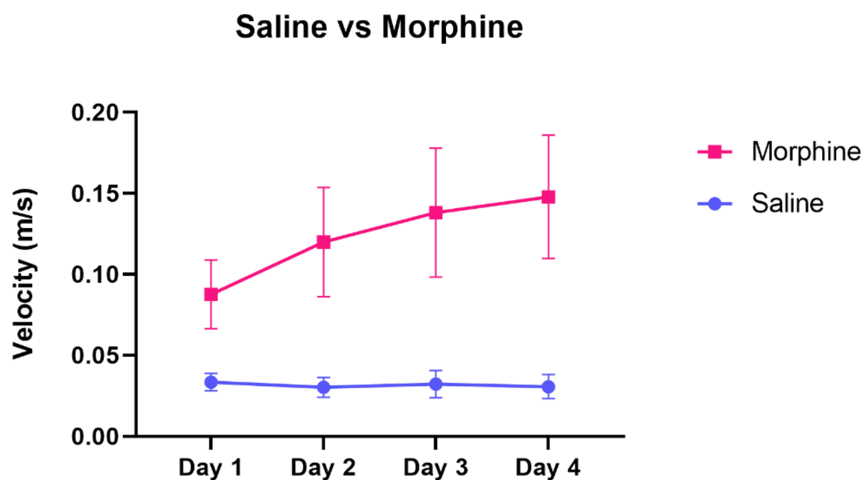

Figure 10. Two-way ANOVA showed a significant difference in the mean velocity of the mice between different injections. Each mice received 4 morphine and 4 saline injections in the altering days. The data shows the mean velocity of all mice receiving morphine and saline for the first, second, third and fourth time. The error bars represent the standard deviation.

4.4 Head Twitch Response

The used 1.0 mg/kg dose of LSD produced a significantly increased number of head twitch responses compared to the saline (Fig. 10; $t(4.13)= 7.56$, $p= 0.0014$) during the 10- minute observation period.

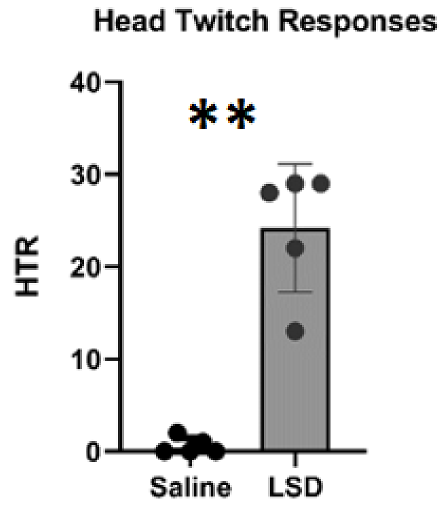

Figure 10. Unpaired two-tailed t-test showing the difference in head twitches between the treatment groups during 10 minutes after the injections. The means are shown and each dot represents an individual value. The error bars represent the standard deviation.

5 Discussion

In this project we investigated how a single dose of LSD affects the brain BDNF expression and extinction learning in mice. We used ELISA to study how BDNF protein concentrations change within 48 hours of the LSD administration in brain areas known to be important for extinction learning, amygdala and cortex (Quirk and Mueller, 2008). To compare the molecular level changes to behavioral outcomes, we examined extinction learning after the LSD treatment in two different Pavlovian conditioning paradigms, the contextual fear conditioning and the conditioned place preference. In our experimental settings, the single dose of LSD had no notable effects on the BDNF expression or on extinction learning in either of the paradigms.

It has already been shown that a single dose of psychedelics increase brain BDNF mRNA expression and *Bdnf1* gene transcription in rodents (Desouza et al., 2021; Gewirtz et al., 2002; Vaidya et al., 1997). In humans, clinical trials show increased plasma BDNF protein levels up to 48 h after psychedelic administration (Almeida et al., 2019; Hutten et al., 2020). In our data, there were no significant differences between the treatment groups in either of time points. However, the Comparison of the 24 h and 48 h cortex samples revealed a difference in the BDNF protein levels in the LSD treated groups. BDNF levels were significantly higher after 48 h than the comparator level at 24 h. Interestingly, the saline groups' values remained approximately unchanged. This hints that there could be some time-dependent changes caused by LSD, possibly related to long-term plasticity. However, based on the prior collaboratory finding in the lab (Moliner et al., 2020), we expected to see higher BDNF protein levels in the LSD treated cortex samples after 24 h of injections compared to the saline treated group. Contrary to our expectations, the LSD groups' average concentration was in fact lower in the 24 h time point. In addition, the detected total BDNF levels were unexpectedly low for both the amygdala and the cortex samples, in both treatment groups (all under 100 pg/ml). This indicates potential problems with the antigen retrieval and can be considered as a one limitation of this study. Within-group variations were also high, which further limits the possible conclusions. To make sure that the used LSD worked as supposed, we measured the head twitch responses caused by the given LSD dose as a control experiment. The observed head twitch responses were increased by the LSD treatment as reported in prior publications from our lab and elsewhere (Elsilä et al., 2020; Halberstadt and Geyer, 2013) excluding a defective drug as a source of error.

To this day, most preclinical studies have been focusing on the BDNF gene or mRNA expression, or studying BDNF protein level changes after multiple doses (Colaço et al., 2020). Although the effect on BDNF protein levels after a single dose has been studied in humans in similar time points as in

our study design, the correlation between brain and plasma BDNF protein levels is still highly unclear. More research is needed to unravel the strength and duration of the suggested effect of a single dose of LSD on protein BDNF levels.

Psilocybin and DMT have been demonstrated to facilitate fear extinction when administered before memory retrieval (Cameron et al., 2018; Catlow et al., 2013). Our earlier data from a collaboration suggests that LSD may have similar enhancing effect on the extinction learning after the reinstatement of the extinguished fear memory (Moliner et al., 2020). We replicated the study design but included subjects of both sexes this time. To our knowledge, there is currently only one publication using both sexes in a study combining psychedelics and fear extinction (Cameron et al., 2019). Generally in fear conditioning experiments, female rats tend to show less freezing (Russo and Parsons, 2021; Shanazz et al., 2022). In our data we could see a similar trend, although the difference between sexes was not statistically significant.

Unlike in our collaboration data with Moliner et al., we found no significant difference between the treatment groups during the first three-day extinction. We also expected to see a difference between the treatment groups during the extinction following the reinstatement of fear memory. We did not however detect any statistically significant differences after the second extinction phase either. The reinstatement produced a significant and similar increase in the freezing behavior in both groups, indicating that LSD did not affect the consolidation or retrieval of the fear or extinction memory. Differences between our study design and the earlier study included the use of both sexes and placing the mice in reversed light-dark cycle. We chose to house the animals in reverse light-dark cycle so that we could perform the behavior test during their active circadian period, i.e. the lights-off time. As mice are nocturnal animals, it could be assumed that the animals show enhanced learning when tested during their active hour. Mice tested on dark have been reported to express slower acquisition of the conditioning, but faster extinction (Chaudhury and Colwell, 2002).

Altogether, our data suggests that the earlier findings of LSD's effect on the extinction of conditioned fear might not be widely repeatable. Our prior collaboratory research found that LSD enhances the extinction of fear memory (Moliner et al., 2020). The data is supported by few published studies, although they are mostly done with psilocybin (Catlow et al., 2013; Du et al., 2023). While all serotonergic psychedelics are considered to mediate their main effects through the same 5-HT_{2A} receptor, they differ in the pharmacological level. Unlike psilocybin, LSD interacts with both serotonin and dopamine receptors (Passie et al., 2002; Watts et al., 1995). Because most of the research is focused on the psychedelics relation to the serotonin system, it is still largely unknown what this difference means in the overall effect. In fact, some studies using different psychedelics, like 2,5-Dimethoxy-4-iodoamphetamine (DOI) (Pędzich et al., 2022) or DMT (Cameron et al., 2018), show

contradictory results in fear conditioning paradigm. Pędzich et al. (2022) report that DOI did not affect the fear extinction in auditory fear conditioning, although it reduced the fear expression in mice. In the case of DMT, Cameron et al. (2018) show that acutely administered DMT accelerated cued, but not contextual, fear extinction. However, both Pędzich et al. (2022) and Cameron et al. (2018) administered the drug shortly before experiments, focusing on the acute effects of psychedelics. Our longer time point aimed to target the BDNF related plasticity mechanism, which is currently not well studied. As described above, the used LSD was validated with head twitch response measurements. Although interestingly, our collaboratory data implies that head twitch responses persist even when the proposed plasticity mechanisms are impaired (Moliner et al., 2020). To provide further understanding on if, and how, LSD facilitates extinction learning through increased neural plasticity, more research is needed.

Despite the differences in the unconditioned stimulus, fear- and drug-related extinction are thought to have an overlap on the neurocircuit level (Goode and Maren, 2019). Although we could not replicate the earlier findings in the fear conditioning, we continued to investigate whether similar phenomena can be seen in conditioned place preference paradigm. Compared to the aversive fear conditioning, conditioned place preference associates a rewarding stimulus to a conditioned context (Tzschentke, 2007). Due to the positive reinforcing effect, conditioned place preference is widely used in modelling addiction-like behavior. It can be used to study the rewarding effects of drugs, but also features of relapse (Goode and Maren, 2019). In our experiment, we aimed to use conditioned place preference to examine the extinction of morphine-induced place preference, drug-induced reinstatement, and LSD's potential effects on those. Due to unexpected challenges, the experiment never continued to the extinction and reinstatement phase. After eight days of conditioning, we could not reliably detect the formation of the place preference. Data from the habituation showed us that the blue acrylic floor was initially significantly more preferred by the females and close to significantly more preferred in males as well. We have observed this trend before, but not in a significant way nor has it ever disturbed the formation of place preference when using an unbiased experimental design such as this (van den Broecke & Elsilä, unpublished data). This was also the reason why no pre-test to assess initial material preferences was carried out.

It is unclear what caused the disturbance to the formation of the morphine- induced place preference. One possible distraction is using both sexes in each trial with an assumption that exposure to olfactory and auditory cues of the opposite sex might be rewarding enough to disrupt the formation of drug-context association. Until this experiment, our lab has studied conditioned place preference only with males. However, successful conditioned place preferences performed with both female and male rodents can be found from the literature (Yates et al., 2021). From information received through personal communication with Professor Yates, we can point out few differences between

their and our study designs. Yates et al. (2021) used chambers enclosed in a sound attenuating cabinet and animals' exposure to each other was limited. During our handling and trials, we however opened the top lids of the cages to habituate the mice to each other's odors and sounds, in line with other studies of Pavlovian conditioning actively using sex as a biological variable (Laine et al., 2022; Laine M. personal communication, 2023). We divided the floor materials and chambers between sexes, but due to their previous use some odors may have remained in the materials. We chose not to use hydrogen peroxide for cleaning and odor removal since it has been observed to have a strong effect on the behavior of the mice during the experiment in our lab (van den Broecke & Elsilä, unpublished data). There is a small possibility that the habituation for smells was not sufficient, and this may have disturbed the protocol.

To make sure that the used morphine was sufficient to produce an effect and that the injections were successful, we analyzed the velocity data received from the conditioning sessions. Together with Straub tail effect, increased locomotor activity is a well characterized effect of morphine in mice (Gupta et al., 1988; Patti et al., 2005). Our data clearly showed a higher activity in the mean velocities after morphine injections compared to the saline injections. In addition to increased locomotor activity, Straub tail reaction was regularly detected during the conditioning phase while not systematically recorded (data not shown). Together these observations indicates that the problem was not the morphine itself or insufficient injection method. The most likely explanation is that we used an unbiased study design in a biased context. Although the mice show clear response to the morphine, the induced place preference could not be detected with this study design due to the attenuating effect of the initial preference, which was not initially assumed based on the previous experiments with the same floor materials.

6 Future perspectives

For future considerations, the most crucial steps of the ELISA methods should be evaluated more precisely. In this case, the acidic treatment for the samples seems to be sensitive for time and technique. Also, testing the ELISA samples in triplicates instead of duplicates would increase the accuracy. Furthermore, the number of samples was limited by material restrictions. Larger group sizes could provide greater consensus. In plasma, increased BDNF protein concentrations have been measured as early as 4 h after the administration of psychedelics (Becker et al., 2022). Collecting brain samples from both earlier and later than 24 h of injections could help to assess the time window of speculated BDNF protein level changes and the best timing for behavioral experiments.

To avoid the covering effect of initial preference in the conditioned place preference, the baseline preference should be measured before choosing a study design. In case of significant initial preference, choosing a biased study design (pairing the drug with the less preferred material) may be beneficial. When performing conditioned place preference with morphine, both biased and unbiased designs are known to provide similar outcomes (Blander et al., 1984). This consideration taken into account, the study will be performed again in the future to evaluate the effect of using both sexes. To further study how LSD effects extinction learning, expanding the experimental setup from Pavlovian learning to other forms of extinction can provide wider understanding. For example, studying LSDs effect on extinction from e.g. lever press behavior could be a way of expanding the hypothesis to operant learning.

7 Conclusions

The therapeutical effects, let alone the molecular mechanisms, of psychedelics in the treatment of addictions are still poorly understood. We hypothesized, that by combining the ELISA measurements of the known plasticity marker, BDNF, with the extinction learning paradigms, we could show a meaningful correlation between the molecular and behavioral level changes. Contrary to our hypothesis, we did not detect significant changes in the BDNF protein levels or behavioral paradigms after the LSD injections in our experimental design. To our knowledge, this is the first study including amygdalar brain regions and the 48-h time point in the case of cortex samples in the BDNF protein level measurements. Despite the central role of amygdala in Pavlovian conditioning and extinction learning, little research has been done in determining the changes in BDNF protein concentrations after psychedelic administration in this brain region. Although no differences were observed between the treatment groups, the statistically significant change between the 24 h and 48 h LSD treated cortex samples demonstrate the importance of further studying larger scale of brain areas and the time-dependent changes. These results are however speculative, due to the suspected challenges in the epitope release. In the case of the fear conditioning, the failure to reproduce the earlier results calls for methodological observation. As the only major changes in our study design were the inclusion of both sexes and placing the mice in the reversed light-dark cycle, considering the influence of remaining olfactory cues and the effect of circadian rhythm needs more careful estimations. The unfortunate pretermination of the conditioned place preference leaves the need for future research on psychedelics' possible effects on the extinction of appetitive conditioning.

Acknowledgements

I would like to express my profound gratitude to Lauri Elsilä for the support and guidance throughout this project. This thesis would not have been possible without his help and feedback. I also want to thank Prof. Esa Korpi and the department of pharmacology for giving me this wonderful opportunity to be a part of the research. Also, a warm thank you to Dr. Eero Castrén's lab for collaboration, and to Rafael Moliner, Sulo Kolehmainen and Paula Pastor, who kindly and patiently familiarized me with the biochemical methods.

Author contributions

Writing, investigation, formal analysis and visualization: Liisa Ilkka.
Supervision, conceptualization, methodology, investigation (brain tissue microdissections) and formal analysis (head twitch responses): Lauri Elsilä.
Supervision: Esa Korpi

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