The Acute Effects of LSD on Reward Behavior in Mice with Intracranial Self-Stimulation

Master's Thesis

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ABSTRACT

Background: Alcohol dependence is a chronic severe substance use disorder that has devastating personal and public health consequences. The efficacy of the current pharmacotherapy options for the treatment of alcohol dependence are modest at best, therefore better alternatives are greatly needed.

Lysergic acid diethylamide (LSD) has shown promise in treatment of alcohol dependence in several clinical trials. A sigle high dose of LSD has been suggested to have a treatment effect that last for at least six months, indicating a remarkably better efficacy than the currently available methods. LSD itself has been reported to have a low addiction potential. In mouse models, acute LSD has been demonstrated to reduce ethanol consumption. Yet, the mechanism of action behind these effects has remained largely unknown. LSD is an agonist of serotonin's 5-HT_{2A} and 5-HT_{2C} receptors which have been shown to modulate the dopaminergic activity of the reward circuitry, a crucial brain area in the initiation of addiction.

Intracranial self-stimulation (ICSS) is a procedure for a quantitative assessment of reward behavior in animal models. In ICSS, laboratory rodents self-administer electric stimulation to the dopaminergic pathways of the reward circuitry inducing a reinforcing effect similar to drug reward.

Aim: The aim of the current body of work was to use ICSS to assess the acute effects of LSD on reward behavior in C57BL/6JRj mice. This was done to improve the understanding of the mechanism of action of LSD and to evaluate whether the ethanol-consumption-reducing effect of LSD in mice is mediated through the reward mechanism.

Methods: Bipolar electrodes targeting the medial forebrain bundle were implanted in the brains of C57BL/6JRj mice in a stereotaxic surgery. The animals were trained to acquire the self-stimulation in the discrete-trial current-intensity procedure. First, the possible dose-dependent acute effects were tested with three different doses of LSD. Next, the acute effect of LSD on amphetamine-induced changes in ISCC were tested.

Lastly, a small preliminary test on the effects of LSD on lipopolysaccharide (LPS) -induced changes on ICSS were conducted.

Results and conclusions: Acute LSD did not affect reward behavior in ICSS on any of the tested doses. Accordingly, LSD did not affect the facilitation of ICSS induced by acute amphetamine. The results of the LPS experiment were likely to be skewed by the development of tolerance to LPS, therefore the evaluation of the possible effect of LSD was not possible. These findings suggest that the previously reported LSD-induced reduction in ethanol consumption in mice, is not mediated through alteration of the reward mechanism. At the same time, these findings provide further evidence supporting the suggestion that LSD itself does not induce facilitation of the reward circuitry needed for the development of addiction.

Keywords: 5-HT_{2A}, 5-HT_{2C}, addiction, alcohol dependence, amphetamine, ethanol, intracranial self-stimulation, lipopolysaccharide, lysergic acid diethylamide, reward circuitry

ABBREVIATIONS

5-HT	5-hydroxytryptamine
ANOVA	Analysis of variance
Amph	Amphetamine
DA	Dopamine
DMT	Dimethyltryptamine
DOI	2,5-dimethoxy-4-iodoamphet-amine
DSM-5	The Diagnostic and Statistic Manual of Mental Disorders, Fifth edition
ICSS	Intracranial self-stimulation
IP	Intraperitoneal
ITI	Intertrial interval
LH	Lateral hypothalamus
LPS	Lipopolysaccharide
LSD	Lysergic Acid Diethylamide
NAc	Nucleus accumbens
PFC	Prefrontal cortex
Sal	Saline
SERT	Serotonin transporter
SNP	Single nucleotide polymorphism
TH	Reward threshold
VTA	Ventral tegmental area

1 INTRODUCTION

Alcohol use disorders are a massive public health issue which may have devastating consequences for the personal lives of the patients and their families. According to World Health Organization (2018), harmful use of alcohol causes 3 million deaths every year and more than 5% of the global disease burden. In Finland, on average nearly 34 000 people per year have periods of inpatient care because of alcohol related diseases (National Institute for Health and Welfare, 2018). The overall public cost of alcohol abuse in Finland is estimated at around 900 million euros per year.

Current pharmacotherapy options for the treatment of alcohol dependence include disulphiram, acamprosate and opioid antagonists, such as naltrexone. These are used together with psychosocial therapy, but the treatment results are modest and therefore better alternatives are immensely needed (Miller, Book and Stewart, 2011)

Lysergic acid diethylamide (LSD), has shown potential in the treatment of alcohol dependence and other substance use disorders in several clinical studies, as well as in non-clinical settings (Garcia-Romeu et al., 2019; Bogenschutz and Johnson, 2016; Krebs and Johansen 2012). In addition, animal studies have shown that acute administration of LSD reduces ethanol consumption in mice (Alper *et al.*, 2018, Elsilä and Martti, unpublished data). LSD is a substance best known for its profound consciousness-altering psychoactive effects, because of which it has been widely used as a recreational drug. Interestingly, even though the drug is legally classified in the most restrictive classes along with substances like heroin, LSD itself is considered to have a very low addiction potential (Nichols, 2018).

Despite the promising clinical and preclinical data, the mechanism of action behind the treatment effect of LSD is still largely unknown. LSD, like other psychedelic drugs (also referred to as classical hallucinogens), is a potent agonist or a partial agonist of serotonin's 5-HT_{2A} and 5-HT_{2C} receptors (Passie *et al.*, 2008). These receptors are known

to modulate the dopaminergic activity in the reward circuitry; the key brain area in the initiation of addiction (Bortolozzi *et al.*, 2005; Katsidoni, Apazoglou and Panagis, 2011). All drugs of abuse raise the dopamine (DA) level in the reward circuitry beyond normal physiological level (Koob and Volkow, 2016). This surge of DA mediates the rewarding and reinforcing effects of drug intake. Therefore, it is of interest to see whether LSD alters the behavior related to the functioning of the reward circuitry.

Intracranial self-stimulation (ICSS) is a procedure used for a quantitative assessment of reward behavior in animal models (Stoker and Markou, 2011). In ICSS, the animals self-administrate an electric stimulus through an electrode implanted into the medial forebrain bundle, which includes the main reward pathway of the brain. Here, I have used ICSS to investigate the acute effects of LSD on reward behavior in mice.

2 REVIEW OF LITERATURE

2.1 Lysergic acid diethylamide (LSD)

LSD (Figure 1.) is an indolamine structured psychedelic drug, which shares its pharmacological properties with other psychedelics, such as psilocybin, mescaline and dimethyltryptamine (DMT) (Nichols, 2016). For several decades LSD has been stigmatized by its use as a recreational drug due to its profound psychoactive effects (Nichols, 2018). Within the last ten years, a new interest on the therapeutic potential of LSD has emerged, as small clinical trials with psychedelics have shown promise in treatment of psychiatric conditions including major depression and substance use disorders (Bogenschutz *et al.*, 2015; Bogenschutz and Johnson, 2016; Carhart-Harris and Goodwin, 2017a, 2017b) .

2.1.1 History

LSD was first synthesized in 1938 in Sandoz Laboratories, Basle, Switzerland by chemist Albert Hofmann (Hofmann, 1970a). It was one of the molecules Hoffman produced while investigating partially synthetic derivates of lysergic acid, the basic structural element of ergot alkaloids, as part of a systematic process for finding new molecules with possible therapeutic properties. At the time, the molecule was not found particularly interesting and it was forgotten for some years. In 1943, Hofmann, however, decided to produce a new batch of LSD. While working on the last stage of the synthetization process, removal of the inactive isomer by columnar chromatography, he serendipitously got exposed to the psychoactive properties of LSD (Hofmann, 1970b). In the detailed report, written a few days later to the Head of Pharmaceutical Department of Sandoz Laboratories, he described the subsequent experience as “an uninterrupted stream of fantastic images of extraordinary plasticity and vividness accompanied by an intense, kaleidoscope-like play of colors” (Hofmann, 1970a).

After this first, unintentional, self-experiment of LSD, Hofmann conducted a pre-planned experiment to confirm his suspicion that it actually was LSD that had caused his unexpected intoxication (Hofmann, 1970b). Hofmann started the experiment with 0,25 mg peroral dose of LSD tartrate, the lowest dose he could possibly think of having any effect, as no drug at the time was known to be pharmacologically effective on below 1 mg doses. As a result, he underwent considerably stronger psychoactive effects than the previous time. These effects manifested e.g. as an experience of distorted perception and synaesthesia that lasted for several hours. In later experiments 0.03 mg to 0.05 mg of LSD tartrate was confirmed as an effective dose for humans (Hofmann, 1970b).

Eventually, Sandoz made LSD available for research purposes and physicians as an experimental drug under a trade name *Delysid* (Nichols, 2018). The indications stated on the drug label were analytical psychotherapy and induction of short term model psychosis in normal patients. In the instructions of use, the psychiatrist was encouraged to take *Delysid* him/herself to be “able to gain an insight into the world of ideas and sensations of mental patients”.

LSD was in active experimental clinical use in 1950's and 1960's when it was used for psychedelic assisted psychoanalysis, for the treatment of alcohol dependence and other addictions and for psychiatric therapy of cancer patients (Nichols, 2018). Numerous research papers were published during that time; by 1963 there were already over a thousand of them. By 1970, practically all clinical research and therapeutic use of LSD came to a halt after it was declared a Schedule I drug in the United States. In most countries, LSD is still legally categorized in the most restrictive classes, alongside substances like heroin.

2.1.2 Pharmacological properties and pharmacodynamics of LSD

All psychedelic drugs, including LSD, are known to be potent serotonin (5-hydroxytryptamine; 5-HT) receptor agonists or partial agonists (Passie *et al.*, 2008; Nichols, 2016; López-Giménez and González-Maeso, 2018). The evident structural similarity between serotonin and LSD (Figure 1.), and the detection of serotonin in mammalian brain ten years after the discovery of LSD, indeed advanced the understanding of the neurochemical bases of human psyche and psychiatric disorders (Nichols, 2018).

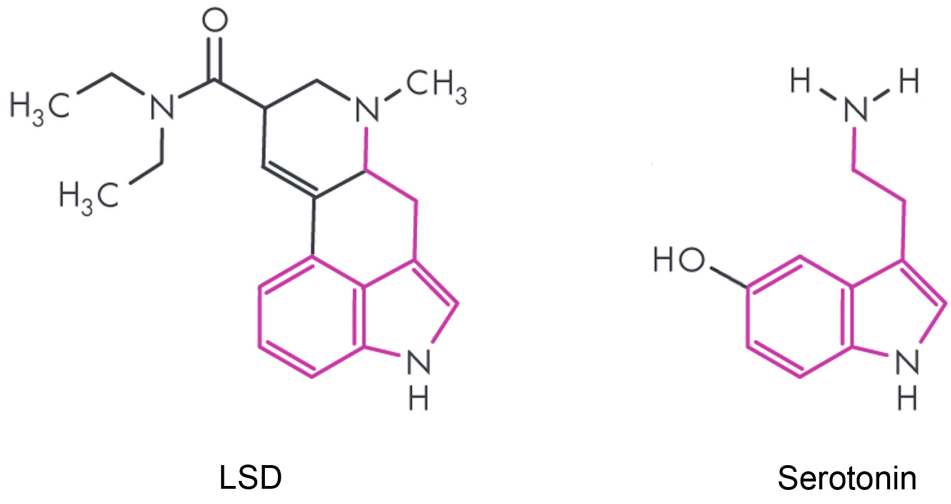

Figure 1. The molecular structures of lysergic acid diethylamide (LSD) and serotonin. The structural similarity (the indolamine structure) of the two molecules has been high-lighted with pink.

LSD has a high affinity (Table 1.) to many G-protein coupled serotonin receptors, predominantly 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C}. While the binding affinity to 5-HT_1 receptors is the highest, these receptors have not been found important for the pharmacodynamics of LSD, whereas 5-HT_2 receptors seem to mediate its most prominent effects (Nichols, 2016). Of the 5-HT_2 receptor family, 5-HT_{2A} is the subtype known to be responsible for the unique psychoactive effects of psychedelics. 5-HT_{2C}, on the other hand, is known to have a role in alteration of DA transmission, which I will look into in more detail in a later section of this thesis (Browne *et al.*, 2017; Canal and Murnane, 2017)

Table 1. Binding affinities of LSD. All values are K_i (nM). (Passie *et al.*, 2008; Liechti, 2017)

5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	5-HT_{5A}	5-HT_6	5-HT_7
1.1	3.9	1.4	93	2.7	30	5.5	9	2.3	6.6
SERT	Alfa	$Beta_1$	$Beta_2$	D_1	D_2	D_3	D_4	D_5	H_1
>3000	220	140	740	180	120	27	56	340	1.540

5-HT = 5-hydroxytryptamine (serotonin) receptor, Alpha = adrenergic alpha receptor, Beta = adrenergic beta receptor, D = dopamine receptor, H = histamine receptor, SERT = serotonin transporter.

The fact that the dream-like alteration of consciousness and other subjective psychoactive effects of LSD in humans are mediated by 5-HT_{2A} receptor, has been demonstrated in numerous studies (Nichols, 2016; Kraehenmann *et al.*, 2017; Liechti, 2017; Preller *et al.*, 2017; Barrett *et al.*, 2018). The most prominent evidence supporting the role of 5-HT_{2A} is the fact that the psychoactive effects of psychedelics can be blocked completely by the selective 5-HT_{2A} antagonist ketanserin. In rodent models, ketanserin blocks the psychedelic-induced head-twitch and wet-dog-shake behaviour, as well as the discrimination cue effect in a drug discrimination paradigm (Glennon, Young and Rosecrans, 1983; Buchborn *et al.*, 2015; Nichols, 2016).

The induction of head-twitch and wet-dog-like shaking behaviour in rodents is a distinctive feature of 5-HT_{2A} agonist psychedelics and is considered to be an indicator of the action of these compounds, corresponding with the psychoactive effects in humans (Canal and Morgan, 2012; Hanks and González-Maeso, 2013). Head-twitch response is

regarded as a result of metabotropic glutamate mGlu2/3-receptor-sensitive glutamate release induced by frontocortical 5-HT_{2A} activation. This is supported, for instance, by the mouse studies by Moreno *et al*. (2011, 2013), which have shown that the head-twitch response is not induced in mGluR2 knock-out mice and is decreased in the mice treated with mGlu2/3 receptor antagonist LY341495.

It was noticed early on that a repeated administration of LSD on 3 to 5 consecutive days, causes tolerance to the effects of the drug in both humans and animals (Isbell *et al.*, 1961; Murray, Craigmill and Fischer, 1977; Nichols, 2016). This has been shown to result from the down regulation of cortical 5-HT_{2A} receptors (Buckholtz, Freedman and Middaugh, 1985; Buckholtz, Zhou and Freedman, 1988; Buckholtz *et al.*, 1990; Gresch *et al.*, 2005). Due to their shared pharmacology, also a cross-tolerance between LSD and other psychedelics occurs (Isbell *et al.*, 1961; Wolbach, Isbell and Miner, 1962; Kovacic and Domino, 1976). The development of tolerance might also contribute to the fact that, despite their legal status, psychedelics are considered to have a very low addiction potential (Bogenschutz and Johnson, 2016).

The clinical findings about the long-lasting treatment effect of a single high dose of LSD and other psychedelics, have indicated that neural and synaptic plasticity might have a role in the mechanism of action of these compounds. In their enlightening study, Ly *et al.* (2018) indeed demonstrated that psychedelics do promote neural plasticity both *in vitro* and *in vivo*, and that this effect, as well, is mediated through 5-HT_{2A} activation and blocked by ketanserin. In cultured rat cortical cells, LSD, DMT and 2,5-dimethoxy-4-iodoamphetamine (DOI) were shown to increase neurite growth, dendritic arbor complexity, dendritic spine growth and synapse formations which all can be considered hallmarks of neural plasticity. 24-hour treatment with LSD nearly doubled the number of dendritic spines per 10 μm in mature rat cortical cultures. Moreover, the spine density increased significantly in the prefrontal cortex (PFC) of adult rats 24 hours after 10 mg/kg IP administration of DMT. In humans, 5-HT_{2A}-mediated increased plasticity has been suggested as a possible mechanism behind the increased psychological flexibility and

suggestibility that seem to have on important role in the therapeutic ability of psychedelics (Carhart-Harris and Nutt, 2017).

The modern neuroimaging studies, using e.g. functional magnetic resonance imaging and blood oxygen level dependent measure, have revealed that LSD seems to alter the functional connectivity and thus the information flow of the brain in humans (Carhart-Harris *et al.*, 2016; Tagliazucchi *et al.*, 2016). The global connectivity from frontal, parietal, and inferior temporal cortical areas and thalamus has been shown to increase. The cortical areas with increased connectivity, overlap with the areas of high 5-HT_{2A} density. In two recent brain imaging studies, Preller et al (2018, 2019) showed that LSD increases sensory-somatomotor brain-wide and thalamic connectivity and alters connectivity within the cortico–striato–thalamo-cortical pathways increasing the excitatory connectivity from thalamus to posterior cingulate cortex through 5-HT_{2A} activation. LSD also reduced the effective connectivity from striatum to thalamus independent of 5-HT_{2A} activation.

Although, it is evident that LSD and other psychedelics act through 5-HT_{2A} agonism or partial agonism, there is no clear consensus on which signaling pathway mediates their therapeutic or psychoactive effects (Nichols, 2016). It is known that, depending on a ligand, 5-HT_{2A} can activate different signaling molecules, a phenomenon called functional selectivity. Therefore, it is possible that the signaling cascade activated by LSD differs from the one activated by endogenous serotonin and other ligands of the same receptor.

2.1.3 Therapeutic potential in treatment of addiction

Most of the well over a thousand studies conducted with LSD during the first wave of psychedelic research, do not meet the current scientific standards, most often for the lack of control group or insufficient follow-up. However, a meta-analysis of six randomized controlled trials from between 1966 and 1970 demonstrated LSD having a significant therapeutic effect in treatment of alcohol dependence (Krebs and Johansen,

2012). According to the authors, a single high dose (210-800 μg) of LSD had a treatment effect that lasted for at least six months, suggesting notably higher efficacy and better treatment outcomes than the currently available pharmacological treatments.

A recently published systematic review of all controlled and randomized clinical trials that assess the potential use of LSD in psychiatry, included altogether eleven studies, majority of which concerned the treatment of alcohol dependence (Fuentes *et al.*, 2020). Six of these studies were also included in the previously mentioned meta-analysis. In line with Krebs and Johansen, the authors of the review concluded that LSD may be beneficial in treatment of alcohol dependent patients.

At the time of writing this, to my knowledge, no modern clinical trials on LSD in treatment of alcohol dependence or other substance abuse disorders have been published. However, pilot studies with psilocybin have shown promising results for treatment of alcohol and nicotine addictions (Johnson *et al.*, 2014; Bogenschutz *et al.*, 2015). An open-label proof-of-concept study of 10 alcohol-dependent participants showed significant and long-lasting reduction of drinking days after psilocybin assisted psychosocial therapy (Bogenschutz *et al.*, 2015). Moreover, to asses the effect of psychedelic use on alcohol use disorder in non-clinical natural setting, Garcia-Romeu *et al.* (2019) conducted an online survey that assessed the cessation and reduction of alcohol misuse after psychedelic use. According to the answers of the 246 respondents who, based on the questioner, met The Diagnostic and Statistic Manual of Mental Disorders, Fifth edition (DSM-5) diagnostic criteria for severe alcohol use disorder before psychedelic use, only 16 met the criteria after the reference psychedelic use.

In mice, a single 50 μg/kg dose of LSD has been shown to reduce ethanol consumption and preference in a two-bottle choice alcohol drinking paradigm, whereas 25 μg/kg dose was not significantly effective (Alper *et al.*, 2018). Neither of the doses affected the total fluid intake. The high-dose effect on the ethanol consumption lasted throughout the whole 46-day follow-up period. According to unpublished preliminary results from Lauri Elsilä of our research group, the acute administration of 100 μg/kg of LSD lowers the consumption of ethanol in mice but does not have long term effects. In this experiment,

the drinking habit was established by the Drinking in the Dark protocol, in which the animals had access to ethanol for two hours at the dark period on four days per week for four weeks before the test. Conversely, a recent study that used the alcohol deprivation model of relapse did not find LSD nor psilocybin effective in preventing a relapse in rats (Meinhardt *et al.*, 2020). Both substances were tested with two high doses. First dose was administered seven days before, and the second one on the day of reintroducing ethanol. For LSD, the high dose was 320 μg/kg. In addition, repeated low and medium doses were tested with no significant effect.

2.2 Neurobiology of addiction

Addiction is a chronic, relapsing psychiatric disorder and a brain disease caused by a repeated exposure to an addictive substance (Koob and Volkow, 2016). It is characterized by compulsive drug or alcohol seeking and uncontrolled, excessive intake despite harmful consequences. The term addiction, is generally used as a synonym to DSM-5 diagnostic term "severe substance use disorder" (Volkow, Koob and McLellan, 2016).

2.2.1 The reward circuitry

"Drive opium off the ship, and it hides in the engine-room." French author Jean Cocteau wrote these words in the end of 1920's at a rehabilitation clinic, while trying to recover from his opium addiction (Cocteau, 1990). He was describing a distinctive feature of an addiction; the persisting neurobiological changes that remain in the brain long after the drug has left the system. The initiative events of these changes take place in the reward circuitry (Figure 2.), most importantly in the mesolimbic dopaminergic pathway projecting from the ventral tegmental area (VTA) to nucleus accumbens (NAc) (Wise, 2008; Korpi *et al.*, 2015; Volkow and Morales, 2015).

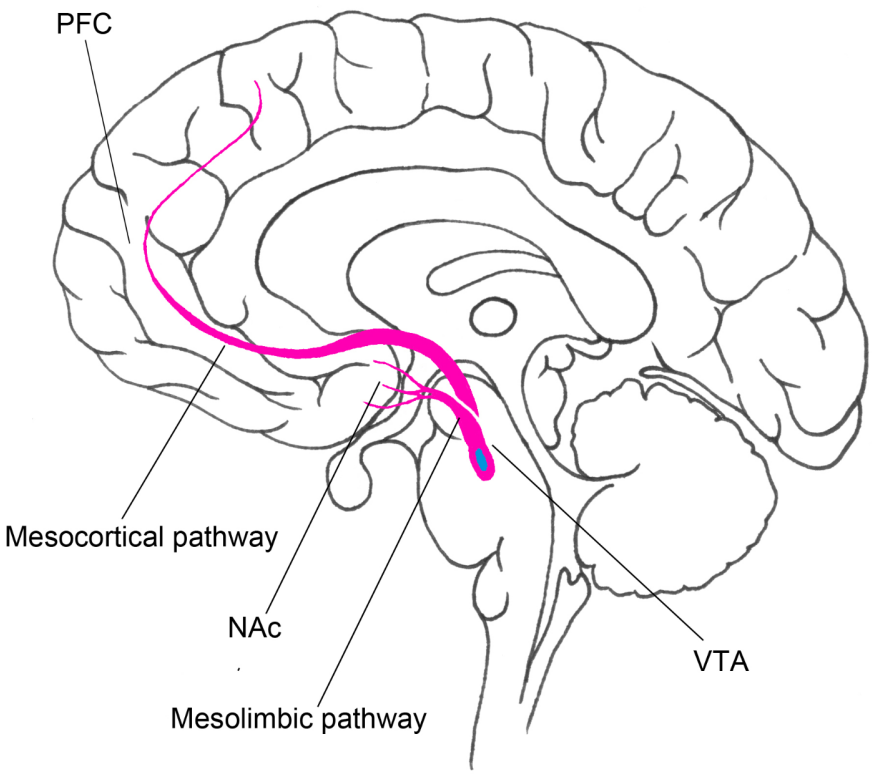

Figure 2. The dopaminergic pathways of the reward circuitry in a sagittal image of a human brain. NAc = nucleus accumbens, PFC = prefrontal cortex, VTA = ventral tegmental area.

All drugs of abuse increase the phasic firing of the DA neurons in the VTA, leading to a rapid and large increase of DA and activation of the low-affinity D_1 receptors in the NAc shell. (Volkow and Morales, 2015). The surge of DA leads to alteration of glutamatergic synaptic transmission in the VTA, and eventually, after repeated exposure also in the NAc, resulting in long term changes in the functioning of the reward circuitry (Lüscher and Malenka, 2011).

Different substances increase the dopaminergic activity in the VTA by different mechanisms. For instance, as a monoamine reuptake inhibitor, cocaine blocks the reuptake of DA from the synaptic cleft, whereas monoamine releaser amphetamine does the same, but additionally enters the DA carrying vesicles in the cell causing DA efflux (Lüscher, 2016). For alcohol, the DA increasing mechanism is not fully understood

and several mechanisms are likely to be involved. It has been suggested that alcohol affects the gamma-aminobutyric acid (GABA) $GABA_A$ receptor function inhibiting the GABAergic transmission in the VTA which results in increased firing of the VTA DA neurons and disinhibition of the DA release in the NAc (Figure 3.) (Gilpin and Koob, 2008; Korpi *et al.*, 2015).

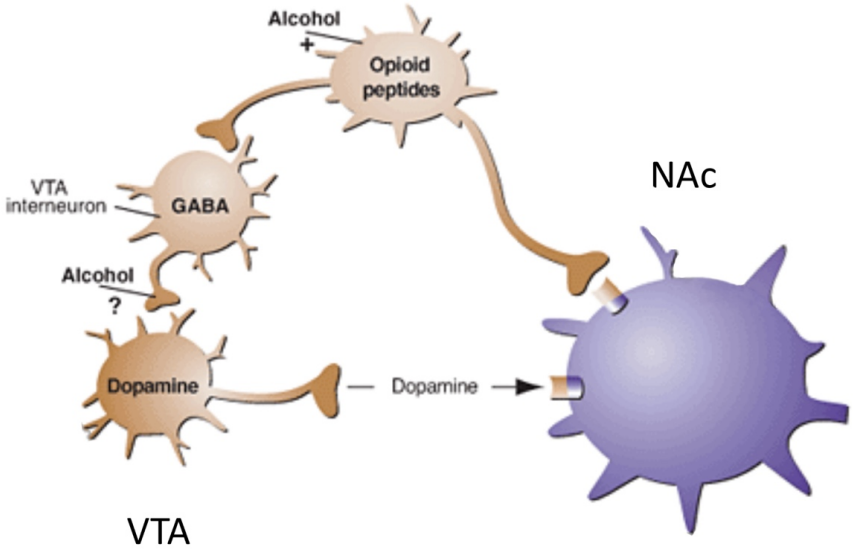

Figure 3. One of the suggested mechanisms for alcohol-induced DA increase in the NAc is disinhibition through GABAergic interneurons in the VTA. GABA = gamma-aminobutyric acid, NAc = Nucleus accumbens, VTA = Ventral tegmental area. (Adapted from Gilpin and Koob, 2008)

2.2.2 Three stages of addiction

Addiction can be thought of as a cycle that can be divided in three stages that all involve a distinctive behaviour and an imparting neurocircuitry (Koob and Volkow, 2016). These stages are binge/intoxication, withdrawal/negative affect and preoccupation/ anticipation.

The substance-induced supraphysiologic surge of DA in the NAc is the key element of the binge/intoxication stage of the addiction cycle (Koob and Volkow, 2016). This recruitment of the reward circuitry is responsible for the reinforcing effects of the drug

intake and the drug-induced euphoria. The activation of the low-affinity D_1 receptors is required for the drug reward, whereas high-affinity D_2 receptors, which are activated by the normal physiological DA levels, are not sufficient for addiction-initiating reward and are associated with normal motivation drive (Trifilieff *et al.*, 2013). In addition to DA, other neurotransmitter systems, such as opioid peptides, 5-HT, GABA and glutamate have a role in drug reward (Koob and Volkow, 2016). The role of the 5-HT_2 receptor activation will be looked into later in this thesis.

The phasic firing of the VTA neurons and the activation of the reward mechanism can also be induced by an incentive salience (Koob and Volkow, 2016). After repeated administration, the rewarding effect caused by the drug may become paired with a previously neutral stimulus, in which case an exposure to this drug related cue stimulates the burst of DA and the reward effect on its own.

Withdrawal/negative affect stage is characterized by dysphoria, loss of motivation towards natural rewards and chronic irritability (Koob and Volkow, 2016). Avoidance of these aversive mood-states leads to negative reinforcement of addiction. Extended amygdala and habenula are the main brain areas involved in withdrawal/negative affect. Activation of stress systems, such as elevated release of corticotropin-releasing factor, also contribute to this stage. During withdrawal from chronic administration of any addictive drug, animal models have shown increased stress and anxiety related responses and desensitization of the reward system which manifests as elevation in reward thresholds (TH) (Cryan, Hoyer and Markou, 2003). These behavioral changes are, at least partially, result from within-system cellular level neuroadaptation, in which the physiological attempt to neutralize the abnormally high levels of DA caused by drug intake, results in abnormally low level of the neurotransmitter in withdrawal (Koob and Volkow, 2016). According to Volkow and Morales (2015), human brain imaging of individuals with addictions, as well as several animal models of chronic administration of addictive drugs, have shown down regulation of D_2 receptors in striatum, including the NAc.

Preoccupation/anticipation stage is considered to play an important role in the relapsing nature of addiction (Koob and Volkow, 2016). In terms of behavior, this stage is characterized by constant craving of the drug, inability to concentrate on non-drug-related matters and drug-seeking after abstinence. In this stage the dopamine transmission is, again, higher. The PFC, which is the main area involved in this stage, sends glutamatergic projections to the dopaminergic neurons of the VTA increasing the DA level in the NAc. This might also contribute to incentive salience process and depleted goal-directed behavior.

2.3 5-HT_2 receptors and reward

As mentioned earlier, while dopaminergic system has a major role in reward, other neurotransmitter systems are involved in it as well. Serotonergic system is known to alter DA release in reward circuits (Howell and Cunningham, 2015). The modulatory role of serotonin has been shown, for instance, with serotonin transporter (SERT) inhibitors that increase the extracellular level of serotonin by selectively inhibiting its reuptake. SERT inhibitors, such as citalopram and fluoxetine, are widely used for treatment of depression and other mood disorders. According to several studies, drugs from this group attenuate drug-induced increases of DA levels and supress behavioural effects, reinforcing effects, self-administration and reinstatement of psychostimulants in both rodents and non-human primates (Howell and Cunningham, 2015; Canal and Murnane, 2017). However, substance abuse related clinical trials with SERT inhibitors have not been able to demonstrate strong enough efficacy in humans (Howell and Cunningham, 2015).

LSD's target receptors 5-HT_{2A} and 5-HT_{2C}, are also well known to have modulatory effects on DA release in the reward circuitry (Howell and Cunningham, 2015). 5-HT_{2A} activation enhances the dopaminergic activity in the VTA, whereas 5-HT_{2C} receptor activation has been shown to inhibit mesolimbic DA release. Some of the evidence supporting these roles is reviewed herein.

2.3.1 Role of 5-HT_{2A}

Extensive number of studies have demonstrated the DA and reward enhancing role of 5-HT_{2A} receptor activation. Systemic administration of 5-HT_{2A} agonist psychedelic DOI increases the firing rates of the DA neurons in the VTA and the DA release in the PFC as does local administration of DOI in the medial PFC (Bortolozzi *et al.*, 2005). This effect can be reversed with 5-HT_{2A} antagonists M100907 and ritanserin (Bortolozzi *et al.*, 2005). Apparently, the increase of DA firing rate in the VTA happens primarily via prefrontal 5-HT_{2A} receptors and through projections from the PFC to VTA. Also, amphetamine-induced elevation of DA level in the NAc has been shown to be potentiated by systemic administration of DOI (Kuroki, Meltzer and Ichikawa, 2003). This effect, as well, was blocked by M100907. Several other studies have shown 5-HT_{2A} antagonists attenuating cocaine and amphetamine-induced increases in the extra-cellular DA levels (Howell and Cunningham, 2015). In several behavioural studies with rodents and non-human primates, 5-HT_{2A} antagonists have been shown to attenuate the reinstatement of cocaine self-administration by either cocaine or drug related cues (Müller and Homberg, 2015).

Findings about genetic risk factors for drug addiction further support the evidence of the important role of 5-HT2A in the development of addiction. It has been suggested that a single nucleotide polymorphism (SNP) rs6313 in 5-HT_{2A} encoding HTR2A gene is associated with heroin dependence, whereas SNP rs6561333 in the same gene has been associated with cocaine dependence (Müller and Homberg, 2015).

The fact that 5-HT_{2A} activation enhances the mesolimbic dopaminergic activity seems to be in contradiction with the possible therapeutic effect that 5-HT_{2A} agonist psychedelics seem to have on substance use disorders. It is also in conflict with the suggested non-addictive nature of these compounds. Therefore, there is a reason to assume that the activation of 5-HT_{2C} plays a bigger role in these properties.

2.3.2 Role of 5-HT_{2C}

The hypothesis that 5-HT_{2C} receptor activation inhibits mesolimbic DA release and supresses reward system function is supported by numerous studies. The 5-HT_{2C} receptor agonist CP809101 reduces locomotor activity and responding for conditioned reinforcer in mice, whereas antagonists enhance these behaviours (Browne *et al.*, 2017). In rats, 5-HT_{2C} agonists have also been shown to reduce responding to conditioned reinforcer, both on the basal level and when the behaviour is potentiated with methylphenidate (Fletcher *et al.*, 2017). 5-HT_{2C} agonist WAY 163909 has been shown to dose dependently suppress cocaine self-administration and cue-evoked reinstatement of cocaine (Cunningham *et al.*, 2011) Lorcaserin, a 5-HT_{2C} agonist approved for clinical use in treatment of obesity in United States but not in Europe, dose-dependently reduces cocaine self-administration and a variety of other cocaine-induced behaviours in rats (Harvey-Lewis *et al.*, 2016). Lorcaserin, has also been shown to attenuate intracranial self-stimulation and to block the reward-enhancing effects of nicotine (Zeeb, Higgins and Fletcher, 2015). According to a review by Howell and Cunningham (2015), data from several studies suggests that 5-HT_{2C} agonists have potential to reduce subjective and reinforcing effects of cocaine if a patient with a substance abuse disorder is exposed to the drug during recovery.

In terms of neurochemistry, in mice, systemic administration of 5-HT_{2C} agonists reduces both basal level and drug-induced DA release in the NAc (Browne *et al.*, 2017; Canal and Murnane, 2017). Conversely, according to a recent study with rats, lorcaserin does not seem to affect DA release from the NAc and slightly increases the firing rate of the VTA DA neurons (De Deurwaerdère *et al.*, 2020).

2.3.4 The Distribution 5-HT_{2A} and 5-HT_{2C} receptors

What would explain the thoroughly opposite effects these two receptors have on mesolimbic DA release and reward? In the mesocorticolimbic system, 5-HT_{2C} receptors seem to be predominantly expressed in GABAergic interneurons, whereas 5-HT_{2A} receptors are more densely expressed on DA and glutamate neurons (Howell and Cunningham, 2015). The distribution of these receptor subtypes on different types of

neurons is a probable explanation for the oppositional effects they have on DA transmission. It has been suggested that 5-HT_{2C} mediated GABAergic inhibition of DA neurons would mainly take place in the VTA and PFC, resulting in reduced firing from the VTA to NAc (Figure 4.). In contrast to this, Canal and Murnane (2017) state that the inhibition is actually localized in the NAc shell. They propose a hypothesis that the 5-HT_{2C} activation on GABAergic medium spiny neurons in the NAc shell modulates the voltage-gated potassium Kv1.x channels, and by doing so counteracts the decrease in medium spiny neurons activity caused by psychostimulants, such as cocaine (Canal and Murnane, 2017).

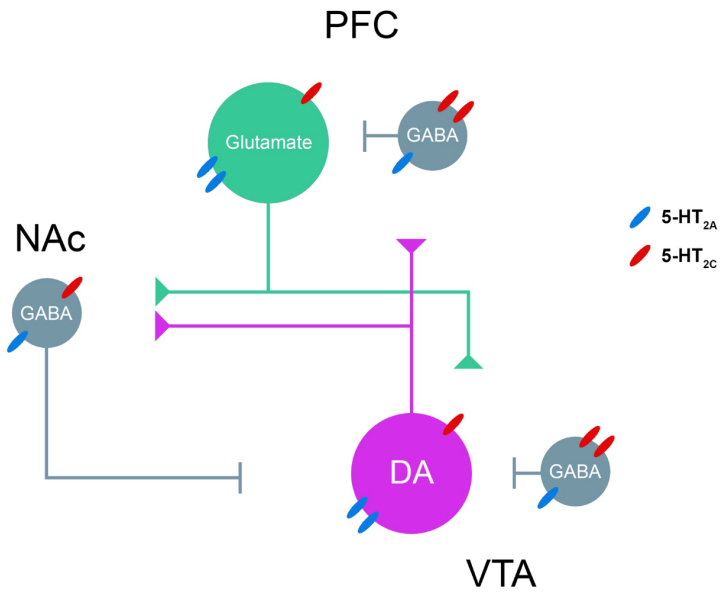

Figure 4. The suggested distribution of 5-HT_{2A} and 5-HT_{2C} receptors in the reward circuitry. 5-HT_{2C} receptors have been proposed to be more abundant on the GABAergic interneurons of the VTA and PFC. NAc = Nucleus accumbens, PFC = prefrontal cortex, VTA = Ventral tegmental area. (Modified from Howell and Cunningham, 2015)

2.4 Intracranial self-stimulation (ICSS)

Intracranial self-stimulation (ICSS) is a behavioural procedure in which laboratory animals self-administer electrical stimulation through electrodes implanted into the brain areas involved in reward and motivation (Carlezon and Chartoff, 2007; Negus and

Miller, 2014). ICSS, sometimes referred to as brain stimulation reward, is used for a quantitative assessment of reward and anhedonia, most often with pharmacological manipulations.

ICSS originates from 1950's and since then it has been an important tool for studying the neurobiology of reward. Its discovery by James Olds and Peter Milner was, as so often happens in science, a result of a lucky accident (Olds and Milner, 1954; Milner, 1989). Olds happened to misplace an electrode that was intended in the rat's reticular formation and was supposed to cause avoidance behaviour. Instead of avoidance, the stimulation through this electrode had a potent reinforcing effect. With further investigation Olds and Milner found out that instead of reticular formation the electrode was placed in the septal area of the brain.

In later studies, the medial forebrain bundle (MFB) at the level of the lateral hypothalamus (LH) (Figure 5. A) was recognized as the site in which the stimulation causes the strongest reinforcing effect (Olds and Olds, 1963; Wise, 2005; Negus and Miller, 2014). The MFB is a group of fibers that contains the dopaminergic mesolimbic pathway from the VTA to NAc, known, as described earlier, as a crucial site in neurobiology of addiction. The stimulation of the MFB in ICSS is thought to directly activate descending myelinated neurons that originate in the LH, or other more rostral regions. These neurons project to the VTA, thus the stimulation is considered to indirectly activate the unmyelinated mesolimbic DA neurons from the VTA to NAc (Figure 5. B) (Negus and Miller, 2014).

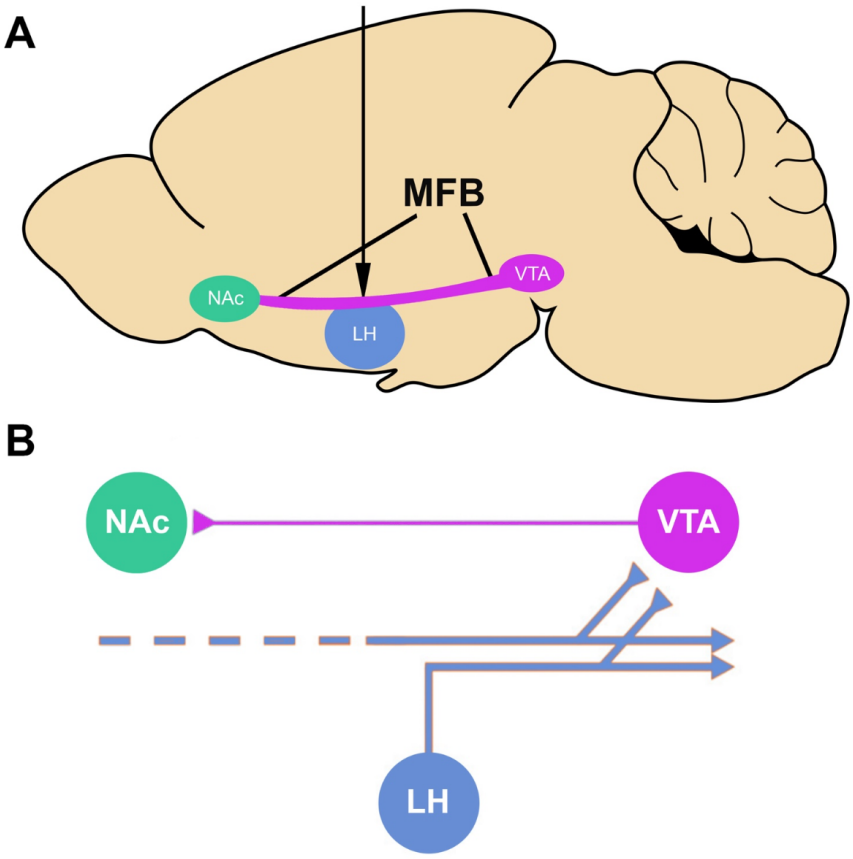

Figure 5. The strongest reinforcing effect with ICSS is achieved when the electrode is placed in MFB at the level of LH (A) Location of the MFB and the implantation site of the ICSS electrode in a sagittal section of a mouse brain. (B) A graphical diagram of the neurons that are considered to involve in ICSS (Modified from Negus and Miller, 2014). LH = lateral hypothalamus, NAc = nucleus accumbens, VTA = ventral tegmental area.

ICSS procedure can be applied to both rats and mice (Stoker, Astrid K.; Markou, 2011; Stevens Negus and Miller, 2014). Rats typically self-administer the stimulation by pressing a leaver, whereas mice do it by turning a wheel. The aim of the procedure is to determine a threshold level for the reinforcing effect of the stimulation. Most common ICSS protocols are the discrete-trial current-intensity method in which the amplitude of the stimulation varies, and the rate-frequency curve-shift method in which the frequency of the pulses is the changing variable. In this master's thesis project, the former one is used and explained in detail in Methods.

2.4.1 ICSS facilitation and attenuation

ICSS is most commonly used to asses an impact of pharmacological substances on reward behaviour. It has proved to be a useful instrument for an evaluation of the abuse liability of drugs. Numerous studies have shown that drugs of abuse sensitize the reward system, which in ICSS shows as increase of the response rate and by lowering of the TH (for an extencive review see Negus and Miller, 2014). Below, I will give some examples on how pharmacological manipulations may alter ICSS.

Facilitation of ICSS has been most explicitly observed with psychostimulants, such as monoamine reuptake inhibitor cocaine and monoamine releasers amphetamine and methamphetamine. The increase in response rates and lowering of the TH have been demonstrated with both rats and mice and with both of the most common ICSS procedures (Esposito, Motola and Kornetsky, 1978; Esposito, Perry and Kornetsky, 1980; Bain and Kornetsky, 1987; Gill, Knapp and Kornetsky, 2004; Bauer *et al.*, 2013). The facilitation of ICSS can be seen directly after an acute administration of cocaine or amphetamine, and it maintains after repeated administration. Withdrawal from chronic administration of these substances, on the other hand, causes attenuation of ICSS, manifested as elevated THs (Lin, Koob and Markou, 2000; Paterson, Myers and Markou, 2000; Cryan, Hoyer and Markou, 2003; Stoker and Markou, 2011).

With opioids, the results have been more complex and mixed (O'Neill and Todtenkopf, 2010; Altarifi, Rice and Negus, 2013; Negus and Miller, 2014). It seems that acute administration of opioids attenuates, whereas repeated administration facilitates ICSS (Altarifi, Rice and Negus, 2013). The time point of the ICSS session after acute administration of morphine has been reported to be crucial in detecting the rewarding effects of the drug in ICSS (O'Neill and Todtenkopf, 2010). Three hours after the morphine administration has been suggested as the optimal time for ICSS. It has been speculated that the sedating effect of morphine would outplay the reward behaviour in ICSS if the session is conducted too soon after the administration.

Ethanol, which has both rewarding and aversive effects, has shown facilitation of ICSS with lower (1.0 g/kg), and attenuation with higher (>1.0 g/kg) doses in rate-frequency curve-shift procedure with rats and discrete-trial current-intensity procedure with mice (Kornetsky *et al.*, 1988; Barkley-Levenson, Der-Avakian and Palmer, 2020).

Whereas facilitation of ICSS is considered a representation of the drug reward and drug-induced euphoria, attenuation of the response rate and the increase of the TH are seen as a model for anhedonia. As mentioned earlier, the attenuation of ICSS can be seen after withdrawal from drugs of abuse, but it can also be induced by administration of certain drugs, such as kappa-opioid receptor agonist U-69593, which is considered to cause depression-like negative mood-states in humans and animals (Todtenkopf *et al.*, 2004; Tomasiewicz *et al.*, 2008; Bruijnzeel, 2009). Another molecule known to evidently reduce response rate in ICSS, is lipopolysaccharide (LPS) (Borowski *et al.*, 1998; Barr *et al.*, 2003; Van Heesch *et al.*, 2013, Lainiola, Hyytiä and Linden, unpublished data). LPS is a component of the outer membrane of gram-negative bacteria which, when systemically administrated, activates proinflammatory cytokine release causing sickness-reaction and anhedonia in rodents (Van Heesch, 2014).

2.4.2 ICSS with 5-HT_2 receptor ligands

Apparently, James Olds himself did ICSS experiments with LSD a few years after the discovery of the procedure, but unfortunately these experiments are poorly documented. In these studies, the effect of 0.2 mg/kg of LSD on self-stimulation was tested in rats with electrodes implanted in three different locations (Olds and Eiduson, 1959). The remaining one-page paper where the results are shortly explained, tells that LSD had substantial rate-depressing effect when the electrode was implanted in the sub-cortical cell masses of rhinencephalon but lesser effect when the electrode was in hypothalamus.

A recent study by Sakloth *et al* (2019) investigated the effects of acute LSD, mescaline and psilocybin on rate-frequency curve-shift ICSS procedure in rats. With LSD, also repeated administration and interactions with kappa-opioid receptor agonist U-69593

and psychostimulant methamphetamine were tested. The acute administration of high doses of these psychedelics produced ICSS attenuation, whereas with lower doses the results showed only inconsistent and weak facilitation. For LSD the high dose was 0.32 mg/kg. 7-day repeated LSD administration showed similar dose dependent results as acute administration, with no evidence of development of tolerance or increased facilitation of ICSS. Repeated LSD did not affect the basal ICSS levels nor methamphetamine-induced facilitation of ICSS. However, the repeated administration of higher doses of LSD significantly reduced the ICSS attenuation induced by U-69593.

Katsidoni, Apazoglou and Panagis (2011) studied the effects of 5-HT_{2A} and 5-HT_{2C} agonists and antagonists on ICSS with rats. Based on their results, systemic administration of the 5-HT_{2A} agonist TCB-2 or 5-HT_{2C} agonist WAY-161503 both increased the TH levels in ICSS. WAY-161503 was also able to hinder the cocaine-induced facilitation of ICSS. The 5-HT_{2A} antagonist R-96544 and the 5-HT_{2C} antagonist SB-242084 did not affect the ICSS on their own, but they completely blocked the reward attenuating effects of the corresponding agonists.

As mentioned earlier in this thesis, 5-HT_{2C} agonist lorcaserin has also been shown to attenuate ICSS and abolish the nicotine-induced facilitation of reward in rats (Zeeb, Higgins and Fletcher, 2015). The same study demonstrated that the TH-increasing effect of lorcaserin was blocked by the 5-HT_{2C} antagonist SB-242084. Interestingly, ICSS with lorcaserin was also performed with rats that had the electrodes implanted into dorsal raphe nuclei instead of the MFB. The results of these rats were similar to those of the ones with the electrode implanted in the MFB.

3 AIMS

Firstly, the aim of this study was to assess the acute effects of LSD on reward behavior in mice with ICSS and, furthermore, to see if LSD alters the amphetamine- and LPS-induced changes in ICSS. This was done to advance the understanding of the mechanism of action behind the potential therapeutic properties of LSD in treatment of alcohol use disorders and to evaluate whether the acute effects of LSD on ethanol consumption in mice are mediated through the reward mechanism. Secondly, the aim was to successfully train the mice to acquire ICSS, in order to make the above-mentioned experiments feasible and to test the suitability of the ICSS procedure for this type of experimenting with the current mouse line, programs and apparatus.

4 HYPOTHESIS

As a serotonin 5-HT_{2A} and 5-HT_{2C} agonist, LSD is likely to modulate the mesocorticolimbic DA activity and thus the functioning of the reward circuitry. Therefore, it is reasonable to hypothesize that LSD could alter the reward behavior in ICSS. Knowing the suggested therapeutic properties of LSD in alcohol use disorders, the acute effect it has on ethanol consumption in mice and the non-addictive nature of psychedelic drugs, the possible alteration of ICSS could be assumed to take place predominantly through 5-HT_{2C} mediated desensitization of the reward mechanism. Based on what is known about the actions of 5-HT_{2C} agonists, such as lorcaserin, in ICSS, it could be presumed that LSD might also alter amphetamine-induced facilitation of ICSS.

On the other hand, the withdrawal/negative affect state of addiction involves negative mood-state, anhedonia and desensitization of the reward circuitry. In clinical trials, LSD has also shown promise in treatment of depression and in a rat model, LSD reduced the ICSS attenuation induced by negative-mood-state-causing kappa-opioid receptor agonist U-69593 (Carhart-Harris *et al.*, 2017; Sakloth *et al.*, 2019). Thus, it could be

assumed that LSD would have a reversing impact on LPS-induced anhedonia and attenuation of ICSS.

5 MATERIALS AND METHODS

5.1 Subjects

The surgery was carried out for 20 male C57BL/6JRj (Janvier Labs, Saint Berthevin, France) mice. At the time of the arrival to the animal facility, the animals were approximately 8 weeks old. The mice were housed in pairs before and singly after the surgery in GR500 IVC cages (Tecniplast, Buguggiate, Italy). The animals were kept under 12-hour light/dark cycle with light on at 6 am and *ad libitum* access to basic rodent chow (Teklad) and water.

The cages of the mice were changed once a week before the weekend by the experimenter to make sure that the inevitable stress caused by the cage change would not affect the ICSS results. The mice were weighed once a week before the testing days. The weighing was performed after the ICSS session to ensure that the weighing would not affect the TH. The mice were always handled and taken out of their cages with cupped hands and never by lifting from the tail to minimize the stress caused by the handling.

All animal procedures in this project were done according to 3R principals and the guidelines of the National Institute of Health's Guide for the Care and Use of Laboratory Animals. The use of laboratory animals for these experiments was approved by Animal Experiment Board in Finland (approval code ESAVI/1172/04.10.07/2018).

5.2 Surgery

The stereotaxic surgery was performed by Lauri Elsilä three weeks after the animals had arrived in the facility. Each mouse was anesthetized with isoflurane (Vetflurane 1000 mg/g, Virbac Animal Health, Carros, France) and attached to a stereotaxic frame. The

scalp of the mouse was opened and after a small craniotomy a 6 mm (cut below the pedestal), 0.008 inch diameter, 2 channel (bipolar), stainless steel electrode (MS303/2-B/SPC, Plastic One, Roanoke, Virginia, United States) was implanted into the right side of the head (coordinates -1.6 AP, -1.0 ML, -5.3 DV, mm relative to bregma) targeting the medial forebrain bundle. Two small anchor screws were attached to the skull near the electrode and the screws and the electrode were embedded in dental cement in order to hold the electrode in place. The wound was closed with sutures and the mouse was left to recover with proper analgesia with carprofen (5 mg/kg; Norocarp Vet 50 mg/ml, Norbrook Laboratories Ltd., Monaghan, Ireland) and buprenorphine (0.05 mg/kg; Temgesic 0.3 mg/ml; Indivior Ltd., Chesterfield, VA, USA).

5.3 Habituation

To minimize the stress which might have affected the well-being of the animals and consequently could have had an impact on the results, the mice were habituated to handlining by a six-day step by step protocol described in detail in Table 2. The mice were also habituated to immobilization and intraperitoneal (IP) injections by giving them saline injections once per day on two to four days before the drug testing.

Table 2. The six-day protocol for habituating the mice to handling. All the steps of the protocol were carried out with each subject separately.

DAY 1	The experimenter held her hand still for 2 min and then moved it around slowly for 1 min.
DAY 2	The hand was kept still for about 20 s and then moved around, gently touching the mice, for 2 min.
DAY 3	The hand was first kept still for 30 s and then moved around the cage for the same amount of time. Then, for 2 min, the mice were repeatedly taken on the hand inside the cage and let go back in the cage.
DAY 4	The mice were taken on the hand, held there for a moment and let go back in the cage. After this the mice were taken on the hand again and taken out of the cage letting them sniff and get familiar with the experimenter.
DAY 5	The previous step was repeated after which the mice were taken out of their cages with the same cloth that was going to be used when putting them in the ICSS chambers. Attaching the ICSS cable on the electrodes was practiced with a loose ICSS cable.
DAY 6	The mice were lifted out of their cages with the cloth and put in the ICSS chambers for 5 min. Attaching the cable was practiced again and all the mice were weighed.

5.4 ICSS apparatus and software

ICSS operant chambers (Figure 6.), PHM-152 constant current stimulators and SOF-700RA-5 software package (MedAssicoates Inc., Fairfax, Georgia, United States) were provided by the Department of Pharmacology, University of Helsinki. The operant chambers were equipped with a wheel manipulandum, a metal rod floor, a metal tray for the saw dust under the floor, a flexible 18 cm long plastic-coated bipolar cable (305-305 C, Plastic One) and a two channel commutator (SL2C/SB, Plastic One) to connect the cable to the stimulator.

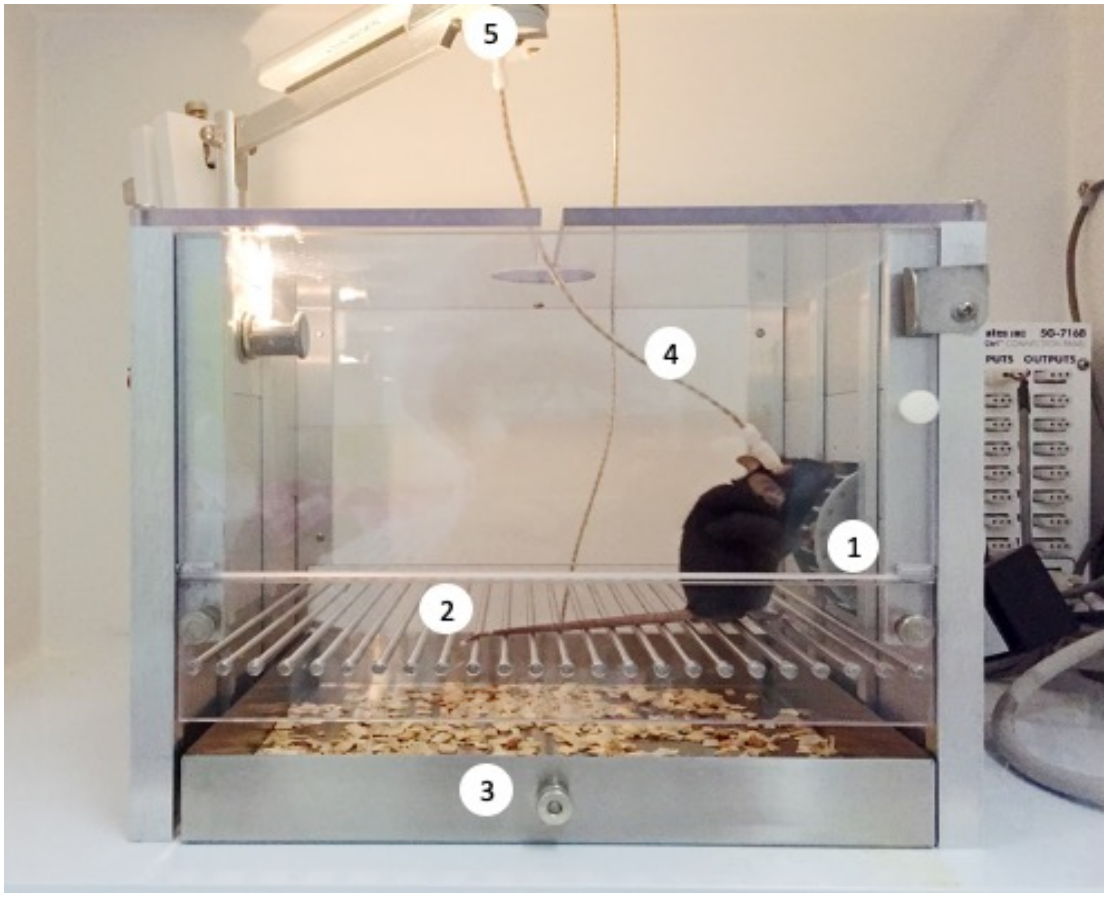

Figure 6. ICSS operant chamber. 1. The wheel manipulandum. 2. The metal rod floor. 3. The metal tray. 4. The bipolar cable. 5. The commutator. The subject is turning the wheel manipulandum to obtain a stimulation.

5.5 Stimulation

Each stimulation consisted of a train of symmetric biphasic cathodal and anodal square wave pulses, the delay times between these two and the delay times between the pulse pairs, as seen in Figure 7. The duration of each train was set to 500 ms, the delay between negative and positive pulses was set to 100 μs and the pulse width of each individual pulse to 200 μs. The number of the biphasic pulses given during each trial and the delay time between the pulse pairs depended on the selected frequency, which in this case was set to 50 Hz or 100 Hz for all except one mouse, which responded best at 150 Hz. The intensity of the stimulation was varied by adjusting the amplitude of the pulses.

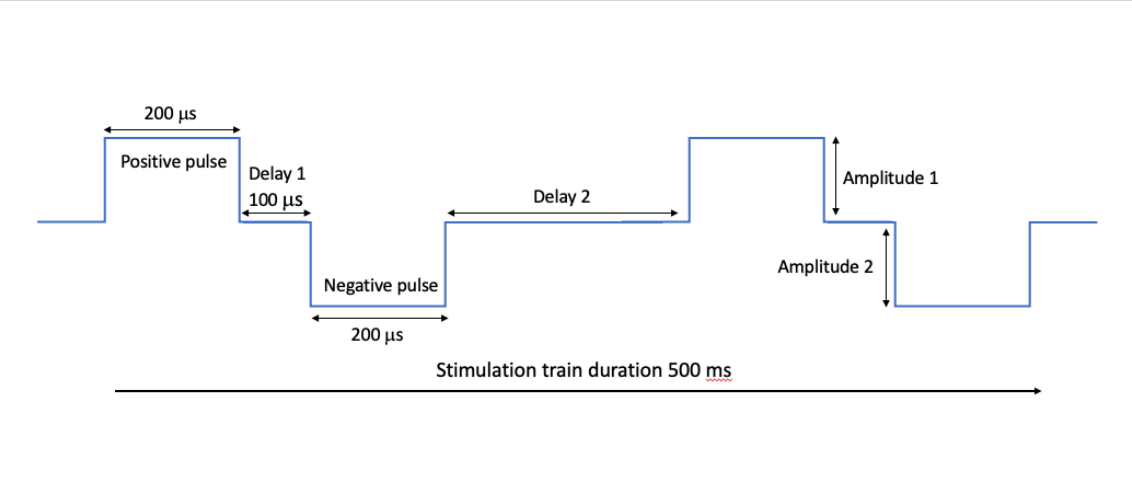

Figure 7. A fraction of a stimulation train. Duration of the delay 2 depends on the selected frequency. Amplitudes of the pulse 1 (positive pulse) and pulse 2 (negative pulse) are identical.

5.6 ICSS training

The ICSS training protocol was modified from the mouse ICSS protocol previously described by Stoker and Markou (2011) and Gill, Knapp and Kornetsky, (2004) The protocol is based on the discrete-trial current-intensity method, in which the amplitude of the pulses is the changing variable and the frequency of the pulses is fixed.

ICSS training was performed in three phases which were FR1, Detection and Reward. Each phase is described in more detail below and in Table 3. Before each ICSS session the mice were brought to the ICSS room and the filters of the individually ventilated cages were removed. The mice were let to habituate in the room for minimum 30 min before starting the session. The training, and the basal ICSS sessions between the testing days, were performed five days a week with two days break during the weekends.

Table 3. ICSS programs used in the training and testing.

Program	FR1	FR1 short	Detection short	Detection	Reward	Reward Mid
Aim	To train the mice to turn the wheel to receive a reward.	To train the mice to turn the wheel to receive a reward after slow learning in FR1.	To train the mice to respond to the non-contingent stimulus.	To detect the amplitude that the mouse responses to.	Establishing the constant basal TH	Maintaining the constant basal TH/ Testing
Time between the stimulation trains	5 s time out	2.5 s time out	ITI 1-2 sec	ITI 2.5-7.5 s	ITI 2.5-7.5 s	ITI 7.5-12.5 s
Non-contingent stimulus	-	-	+	+	+	+
Amplitude of the contingent stimulus	Fixed	Fixed	Fixed	Fixed	Same as the non-contingent stimulus	Same as the non-contingent stimulus
Proceeding to the next program	After 2-4 day to encourage the learning -> FR1 short	100 stim /10 min. -> Detection short	30 rewards/ session -> Detection	30 rewards/ session -> Reward	Sufficiently stable TH and >30 rewards/ session -> Reward Mid	SD of 3-day mean TH <10% of the mean TH-> Testing

5.6.1 FR1

The mice were trained to turn a wheel manipulandum on a fixed ratio 1 schedule of reinforcement in which each ¼ turn of the wheel caused an electric stimulus. The area where the mice could move in the chamber was restricted with a curved plastic sheet

so that the mice would stay closer to the wheel. The training was started with frequency 50 Hz, amplitude 80 μA and 5 s time out between the stimulations for all mice. The frequency and the amplitude where constant for each session, but, if necessary, they were varied between the sessions by 50 Hz or 20 μA respectively, so that the intensity would be reinforcing for the mouse. The time out was changed to 2.5 s after the first few days to encourage the learning. Once the mouse had learned to earn 100 reinforcement stimuli within 10-15 min, it proceeded to the detection program. Reaching this state took 5 to 7 training days depending on a mouse.

5.6.2 Detection and Reward

The aim of the Detection phase was to train the mouse to respond to a non-contingent stimulus by turning the wheel manipulandum, and to detect the threshold of the amplitude that the mouse responses to. The aim of the Reward program was to establish and maintain the constant basal threshold of the reward.

Both Detection and Reward programs consisted of descending and ascending trial blocks in which one individual trial equaled one stimulation described earlier. Between the trial blocks the amplitude of the pulses was either reduced or increased depending on the direction. In each trial block the mouse received five non-contingent stimuli, each one of them followed by a 7.5 s time window during which the mouse could respond by turning the wheel to receive a contingent stimulus. After the time window, or 2 s after the mouse had responded positively to the non-contingent stimulus, followed an intertrial interval (ITI) which is a time-out period varying randomly from 1 to 12.5 s depending on a training schedule as seen in Table 3. If the mouse turned the wheel during ITI, a new ITI followed as a "penalty" period before the next non-contingent stimulus was received.

The figure in Supplement 1. illustrates an example of an ICSS session. Each training session started with descending trial blocks which were reversed to the ascending ones after the mouse had reached the lower limit of the reward and responded to less than 3 out of 5 non-contingent stimuli in two consecutive trial blocks. With ascending trial

blocks the direction was reversed after the mouse had responded to at least 3 out of 5 non-contingent stimuli in two consecutive trial blocks. During the descending trial blocks the amplitude of the stimuli was reduced 5 μA between the trial blocks and during the ascending trial blocks the amplitude was increased for the equal amount. The direction was reversed four times during each training session.

The variables selected for each mouse before starting the program are shown in the table in Supplement 2. The amplitude of the stimuli of the first trial block of the training session was set 20 to 30 μA higher than the expected TH value. The estimate was based on the average TH value of the previous session. If the amplitude is set too high in the beginning of the program, the animal might receive an aversive stimulus and not respond, which would lead the program to interpret current amplitude as the lower limit of reward and raise the amplitude until it reaches the pre-set maximum value. This would potentially cause misleading results and discomfort for the animals.

In Detection program the amplitude of the contingent stimulus, which the mouse received after responding to the non-contingent one, had the same pre-set value through the whole training session. The pre-set amplitude was selected based on the previous training sessions and was aimed to be on a clearly reinforcing level.

In the Reward program the amplitude of the contingent stimulus was the same as the amplitude of the non-contingent one. Otherwise the basic principal of the Reward program and the pre-selected variables (excluding variable for stimulation 2) were the same as in the Detection program.

The mice started the Detection training with a 1 to 6 s ITI program and proceeded to a longer 2.5 to 7.5 ITI program after learning to earn at least 30 contingent stimulations during one session. When at least the same number of contingent stimulations per session were earned in the longer ITI program, the animals proceeded to the Reward phase.

The ITI time used in the beginning of the Reward training was 2.5 to 7.5 s. After 6 to 14 days of training with the shorter ITI time, when the animals had a sufficiently acquired

earning the reward stimulations and the TH started to stabilize, the mice proceeded to a longer 7.5 to 12.5 s ITI Reward program which was also used in the testing phase. When the standard deviation of the daily mean THs was less than 10% of the last three days' mean TH, the mice proceeded to the testing phase.

The TH is the most important parameter obtained with ICSS as it tells at which level of intensity the subject finds the stimulation rewarding. The training session image in Supplement 1. shows how the mean TH of each ICSS session is calculated. The parameters that were collected and analyzed after each training and testing session were the mean TH of the session, the mean latency time (the time between the non-contingent stimulus and the response), total number of reward stimulations, ITI response per minute, the total number of responses and the session duration. The standard deviation and the mean of the last three days' mean TH values were calculated daily.

5.7 Drugs

The drugs used in the experiments were LSD 50, 100 and 200 μg/kg (Sigma-Aldrich Corp., Saint Louis, Missouri, United States), D-amphetamine sulphate 3.0 mg/kg (Smith Kline & French Laboratories Ltd, London, United Kingdom) and LPS 0.05 mg/kg (*E.coli* strain 0111:B4, Sigma-Aldrich Corp., Saint Louis, Missouri, United States) All drugs were dissolved in sterile saline and administered as IP injections. Saline vehicle was used as a control in all experiments.

5.8 Experimental design

5.8.1 The acute effects of LSD on ICSS

Each mouse received three different IP administered doses of LSD (50, 100 and 200 μg/kg) and one dose of saline control in random order. The experimenter was blinded for the treatments. After the drug injection, the mice had two consecutive ICSS Reward program sessions. The mice were placed in the operant chambers directly after the drug administration, attached to the cables, the ICSS Reward program was started and repeated immediately after the first session had finished.

Testing was performed once a week. Between the testing sessions, the mice had a daily ICSS basal session on weekdays and a two-day break on the weekends. The basal sessions followed the same protocol as during the training phase to regain the stable baseline TH. With some mice, seven days was not enough for the TH to stabilize after the testing, in which case they were given more basal session days between the testing sessions.

5.8.2 The acute effects of LSD on amphetamine-induced changes in ICSS

Each mouse had three different combinations of two injections with minimum one week in between the administrations. The combinations were amphetamine (3.0 mg/kg) and LSD (100 μg/kg), amphetamine (3.0 mg/kg) and saline vehicle and two injection of saline vehicle. The experimenter was blinded for the treatments. The IP injections were given consecutively on the right side of the peritoneal area. After the drug treatment the mice were placed in the operant chambers and the Reward program was run twice as in the previous experiment.

5.8.3 The acute effects of LSD on LPS-induced changes in ICSS (preliminary test)

Each mouse received an IP injection of LPS (0.05 mg/kg) followed by an IP injection of LSD or saline vehicle 4 hours later. All the mice received both combinations and served as their own controls. Water consumption and the body weight were measured before and after the LPS administration by weighing the water bottles and the animals in four different time points; at 4 pm the day before the administration of LPS, at 6:50 am right before the administration of LPS and 4 pm and 6.50 am after the administration of LPS. Two control water bottles were weighed at the same time points to detect the average amount of leakage.

5.9 Data analysis

All data analysis was performed with GraphPad Prism8 (GraphPad Software, LLC, San Diego, California, USA). The results of the dose dependent effects of LSD and the LSD-amphetamine experiments were analyzed using repeated measures one-way analysis of variance (ANOVA) with Geisser-Greenhouse correction and Bonferroni's multiple comparisons test with individual variances computed for each comparison. A two-tailed paired t-test was used to analyze the results of the LSD + LPS experiment. The level of significance for all the results was set to $P < 0.05$.

6 RESULTS

6.1 ICSS training

On average, 43 ICSS training sessions were needed before the THs were stable enough for the subjects to proceed to the testing phase. The amount of days each mouse spent in each phase of the training can be seen in Table 4. The values of the main parameters in the end of the training phase are shown in Table 6. 11 out of 20 mice completed the

training and entered the first experiment, but two of these had to be excluded during the experiment. The Table 5. shows the reasons for all exclusions. The brains of the excluded animals were removed and frozen in -70 °C. After the last experiment, the locations of the electrodes in all the brains were examined. The locations are shown in the figure in the Supplement 3. The Figure 8. shows the number of days that was required for each step of the entire project.

Table 4. The number of days each subject required in each phase of the ICSS training.

ID	Surgery	FR1	FR1 short	Detection short	Detection	Reward	Reward Mid	Test	Exclusion
1	+	3	2	1	1	6	24	+	
2	+	2	2						+
3	+	3	2	1	1	9	26	+	
4	+	3	2	1	1	8	32	+	
5	+	3	3	11	4	2	29	+	+
6	+	3	6	1	1	14	4		+
7	+	3	5						+
8	+	3	3	11	4	3	45		+
9	+	3	3	1	1	7	29	+	
10	+	3	3	1	1	6	30	+	
11	+	3	3	1	1	5	43		+
12	+	3	3	1	1	5	24	+	
13	+	4	1	1	1	6	28	+	
14	+	2	1						+
15	+	3	4						+
16	+	3	2	1	1	12	18	+	
17	+	3	2	1	1	8	35	+	+
18	+								+
19	+	5		1	1	7	31		+
20	+	3	4	3	1	6	25	+	

Table 5. Causes of exclusions. The numbers indicate the number of the subjects excluded.

	Did not recover well after surgery	Did not acquire icss	Unstable TH	Too high iti/min	Lost the electrode	Declining health
Number of the mice	1	5	2	1	1	1

Table 6. Values of the main ICSS parameters in the end of the training phase for all the subjects that entered the testing phase.

ID	Frequency Hz	Mean TH µA	Mean ITI/min	Mean latency s	Starting amplitude µA	Max amplitude µA
1	50	49	7	3	75	105
3	50	85	15	3	115	145
4	100	32	3	3	60	90
5	150	94	4	4	115	145
9	100	70	1	3	95	125
10	100	61	7	3	80	110
12	100	50	11	3	80	110
13	50	30	6	3	65	90
16	100	46	2	3	75	105
17	50	35	28	3	70	100
20	50	193	3	3	215	230

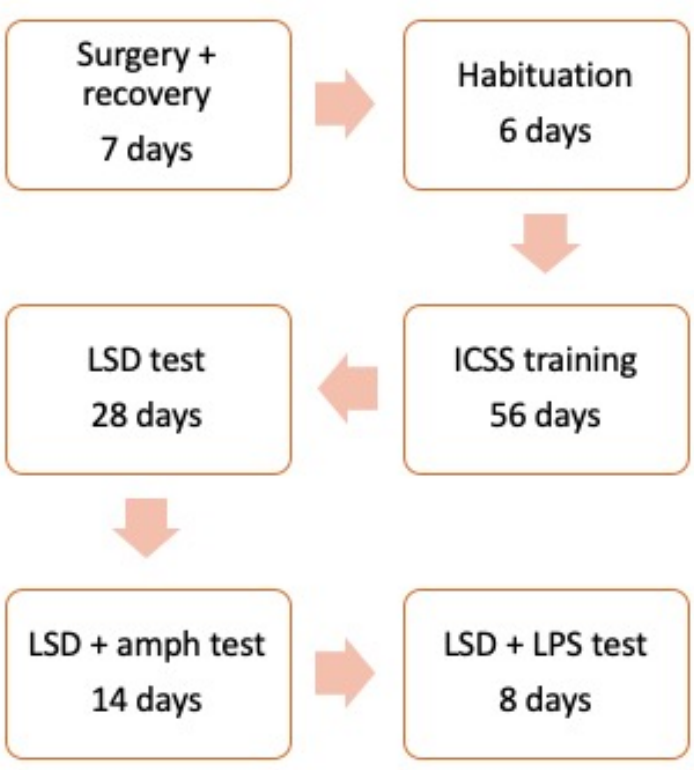

Figure 7. The number of the days required for each step of the project. Between the experiments, there was a minimum seven-day wash-out period. The amount of the training days and the basal sessions needed between the testing days varied between the animals. This timeline shows the number of days for the first mice that entered the testing phase.

6.2 The acute effects of LSD on ICSS

As seen in Figure 9., the repeated measures ANOVA of ICSS results of acute effects of LSD, did not show significant effect on the TH [session 1: $F(2.335, 18.68) = 1.553$, $P = 0.2371$, session 2: $F(1.983, 15.86) = 0.3811$, $P = 0.6875$] , ITI response rate [session1: $F(1.625, 13.00) = 0.6757$, $P = 0.4962$, session 2: $F(1.614, 12.92) = 0.1102$, $P=0.8563$] or mean latency time [session1: $F(2.395, 19.16) = 0.6031$, $P = 0.5857$, session 2: $F (2.525, 20.20) = 1.125$, $P = 0.3552$] on the tested doses on either of the two ICSS sessions conducted on the days of testing. On the second session, it could be noticed that the individual TH values were closest to basal level with the two highest doses of LSD, whereas with saline and the 50 μg/kg dose the individual results where more scattered (standard error of mean (SEM): Sal = 7.461, 50 μg/kg = 7.589, 100 μg/kg = 3.692, 200 μg/kg = 4.292) .

Figure 10. shows the TH values 24 hours after the LSD treatment. No significant differences were observed between the treatments in the repeated measures ANOVA [$F (2.039, 16.31) = 0.9597$, $P = 0.4052$]. However, the TH values were more scattered after the LSD treatment than after the saline treatment (SEM of the second session TH: Sal = 3.165, 50 μg/kg = 6.282, 100 μg/kg = 4.598, 200 μg/kg = 7.151).

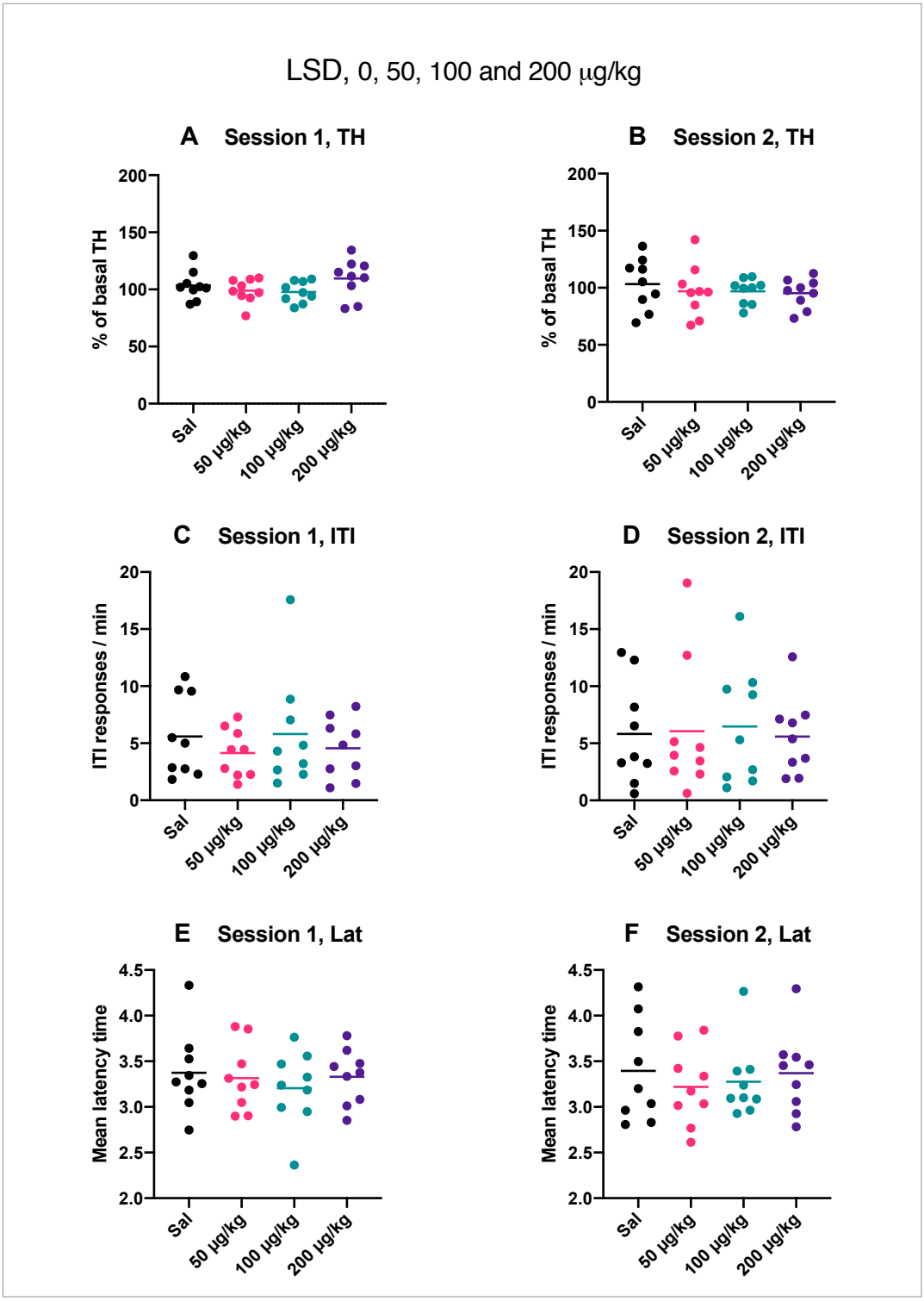

Figure 9. LSD did not affect the reward TH, ITI responses or mean latency time on any of the tested doses. N=9. The results of the repeated measures ANOVA. (A) 1st session TH. (B) 2nd session TH. (C) 1st session ITI. (D) 2nd session ITI. (E) 1st session latency time. (F) 2nd session latency time. The scattered dots present the values of the individual subjects. The line is at the mean. The TH values are presented as percentages of the basal level TH, ITI values present the number of responses per minute and mean latency time is presented in seconds. TH=reward threshold, ITI=intertrial interval, Lat=mean latency time, Sal=saline control.

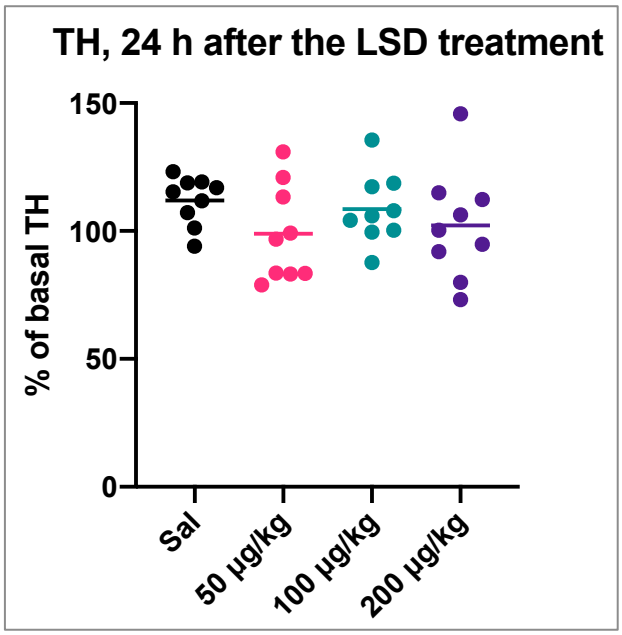

Figure 10. The TH values 24 hours after the LSD treatment. N=9. The results of the repeated measures ANOVA. The scattered dots present the values of the individual subjects. The line is at the mean. The TH values are presented as percentages of the basal level TH. TH=reward threshold, Sal=saline control.

6.3 The acute effects of LSD on amphetamine-induced changes in ICSS

Figure 11. shows the main results of the repeated measures ANOVA and the Bonferroni's multiple comparisons test of the acute effects of LSD and amphetamine in ICSS. In the second ICSS session of the treatment day, amphetamine significantly reduced the TH compared to the control, both with saline and LSD [$F(1.954, 11.73) = 9.366$, $P = 0.0038$, sal-sal vs. amph-sal: $P = 0.0312$, sal-sal vs. amph-LSD: $P = 0.0245$]. LSD did not have a significant effect on amphetamine-induced decrease of the TH in either of the sessions.

The number of ITI responses per minute dropped noticeably after the amphetamine treatment, with and without LSD, in both testing day ICSS sessions [session 1: F(1.012,

6.073) = 7.223, *P* = 0.0355, session 2: F(1.139, 6.833) = 22.70, *P* = 0.0019]. The *post hoc* analysis showed significance between the treatments in the second session (sal-sal vs. amph-sal: *P* = 0.0120, sal-sal vs. amph-LSD: *P* = 0.0048). There were no significant differences between the amphetamine-saline and amphetamine-LSD treatments.

In the results of the second session, the treatment with amphetamine and LSD significantly lengthened the latency times compared to the saline-saline treatment, but no significance was observed when compared to amphetamine-saline treatment [F(1.686, 10.12) = 5.272, *P* = 0.0309, Sal-Sal vs. Amph-LSD: *P* = 0.0193].

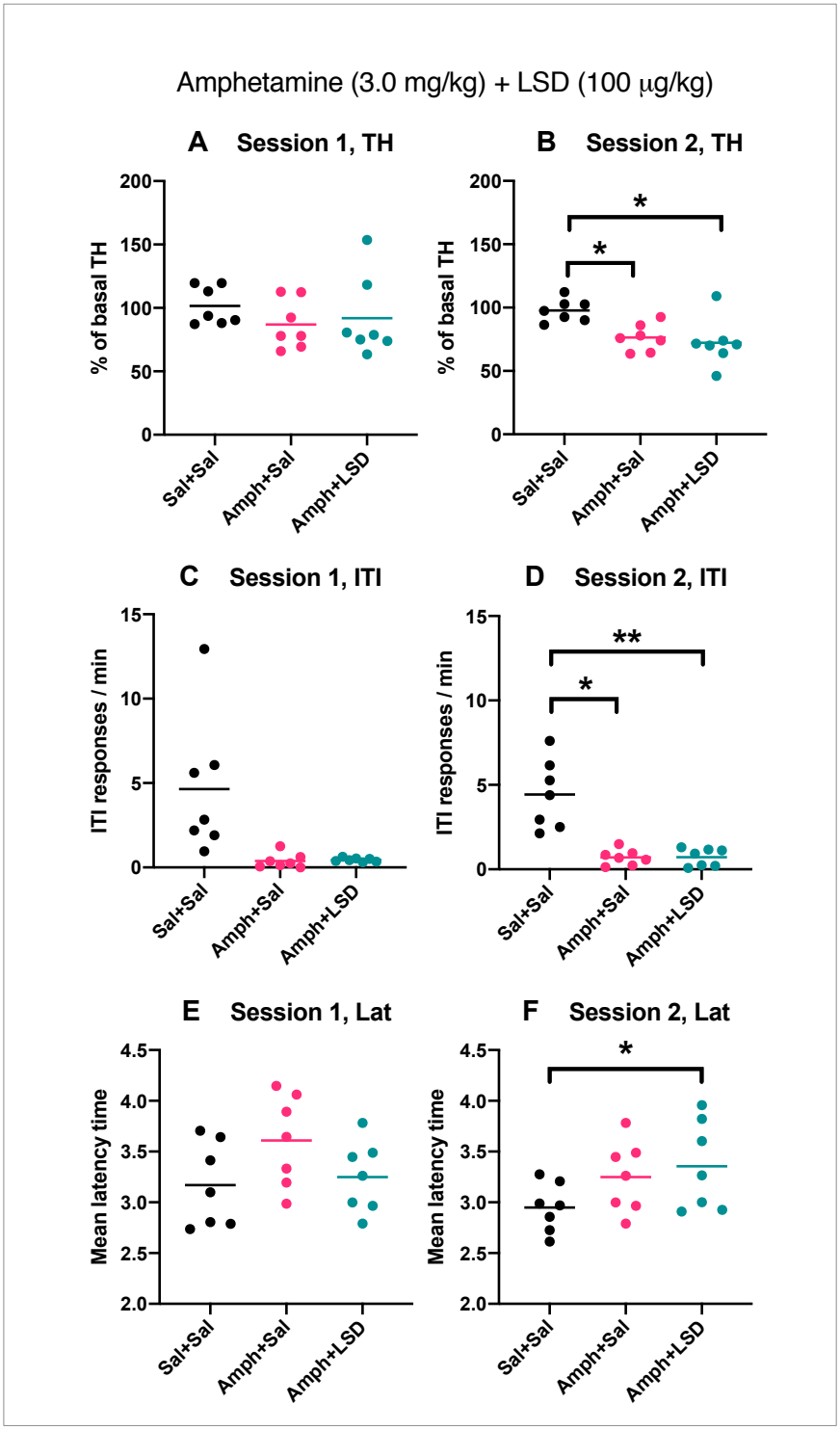

Figure 10. LSD did not affect the amphetamine-induced changes in ICSS.
N=7. The results of the repeated measures ANOVA and Bonferroni's multiple comparisons test with individual variances computed for each comparison. (A) 1st session TH. (B) 2nd session TH. (C) 1st session ITI. (D) 2nd session ITI. (E) 1st session latency time. (F) 2nd session latency time. The scattered dots present the values of the individual subjects. The line is at the mean. The TH values are presented as percentages of the basal level TH, ITI values present the number of responses per minute and mean latency time is presented in seconds. Amph =amphetamine, TH = reward threshold, ITI = intertrial interval, Lat = mean latency time, Sal = saline control, *= $p<0.05$, **= $p<0.005$

6.4 The acute effects of LSD on LPS-induced changes in ICSS

This experiment was a pilot test conducted with four animals. The results were analyzed by comparing the two different treatments (LPS-saline and LPS-LSD), and also by comparing the results of the first and the second LPS treatment. Two of the mice received the LSD after the first LPS treatment, and the other two after the second LPS treatment. Figures 12. and 13. show the results of this experiment.

The TH was increased above basal level after LPS-saline, as well as LPS-LSD treatment. The increase appeared moderately lower and the ITI response rate higher after the LPS-LSD treatment, yet according to the two-tailed paired t-test these changes were statistically non-significant (second session, TH: P = 0.4802, ITI: P = 0.3467, Lat: P = 0.9265)

The analysis of the results by the order of the LPS treatments, showed that the increase of the TH was lower after the second LPS treatment, the difference being significant in the first ICSS session of the treatment day (P = 0.0395). Also, the number of the ITI responses was higher and the latency time shorter after the second LPS administration, although these differences were non-significant (second session, TH: P = 0.0575, ITI: P = 0.0957, Lat: P = 0.1438).

Figure 14. shows the water consumption after the LPS treatments. The water consumption was higher and closer to basal level after the second LPS administration. After the LSD treatment, the water consumption was reduced. Neither of these results were statistically significant

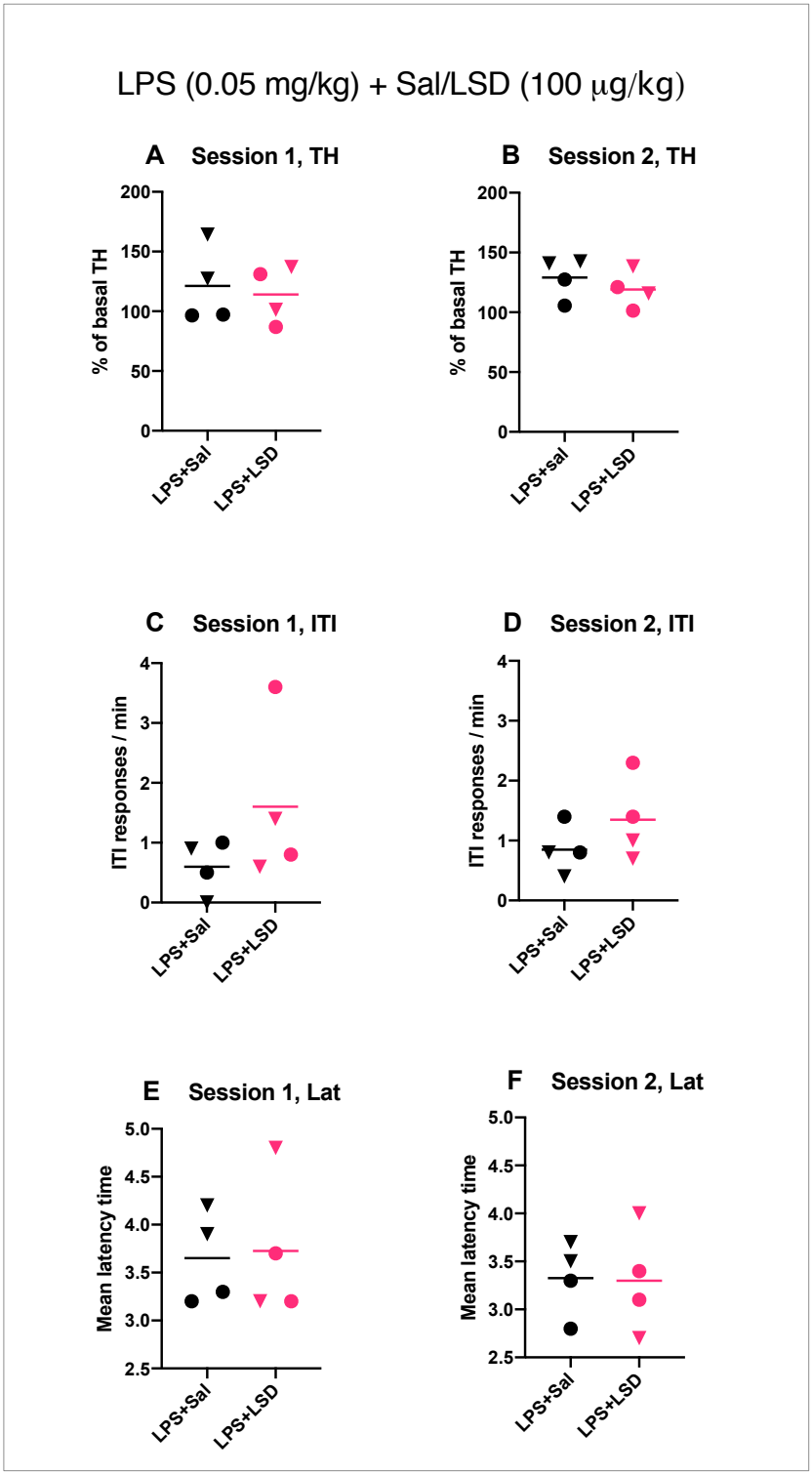

Figure 12. The results of the LSD-LPS experiment by the treatment.
N=4. The results of the two-tailed paired t-test. (A) 1st session TH. (B) 2nd session TH. (C) 1st session ITI. (D) 2nd session ITI. (E) 1st session latency time. (F) 2nd session latency time. The scattered symbols present the values of the individual subjects. The triangular symbols mark the mice that received LPS for the time. The line is at the mean. The TH values are presented as percentages of the basal level TH, ITI values present the number of responses per minute and mean latency time is presented in seconds. TH=reward threshold, ITI=intertrial interval, Lat=mean latency time, Sal=saline control, LPS=lipopolysaccharide.

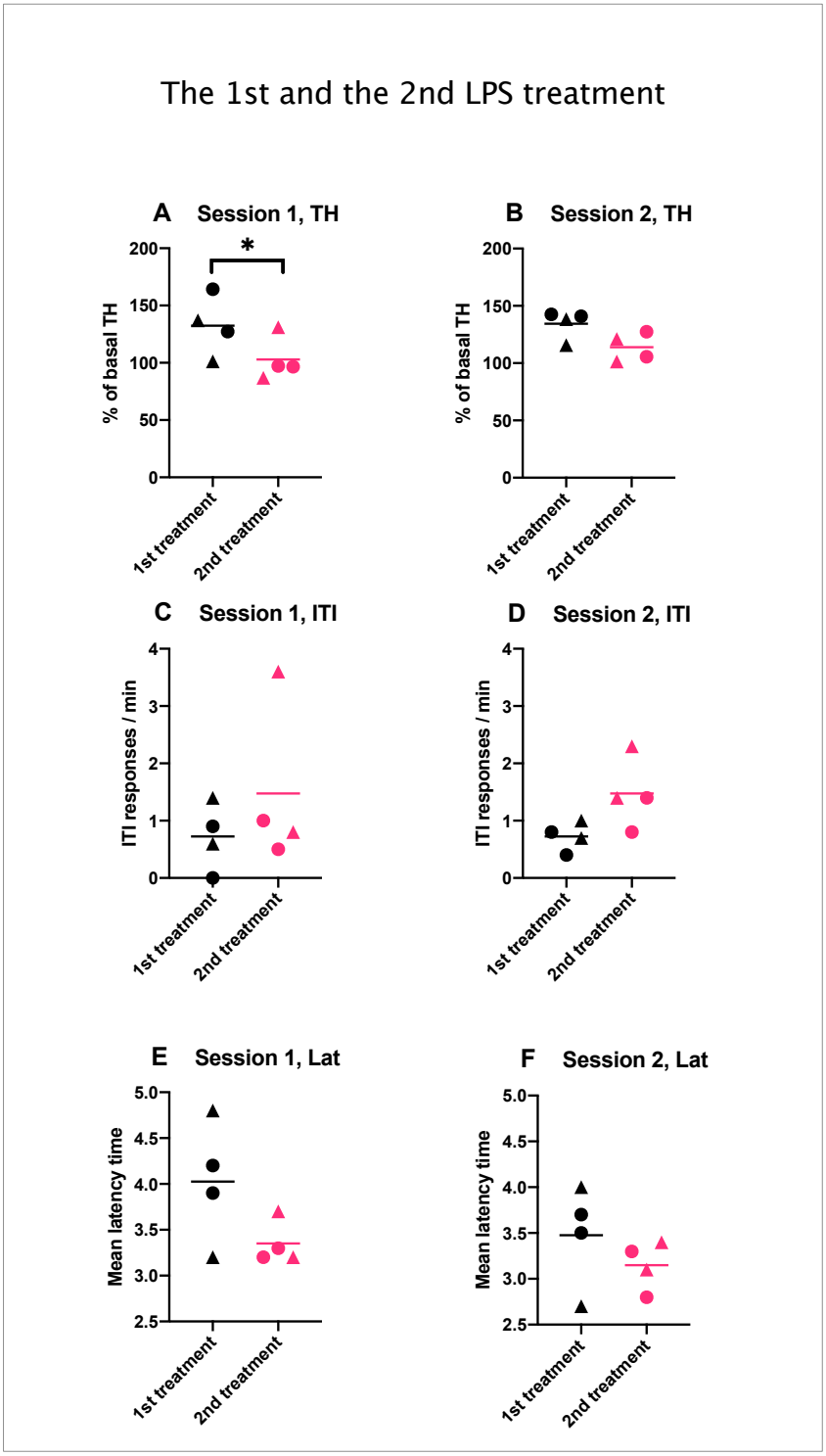

Figure 13. The effects of the first and the second LPS administration.
N=4. The results of the two-tailed paired t-test. (A) 1st session TH. (B) 2nd session TH. (C) 1st session ITI. (D) 2nd session ITI. (E) 1st session latency time. (F) 2nd session latency time. The scattered symbols present the values of the individual subjects. The triangular symbols mark the mice that were treated with LSD. The line is at the mean. The TH values are presented as percentages of the basal level TH, ITI values present the number of responses per minute and mean latency time is presented in seconds. TH=reward threshold, ITI=intertrial interval, Lat=mean latency time, Sal=saline control, LPS=lipopolysaccharide,
*= $p<0.05$.

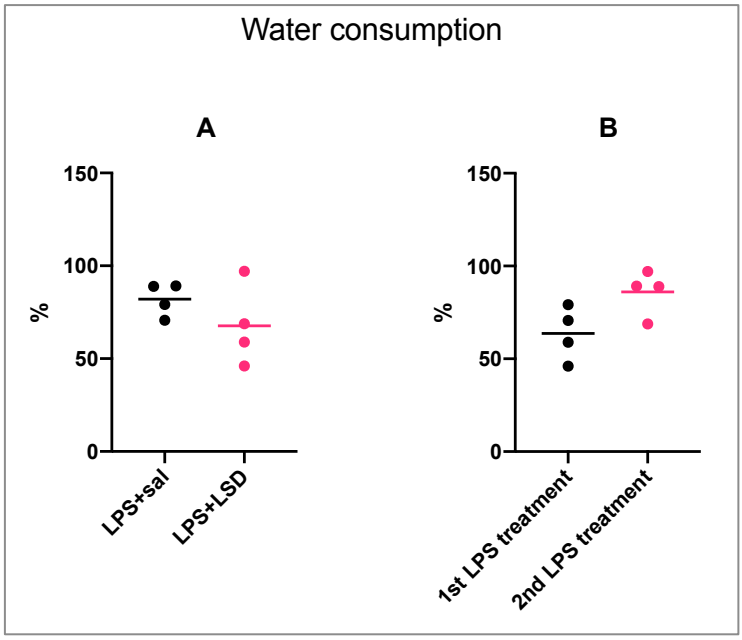

Figure 14. Water consumption after the LPS treatments.
N=4. The results of the two-tailed paired t-test. The water consumption is shown as a percentage of the basal level water consumption. (A) The water consumption after the LPS-sal and LPS-LSD treatments. (B) The water consumption after the first and the second LPS treatment. The scattered dots present the values of the individual subjects. The line is at the mean. Sal=saline control, LPS=lipopolysaccharide.

6.5 The basal TH during the testing phase

Figure 15. shows the three-day mean TH values on four different time points of the testing phase for the four subjects that went through all three experiments. By the end of the last experiment the animals had received LSD five times (1x50 μg/kg, 3x100 μg/kg and 1x200 μg/kg) with minimum seven days between the administrations. The repeated LSD treatment did not result in any apparent trend in the basal TH.

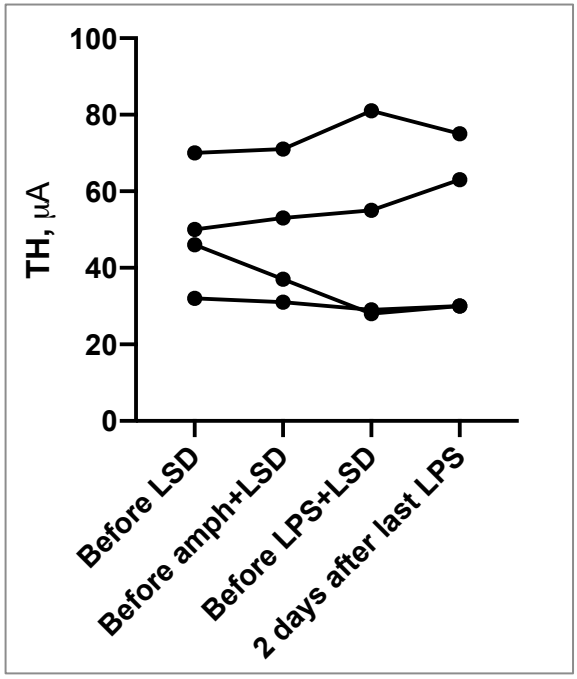

Figure 15. The three-day mean TH values on four different time points during the testing phase. The values are shown for the four mice which completed all of the three experiments. Amph = amphetamine, TH = reward threshold, LPS = lipopolysaccharide.

7 DISCUSSION

To my knowledge, this is the first discrete-trial current-intensity ICSS study assessing the effects of LSD in mice. The only other modern study in which the effects of LSD have been tested in ICSS was performed with rats and with a different ICSS protocol. This recent rate-frequency curve-shift protocol study by Sakloth *et al.*(2019) concluded that the curve-shift to the right and the reduced response rate induced by a high dose of LSD supports the earlier reported low addiction liability of the drug. In the current body of work, a similar kind of attenuation of ICSS, which in discrete-trial current-intensity protocol would show as a higher TH, was not observed. One probable explanation for the differing results is the higher dose of LSD used by Sakloth *et al*. However, even though LSD failed to induce attenuation of ICSS reward in the current experiment, it is worth noting that there was no evidence of facilitation of the reward behavior either. This finding further supports the previous observations about non-addictive nature of LSD.

As expected based on earlier results (e.g. Esposito, Perry and Kornetsky, 1980), amphetamine induced a robust facilitation of ICSS reward which manifested as reduced TH. The reduction of ITI responses was in line with the previous results from our laboratory (Lainiola, Hyytiä and Linden, unpublished data). LSD had no effect on these amphetamine-induced changes. The reduced ITI responses may be a consequence of amphetamine-induced improved attention reported before in mice, as well as in humans (MacQueen *et al.*, 2018). Amphetamine-LSD treatment increased the latency time, although a milder, statistically non-significant, increase was also seen after the amphetamine-saline treatment. The longer latency time observed after amphetamine treatments is also consistent with the previous unpublished results from our research group. These results were obtained with C57BL/6J mice, whereas earlier studies with rats, conversely, reported shorter latency times after acute amphetamine (Lin, Koob and Markou, 2000; Paterson, Myers and Markou, 2000). The observation that amphetamine seems to slow down the responding to the non-contingent stimulus in C57BL/6J mice, is not easy to interpret. Slower motor functions as a result of an amphetamine treatment seem incompatible with general stimulatory effects of the drug. Although, there is a possibility that the responding has been slowed down by increased activity e.g. running around the operant chamber. However, based on these results, LSD seems to mildly amplify the amphetamine-induced increase of the latency time.

Whereas several 5-HT_{2C} agonists have been shown to attenuate ICSS and block the drug induced facilitation, based on these results, this does not seem to take place with LSD. Presumably, the effect of 5-HT_{2C} activation may be abolished by the oppositional effect of the 5-HT_{2A} activation. On the other hand, as LSD did not induce facilitation of ICSS either, the DA-release-enhancing effect of 5-$HT_{2A.}$ activation does not seem to be plainly overpowering the effect of 5-HT_{2C} activation. It could be hypothesized that higher doses of LSD might emphasize the role of 5-HT_{2C} activation, which would explain the reduced response rates observed earlier by Sakloth *et al.* (2019).

In the LPS-LSD experiment, the subjects treated with LSD after LPS administration had a mildly lower TH and a higher ITI response rate than the ones treated with saline after

LPS. However, the analysis of these results by the order of the LPS administration showed that after the second LPS administration the TH values were significantly lower, ITI responses increased and latency times were shorter than after the first administration. This suggests that the LPS-induced anhedonia might have been milder after the second administration. It has been reported before that a daily exposure to LPS on consecutive days causes tolerance to its anhedonic effects (Barr *et al.*, 2003). Despite the seven-day wash-out time between the LPS administrations, these results suggest that the mice had developed tolerance to LPS, and the treatment order had more impact on the results than the combination of the drugs. This is further supported by the observation that the water consumption was higher after the second LPS treatment. Interestingly, when the water consumption was viewed by the drug treatment, LSD administration seems to have had the opposite effect, reducing the consumption. However, because of the possible tolerance to LPS and the low number of the subjects, further conclusions or a proper evaluation of the possible effects of LSD are not possible based on these results.

For the four mice that went through all three experiments, the baseline TH remained relatively stable until the end of the testing period. The fact that the basal THs did not show any clear trend of change after repeated LSD, amphetamine and LPS treatments, supports the idea that the same animals may be used in multiple experiments, once trained to acquire ICSS. As the ICSS training of the animals is a highly time-consuming process, this observation may be of value regarding future experiments.

A large number of mice had to be excluded during the ICSS training and the testing phase. The majority of these did not acquire the self-stimulation and appeared to be either oblivious to the stimulation or clearly avoiding the wheel and finding the stimulation unpleasant. Most typical reason for this, according to Stoker and Markou (2011), is the misplacement of the electrode. In this case however, most electrodes appeared to be correctly implanted into the LH as seen in the Supplement 3. The mice used in these experiments were obtained from a different provider than the C57BL/6J mice that were used in earlier ICSS experiments in our laboratory, which might imply

that these particular animals were not ideal for ICSS. In addition, some other modifications, such as lighter plastic cables and variations to the ITI times of the ICSS programs, took place and may have affected the training results. In general, with the successfully trained animals, the ICSS method used in these experiments proved to be well suited for the assessment of pharmacological manipulations of the reward behavior, as was clearly seen in the amphetamine and LPS experiments.

7.1 Conclusions

In summary, it can be concluded that acute LSD does not affect reward behavior in ICSS on the tested doses. Accordingly, LSD does not affect the facilitation of ICSS induced by acute amphetamine. These main findings of the current body of work suggest that the previously reported LSD-induced reduction in ethanol consumption in mice is not mediated through alteration of reward mechanism. At the same time, these findings provide further evidence supporting the suggestion that LSD itself does not induce facilitation of the reward circuitry needed for the development of addiction. Considering the possible therapeutic use of LSD, low addiction potential is a highly important factor regarding its safety.

7.2 Future prospects

As the results of the amphetamine-LSD experiment imply, the possible efficacy of LSD in treatment of substance use disorders does not seem to lie in the acute alteration of the functioning of the reward mechanism in the binge / intoxication stage. However, it would be of interest to have another look on the possible effects of LSD in the anhedonic withdrawal / negative affect stage. This could be done by assessing the effects of acute LSD with ICSS in amphetamine withdrawal and also, by conducting another LPS-LSD experiment, this time with separate groups of animals for each treatment to avoid the development of tolerance to LPS. In addition, it would be worthwhile conducting ICSS experiments with the more selective 5-HT_{2A} agonist 25CN-NBOH (Jensen *et al.*, 2017). 25CN-NBOH has the highest known selectivity to the 5-HT_{2A} receptor, therefore it would be compelling to test the effects of this molecule in ICSS to see whether they differ from

those of the less selective LSD. Also, more selective 5-HT_{2C} agonists, such as lorcaserin, that at least in normal therapeutic doses, lack the psychoactive effects of psychedelics, offer an interesting target for further research for finding more effective pharmacotherapies for the treatment of alcohol dependence and other addictions.

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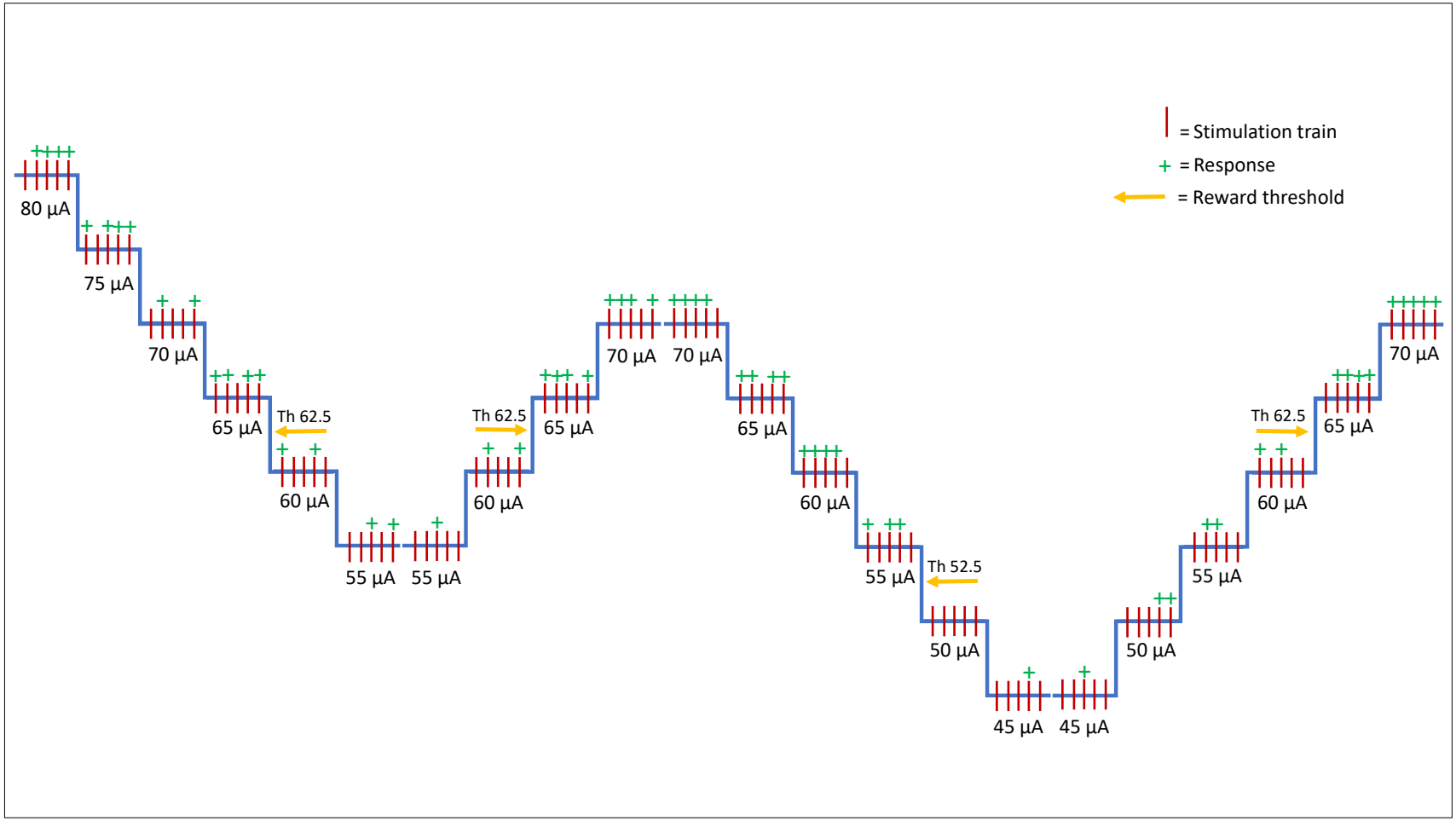
SUPPLEMENT 1



An example of an ISCC session. In this example the mouse has been performing a reward phase ICSS session with frequency 100 Hz and starting amplitude 80 µA. The session has started with descending trial blocks and the amplitude has been reduced 5 µA between each trial block. After the amplitude 65 µA the mouse has not responded positively (responded to less than 3 out of 5 trials) for two consecutive trial blocks after which the direction has changed to ascending. At 65 µA the mouse has started to respond positively again and has done so for two consecutive trial blocks after which the direction has changed again. The four threshold values obtained on this session have been, 62.5 µA, 62.5 µA, 52.5µA and 62.5 µA, which makes the average threshold of the session 60 µA. The length of this session has been 31 minutes.

SUPPLEMENT 2

The variables selected before each ICSS session.

Variable	Description	Used value
Step size	The amount (in µA) that the amplitude was increased or decreased during training or testing.	5 µA
Pulse width #1	The duration of the positive pulse.	200 µs
Pulse amplitude #1	The amplitude (intensity) of the positive pulse.	60-240 µA
Pulse delay	The delay between positive and negative pulses.	100 µs
Pulse width #2	The duration of the negative pulse.	200 µs
Pulse amplitude #2	The amplitude (intensity) of the negative pulse.	60-240 µA
Frequency	The frequency of the pulse pairs.	50-150 Hz
Pulse train duration	Entire duration of the stimulation train.	500 ms
Pulse amplitude for Stim2	This variable was used only in the detection program. It determined the amplitude of the non-contingent stimulus that remained constant during the session.	60-240 µA
Starting block type	Start with descending or ascending trial blocks.	Descending
Response time	Time window for response to non-contingent stimulus.	7.5 s
Min stimulation amplitude	Minimum amplitude of a stimulation to be supplied.	5 µA
Max stimulation amplitude	Maximum amplitude of a stimulation to be supplied.	90-250 µA

SUPPLEMENT 3

A graphical presentation of the electrode placement.