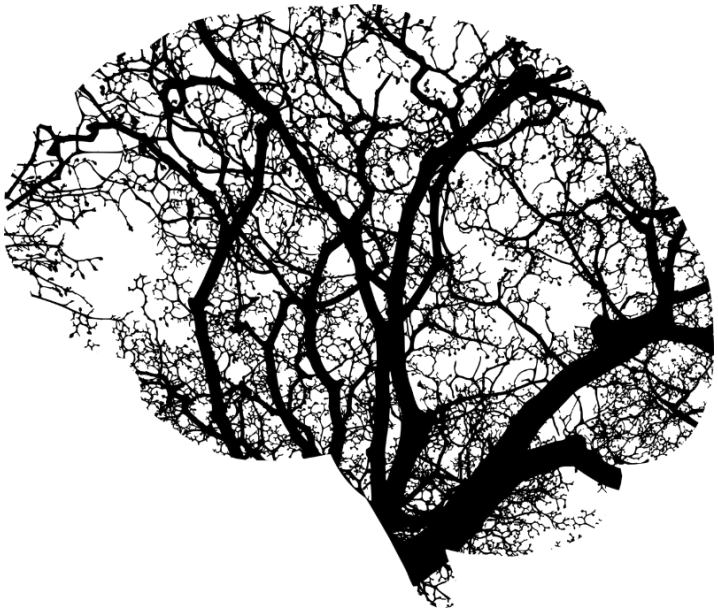
AMPHETAMINE INDUCED NEUROPLASTICITY IN BRAIN REGIONS INVOLVED IN CONDITIONED DRUG EFFECTS AND RELAPSE



- FINAL BACHELOR THESIS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY -

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Work based on the results obtained during my extracurricular internship at the department of pharmacology of the Institute of Neuroscience and Physiology (University of Gothenburg), under the mentorship of Prof. Louise Adermark.

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1. ABBREVIATIONS

ATS	Amphetamine-type stimulants
BLA	Basolateral amygdala
nAc	Nucleus accumbens
VTA	Ventral tegmental area
DA	Dopamine
GABA	Gamma-aminobutyric acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
LMA	Locomotor activity
aCSF	Artificial cerebrospinal fluid
I/O	Input/Output
PPR	Paired pulse ratio
fEPSPs	Field excitatory postsynaptic potentials
sEPSCs	Excitatory postsynaptic currents

2. ABSTRACT

Amphetamine addiction is associated with a continued use even though it can lead to negative consequences and a high risk of consumption relapse. The mechanisms underlying the development of drug addiction have not yet been established, but neuroplasticity in brain circuits involved in the regulation of incentive motivation and reward-based behaviour may play a key role. The following project aims to study the occurrence of long-lasting neuroadaptations induced by repeated amphetamine exposure in the subregions of the brain basolateral brain amygdala (BLA) and nucleus accumbens (nAc) in male rats. For this purpose, monitored arenas have been used to study changes in locomotor activity caused by amphetamine exposure, as well as electrophysiological techniques, such as field potential recordings and single cell recordings (voltage clamp) to monitor neurochemical alterations.

Statistical analyses suggest the appearance of neuroadaptations inducing behavioural sensitization after one week of amphetamine administration. Moreover, BLa and nAc exhibited neuroadaptations involving an increase in the GABAergic inhibitory tone leading to a reduction in the probability of neurotransmitter release and a suppressed neuronal excitability. The observed neurological alterations could imply important long-term health risks, in addition to altering different signalling circuits that drive the reward-seeking responses, closely associated to the BLA and nAc.

Key words: amphetamine, drug addiction, mesolimbic system, reward-seeking behaviour, dopamine, basolateral amygdala, nucleus accumbens, behavioural sensitization, neuroadaptations, neuroplasticity, electrophysiology, field potential recordings, single potential recordings, voltage clamp, GABAergic tone, neuronal excitability.

3. INTRODUCTION

3.1 Amphetamine and its usage

Drugs have been used through the history by many civilisations and for many different purposes, such as religion, rituals, magic, medicine, or just for fun. There are indications that natural drugs were already used in civilizations such as in Mesopotamia or the ancient Egypt and have been used throughout time on many other occasions. For example, it has been proven that cannabis was already cultivated in China more than 4000 years ago, and that the Inca empire in Central America used cocaine leaves as an analgesic (Corrêa de Carvalho, et al. 2007). Nowadays, consumption of drugs has increased a lot and many new types of synthetic drugs have arisen due to the improvement and availability of chemical synthesis techniques and protocols.

The following project focuses on amphetamine (α-methylphenethylamine), see structure in **figure 1**. This psychoactive molecule was discovered in 1910, but it was not until 1927 when it was first synthesised in the lab by G. A. Alles, trying to find a substitute for the plant alkaloid ephedrine (Heal, et al. 2013), which was used for treating asthma and upper respiratory infections (Nader, et al. 2020). Amphetamine is a psychoactive molecule derived from β-phenylethylamine (Carvalho, et al. 2012). It is a phenethylamine that has a single chiral center and two different active optical configurations: levo- and dextroamphetamine, where the second one is the most potent isomer (Heal, et al. 2013).

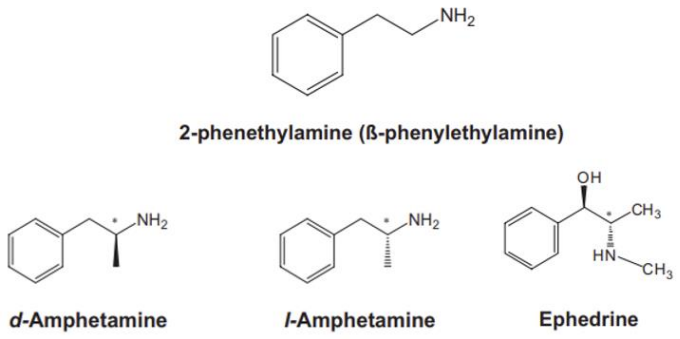

***Figure 1**. Chemical structures of β-phenylethylamine, d-amphetamine, l-amphetamine and ephedrine. Source form (Heal,. et al. 2013).*

Amphetamine-type stimulants (ATS) consumption and production has been increasing throughout the last years on a global scale according to the UNODC (United Nations Office on Drugs and Crime). In accordance with their last study (made in 2022), in 2020 there were

around 34 million people recorded as amphetamine users, while the total production of ATS (methamphetamine, amphetamine, ecstasy and others) reached the total amount of 525 tones. That represents a 15% increase in terms of production comparing the data from 2019. The annual evolution of ATS production is shown in **figure 2**.

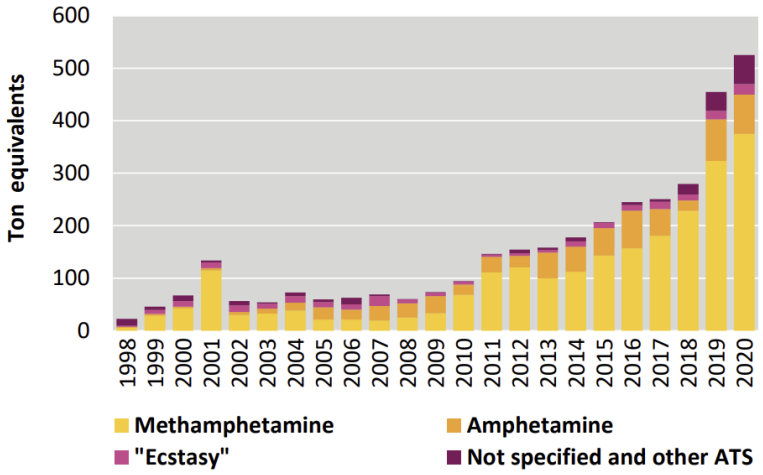

***Figure 2**. Global quantity of amphetamine-type stimulants sized (ATS), years 1998 to 2020. Source from UNODC, World Drug Report 2022 (United Nations publication, 2022).*

Regarding the trafficking of ATS, as shown in **figure 3**, methamphetamine has been leading for the past years. During the period 2016 to 2020, 72% of the ATS sized corresponded to methamphetamine, followed by amphetamine (17%) and to a lesser degree “ecstasy” (4%).

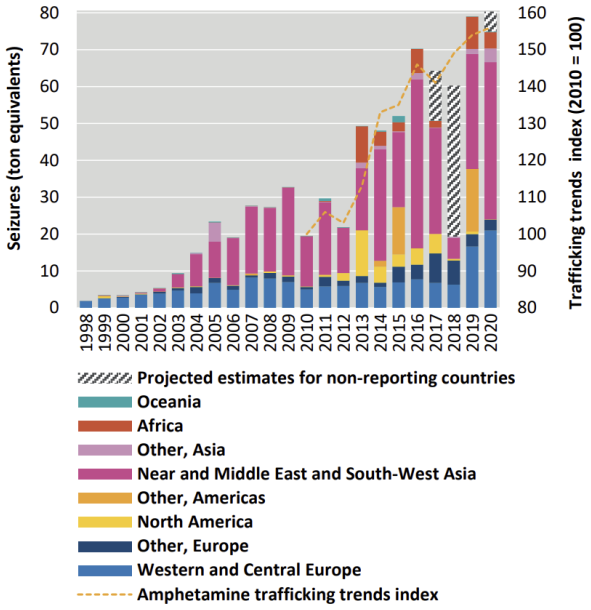

***Figure 3**. Quantities of amphetamine seized and reported qualitative trends in amphetamine trafficking, year 1998 to 2020. Note: The trafficking trends index is based on qualitative information on trends in*

amphetamine trafficking reported by Member States. The trend line is calculated on the basis of the number of countries reporting increases minus the number of countries reporting decreases (2 points for "large increase", 1 point for "some increase", 0 points for "stable", -1 point for "some decrease", -2 points for "large decrease"). Source from UNODC, World Drug Report 2022 (United Nations publication, 2022).

On the European context, studies conducted by The European Monitoring Centre for Drugs and Drug Addiction (EMCDDAF) in 2022 showed that the countries with the highest share of population using amphetamine were Austria (1,8%), Croatia (1,8%), and Finland (1,7%). Countries such as Spain (0,7%) or Sweden (0,7%) were close to the European average, while countries like Italy (0,1%), Slovakia (0,1%), or France (0,3 %) showed very low rates (UNODC, World Drug Report 2022; United Nations publication, 2022).

3.2 Health consequences associated with amphetamine consumption

Prolonged or short-term use of amphetamine can have different consequences on the human body according to the Mount Regis Center (2023). Some of the short-term effects are memory loss, aggressive behaviour, paranoia, psychosis, hallucinations, locomotor problems, convulsions, among others. Amphetamine has shown neurotoxic effects by interacting with the serotonergic and dopaminergic neuronal signalling pathways. Both mentioned neurotransmitters are related to the neurobiological mechanisms of aggressive, defensive, social, and sexual behaviour (Miczek, et al. 1989). Amphetamines are therefore associated with extreme changes on the user's temperament, ranging from sudden switch to aggressiveness to a complete withdrawal from any social intercourse. Symptoms vary depending on other factors, such as pharmacological, behavioural, environmental, and genetic determinants (Miczek, et al. 1989).

In a longer-term context, amphetamine may help to develop substance-induced psychotic disorders, substance-induced sleep disorders (insomnia, decrease of the REM phase or excessive sleepiness), and substance-induced anxiety disorders. It can also lead to substance-induced major depressive episodes caused by its withdrawal (DSM–5; American Psychiatric Association, 2022).

3.3 Neurobiological underpinning of amphetamine reward and addiction

3.3.1 Mechanism of the amphetamine and the dopamine system

Amphetamine has a similar chemical structure to the group of neurotransmitters of catecholamines in the central nervous system (dopamine, serotonin, and norepinephrine). Its pharmacological effect is therefore determined by this similarity in the 3D structure, which causes amphetamine to alter the mechanism of uptake and transport of monoamines in the presynaptic nerve terminals.

The translocation of monoamines is regulated by their synthesis, release, reuptake, and catabolism rates. However, amphetamine competes with endogenous monoamines for transport into nerve terminals via specific transporters. The greater the number of amphetamine molecules in the synaptic space, the more of them will be transported into neurons rather than the endogenous monoamine. Once inside the terminals, moreover, amphetamine displaces monoamines from the cytosolic pool, and prevents their translocation to the neurotransmitter storage vesicles of the synaptic terminals.

The increase of amphetamine in the monoamine pools of synaptic terminals reverses the process of neurotransmitter reuptake. Therefore, instead of neurotransmitters being incorporated into the neurons, they are pumped into the synaptic space outside the cell (Heal, et al. 2013).

To a more organismic level, exposure of experimental animals to psychostimulant drugs appears to have an effect on increasing locomotor activity, known as behavioural sensitisation (Vestin, et al 2022). Previous studies have already shown that amphetamine or cocaine exposure leads to locomotor sensitization and promotes self-administrative impulses, each time strengthening the stimulus-reward relationship conditioned by the mesolimbic system (Everitt, et al. 2002).

3.3.2 The dopaminergic mesolimbic system and reward

The mesolimbic system, also known as the reward system, is responsible for activating and processing the physiological and cognitive processing of reward. This process allows associating various external or internal stimuli (sex, drinking, food, substance abuse...) with reward or a positive outcome (Shultz, 1998). The main regulator for the reward response is

dopamine (DA), which is secreted by dopaminergic neurons originating in the midbrain (Lewis, et al. 2021), see **figure 4**. Dopamine is not only important for reward and reinforcement, but it also has important functions related to locomotion, wakefulness, and as a fact, it also plays an important role in synchronising the circadian rhythm of the mother and her offspring (Grippo, et al. 2016). Moreover, dopamine plays an important role in synaptic plasticity, and increased dopamine signalling may thus give rise to neuroadaptations that could outlast the presence of the positive stimuli/drug.

As it is shown in **figure 4**, the mesolimbic system is composed of several brain structures: different projections of midbrain dopamine neurons of the ventral tegmental area (VTA) connect to the striatum, prefrontal cortex, amygdala, hippocampus, and other structures of the limbic system (Lewis, et al. 2021). Diverse pharmacological agents, such as the ones used for the treatment of schizophrenia, or some stimulant drugs, such as amphetamine or cocaine, act on the dopamine receptors of the mesolimbic system. In the first case, anti-schizophrenia agents are dopamine antagonists, thus blocking the receptors and decreasing the reward response. While some stimulant drugs are dopamine agonists, increasing the response initiated by the activation of dopamine receptors, thus increasing the feeling of reward. Moreover, micro-dialysis studies have shown that addictive drugs increase the concentration of extracellular dopamine in parts of the brain, such as the nAc (Di Chiara, et al. 2004).

The mesolimbic system is in this sense activated by different substances that should exert a rewarding sensation in the body, however, some addictive substances, such as alcohol, tobacco or drugs like amphetamine or cocaine hijack the mesolimbic system by offering a reward without an obvious biological function (Lewis, et al. 2021).

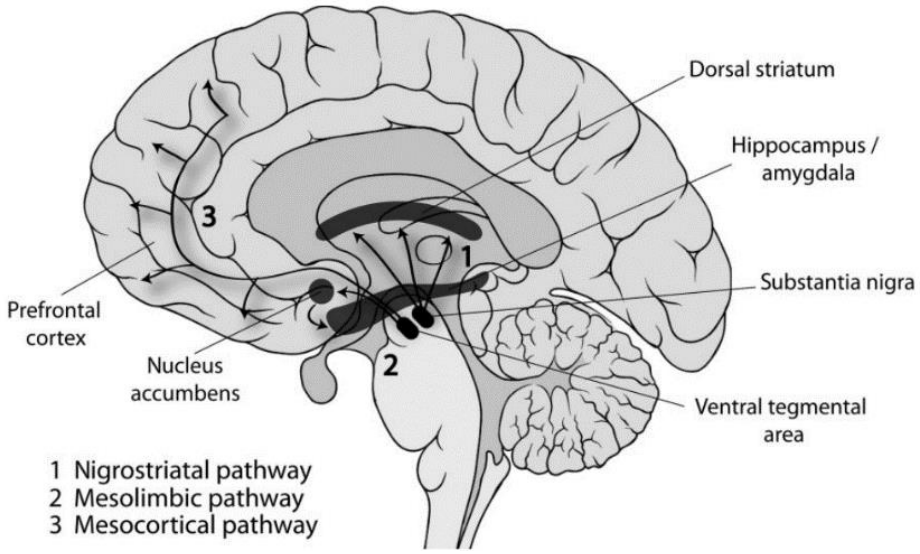

***Figure 4**. Schematic overview on the three main dopaminergic pathways in the brain. The project is focusing on the mesolimbic pathway, originating from the ventral tegmental area (VTA) to other regions, such as the BLA. Source from (Horstmann, et al. 2015).*

3.3.3 Dopamine signalling during reward

When a reward is anticipated, the dopaminergic neurones in mesolimbic system increase notably their activity (Salamone, et al. 2012).

Dopamine is a monoamine catecholamine, which action is mediated by G-protein coupled receptors, expressed in the central nervous system. There are 5 different types of dopamine receptors, encoded in different genes and with different functions: D1 (excitatory) and D2 (inhibitory), expressed on neurons in the amygdala for example, and related to memory, attention, learning, locomotion, and reward response; D3 (excitatory) and D4 (inhibitory), related to cognition, impulse control, and sleep; and last, D5 receptor, related to decision-making and cognition (Bhatia, et al. 2022)

Neurons that transmit signals via dopamine do so in two different ways: in a "tonic" mode or in a "phasic" mode. In tonic mode, neurons maintain a steady, baseline level of dopamine in downstream neural structures that is vital for enabling the normal functions of neural circuits. In phasic mode, on the other hand, neurons suddenly increase or decrease their dopamine release for a short period of time, causing large changes in the concentration of dopamine in downstream neural structures. The phasic mode is activated by various types of rewards and

reward-related sensory cues, but promotes and reinforces immediate reward seeking (Bromberg-Martin, et al. 2010).

Conventional theories for dopamine in the context of reward signalling propose a learning model. The following diagram (**Figure 5**) shows in red how neurons respond with a phasic excitation when a reward situation turns out to be better than the system predicted. That causes the mesolimbic system to learn from the experience, and previous as well as future actions, will be reinforced in response to a new exposure to the stimulus.

On the other hand, shown in blue, the opposite effect is produced, a phasic inhibition occurs when a situation's reward value becomes worse than predicted. Consequently, the mesolimbic system will learn from the specific reward stimulus to punish previous actions or suppress future ones. This self-learning model allows the system to promote or suppress future reward-seeking actions (Bromberg-Martin, et al. 2010).

On this process, dopaminergic neurons play vital role, they classify stimuli with an appetitive value, which can be reward labelled as positive or negative. They are activated when rewards are better than predicted, while they remain inactivated with rewards that are worse than predicted (Schultz, 1998).

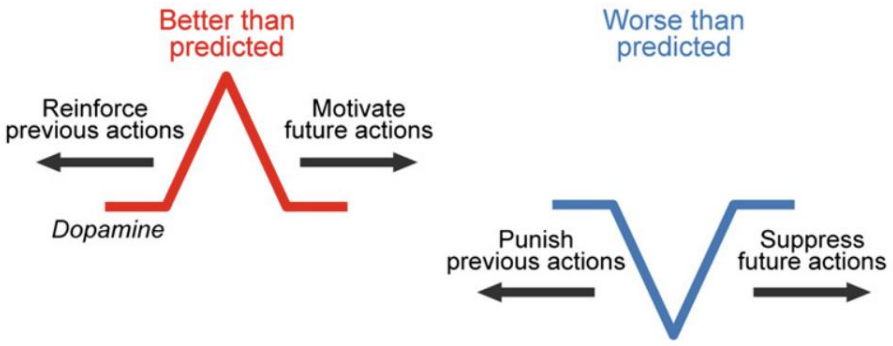

***Figure 5**. Conventional theories of DA reward signals. Diagram illustrating how the mesolimbic system learns from a stimulus depending on whether it is better or worse than predicted, in order to regulate future reward-seeking actions. Source from (Bromberg-Martin, et al. 2010).*

Regarding the above, recent studies have shown that repeated administration of psychostimulants appears to increase the reward threshold by attenuating dopaminergic signalling, eventually leading to higher drug use urges (Danielsson, et al. 2021).

Dopaminergic neurons are in turn tonically regulated by other neurotransmitters related to the mesolimbic system, such as glutamate and gamma-aminobutyric acid (GABA). Glutamate receptors are localised in neurons of the central nervous system, but also in non-neural cells and mediate both fast excitatory synaptic transmission through ligand-gated ion channels, and slower transmission through G-protein coupled receptors (Traynelis, et al. 2010). Glutamate receptors may be ligand-gated ion channels, such as AMPA receptors or NMDA receptors, that contain a recognition site for agonist, but there are also several forms of metabotropic glutamate receptors that modulate neurotransmission via G-protein signalling (Purves, et al. 2001).

GABA is the main inhibitory neurotransmitter in the mammalian nervous system. It is synthesised in the glutamine-glutamate-GABA metabolic pathway and is therefore a transformed product of glutamate (Petroff, et al. 2002). GABA activates both ligand-gated receptor, such as the $GABA_A$ or GABA-rho receptor, and G-protein coupled receptors, such as the $GABA_B$ receptor (always inhibitory) (Olsen, et al. 1999). In this project, we primarily outlining amphetamine-induced changes in signalling through AMPA and $GABA_A$ receptors.

3.4 What are neuroadaptations?

An addiction is defined from a behavioural point of view as the self-administration of substances which in long term may have medical and social consequences, and which create a dependency on them. Two different processes modulate the course of an addiction: neuroadaptations and reinforcement.

Roberts and collaborators (1997) define neuroadaptation largely as the modulatory processes by which initial drug effects are either enhanced (i.e., sensitization) or attenuated (i.e., counteradaptation) by repeated exposure to drugs of abuse. Drug-related responses (i.e., reinforcement) are modulated by the neuroadaptive changes that occur with repeated drug exposure.

Additionally, they also define reinforcement as a theoretical construct by which a stimulus (e.g., an unconditioned stimulus, such as the drug itself or drug withdrawal, or a conditioned stimulus, such as drug-taking) increases the probability of a response (e.g., continued use of the drug)

These conditions combined appear to promote the initial, short-term responses (acute) to the drug and the development of long-term (chronic) cravings that lead to drug addiction.

Moreover, some neuroadaptive changes may be permanent and cause discomfort during withdrawal that can lead to relapse (Roberts, et al. 1997). In this project sustained changes in neurotransmission are elicited by a brief period of amphetamine exposure.

3.4 Role of basolateral amygdala and nucleus accumbens in addiction

The amygdala is a collection of neurons located deep in the temporal lobes of complex vertebrates, including humans. It is specialized for input and processing of emotion, and it is associated with emotional memory (Yang, et al. 2017). It is divided in two parts: the basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA) (Vestin, et al. 2022). The nucleus accumbens is the core structure of the ventral striatum in the brain, and it is known for mediating motivational and emotional processes. It responsible for translating reward-predicted information from the amygdala into goal-directed behaviour. In response to external stimuli, the BLA communicates with the nAc via direct excitatory projections to evoke a response (Ambroggi, et al. 2008).

The following project focuses on BLA and nAc, which play an important role in receiving, processing, and learning information from the reward system. Specifically, they are strongly implicated in adaptive, goal/reward-directed behaviour and appetitive conditioning, which relates to learning the beneficial biological value of stimuli of the reward system (Wassum, et al. 2015; Vestin, et al. 2022). Because the BLA and the nAc are focal points for memory and learning in the mesolimbic system (regulated mainly by dopamine), it is an interesting region of the brain for studying drug addiction, and the neuroadaptations involved.

The BLA contains glutamatergic pyramidal output neuron and heterogeneous populations of GABAergic interneurons (Polepalli, et al. 2020). Long-term use of amphetamine has recently been found to alter structurally and functionally the synaptic connectivity within the BLA neuronal network, through long-term alteration of GABA interneuron signalling (Rademacher, et al. 2015). Moreover, in previous studies performed by Danielsson and colleges (2021), it has been observed that exposure to amphetamine increased drastically the dopamine levels in the brain subregion nAc (See **figure 6**).

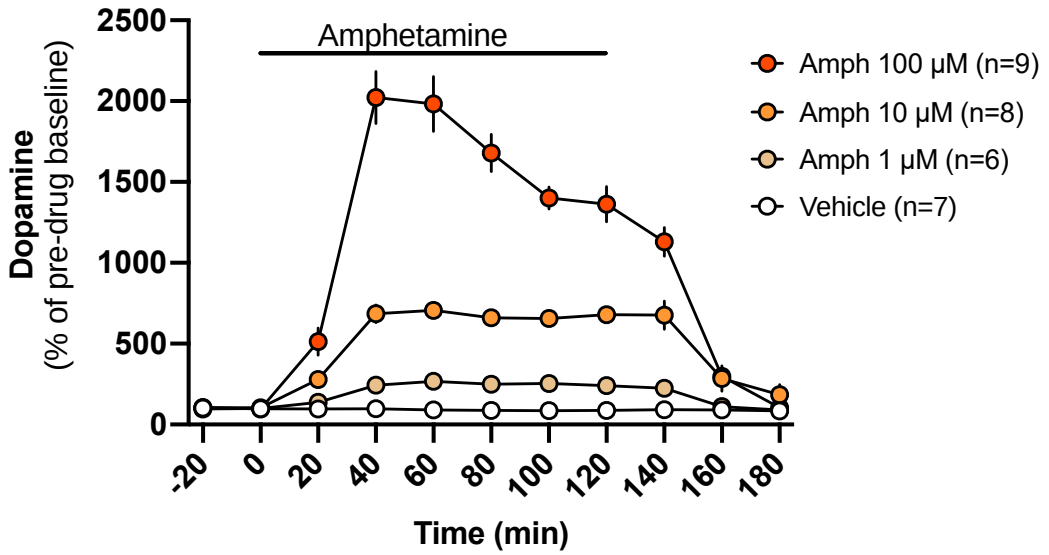

Figure 6. Amphetamine robustly increases dopamine levels in the nucleus accumbens. *In vivo micro-dialysis performed in awake and freely moving rats demonstrates that local administration of amphetamine robustly increases dopamine levels in the nucleus accumbens in a dose-dependent manner. Source from (Danielsson et al., 2021).*

3.6 Study approaches and techniques for assessment of brain drug effects

A common way of studying neuroadaptations elicited by drugs of abuse in different parts of the brain is using electrophysiological techniques. Electrophysiology stets its target on exploring the electrical activity of living neurons *in-vivo*, *in-vitro* and *ex-vivo* and tries to understand the cellular and molecular processes of neuronal signalling, both in an intra- and intercellular level (Carter, et al. 2010). In the present study two electrophysiological techniques are used: field potential recordings, and single cell recordings (patch camp).

Field potential recording is a technique that uses micro-electrodes in order to record differences in the electric potential in the neuronal extracellular space (see **Figure 7**). By stimulating the part of the tissue of interest, this technique is able to record the amplitude of representative field excitatory postsynaptic potentials (fEPSPs). Moreover, field potential recordings allow researchers to study synaptic plasticity related to different stimuli in both long and short term.

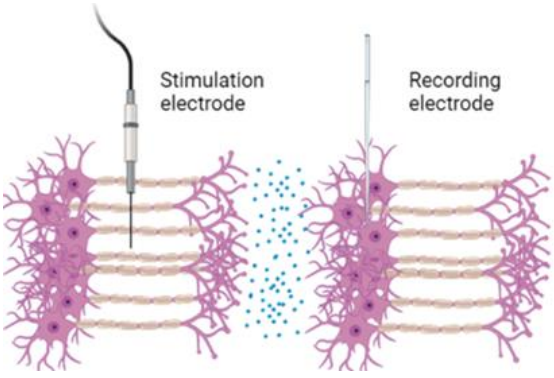

Figure 7*. Diagram of field potential recording. Source from biorender.com*

On the other hand, single cell recordings (patch camp), as the name says, is a technique that records changes in voltage and current by attaching a single electrode to the cellular membrane of a single neuron aiming to understand and evaluate ion channel behaviour. The microelectrode used for the technique forms a high resistance seal with the cellular membrane and the patch of the membrane containing the ion channel of interest is removed. The process is described in (**Figure 8**).

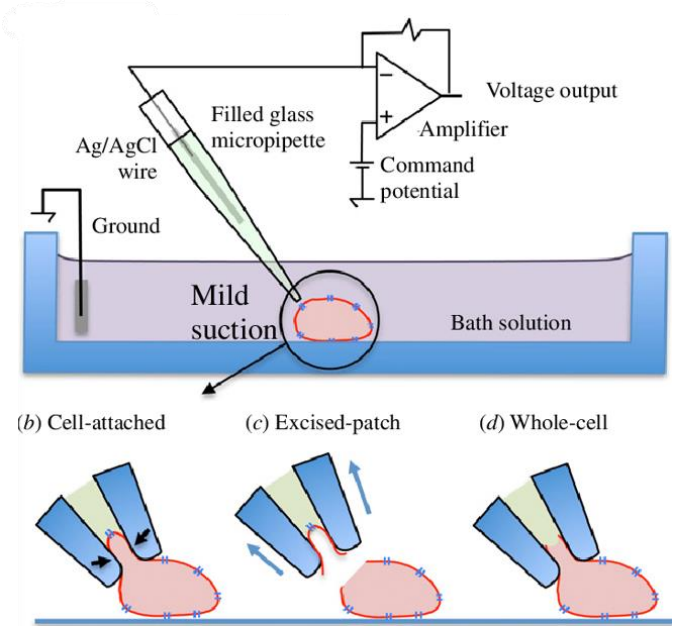

***Figure 8**. Single cell recording (patch camp) procedure. Conventional approach utilizing a glass micropipette electrode on a cell adhered to a solid support (belonging tissue) arranged in various recording configurations: (b) cell-attached, (c) excised-patch and (d) whole-cell mode. Source from (Yobas, et al. 2013).*

4. OBJECTIVES

Amphetamine addiction is associated with continued use despite negative consequences, and a high risk of relapse. The mechanisms underlying the development of drug addiction are not well established yet, but neuroplasticity in brain circuits involved in regulating incentive motivation and reward-based behaviour may play a key role. The overall aim of this project is to define if repeated exposure to amphetamine for a short period of time is sufficient to produce long-term neuroadaptations in the basolateral amygdala and the nucleus accumbens, brain sub regions linked both to emotional regulation, conditioned drug effects and drug relapse.

To this end, male Wistar rats received either controlled amphetamine injections (2.0 mg/kg) or vehicle injections for five days. Furthermore, the occurrence of sustained neuroadaptations still present after two weeks of amphetamine abstinence were outlined using electrophysiological slice recordings. Behavioural sensitization to the locomotor stimulatory properties of amphetamine was monitored also as a proxy for drug-induced aberrant dopamine signalling and behavioural transformations.

5. MATERIALS AND METHODS

5.1 Experiment description

The following experiment studies the behavioural sensitization and neuroadaptations induced in the BLA and nAc by the administration of amphetamine to a group of male Wistar rats. For this purpose, 18 rats received 5 injections in one week with either amphetamine (2.0 mg/kg, n = 9) or saline (0.9 % NaCl, n = 9). A diagram of the experiment can be assessed in **figure 9**.

On both the first day of the experiment (Thursday) and the last day (Wednesday), locomotor assessments of all rats were performed. Fourteen days after the last injection, *ex-vivo* electrophysiological recordings of 12 of the rats were started (Amphetamine: n = 6 ; Vehicle: n = 6). The delay before recordings was conducted in order to outline sustained neurophysiological transformations that were not only present due to acute withdrawal.

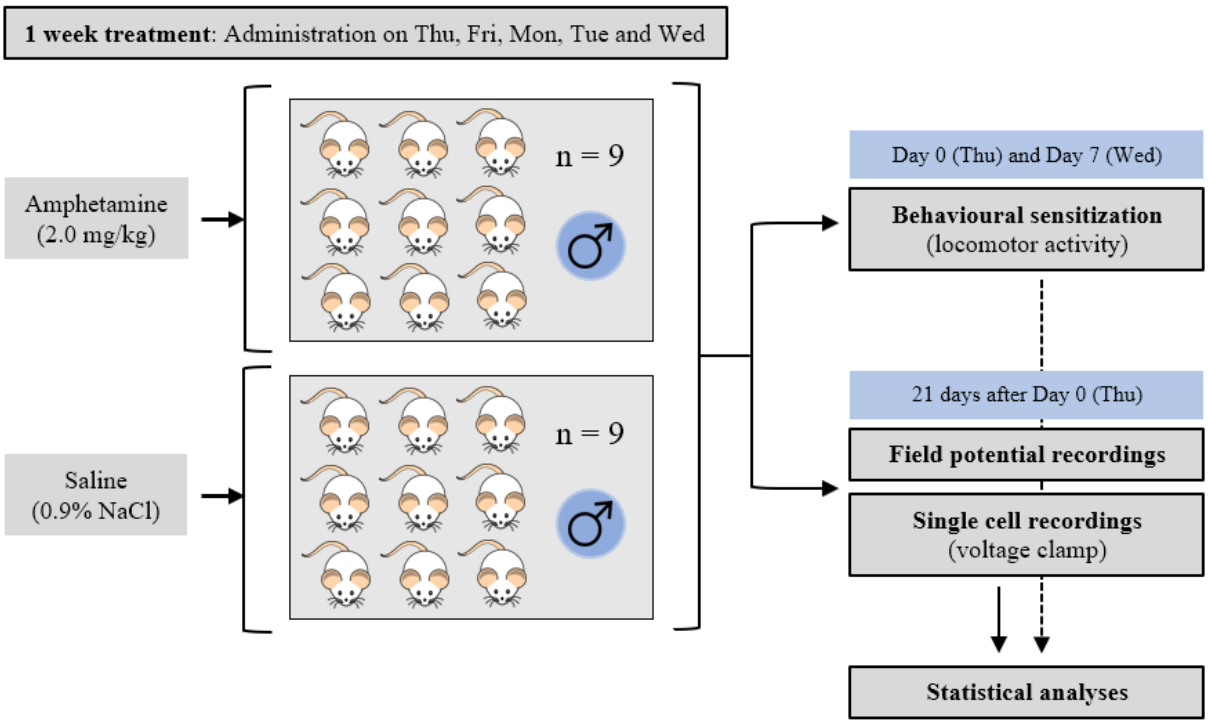

***Figure 9**. Diagram of the experimental design for the amphetamine experiment. (Own design)*

5.2 Animals

Wistar Rcc Ham rats (n = 18) weighing between 180-200 g on arrival (about 12 weeks old) were used. They were sent by Envigo (Netherlands).

They were kept in standard rat cages (55 x 35 x 20 cm) at a constant room temperature of 22 ºC, 65% humidity and a light/dark cycle of 12 hours. The animals had *ad libitum* access to food and water. Prior to treatment, the rats were habituated to their new space for one week, during the last 3 days of which the animals were handled twice daily.

5.3 Amphetamine treatment

Male rats (n = 18) were randomly divided into two groups (Amphetamine: n = 9 ; Vehicle: n = 9). One group was injected intraperitoneally with amphetamine (2.0 mg/kg, dissolved in 0.9% NaCl) and the other group with vehicle (0.9% NaCl). The injections were carried out in five sessions over one week (Thursday, Friday, Monday, Tuesday, and Wednesday).

Rats were divided into cages in groups of three, where there were either two amphetamine-treated and one vehicle-treated rats or one amphetamine-treated and two vehicle-treated rats.

5.4 Assessment of locomotor activity

To study amphetamine-induced behavioural sensitisation, the rats were placed in monitored arenas that allow analysis of locomotor activity (LMA) on the first day of treatment (Thursday) and the last day (Wednesday). The monitored arenas (40 × 40 cm, Med Assoc, Fairfax, VT, USA) are sound-proof, ventilated and illuminated. They allow vertical and horizontal ambulatory movements to be recorded by a two-layer grid of photocell beams. For testing, rats were habituated to the arenas 30 minutes prior to injection, and locomotion was registered for an additional 30 minutes after injection.

Figure 10. *Locomotor activity monitored arena (40 × 40 cm, Med Assoc., Fairfax, VT, USA).*

5.5 Electrophysiology

5.5.1 Brain slice preparation

The rats were sacrificed by decapitation after being anaesthetised with isoflurane. The brains were introduced in ice-cold modified artificial cerebrospinal fluid (aCSF) (in mM; 220 sucrose, 2.0 KCl, 6.0 MgCl2, 0.2 CaCl2, 26.0 NaHCO3, 1.3 NaH2PO4, 10 D-glucose) for 5 minutes.

Using a vibratome (LEICA VT 1200S), slices of 250 μm or 300 μm thickness were cut. These were conditioned for 30 min at 30 ◦C in regular aCSF solution (in mM; 124 NaCl, 4.5 KCl, 2.0 CaCl2, 1.0 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, 10 D-glucose). The slices used for electrophysiology were then left to oxygenate in regular aCSF solution at room temperature.

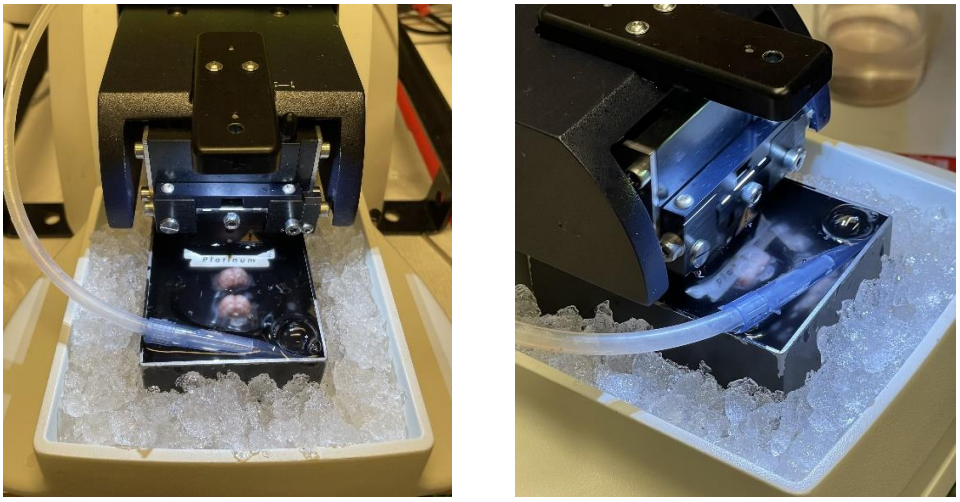

***Figure 11**. Two brains of Wistar rat being sliced by the vibratome(LEICA VT 1200S).*

5.5.2 Field potential recordings

The slices were positioned in the filed potential recording chambers with perfused pre-warmed aCSF (2 ml/min, 30 ◦C), oxygenated with a mixture of 95% O2/5% CO2. The equipment measures the activation of AMPA receptors in BLA (Vestin, E. et al. 2022). For recordings, two stimulation electrodes (World Precision Instruments, FL, USA; type TM33B) were positioned ventrally at 0.2-0.3 mm from the recording electrode (borosilicate glass, 2.5-4.5 MΩ, World Precision instruments).

To study the long-lasting effects on synaptic output, the Input/Output (I/O) stimulation protocol was performed, in which the tissue is afferently stimulated gradually to detect the maximum response amplitude. To determine the effects caused by drug administration on neurotransmitter release, the paired pulse stimulation protocol (50 ms interpulse interval) was applied, which records two consecutive pulses in order to reckon paired pulse ratios (PPRs), calculated by dividing the second pulse (PS2) with the first pulse (PS1). The obtained ratio gives information of the synaptic release probability, in which:

- PPR ratio < 1: there is a high neurotransmitter release probability (inhibition).
- PPR ratio > 1: There is a low neurotransmitter release probability (facilitation).

To measure the response to stimulation of synaptic activity when different drugs were perfused, the electrostimulation was set at 60% of the maximum stimulation obtained during the I/O protocol. A stable baseline was then recorded before perfusion of the different drugs for 10 minutes.

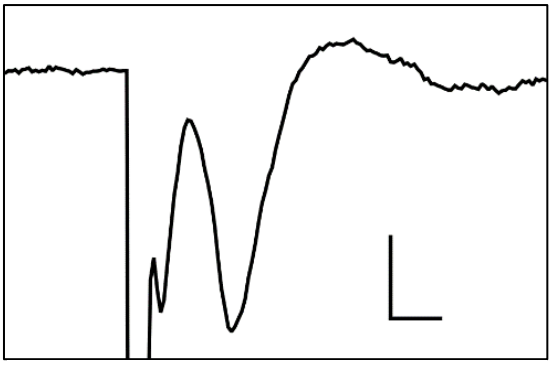

Figure 12. *Example trace of a field potential response recording in the BLA.*

Once the baseline was recorded, the brain slices were perfused with bicuculline, (dissolved in H_2O to 20 mM and diluted in aCSF to 20 μM), 20 minutes. Next, the I/O protocol was run again to assess synaptic output. **Figure 12** shows an example trace of response recorded by the field potential equipment.

Bicuculline acts in the nervous system as a $GABA_A$ antagonist (ionotropic receptor), thereby decreasing the cognitive response (Poulos, et al. 2008).

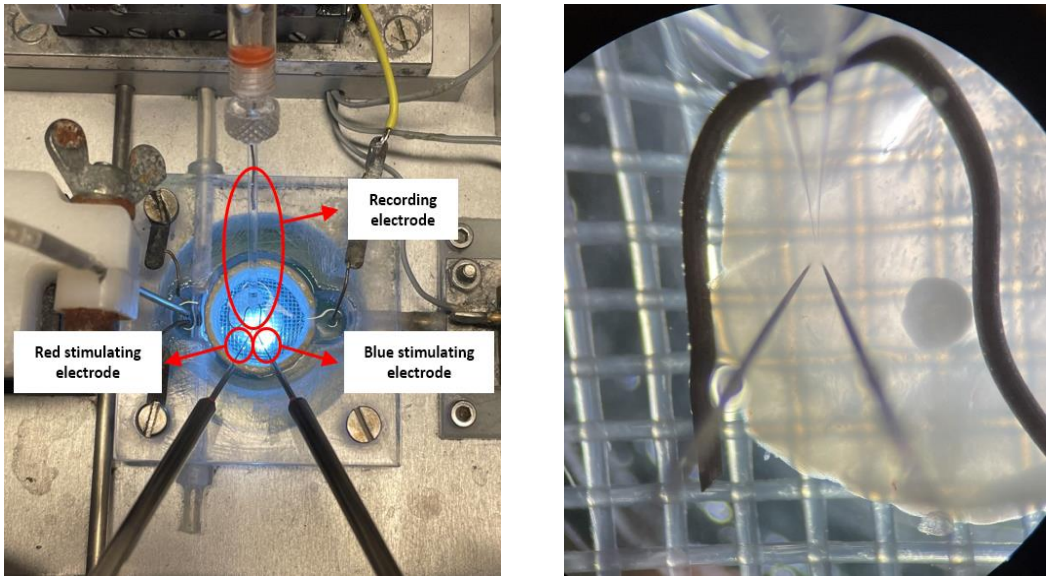

Figure 13*. Field potential recording chamber where electrodes can be seen (left) and closer view to the positioning of the electrodes on the brain slice (right).*

5.5.3 Single cell recordings

The brain slices were positioned in the recording chamber of the patch camp equipment, always under a constant flow of aCSF (33-34 ºC, 2 ml/min).

The region of interest (BLA) was located through a 10×/0.30 objective attached to a Nikon Eclipse FN-1 microscope. The recording electrodes are made of borosilicate glass (outside diameter 1.5 mm, inside diameter 0.86 mm, Sutter instruments, Novato, CA, USA), and pulled to a resistance between 2.5 and 5 MΩ using a Flaming Brown micropipette puller (Model P-97, Sutter instruments, Novato, CA, USA). The electrode was filled with an internal solution containing (in mM; 135 K-Glu, 20 KCl, 2 MgCl2, 0.1 EGTA, 10 Hepes, 2 Mg-ATP and 0.3 Na-GTP).

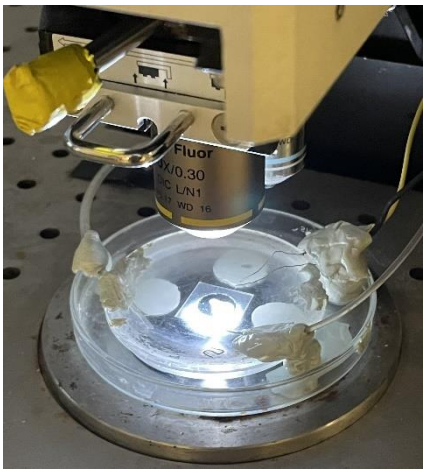

Figure 14*. Slice of Wistar rat brain ready for single cell recording.*

Excitatory postsynaptic currents (sEPSCs) were measured as performing voltage clamp, where single neurones were clamped at -65 mV using a MultiClamp 700B amplifier (Molecular Devices, Axon CNS, San Jose, CA), digitized at 10 kHz and filtered at 2 kHz using Clampex (Molecular devices).

5.6 Data analysis

Locomotor activity data was analysed by performing 2-way ANOVA.

Data obtained from field potential recordings and single cell recordings was assessed using the Electrophysiology Data Acquisition and Analysis Software Clampfit, version 10.4 (pClamp - RRID:SCR_011323) and Microsoft Exel.

Data analysis was performed in GraphPad Prism version 9.2.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. All the presented data is shown as mean ± SEM. In order to find significance between groups 2-way ANOVA, or paired/unpaired t-test was performed, considering statistically significant values under $P < 0.05$.

6. RESULTS

6.1 Locomotor activity

Locomotor activity was monitored on male rats (n = 18) on the first day of treatment with amphetamine injections (Thursday) and on the last day of treatment (Wednesday) when the fifth injection was administrated.

Two-way ANOVA of the horizontal movements reveals a trend towards a difference in locomotor activity ($F_{(1, 16)} = 2.371$; $P = 0.0537$) in the rats after the first injection and the fifth injection, showing that repeated amphetamine exposure produces behavioural sensitisation in male rats. In addition, the values were significantly higher than in the vehicle group (see **figure 15A**).

Behavioural sensitization was also observed in vertical movements, where rats repeatedly exposed to amphetamine showed significantly elevated locomotor activity ($F_{(1, 16)} = 5.411$; $P = 0.0335$) compared to those injected on the first day of injection (see **figure 15B**).

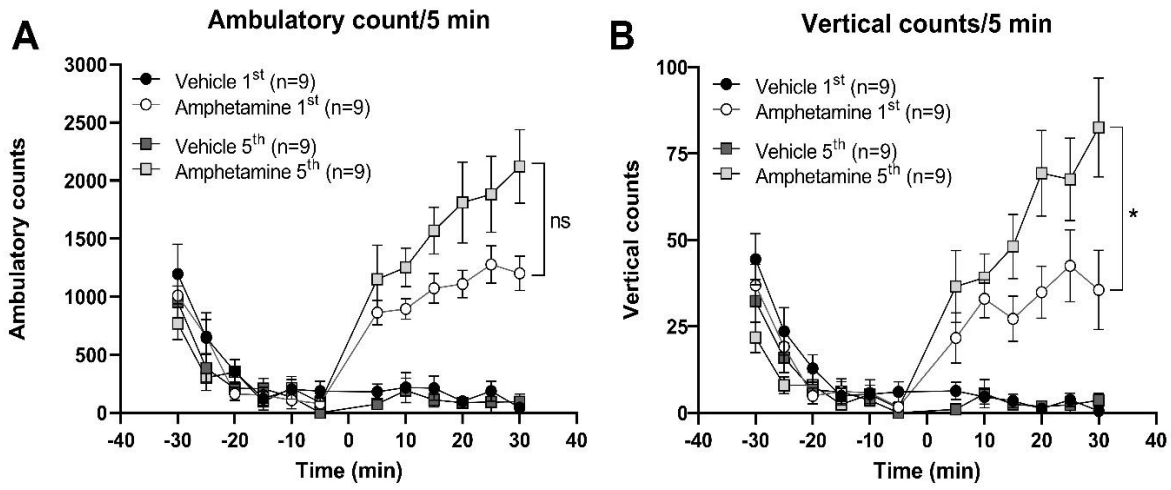

Figure 15. Short-term exposure to amphetamine induces long-term behavioural sensitization in male rats*. Locomotor activity was recorded using monitored locomotor arenas the first day of amphetamine treatment (first injection) and the last day (fifth injection). A) A trend towards an enhanced horizontal locomotor activity was recorded as well. B) Significantly enhanced vertical locomotor activity was monitored comparting the recordings after the 1st and the 5th amphetamine injection. The data represents the mean values ± SEM. Significant differences between amphetamine and vehicle treated animals (*P < 0.05). n = number of animals.*

6.2 Electrophysiology

6.2.1 Single cell recordings

Voltage clamp was held only in BLA. The results after performing an unpaired t-test reveal a significantly lower mean in rats treated with amphetamine in the induction of action potential frequency (t = 3,155, df = 34; P = 0.0034) (**figure 16A**). In contrast, no significant differences were observed in the amplitude of the recorded action potentials between the groups (t = 0.2387, df = 33; P = 0.8128) (**figure 16B**).

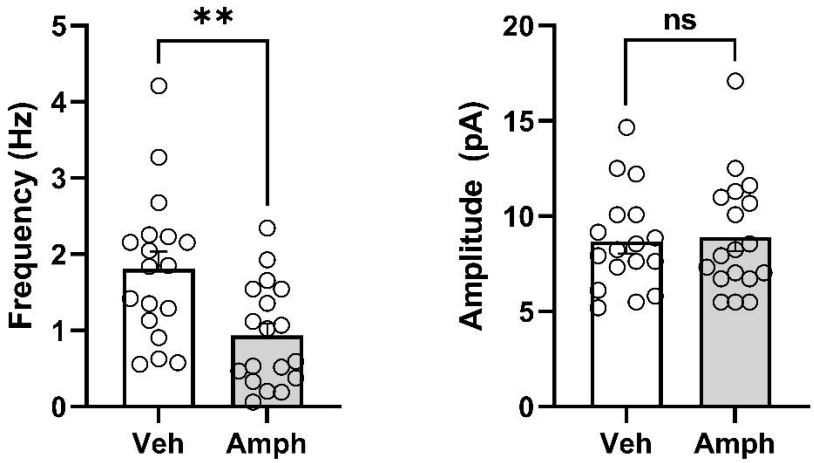

Figure 16. Decrease in post-synaptic amplitude of the responses in BLA indicates a reduced probability of neurotransmitter release*. A) Amphetamine-treated rats present a lower frequency in the voltage clamp recordings. B) No significant difference in peak amplitude has been spotted between groups. The data represents the mean values ± SEM. Significant differences between amphetamine and vehicle treated animals (**p < 0.01). (Amphetamine: n = 18; Vehicle = 18).*

6.3.2 Field potential recordings

Field potential recordings were conducted 14 days after one week treatment of with amphetamine injections. Data was gathered from the following parts of the brain: BLA and nAc.

- ***Basolateral amygdala***

Field potential recordings performed on BLA show a significant decrease in the amplitude of post-synaptic responses during the I/O protocol in amphetamine-treated rats before the bicuculline perfusion ($F_{(1, 29)} = 13.32$; $P = 0.0010$). See **figure 17B**.

Bicuculline perfusion for 20 minutes decreases glutamatergic tone in BLA, hence increasing the amplitude of the recorded responses. I/O performed after the bicuculline wash shows that differences between the amplitude of post-synaptic responses is no longer significant between the amphetamine-treated and vehicle-treated groups of rats (see **figure 17D**). The results suggest the appearance of neuroadaptations reflected in increased glutamatergic tone and reduced excitatory neurotransmission in BLA caused by amphetamine administration.

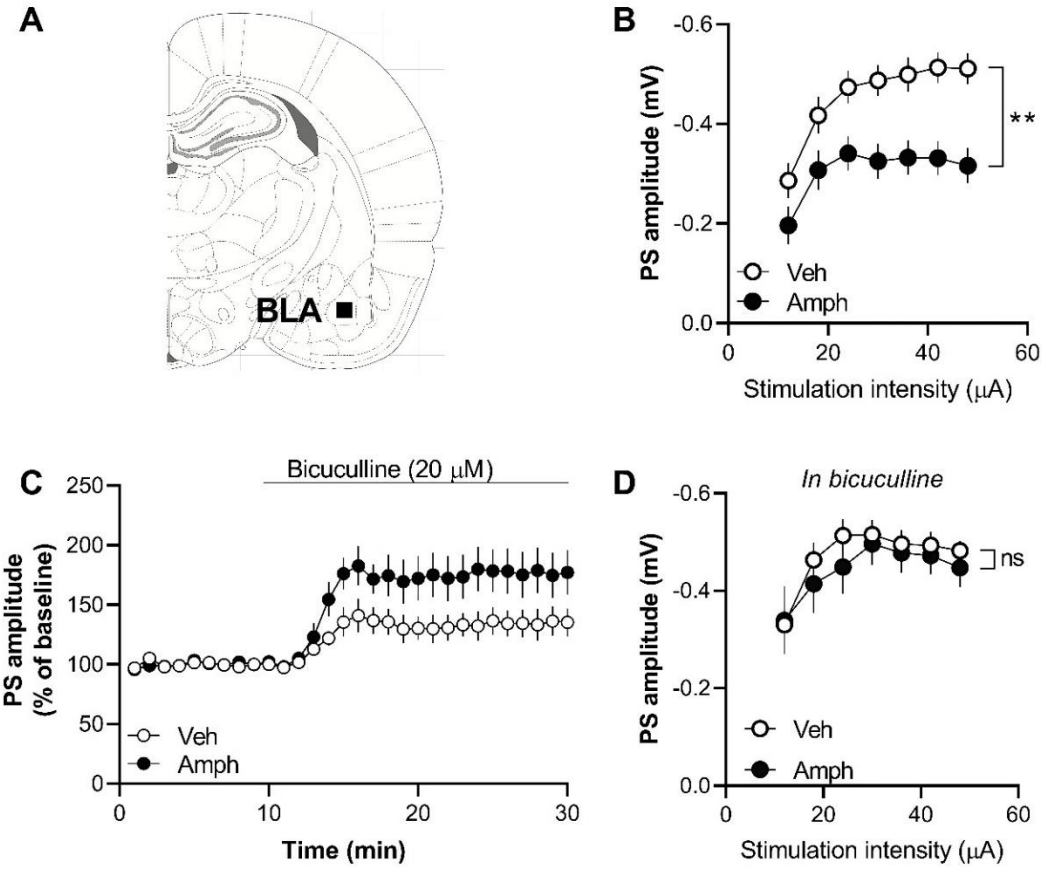

Figure 17. Amphetamine reduces excitatory neurotransmission in BLA as a result of a decreased GABAergic inhibitory tone*. I/O was increased in amphetamine-treated rats after bicuculline perfusion for 20 minutes compared to vehicle-treated ones. A) Location of BLA in the brain slice, B) I/O before bicuculine wash, C) PS amplitude measured before and during the perfusion of bicuculline, D) I/O after bicuculline wash. The data represents the mean values ± SEM. Significant differences between amphetamine and vehicle treated animals (**p < 0.01). Number of total recordings (Amphetamine: n = 17; Vehicle =15), based on 12 animals (Amphetamine: n = 6; Vehicle = 6).*

- ***Nucleus accumbens***

Field potential recordings performed on nAc show a significant decrease in the amplitude of post-synaptic responses during the I/O protocol in amphetamine-treated rats ($F_{(1, 13)}$ = 10,90; P = 0.0057). See **figure 18B**.

A significant difference was assessed by performing a paired t-test in paired pulse stimulation (PPR) (t = 2,463, df = 12; P = 0.0299), situating the amphetamine-treated rats on a value over 1, while the vehicle-treated rats show a value under 1. See **figure 18C.**

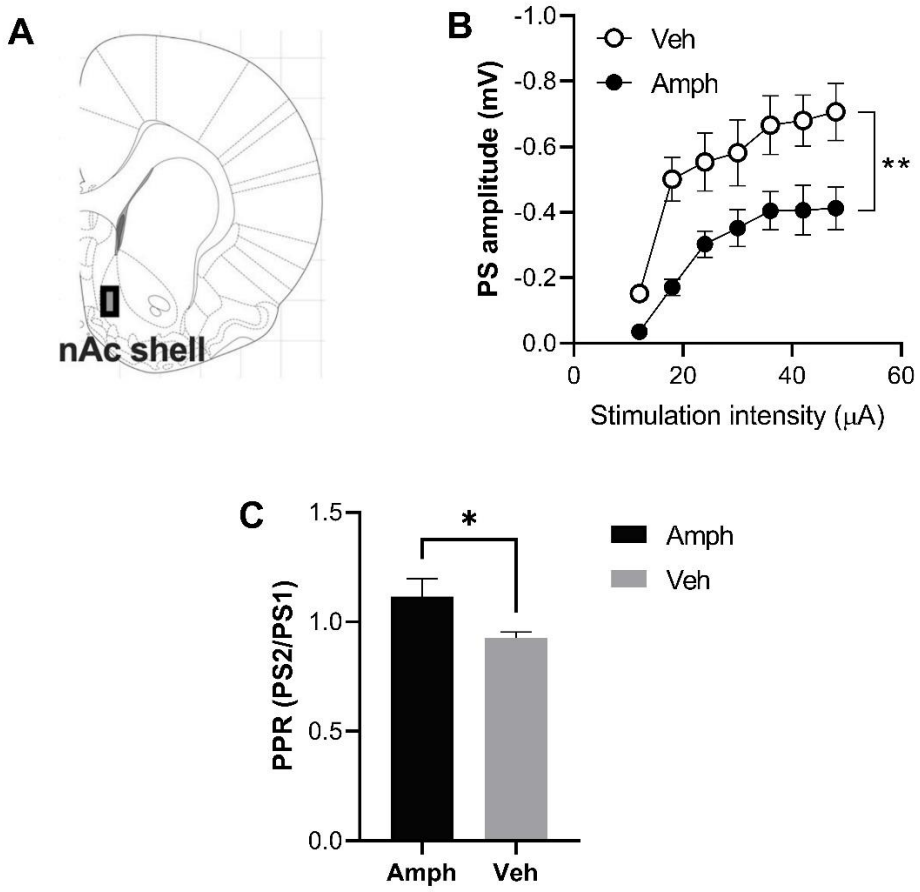

Figure 18. Amphetamine induces a reduced post-synaptic neurotransmission related to decreased neurotransmitter release in nAc*. A) Location of nAc in the brain slice, B) Amphetamine-treated rats show a decreased post-synaptic amplitude, C) Pared pulse stimulation shows a significant difference comparing the two treated groups. PPRS are calculated by dividing the second pulse (PS2) with the first pulse (PS1). The data represents the mean values ± SEM. Significant differences between amphetamine and vehicle treated animals (*p < 0.05 / **p < 0.01). (Amphetamine: n = 8; Vehicle = 6).*

7. DISCUSSION

The aim of this project is to expose the appearance of long-lasting neuroadaptations after repeated amphetamine exposure in male rats. After five amphetamine injections, behavioural sensitization was developed, significant locomotor changes have been observed in vertical movement counts, as well as a trend towards a difference in ambulatory counts. The appearance of neurochemical alterations is supported by the results obtained by voltage clamp in the brain subregion BLA, and by the findings in the field potential recordings, which suggest a decrease in the excitatory response in both brain subregions BLA and nAc, which may be driven by increased GABAergic tone.

The outcome of this project supports the results obtained from studies by Lofti, and collaborators (2022) and Vestin and collaborators (2022), which postulate that repeated exposure to amphetamine produces a long-lasting behavioural sensitization driven by amphetamine-induced neuroadaptations (**figure 15**). Even though the difference in ambulatory counts after 7 days of treatment did not appear significant, there is a clear trend towards expressing behavioural sensitization, which can clearly be probed in the results for vertical counts. Following the line of former studies (Vezina, et al. 2007) that postulate that psychostimulant drugs such as amphetamine and cocaine directly connect behavioural changes (e.g., increased locomotor activity) to midbrain dopamine sensitization, testing the neurochemical consequences arisen by amphetamine on dopaminergic signalling in different brain subregions would be a promising approach for further investigation.

Repeated exposure to amphetamine induces a decreased frequency of action potential on single cells when they are injected with currents (**figure 16**). Voltage clamp recordings show a decrease in frequency that suggests a reduced probability of neurotransmitter release into the synaptic space. Recordings in amplitude offered additional information about the neuroadaptations that might have arisen. The amplitude indicates the number of ions crossing the postsynaptic membrane, followed by activation of the respective receptors. No significant differences in peak amplitude between groups were observed, suggesting that the cause of the decrease in PS amplitude is not related to changes in receptor expression (e.g., AMPA receptors), but due to a reduced probability of neurotransmitter release.

Voltage clamp statements are also supported by the findings assessed in the field potential recordings. The results reflect the neurochemical long-lasting effects induced by short-term administration of amphetamine to male rats. Field potential recordings carried out in the BLA

show a reduction of excitatory neurotransmission due to an increase in the GABAergic tone (**figure 17**). Similar amphetamine-induced alterations in the GABAergic tone have been already observed in other subregions of the brain (Paul, et al. 2016).

In amphetamine-treated rats, GABA-controlled neural processes are impaired. Therefore, a perfusion with bicuculline (GABA antagonist) blocks the GABA inhibitory effect resulting in the recovery of the amplitude of post-synaptic responses to similar values to the ones obtained from vehicle-treated rats. The role of bicuculline in this experiment, is then to inhibit the inhibition of neurotransmission normally caused by GABA by interacting with the recognition sites of $GABA_A$ receptors.

In a more simplified form, blocking the suspected altered signalling pathways (the GABAergic signalling) will expose if they are the cause of a decrease in the responses. In that way, the I/O performed after the bicuculline wash reveals the neuroadaptations developed in BLA by rats exposed to amphetamine (**figure 17D**). Which it is thought be related to an increase in GABAergic tone, which consequently decreases the excitatory response of BLA neuronal tissue.

Both the nucleus accumbens and the basolateral amygdala account greatly for reward-related behavioural learning. The nAc is responsible for translating reward-predicted information from the amygdala into goal-directed behaviour. In response to external stimuli, the BLA communicates with the nAc via direct excitatory projections to evoke a response (Ambroggi, et al. 2008).

Both subregions of the brain are innervated by glutamatergic afferent circuits arising from the cortex, which leads us to think that the effects on neurotransmission could be affected in a related manner after repeated amphetamine exposure. Field potential recordings in nAc during I/O function show a reduction of the amplitude of post-synaptic responses in amphetamine-treated rats (**figure 18B**). As seen by BLA, results suggest that amphetamine modifies the excitatory response by inducing long-lasting neuroadaptations related to an increase in GABAergic tone. Previous studies (Danielsson, et al. 2021) suggest that dopaminergic signalling is significantly reduced as a consequence of amphetamine exposure, a fact that may prompt further in-depth research into how amphetamine affects the relationship between the different neurotransmitter pathways in nAC.

The PPR ratios calculated for nAc provide extra information on the cause of the reduction in the post-synaptic response in amphetamine-treated rats. Pair pulse ratio can be understood as a way of monitor short-term plasticity depending on neurotransmitter release probability.

Results show values of PPR ratios above 1 in amphetamine-treated rats, whereas vehicle-treated rats show values below 1 (**figure 18C**). Values below 1 indicate a reduced likelihood of neurotransmitter release in the synapsis. Only a small part of the neurotransmitter reservoir vesicles stored in the presynaptic terminal are released in response to the first excitatory pulse. Thus, as the second pulse excites the neurons, a greater number of neurotransmitters is released into the synaptic space.

This is related to arisen neuroadaptations affecting the BLA by decreasing its outputs, which was also evidenced by (Vestin, et al. 2022). The increased GABAergic tone observed in the BLA suggests that excitatory outputs are decreased, thereby exposing amphetamine-induced neurochemical alterations that lead to a lower probability of neurotransmitter release at the synapse in nAc.

Although further research remains to be done to elucidate in detail the effects of amphetamine on neurotransmission, the outcome of this experiment suggests the occurrence of neuroadaptations in both BLA and nAc, which induce behavioural sensitisation and generally lead to suppressed neuronal excitability. The underlying motivation for this is likely to be an increase in GABAergic tone leading to a reduction in the probability of neurotransmitter release.

8. CONCLUSIONS

This bachelor thesis aimed to study the long-term neuroadaptations arising from repeated amphetamine exposure in the BLA and nAc brain regions in male rats. Experimentally, locomotor activity monitoring arenas, and electrophysiological techniques such as field potential recordings and single cell recordings have been used. The analysis of the experimental data together with the statistical examination carried out has led to the following conclusions on repeated exposure to amphetamine:

→ Amphetamine produces behavioural sensitisation in both ambulatory activity and vertical movement.

→ Amphetamine does not change the experience of synaptic receptors but reduces the probability of neurotransmitter release in BLA and nAc.

→ Amphetamine decreases excitability and reduces the amplitude of post-synaptic responses in BLA and nAc, due to the appearance of neuroadaptations in the form of a reduction in GABAergic inhibitory tone.

→ Amphetamine reduces the excitatory outputs of the BLA directed to the nAc, thereby exposing amphetamine-induced neurochemical alterations on the nAc as a possible consequence.

→ Amphetamine might alter multiple neuronal signalling pathways (e.g., dopaminergic signalling), therefore diverse future research approaches might arise.

The limitations encountered are the following:

→ Although the anatomy of rats is similar to that of humans, it would be optimal to also study the effects of amphetamine directly in human brains (*post-mortem*).

→ The experiments have been carried out in *ex-vivo*, so the cellular environment is not exactly the same as *in-vivo*, which could cause some alteration in the veracity of the results.

→ No clamp voltage was performed on the BLA, which might have been of interest for comparison with nAc.

As a general assessment, the project has been successful, demonstrating significant results at several levels and being able to give a clear idea of the effects of amphetamine in different subregions of the brain, which can be interpreted in a broader context within the human brain.

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