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Behavioral and neurophysiological transformations elicited by repeated amphetamine exposure in Wistar rats

Degree Project in Medicine

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Abstract

Master thesis, program in medicine.

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Background: Substance use disorder is a chronically relapsing brain disease, causing substantial mortality and morbidity world-wide. Currently, there is no efficacious treatment for substance use disorder. Elucidating the neural underpinnings of substance use disorder can aid in defining new targets for pharmacological treatment.

Objective: To investigate behavioral and neural changes following repeated amphetamine exposure and two weeks of abstinence.

Methods: An experimental study in the Wistar rat. Assessment of behavioral sensitization in the locomotor activity box. Assessment of risk-taking behavior in elevated plus maze. Lastly, electrophysiological recordings of basolateral amygdala, nucleus accumbens shell and medial prefrontal cortex were performed.

Results: Five days of amphetamine-exposure is sufficient to induce behavioral sensitization, this coincided with an increase in time spent in center-zone. After two weeks of withdrawal, we saw no change in time spent in closed arms but a decrease in entries into the closed arms in the amphetamine-treated animals. Electrophysiological field potential recordings showed reduced evoked potential strength in basolateral amygdala (BLA), which was reversed by $GABA_A$-receptor antagonist bicuculline, as well as an increased disinhibition during $GABA_A$-receptor antagonist bicuculline perfusion. Synaptic output from the nucleus accumbens or medial prefrontal cortex were not significantly modulated by treatment.

Conclusion: Repeated exposure to amphetamine produces sustained neuroadaptations in GABAergic signaling in brain regions associated with emotions, which could promote risk-taking behavior during drug abstinence.

Keywords: Substance use disorder. Amygdala. Rat. Locomotor activity. Risk-taking.

1. Introduction

1.1 Amphetamine

Amphetamine is a competitive substrate for monoamine reuptake transporter proteins, effectively increasing concentrations of monoamines in the synaptic terminals (Robertson et al., 2009). The focus of this thesis is the isomer d-amphetamine, which in the central nervous system mainly increase levels of noradrenaline and dopamine, but also to a lesser extent serotonin. In addition, amphetamine increases adrenaline release in the peripheral nervous system (Heal et al., 2013). Important subjective effects of d-amphetamine as described by healthy individuals are *feelings of being stimulated*, *drug liking*, *extraversion*, *positive* mood and *concentration*, together with increases in body temperature, heart rate and blood pressure and dilation of the pupils (Holze et al., 2020). Amphetamine is used in treatment for attention-deficit hyperactivity disorder (ADHD), narcolepsy, waking patients from anesthesia, but it is also used for recreation and for enhancing athletic or professional performance, and as such it has substantial abuse potential (Heal et al., 2013).

1.2 Amphetamine use disorder

Substance use disorder is a debilitating disease, with substantial comorbidity in the form of infectious-, cardiovascular- and psychiatric disease. Individuals suffering from substance use disorder are also overrepresented in cases of homicide, suicide and imprisonment (Farrell et al., 2019). In order to be diagnosed with a substance use disorder, at least two of the following twelve DSM-5 criteria needs to be fulfilled; *tolerance, social or interpersonal problems related to use, withdrawal, using larger amounts or for longer periods than intended, inability*

to quit or control use, spending much time using, seeking drugs or recovering from use, continuing use despite physical or psychological problems related to use, persisting in use despite knowledge that it causes problems at work, school or home, failure to meet important social or professional obligations to use, use in hazardous situations or context leading to self-injury and *craving*. If 2-3 criteria are fulfilled, the individual is diagnosed with a *mild* SUD, 4-5 *moderate* and 6 or more *severe* (Mittal and Walker, 2011).

Despite the negative impact on society, we still lack efficacious treatment for psychostimulant-addicted patients (Farrell et al., 2019). Dissecting the underlying pathological mechanisms is a promising path to uncover novel treatment or improve upon current treatments (Leshner, 1997, Heilig et al., 2021). Modalities for studying the pathological mechanisms in the brain, with the temporal and spatial resolution we can achieve in animal studies, are not accessible for human subjects today. To study changes in brain circuitry, and to pair this with behavior, we need animal studies. (Perry and Lawrence, 2020).

Although many brain regions are affected by amphetamine, this degree-project primarily focuses on the amygdala, nucleus accumbens and medial prefrontal cortex. This is because of these regions' intimate interconnectivity together with relatively recent interest in the context of addiction research as well as the time-constraints given in a degree-project.

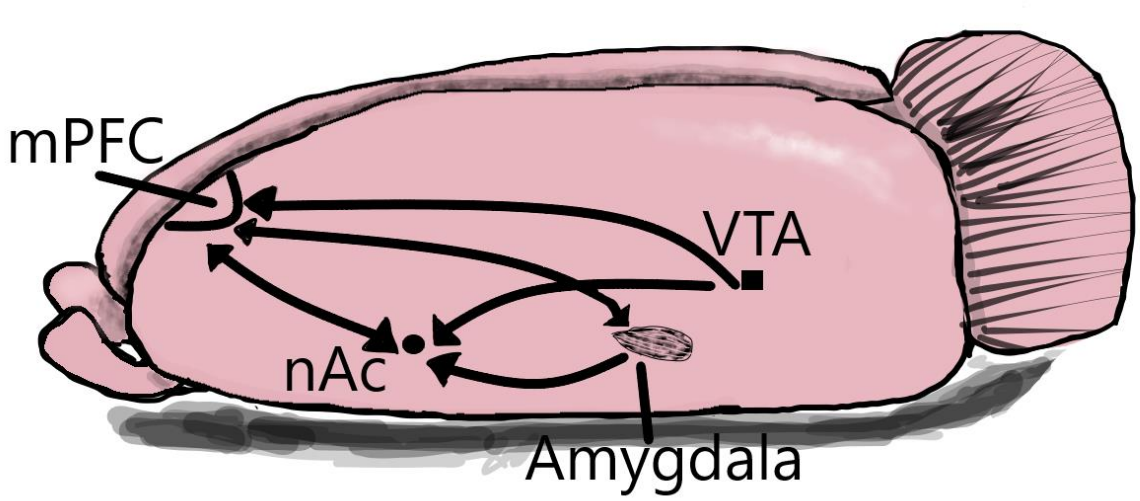

Figure 1. Amygdala interconnectivity in the rat brain. A schematic drawing showing connections to and from amygdala. VTA gives rise to dopaminergic projections to mPFC and nAc. Amygdala and nAc are reciprocally connected to mPFC. Note that not all connections nor the complexity of the connections present are represented in this schematic picture.

1.3 Basolateral amygdala

Amygdala is on the rise as an auspicious therapeutic target in treatment of substance use disorder (Rademacher et al., 2015, Wassum and Izquierdo, 2015, Kruzich and See, 2001) The amygdala is essential for linking an emotional valuation component, for example fear, anxiety or reward, to sensory stimuli, such as environmental cues like smells, sounds or visual input. In substance use disorder, this linking is responsible for triggering craving when an individual encounters drug-related cues (Wassum and Izquierdo, 2015, Goodman et al., 2019, Robinson and Berridge, 1993, Grimm et al., 2001). One subregion of the amygdala, the basolateral amygdala (BLA), has been especially highlighted as a key structure in addiction processes (Wassum and Izquierdo, 2015, Goodman et al., 2019, Whitelaw et al., 1996).

The BLA consist mainly of glutamatergic principal cells. Interestingly, output seems to be predominantly governed by its much smaller, but immensely complex, population of GABAergic interneurons (Krabbe et al., 2018). These interneurons provide feedback, feed forward and lateral inihibition-functionality through their variety of spiking patterns and synaptic connectivity onto each other or principal neurons (Polepalli et al., 2020, Krabbe et al., 2018, Lucas and Clem, 2018). BLA receives massive innervation from cortical subregions, including the medial prefrontal cortex (mPFC), and projects back to mPFC and nucleus accumbens (nAc) shell (Steketee, 2003, Wassum and Izquierdo, 2015) (Fig. 1).

1.4 Nucleus accumbens

Nucleus accumbens is an integral part of the mesocorticolimbic, or reward, system and is repeatedly linked to the reinforcing properties of addicting drugs (Paulson et al., 1991, Robinson et al., 1988). Experimental animal studies of pleasure, and the learning associated with it, has a long history (Thorndike, 1898). However, reward is more than simply pleasure. Reward can be divided into “liking”, “wanting/craving” and “reward learning”, and these subjective emotions reinforce motivated behavior (Berridge and Kringelbach, 2015). Reinforcing properties of many, but not all, drugs of abuse is thought to be in large part derived from dopaminergic projections to the nAc from the ventral tegmental area (VTA) (Vanderschuren and Kalivas, 2000) (Fig. 1).

Located in the ventral striatum, the nucleus accumbens (nAc) is populated mostly by GABAergic medium spiny neurons (MSNs), rich in dopamine receptors (Salgado and Kaplitt, 2015). In addition to VTA, nAc receives projections from mPFC, hippocampus and

amygdala. nAc in turn projects to mesencephalon, the basal ganglia, amygdala and the mPFC (Salgado and Kaplitt, 2015) (Fig. 1).

1.5 Medial prefrontal cortex

The medial prefrontal cortex is responsible for decision-making, long-term memory retrieval and for adaptive behavior (Euston et al., 2012), and implicated in various brain diseases, notably anxiety-related diseases and addiction (Xu et al., 2019). Projections from mPFC have a strong modulatory influence over BLA output, but the mPFC also receives predominantly glutamatergic input from the BLA, both directly onto pyramidal neurons but also to local GABAergic interneurons (McGarry and Carter, 2016). The main cells responsible for output from mPFC are glutamatergic pyramidal cells, which together with locally projecting GABAergic interneurons also receives dopaminergic projections from the VTA, resulting predominantly in inhibition of mPFC output. In function, mPFC appears to be a key mediator of both induction and expression of psychostimulant sensitization (Steketee, 2003).

1.6 Behavioral sensitization to psychostimulants

Repeated administration of psychostimulants, such as amphetamine, progressively transforms neuronal circuits and increases the sensitivity to the behavioral effects by the drug (Segal and Mandell, 1974, Robinson and Becker, 1986). Behavioral sensitization has been proposed to reflect many of the neurochemical changes that are characteristic for drug addiction, and is an established model for investigating drug-induced effects on the function of nervous system (Robinson and Berridge, 1993, Steketee and Kalivas, 2011). The presence of behavioral sensitization further correlates with reinstatement, the animal model counterpart to relapse

(Steketee and Kalivas, 2011, Conrad et al., 2008), thereby supporting a role for these neuroadaptations in addiction. Centrally responsible for the phenomenon of behavioral sensitization is the VTA-nAc projections, while projections from BLA and mPFC modulate these effects (Vanderschuren and Kalivas, 2000).

Behavioral sensitization to amphetamine is a complex phenomenon that engages several neurotransmitter systems, where the role of dopamine and glutamate neurotransmission has been especially acknowledged (Bamford et al., 2008, Vezina, 1996, Jing et al., 2018, Wang et al., 2013, Yoon et al., 2008, Kim and Vezina, 2002, Huang et al., 2020, Robinson and Becker, 1982). Recent research, however, implicate a role for the GABAergic system in sensitization to amphetamines (Wearne and Cornish, 2019), and to the development of amphetamine use disorder (Jiao et al., 2015b).

1.7 Risk-taking/anxiolytic-like behavior and the $GABA_A$-receptor

Anxiety-like, as opposed to risk-taking behavior, has been studied extensively in the open field maze and elevated plus-maze or a combination of these (Seibenhener and Wooten, 2015, Simon et al., 1994, Bi et al., 2013, Déziel and Tasker, 2018, Hogg, 1996, Pati et al., 2018, Simon et al., 2011). Numerous studies implicate both dopaminergic and GABAergic circuitry in BLA, mPFC and nAc in anxiety-related behavior and risk-taking. Dopamine has previously been shown to influence risk-taking behavior in rats. Interestingly, Simon et al. showed that systemic administration of D2-like receptor agonists decreased risk-taking behavior. In addition, D2-like receptor expression in the mPFC had an inverted, u-shaped, negative correlation to risk-taking for reward. Finally, D1-like receptor expression in nAc shell had a

positive correlation with risk-taking behavior for reward (Simon et al., 2011). Activation of mPFC neurons have been shown to decrease anxiety-like behavior in the elevated plus-maze, but not in the open field maze (Pati et al., 2018). Lesions of mPFC have further been shown to increase anxiety-like behavior (Déziel and Tasker, 2018), and the $GABA_A$-receptor antagonist bicuculline has been shown to induce anxiety-like behavior when administered into the mPFC (Bi et al., 2013).

We hypothesize that repeated exposure to amphetamine produces neuroadaptations in subregions of the amygdala, which may contribute to behavioral transformations, including changes in risk-taking behavior, but also drive neuroadaptations in reward related brain regions such as the nAc, and cortical regions such as mPFC.

2. Aim

The aim of this study was to assess behavioral and neurophysiological changes induced by repeated amphetamine exposure followed by abstinence in Wistar rats.

3. Material and methods

3.1 Animals and drug-treatment

Male Wistar rats (Envigo, Horst, Netherlands) (n=18), weighing 200-220g at arrival, were housed in groups of three in standard rat cages (55x35x20 cm), in a controlled environment, 22ºC, 12/12 h light/dark cycle (lights on during day) and 65% air humidity, with free access to standard food pellets and tap water. Food pellet consumption was weighed to approximate food intake. All experimental procedures were conducted during the light cycle. These animals were housed undisturbed for one week and then selected to receive five days of

intraperitoneal injections of either amphetamine (2.0 mg/kg, a comparatively high dose), or vehicle (0.9% NaCl) (Fig. 2). This was followed by two weeks of abstinence from treatment, as we want to study the effects of amphetamine withdrawal on the brain, and not lingering acute effects of the drug. Experiments were conducted in three separate batches of animals. The study was approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden.

Figure 2. Timeline showing the experimental procedure. First, animals arrived at the animal facility and was allowed one week of undisturbed habituation to the facility. Injections of amphetamine (2.0mg/kg, dissolved in 0.9% NaCl), or vehicle (0.9% NaCl), and locomotor activity assessment was then performed after this one week of rest, followed by a two-week period of withdrawal. Elevated plus maze assay was conducted fourteen days after last injection and electrophysiology was performed 15-20 days after the last injection.

3.2 Locomotor activity

During treatment, sensitization to the locomotor-stimulatory properties of amphetamine was recorded in a sound attenuated, dimly lit box (Kungsbacka Mät- & Reglerteknik AB, Fjärås, Sverige) containing a locomotor activity box (40 x 40 cm, Med Assoc., Fairfax, VT, USA). This locomotor activity box is equipped with two infrared light grids, arranged in two layers, mediating measurement of horizontal and vertical activity of the animals. Locomotor activity was recorded with Activity Monitor (Intego, Austin, TX, USA) software. These animals were first allowed to habituate to the room for one hour, then to the locomotor box for 30 minutes, where activity was simultaneously recorded. The same animals were then injected with

amphetamine (2.0 mg/kg, dissolved in 0.9% NaCl), or vehicle (0.9% NaCl), and monitored for another 30 minutes. Measured variables are vertical and horizontal beam breaks. From this data, the software then calculates rearing activity, where the animal breaks the lower and higher grid simultaneously, ambulatory activity, where the animal breaks consecutive photo-cell beams but not adjacent photocell beams repeatedly, and finally stereotypic counts, where the animal breaks adjacent photocell beams repeatedly. We are here interested in seeing the changes in ambulatory activity in the thirty minutes following drug or vehicle administration, as well as the habituation period, where we believe that the first five minutes are the most sensitive to detect change.

3.3 Elevated plus maze

After 14 days of withdrawal, anxiety-like behavior/behavioral disinhibition was monitored in the elevated plus maze (EPM) (Med Associates, St Albans, VT, USA). EPM is an elevated (0.8m) apparatus consisting of two closed (dark) arms and two open (bright) arms, arranged in the shape of a plus. After 1h habituation to the room, every animal is assessed individually for 5 minutes. Measured variables are time spent in open/closed arms or in the center zone as well as the number of times passed from open to closed arms or vice versa. We define total entries as a measure of general activity, decreased time spent on or entries into closed arms as decreased anxiety-like behavior or increase in risk-taking and increased time spent on open arms or increased entries into open arms indicates an increase in risk-taking or decrease in anxiety-like behavior. In the locomotor activity box, an increase in the ratio of activity in center relative to close to walls, is interpreted as an increase in risk-taking or decrease in anxiety-like behavior.

3.4 Electrophysiology

15-20 days after the last injection, electrophysiological recordings in subregions of the amygdala and associated brain regions were performed. All experiments were performed no more than 8 hours after sacrifice.

Brain slice preparation

For this procedure, animals were anaesthetized with isoflurane (AbbVie, Solna, Sweden) and sacrificed by decapitation, followed by swift dissection of the rat cranium and extraction of the brain. The brain was immediately submerged in modified, oxygenated artificial cerebrospinal fluid, aCSF (in mM; 220 sucrose, 2.0 KCl, 6.0 $MgCl_2$, 0.2 $CaCl_2$, 26.0 $NaHCO_3$, 1.3 NaH_2PO_4, 10 D-glucose), followed by slicing into 300-350µm coronal sections in a Vibratome 1200S (Leica Microsystems AB, Bromma, Sweden). Slices were allowed to rest in 32°C oxygenated aCSF (in mM; 124 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 10 D-glucose) for 30 minutes and in room temperature for at least another 30 minutes.

Field potential recordings

One hemisphere of the brain slice was positioned in a recording chamber, and continuously perfused (1.5ml/minute) with oxygenated and heated (30°C) aCSF. Population spikes were evoked with a stimulation electrode (monopolar tungsten electrode, World Precision Instruments, FL, USA) positioned locally in the area of focus. Changes in neuronal excitability was monitored through incremental increase of the stimulation strength, while changes in inhibitory neurotransmission was assessed by pharmacological inhibition of GABA receptors. First, we conducted an input-output protocol. Input-output is a seven-step

ladder of increasing stimulation strength (in mV; 18, 27, 36, 45, 54, 63, 72). Optimally, this gives a function that shows the maximum response amplitude.

To measure the effect of bicuculline on field potential response amplitude, a baseline was recorded for ten minutes. For this baseline, stimulation strength is set to approximately half of maximum recorded amplitude for each recording. Following this, $GABA_A$-receptor antagonist bicuculline (dissolved in H_2O to 20 mM and diluted in aCSF to 20 µM) was perfused continuously for 20 minutes. We then repeated the input-output protocol during bicuculline perfusion for comparison of maximum response amplitude before and after inhibition of $GABA_A$ receptors.

4. Data collection procedures, variable analyses, statistical methods

Clampfit 10.2 (Molecular devices, Axon CNS, CA, USA) and Excel (Microsoft, WA, USA) was used for extraction of PS amplitude of field potentials. Before testing for statistical significance, D'Agostino & Pearson test for normal distribution was performed to ensure normal distribution of data. Unpaired t-test was used to analyze behavioral data, while two-way analysis of variance (ANOVA), as well as unpaired t-test was employed for data deriving from electrophysiological recordings. Prism (GraphPad Software) is used for statistical analysis and data presentation.

5. Results

5.1 Behavioral sensitization

Five days of systemic administration of amphetamine produced behavioral sensitization with respect to the locomotor-stimulatory properties of amphetamine (1^{st} vs. 5^{th} injection: $t_{(8)}=2.46$, $p=0.043$) (Fig. 3B, E). Locomotor activity during the first five minutes of habituation to the activity box was not altered before the first (vehicle vs. amphetamine: $t_{(15)}=0.427$, $p=0.676$) or fifth (vehicle vs. amphetamine: $t_{(15)}=0.467$, $p=0.647$ injection, meaning no detectable sensitization to this environment prior to drug-exposure (Fig. 3C). Ambulatory activity in center zone of locomotor box was increased after fifth compared to the first injection in animals treated with amphetamine, but not in vehicle-treated controls ($F_{(3,20)}=3.494$; post-hoc: vehicle vs amphetamine: 1^{st} vs. 5^{th} injection of amphetamine $q_{(8)}=2.04$, $p=0.049$; post-hoc: 5^{th} injection of amphetamine vs. 5^{th} injection of vehicle $q_{(8)}=4.505$ $p=0.0222$) (Fig. 3D).

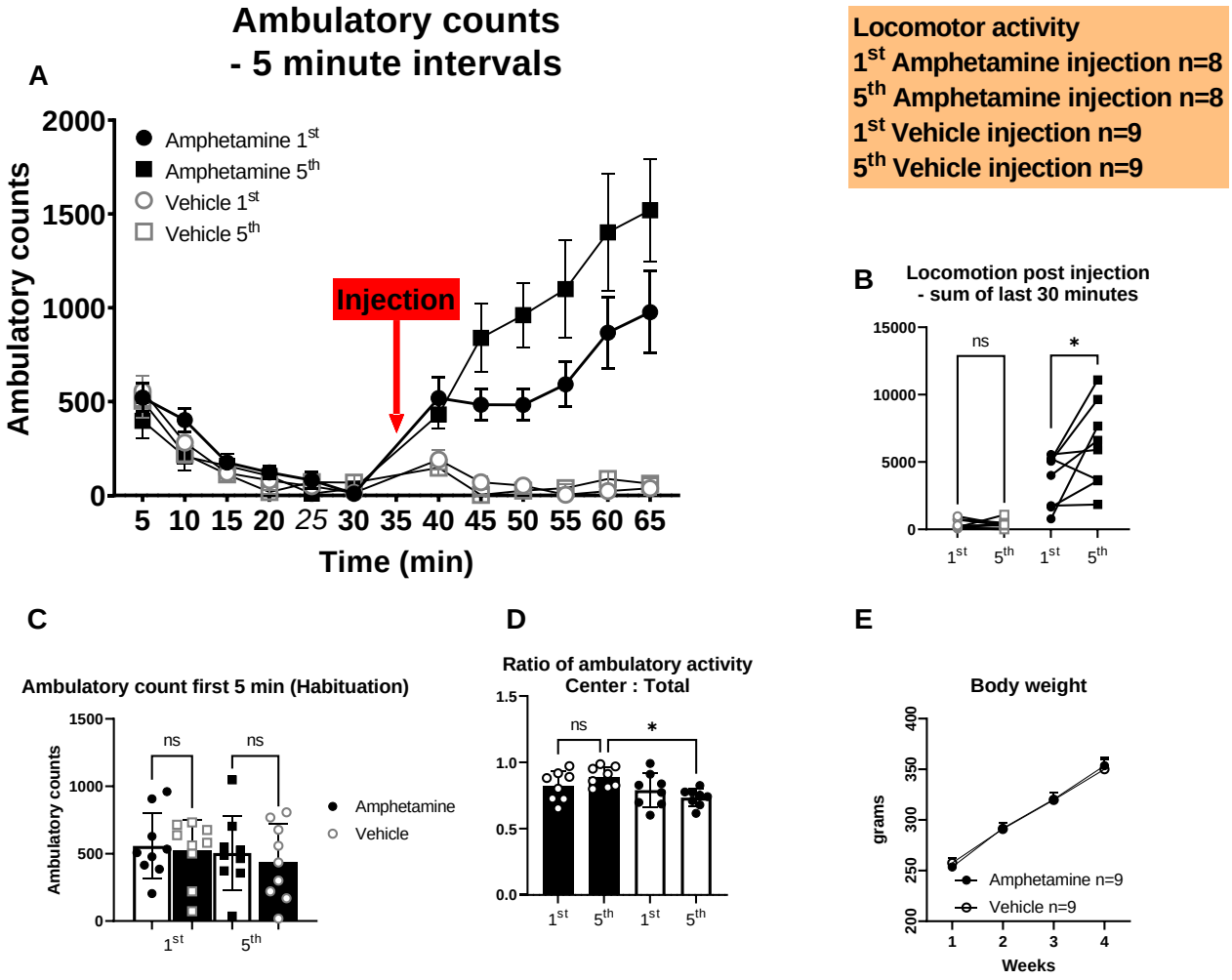

Figure 3. Five days of treatment produces a sensitized response to the locomotor-stimulatory properties of amphetamine. A) Ambulatory activity over time showing 30 minutes of habituation followed by an intraperitoneal amphetamine or vehicle injection. B) Sum of ambulatory activity, measured from t=35 to t=65 min, increased in amphetamine, but not vehicle, -treated animals after repeated injections. C) No inter-group difference in ambulatory activity during habituation to locomotor box during treatment. D) Graph showing ratio of ambulatory activity in center to total activity. A tendency toward increased activity after fifth injection, as compared to the first injection. Increased activity in amphetamine-treated animals after the fifth injection, but not the first injection, as compared to vehicle-treated animals. E) Weight monitoring of animals, weighed once per week. Data are mean values ± SEM, *p<0.05, *** p<0.001.

5.2 Elevated plus maze

To assess risk-taking as opposed to anxiety-like behavior, animals were monitored in an elevated plus maze after fourteen days of amphetamine withdrawal. General activity, as measured by total number of entries, did not differ significantly between groups (amphetamine vs. vehicle: $t_{(16)}=1.01$, $p=0.326$) (Fig. 4A). Assessing animal tendency to explore the respective arm yielded no significant result, neither for time spent on open arms (amphetamine vs. vehicle: $t_{(16)}=1.01$, $p=0.326$), nor time spent on closed arms (amphetamine vs. vehicle: $t_{(16)}=1.45$, $p=0.167$) (Fig. 4B.) However, looking at the number of entries per arm, we saw a significantly decreased proneness of the amphetamine-treated animals to enter the closed arm (amphetamine vs. vehicle: $t_{(16)}=3.01$, $p=0.0082$) (Fig. 4C). Time spent in center zone did not differ significantly between treatments (amphetamine vs. vehicle: $t_{(16)}=1.48$, $p=0.167$ (Fig. 4D)

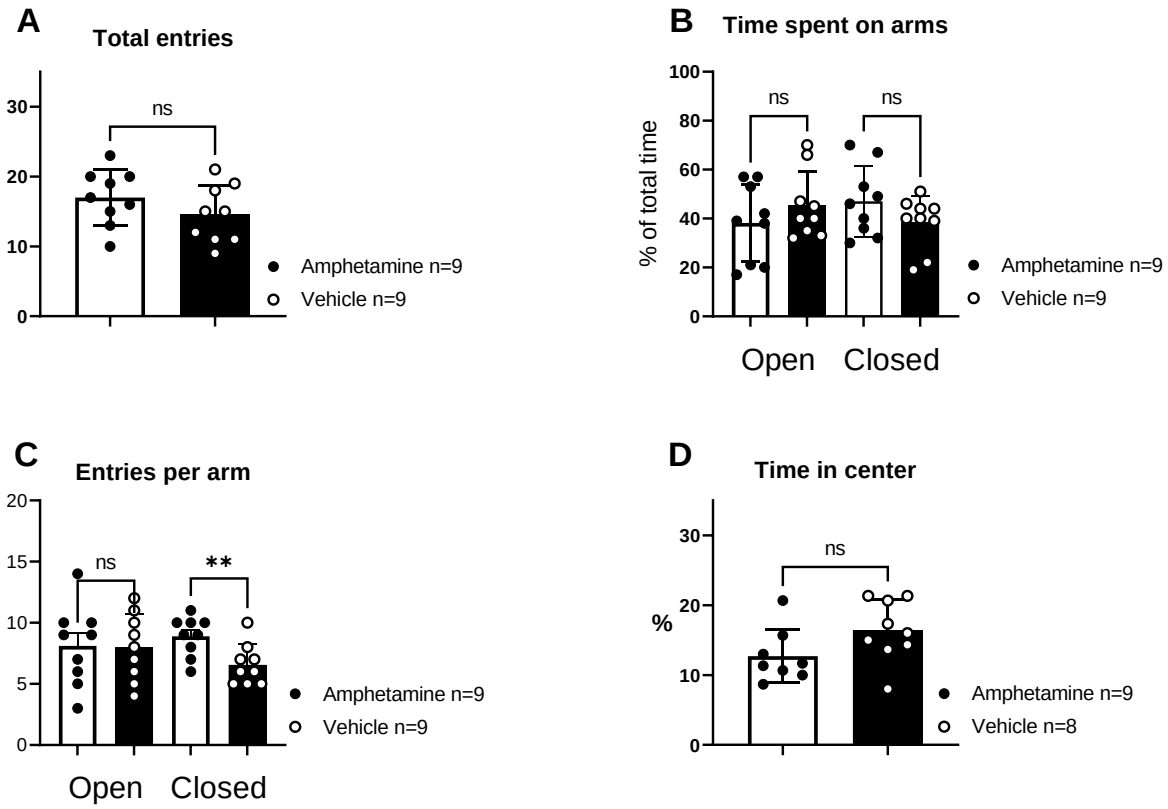

Figure 4. A possible reduction in anxiety-like behavior. A) No significant change in general activity seen after amphetamine exposure and two weeks of abstinence. B) A trend to tend to spend more time on open arms for amphetamine-treated animals after two weeks of abstinence. C) Amphetamine-treated animals, after two weeks of abstinence, show a significantly decreased tendency to enter the closed arm of the elevated plus maze. D) Time spent in center did not differ significantly between groups. Data are mean values ± SEM, ns=not significant, ** $p<0.01$.

5.3 Field potential recordings in the BLA

Two weeks post-treatment, changes in neural transmission was measured in the BLA. $GABA_A$ antagonist bicuculline (20µM) perfusion rendered significantly enhanced disinhibition in the amphetamine-treated animals (vehicle vs. amphetamine: $F_{(1, 18)}=4.8$,

p=0.0419) (Fig. 5A). No significant difference in approximated probability of neurotransmitter release, as measured by paired pulse ratio, in neither aCSF (vehicle vs. amphetamine: $t_{(20)}$=0.745 p=0.465) nor bicuculline (vehicle vs. amphetamine: $t_{(18)}$=1.27 p=0.221) perfusion. (Fig. 5D. In addition, we saw a significantly reduced amplitude of evoked field potentials (vehicle vs. amphetamine: $F_{(1,18)}$=54.8), p<0.001) (Fig 5E), and this was reversed by $GABA_A$ antagonist bicuculline (20µM) (vehicle vs. amphetamine: $F_{(1, 18)}$=0.372, p=0.549) (Fig 5F).

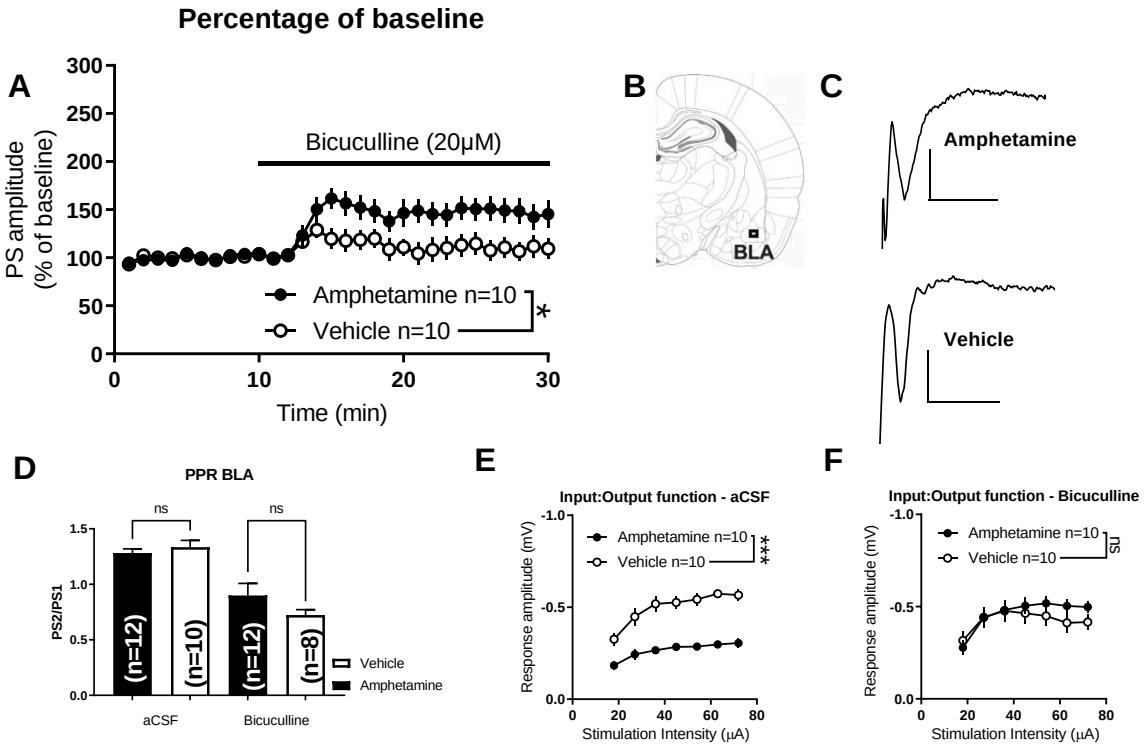

Figure 5. Altered neurotransmission in the BLA and reversal by Bicuculline. A) Disinhibition by GABAA-receptor antagonist bicuculline (20µM) was enhanced in amphetamine-treated animals compared to vehicle-treated animals. B) Illustration showing site of BLA recordings. C) Example traces of recordings in BLA during perfusion of aCSF. Calibration: 0.2 mV, 2 ms. D) No change in approximated neurotransmitter release, as measured by paired pulse ratio (PPR), after treatment. E) Decreased population spike intensity in amphetamine-treated animals. F) Disinhibition of population spike intensity by GABAA-receptor antagonist bicuculline (20µM) in amphetamine-treated animals.

Data are mean values ± SEM, *p<0.05, *** p<0.001, n=number of recordings. Recordings performed on at least 3 animals/treatment.

5.4 Field potential recordings in nAc and mPFC

In a way to assess effects by amphetamine in circuits involving the BLA, recordings were also performed in in nAc shell and mPFC. In nAc shell, there was a trend towards enhanced excitability in brain slices from animals previously treated with amphetamine (vehicle vs. amphetamine: $F_{(1, 37)}$=3.65, p=0.0638) (Fig. 6A). However, we did not see a significant difference between treatment groups in the mPFC (vehicle vs. amphetamine: $F_{(1, 33)}$=0.031, p=0.861 (6C).

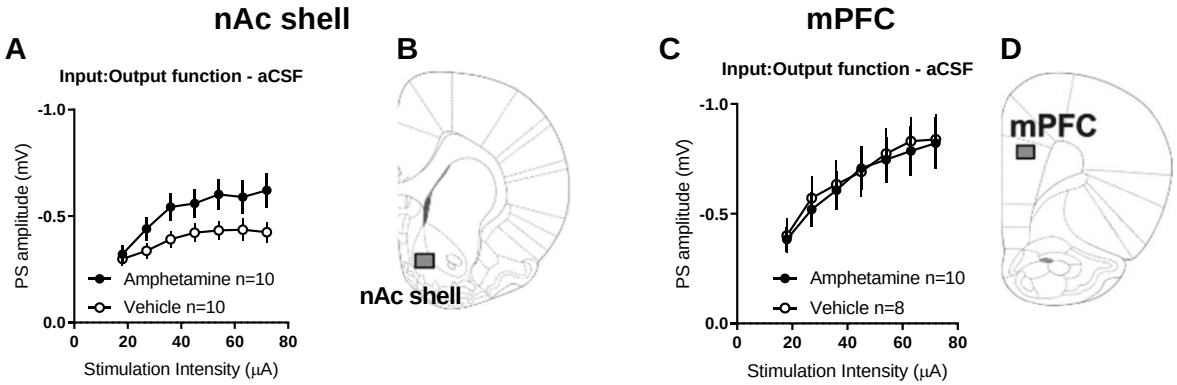

Figure 6. Neurotransmission in brain regions connected to the BLA after repeated exposure to amphetamine. A) We show a trend toward increased excitability in nAc shell in amphetamine-treated animals after two weeks of abstinence. B) Schematic drawing showing location of recordings in nAc shell, coronary section. C) No change in input output function in mPFC. D) schematic drawing showing location of recordings in mPFC, coronary section. Data are mean values ± SEM. *p<0.05, *** p<0.001. n=number of slices, recordings performed in at least three animals per group.

6. Discussion

6.1 Main findings

Five days of amphetamine (2.0mg/kg, dissolved in 0.9% NaCl) exposure induced behavioral sensitization to the locomotor stimulatory properties of amphetamine and increased the time in center zone of locomotor activity box. Following two weeks of abstinence, field potential recordings demonstrated a decrease in activity in the BLA. This coincided with a putative increase in risk-taking behavior, as measured by decreased number of entries into the closed arm of the elevated plus maze.

6.2 Locomotor activity

We show that five days of amphetamine injections are sufficient to induce sensitization to the locomotor-stimulating properties of amphetamine. As nAc and mPFC both are core players in sensitization to the locomotor-stimulatory properties of amphetamine (Vanderschuren and Kalivas, 2000), it is interesting to see that the measurable effect, seen as only as a trend in the nAc shell and not at all in mPFC, seems to be less prominent than that in BLA, which was significantly changed. It should however be noted that the data recordings performed here primarily reflects sustained neuroadaptations without the drug, and that changes in responsiveness to amphetamine were not assessed in electrophysiological recordings. Electrophysiological recordings were also conducted two weeks after the behavior was monitored.

Behavioral sensitization was accompanied by increased locomotion in the central zone of the open field maze, suggesting an increase in risk-taking and possibly a decrease in anxiety-like

behavior. Thigmotaxis, wall-hugging, is a natural behavior to prey animals such as rats and is related to anxiogenic behavior (Carola et al., 2002). Anxiolytic drugs have been argued to be specific in influencing this behaviour (Treit and Fundytus, 1988), but Simon et al. have previously shown that thigmotaxis decreases after amphetamine administration, which is partially in line with the data presented here. A net increase in dopamine signaling enhanced thigmotaxis in their experiment, whereas a net decrease in dopamine signaling attenuated thigmotaxis. Thigmotaxis was also shown to decrease after administration of the anxiolytic drug phenobarbital, a $GABA_A$-receptor positive modulator/agonist. In summary they speculated thigmotaxis to be a behavior governed by an interplay between dopaminergic, serotonergic and GABAergic signaling (Simon et al., 1994). This is somewhat in contrast to our findings here, as amphetamine did not significantly decrease thigmotaxis at first exposure. It did however tend towards decreasing at fifth exposure as compared to first exposure to amphetamine, and decreased significantly at fifth exposure to amphetamine as compared to fifth exposure of vehicle.

However, we cannot know what brain activity that gives rise to this behavior. Amphetamine is known to cause other aberrant behaviors such as lapse in attention (Robinson and Becker, 1986), and d-amphetamine in similar doses to what we use here (1.7mg/kg) have previously been shown to impair attention tasks in rats (Slezak et al., 2018). Perhaps amphetamine treatment causes some unknown cognitive impairment, leading to maladaptive decision-making, which could explain the reduction in thigmotaxis we see here.

6.3 Elevated plus maze

We demonstrate a decrease of closed arm entries in the elevated plus-maze after repeated amphetamine exposure and two weeks of abstinence in the Wistar rat. This could be interpreted as a decrease in anxiety-like behavior. However, as in all scientific endeavors we must pose the question if we measure the right variables, in a correct manner. Perhaps measurements of more variables in the elevated plus maze is needed for a more robust interpretation of the data (Campos et al., 2013). In addition, an approach with combined statistical analysis of measurements in the locomotor activity box and the elevated-plus maze could have yielded even more information (Carola et al., 2002). It is also possible that more animals are required, in order to increase statistical power enough, to yield a significant change.

6.4 Alterations in neural activity

Amphetamine-treatment followed by two weeks of abstinence rendered a distinct decrease in evoked field potentials in the BLA, which was reversed by $GABA_A$ antagonist bicuculline. This indicates an increased GABAergic inhibitory tone on the BLA after two weeks of amphetamine abstinence. An interaction between amphetamine and the GABA system has previously been shown using oocytes, where amphetamine was shown to potentiate inhibitory currents via $GABA_A$ receptors (Hondebrink et al., 2011, Hondebrink et al., 2013). The more probable reason however, is that an area projecting to the BLA either increase excitatory or decrease inhibitory output to BLA.

Other regions of amygdala are the central nucleus of the amygdala (CeA), a main output region mainly consisting of GABAergic projection neurons (McDonald, 1998, McDonald and Augustine, 1993). The CeA does not project directly to the BLA, but feed-forward and feed-back inhibition provided from dopamine D1/D2-receptor expressing intercalated cell masses (Fuxe et al., 2003), could possibly influence the activity in the BLA. These intercalated cell masses also recieve a large amount of projections from mPFC (Vertes, 2004), indicating another factor of excitability of BLA neurons.

We could only discern a trend towards increased excitability in nAc shell. Regarding the nAc, $GABA_A$ receptor and glutamate decarboxylase (GAD), which synthesize GABA, has been shown to decrease in nAc after seven days of methamphetamine injection and self-administration, followed by 28 days of withdrawal (Zhang et al., 2006). Given that there is a difference in our study, which we might have seen with a greater number of recordings or a more adept electrophysiologist, perhaps it is cohesive with these previous findings. That is, if a decrease in GABAergic signaling locally results in increased synaptic output. As BLA projects reciprocally to nAc (Vanderschuren and Kalivas, 2000), perhaps increased excitatory output from BLA onto local GABAergic neurons and give rise to an increased GABAergic tone? However, recently published experiments on rats with synaptotagmin 1-knockdown in prelimbic cortex, that subsequently display increased alcohol preference, show no increase in nAc core excitability, even though there is an enhanced disinhibition in BLA that is reversed by bicuculline perfusion (Barbier et al., 2021).

In the mPFC, Koya et al. has previously shown that phosphorylated extracellular signal-regulated kinase (pERK) levels, a measure of neuronal activity, increase over two-fold in

ventral mPFC (vmPFC) after ten days of self-administration of cocaine and protracted withdrawal. This effect was blocked by $GABA_{A/B}$-agonists and enhanced by $GABA_{A/B}$-antagonists (Koya et al., 2009). We could not show a difference between groups in evoked potentials in the mPFC. As our animals did not self-administrate, nor trained extinction and neural activity is measured with a different method, it is hard to say what could explain the difference. The data presented here does not support an effect but it is possible that this is due to the time-point we have chosen to measure at and how long the animals were exposed to amphetamine.

Previous studies on $GABA_A$ergic signaling connected to psychostimulants are indeed intriguing. Targeting $GABA_A$-receptors systemically with benzodiazepines such as alprazolam or diazepam may have benefit for treating psychostimulant users, as they diminish perceptions associated with taking amphetamine (Jiao et al., 2015a, Rush et al., 2004, Panhelainen et al., 2011), and benzodiazepines can block behavioral sensitization to amphetamine (Ito et al., 2000). Argon, a non-anesthetic noble gas, acts as a $GABA_A$ receptor agonist (as well as μ-opioid receptor antagonist) and can prevent induction of behavioral sensitization (David et al., 2014). Perhaps the neural transformations leading to increased GABAergic tone in the BLA that we see in our experiments can be blocked by concomitant exposure to positive modulators of the $GABA_A$-receptor? Perhaps a focus for future studies can be to block or reverse this transformation of GABAergic neurons in, or projecting to, BLA.

Extinction-training procedures, attempts to reverse the change in animal or human behavior that drugs of abuse cause, are common models of attempting to study relapse prevention.

Amygdala associative memory functionality appears to play a key role in extinction (Grewe et al., 2017, Yuan et al., 2020), and although hard to implement in human clinical trials, previous attempts have been made (Xue et al., 2012, Metcalf et al., 2018, Worley, 2019). Perhaps extinction training using newer treatment-

modalities like virtual or augmented reality (Worley, 2019, Metcalf et al., 2018), paired with pharmacological interventions targeted towards GABAergic or glutamatergic signaling, has some potential for future studies.

Glutamatergic signaling is also involved in the formation of psychostimulant-associated memories. AMPA-receptor levels have previously been shown to be increased in BLA after exposure to methamphetamine and blocking the endocytosis of these receptors hinders strengthening of methamphetamine-associated memories (Yu et al., 2013). In unpublished experiments, we have completely blocked the response seen in BLA with the AMPA-receptor antagonist CNQX. As the responses we see in BLA are glutamatergic, and as we have previously blocked these responses in full with AMPA-receptor antagonists, it may follow target the aberrant glutamatergic signaling of the BLA can be targeted to ameliorate, for example, relapses induced by cues associated with psychostimulants.

6.5 Implications

Enhanced disinhibition of BLA, increased risk-taking under influence of amphetamine and arguably increased risk-taking during two weeks of abstinence. How do we interpret these data? A possibility is that if the system assessing of environmental dangers malfunctions, this could lead to poor risk-management.

We do not currently know what causes these changes in $GABA_A$ergic tone in BLA, or what this signifies. Future studies could investigate if local manipulations of the GABAergic signaling in the BLA can reverse the behavioral change caused by repeated amphetamine exposure.

6.6 Limitations

We show a difference in the amphetamine treated animals' tendency to explore the open arm over the closed arm. The relevance of this result is however clouded by a methodological flaw, as the room where we conduct the EPM-trial is not separated from where researchers record behavior. The animal hears and smells the researcher, the same individual that injected it with amphetamine, potentially influencing the results. This then poses the question: Are the results seen really related to risk-taking?

In all of the performed experiments, researcher laboratory experience and prowess determinates the robustness and validity of data acquired. As all experiments described here were performed by a student, albeit under supervision, the results have to be regarded under this lens.

Post-hoc analysis of a dataset with a multitude of measurements, as we did here with center-zone activity, is always to be looked at with scrutiny. The post-hoc analysis of center-zone activity can, at best, be an indication of which areas are of interest for future studies, not as evidence of an actual difference.

The neurophysiological assessment was conducted in brain slices, in an artificial environment. This affects the validity of our results as the brain regions studied are not

interconnected with other brain regions when we record. Is the result then in a given region really a true representation of the live brain? Perhaps, if all neurophysiological transformations are local and conserved during the treatment. But if given for example that BLA projects onto mPFC and this leads to a net inhibition of mPFC, a transformation that leads to decreased output from the BLA to mPFC could theoretically decrease mPFC output *in-vivo* without detectable changes in a slice-preparation.

It is also important to realize that the method, although having a long history (Renshaw et al., 1940), has limitations like comparatively poorer spatial and functional resolution. It is however reliable, accessible and efficient.

6.7 Ethical considerations

Animal studies are needed in order to map out the intricacies of addiction. This can in turn help us find therapies to relieve the substantial suffering caused by this brain disease. However, we must ask ourselves every step of the way if it is worth the harm caused. In this project, we do not estimate that we cause the animals any unnecessary suffering. Injections can of course cause discomfort, so animals are habituated to handling before injections to ameliorate stress caused by handling. Animals are as well assessed individually for signs of stress, discomfort, or injury. Animals are caged, however in relatively large cages and cared for by professional facility technicians in collaboration with veterinarians which in addition to researchers make sure that animals are tended to in a way that minimizes stress, pain and other discomfort. Before decapitation and for behavioral testing, animals are transported, which can cause stress. Animals are decapitated after deep anesthetization and do not

experience pain or suffering from this, however Isoflurane could be perceived as discomforting or stressing while falling unconscious.

In this work, we use rats for our research. As animal researchers, we work directed by the 3R, reduce, refine, replace. We work to reduce animal suffering by using each individual for a multitude of experiments, as well as carefully handling these animals in a manner that minimizes stress and discomfort. The rats used in this project have been recorded in field potential recordings in other brain regions, with patch-clamp, and we have taken a variety of blood samples in conjunction with the decapitation. We refine our experimental procedure through training of personnel involved in all handling of rats, we plan our experiments to reduce stress in the animals by making sure that animals are as relaxed as possible before each procedure, and by reducing noise and other stressors. The blood samples used in these animals are utilized to connect neurological transformations to blood markers and could therefore be a bridge to non-animal research, as blood markers are available for human studies.

7. Populärvetenskaplig sammanfattning på Svenska

Beroende är en hjärnsjukdom som orsakar omfattande lidande och drabbar människor över hela jorden. En rad beteenden som människor uppvisar vid beroende kan undersökas i djurmodeller och i den här studien har vi använt oss av råttor. Råttan och människans hjärnor är olika men många viktiga kretsar är evolutionärt bevarade. Vi tror att symptom på beroende som vi ser i råttornas beteende kan förklaras med mätbara förändringar i hjärnan. Med en bättre förståelse av hjärnans kretsar så tror vi att vi kan hitta bättre behandlingar för denna utsatta grupp människor.

I det här projektet har vi undersökt hur råtthjärnan påverkas av upprepad amfetaminbehandling följt av amfetaminabstinens. Detta i hopp om att bättre förstå oss på de kretsar i hjärnan som är involverade i amfetaminberoende och beroende i allmänhet. Vi har därför injicerat arton råttor vid fem tillfällen under en period på sju dagar, med antingen amfetamin löst i koksalt eller med endast koksalt, som kontrollgrupp. Vi har mätt förändringen i beteende hos råttor genom två olika beteendeförsök och mätt skillnader i hjärnans kretsar med hjälp av elektrofysiologiska fältpotentialsmätningar.

I våra beteendeförsök har vi sett att råttor som exponerats för amfetamin upprepade gånger rör sig mer och mer. Detta tyder på en tilltagande känslighet för amfetamin vid upprepad exponering, en sensitisering. Dessutom har vi sett att råttor som fått amfetamin är mer benägna att röra sig i öppna områden och inte längs med väggar, vilket råttor normalt sett föredrar. Detta kan tyda på att upprepad exponering för amfetamin har en effekt på råttornas riskbenägenhet. Vi har dessutom sett hur de råttor som fått amfetamin två veckor tidigare har

en benägenhet att röra sig i upplysta områden framför dunkla områden, vilket vi spekulerar kunna bero på ett drogsökande beteende.

Vi har också mätt i ett hjärnområde som bland annat sammankopplar emotionellt värde med kontext och bidrar till att detta lagras som minnen. Ett exempel på detta är kopplingen mellan en omgivning där man får belöning i form av mat – eller ett ljudet av ett rovdjur som efter ett farligt möte kan associeras till rädsla. Beroendeframkallande droger verkar kunna påverka dessa livsviktiga kretsar och detta tror vi leder till att signaler och beteenden som kopplas till drogen kan agera som påminnelser om drogen och leda till återfall.

Sammanfattningsvis kan vi säga att vi i dessa råttor sett att upprepad amfetaminexponering ger upphov till kroniska förändringar i hjärnregioner som är viktiga för emotionella minnen samt en potentiell förändring i risktagande beteende. Vi anser därför att amfetamin och amfetaminabstinens effekter på hjärnan, tillsammans med risktagande beteende, behöver studeras ytterligare då inte tillräckligt är känt om dessa kretsars beskaffenhet och kopplingen till risktagande beteende.

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