Utrecht University

MSc Veterinary Medicine
Animals in Science and Society

Research Report

The role of the Prelimbic prefrontal Cortex and the Basolateral Amygdala in reward seeking under threat of adversity

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February, 2019
15 ECTS Credits
October 2018 – February 2019

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Behavior & Welfare
Behavioral Neuroscience

The role of the Prelimbic prefrontal Cortex and the Basolateral Amygdala in reward seeking under threat of adversity

Article information

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Date: February 2019

Keywords:
Drug addiction, sucrose, amygdala, prelimbic cortex, STA-model, conditioned suppression

ABSTRACT

Addiction is a worldwide problem with an enormous societal, economic and health burden, while treatment options are limited. A hallmark characterizing addiction is persistent drug seeking despite harmful consequences. The loss of control over drug use is mediated by the transition from goal-directed drug seeking and taking to habitual drug use. On the basis of their involvement in impulse-control, goal-directed behavior and modifying behavior on aversive events we investigated the role of the prelimbic prefrontal cortex (PrL) and the basolateral amygdala (BLA) in drug seeking despite negative consequences. The novel 'seeking under threat of adversity (STA)-model' was used, which aims to reflect the situation of human drug addicts. Therefore, we tested the hypothesis that inactivation of either the PrL and the BLA will increase loss of control over reward seeking in this task, meaning seeking behavior will be displayed despite the threat of adversity. Pharmacological inactivation of the PrL and the BLA, using the GABA receptor agonists baclofen and muscimol, did not increase loss of control over drug seeking under threat of adversity. Further research on these and other brain areas involved in addiction needs to be done to understand the underpinnings of this chronic, relapsing disorder and to develop effective treatment strategies for addiction.

Introduction

Relevance research on drug addiction

Addiction is a complex disorder that is characterized by recurrent relapse which reflects loss of control over substance use (Koob & Le Moal, 2005). Substances of abuse, like alcohol, cocaine, heroin etc., have been used by humans for many centuries, and alcohol and tobacco are the most commonly used substances of abuse in the world (Van Laar, 2017; World Health Organization 2014). In western societies such as the Netherlands over 80% of the adult population consumes alcohol on a regular basis, which is the highest percentage alcohol intake of the world (Van Laar, 2017; World Health Organization 2018). Of all individuals consuming alcohol, 5-10% develops alcohol use disorder (AUD) (European Status Report on Alcohol and Health, 2010; Van Laar, 2017). Individual variation of addiction sensitivity is considered to be a result of the degree of alcohol use, genetics and psychological, cognitive, and environmental risk factors (Enoch, 2012, 2013; Jurk et al., 2015) AUD has a tremendous societal, economic and health burden, yet treatment options for AUD are limited in both number and efficacy (Vanderschuren et al., 2017; Volkow & Li, 2005; World Health Organization 2018, 2018). For example, the total cost for addiction in 2010 accounted for more than €65 million in Europe, in the US they appear to be relatively higher (Gustavsson et al., 2011). Overall, alcohol is considered to be the most harmful and costly substance of abuse for individuals and society overall (Nutt et al., 2010). This poses a need to better understand the neurobiological and genetic underpinnings of this devastating disorder, to serve the need to develop effective treatment strategies for addiction.

Addiction is characterized by loss of control over substance of use. During the process of becoming addicted, drugs use escalates from casual consumption to inappropriate use to abuse with compulsive craving for drugs (Everitt & Robbins, 2005, 2016; Pierce & Vanderschuren, 2010), despite the awareness of its adverse consequences (Ahmed, 2012; Vanderschuren & Ahmed, 2017). Resulting in inflexible drug seeking, persistent and insensitive to punishment or devaluation (Everitt & Robbins, 2005). More characteristics of addiction are dependency (Speranza et al., 2004), impulsivity (Winstanley, 2007) and maladaptive memory (Milton & Everitt, 2012). More drug intake ensures neural adaptations that facilitate the downfall to an adaptive state (Vanderschuren & Everitt 2005, 2016; Kalivas & O'Brien 2008).

Substance addiction is mainly driven by changes in the rewarding (mesolimbic and mesocortical) systems of the brain (Koob & Le Moal, 2005). Hence, this neurobiological system is not only involved in general rewarding behaviors, like sex and eating sugar or food, it is also involved in seeking and taking drugs including alcohol and cocaine (Davis and Carter, 2009; Volkow and O'Brien, 2007; Wilson, 2010). Using complex behavioral tasks different aspects of behavior related to rewards are revealed, like motivation to seek for a substance of use (Dalley et al, 2011).

'Substance use despite negative consequences' models

Human research in this field is limited, because it is difficult to investigate specific factors underlying the origin of addiction. Furthermore, it is demanding to execute profound neurobiological research requiring mostly ethically inconvenient operations in humans. Therefore animal models are widely used. The Statistical Manual of Mental Disorders IV (DSM-IV) criteria for addiction are the generally accepted standards used for human. Characteristics of addiction presented in animals are: escalations of drug use, neurocognitive deficits, resistance to extinction, increased motivation of drugs, preference for drugs over nondrug rewards, and resistance to punishment (See table 1), according to Vanderschuren & Ahmed (2013): "The neural machinery that underlies drug seeking and taking is present and can become dysregulated in non-human animals as is does in humans."

Table 1. Appearance of DSM-IV criteria in animal studies of drug addiction. Reproduced from Vanderschuren & Ahmed (2013).

DSM-IV criterion	*Behavioral equivalent in animal studies*
1. *And 2. Tolerance, withdrawal*	Tolerance, escalation of drug use
3. *Using more than intended*	Impaired control, neurocognitive deficits
4. *Difficulty restricting*	Resistance to extinction
5. *Great deal of time spent*	Increased motivation for drug
6. *Other activities given up*	Drug preference over nondrug rewards
7. *Continued use despite problems*	Resistance to punishment

As mentioned above it is hypothesized that there is a shift from goal-directed substance use to habitual use and that there is a breakdown of control over drug use (mediated by the prefrontal cortex). Substance of use despite negative consequences' models are widely used to study the loss of control over drug seeking behavior in animals (Bergman and Johanson, 1981; Grove and Schuster, 1974; Kearns et al., 2002; Limpens et al., 2014, 2015). Negative consequences to test this compulsive behavior could be punishments such as: bitter taste solutions with quinine (Wolffgramm & Heyne, 1995), lithium chloride, resulting in conditioned taste aversion by physical malaise (Dickinson et al, 2002; Miles et al. 2003), and mild electrical foot shocks (Hopf & Lesscher 2014; Vanderschuren et al. 2017). Foot shocks are the most widely used punishments in rodents, they are easily variable in terms of intensity, quantity and probability (Vanderschuren et al. 2017). For example Deroch-Gamonet et al (2004) showed that cocaine seeking behavior can be suppressed by foot shocks that are contingent upon lever pressing for cocaine.

Ignoring alternative pleasures or interests to continue drug use is one of the most important behavioral symptoms of drug addiction, causing deprivation of important occupational, social or recreational activities (DSM-IV). We are using a new probabilistic punishment model which is a variant of existing punishment models (Vanderschuren et al. 2017). It is called the STA-model, which is explained as reward seeking under threat of adversity. During a tone interval there is 25% chance to get a mild foot shock when pressing the lever for a reward. The possibility of being punished in this model reflects the possible negative consequences of taking substance of abuse in human. In the human situation negative consequences caused by addiction (such as losing your job, fall into debts or social isolation) are not always directly contingent with seeking for or taking substance of use, similar to the STA-model. Besides that, the tone in this model is used as a 'warning' signal to simulate the awareness of possible negative consequences of taking drugs. The combination of the possibility of a shock and a warning signal makes this model conforming as accurate as possible to addiction in humans. In this study sugar pellets were used as the reward to study the motivational seeking behavior under threat of adversity.

The neurobiology of reward seeking under the threat of adversity

While substance use is initially driven by the rewarding and motivational aspects of the substance, the use of substances of abuse is thought to become less and less goal-directed with prolonged substance use. Rather, drug use becomes more habitual and compulsive (e.g. reviews by Everitt & Robbins, 2005, 2016 or Koob & Volkow, 2010). Neurobiologically, this change in behavior is thought to be driven by a shift from ventral striatal to dorsal striatal activity and a breakdown of prefrontal cortical control over behavior. Literature shows that with prolonged exposure to alcohol, operant responding for alcohol becomes insensitive to devaluation, thus indicating habitual behavior. The meaningful reward systems in the brain are the mesolimbic and the mesocortical pathway with the origin of the dopamine neurons in the ventral tegmental area (VTA) projecting to brain regions like the ventral striatum (nucleus accumbens

(NAc) and olfactory tubercle), the dorsomedial striatum, the prefrontal cortex (PFC), the hippocampus and the amygdala (Koob & Le Moal, 2005). Drugs of abuse interfere with these brain areas involved in rewarding behavior. For example, inactivation of the dorsolateral striatum (DLS) was shown to restore goal-directed behavior, thus emphasizing the impact of the DLS in alcohol-related habits (Corbit et al., 2012). The amygdala and the medial PFC (mPFC) play a key role in the fear circuit, including acquisition, consolidation, retrieval and extinction of fear memory for example during punished seeking behavior (Marek et al., 2013). Importantly, using a model of punished cocaine seeking in rats, Chen and colleagues (2013) showed that compulsive cocaine seeking was associated with hypoactivity in the prelimbic prefrontal cortex (PrL), a subregion of the prefrontal cortex. Moreover, stimulation of the PrL could restore control over cocaine seeking in these animals. Limpens et al. (2014) showed that inactivation of the PrL could induce compulsive cocaine seeking in rats. Other studies have shown that pharmacological inactivation or lesions of the mPFC results in operant responding for both sucrose and cocaine that is insensitive to potential punishment (Limpens et al., 2015; Resstel et al., 2008) and that inactivation the PrL results in impaired decision-making (Zeeb et al., 2015). The basolateral amygdala (BLA) has been implicated in suppressing punished reward-seeking and can modulate goal-directed behavior via the NAc (Piantadosi et al., 2017). Jean-Richard-Dit-Bressel and McNally (2015) set out the role of the BLA in punishment and showed that the BLA promotes behavioral suppression during punishment. Inactivation of the caudal BLA eliminates the inhibition of lever pressing produced by a contingent foot shock. So, these rats made more active lever-presses during punishment compared to their controls. Moreover, rats with selective lesions to the BLA increased their motivation for cocaine-seeking under punishment and were impaired in the acquisition of conditioned fear (Pelloux, Murray & Everitt, 2013).

The aim of this study was to investigate the role of the PrL and the BLA brain regions in reward seeking behavior under the threat of adversity. In this experiment the PrL and the BLA brain regions were inactivated by GABA-agonists during the operant task using the STA-model, pressing for sucrose with probabilistic punishment during a tone interval. It is hypothesized that inactivation of either the PrL and the BLA will increase loss of control over the task, expressed in an increase of seeking behavior despite the threat of adversity.

Material and Methods

Animals

Male Lister Hooded rats obtained from Charles River (Germany) were used. They arrived weighing 200-250 gram and were 8-10 weeks old. They were individually housed under a reversed 12-hour light/dark cycle (lights on at 07:00 AM) and controlled temperature (20-21 °C) with ad libitum access to water and chow. Prior to surgery, the rats were acclimatized to housing conditions for at least 7 days. The rats were divided in two groups, a group of 12 BLA animals and a group of 12 PrL animals, from which one rat was excluded of the experiment and data analysis due to broken cannulas. All experimental protocols were approved by the Animal Ethics Committee of Utrecht University and conducted in compliance with guidelines provide by the Dutch Law (Wet op de Dierproeven, 1996) and the European regulations (Guideline 86/609/EEC). All reasonable efforts were made to minimize the number and suffering of animals used.

Apparatus

Behavioral testing and training was conducted in six (29.5 cm length x 24 cm width x 25 cm height; Med Associates; Georgia, VT, USA) operant conditioning cambers as schematically shown in figure 1. The operant chambers are each surrounded by a sound-attenuating and light reducing box, containing a fan for ventilation and to mask external sounds. Each chamber is equipped with two 4.8 cm wide retractable levers placed 11.7 cm apart from each other and 6.0 cm above the grid floor, in between them there is a receptacle from which the sucrose pellet reinforcement was delivered. The placement of the active and the inactive lever was counterbalanced between rats to avoid place preference. Each box was outfitted with two cue lights (28 V, 100mA), one above each lever and a house light (28 V, 100mA) on the opposite wall at the left side of the chamber where the toner was also placed (85dB and 2900 Hz). The floors consist of 19 stainless steel rods spaced 1,5 cm apart connected to a shocker (28 V; Med Associates, Georgia, VT, USA) with two output ranges: 0-1 mA or 0-5mA for the delivery of foot

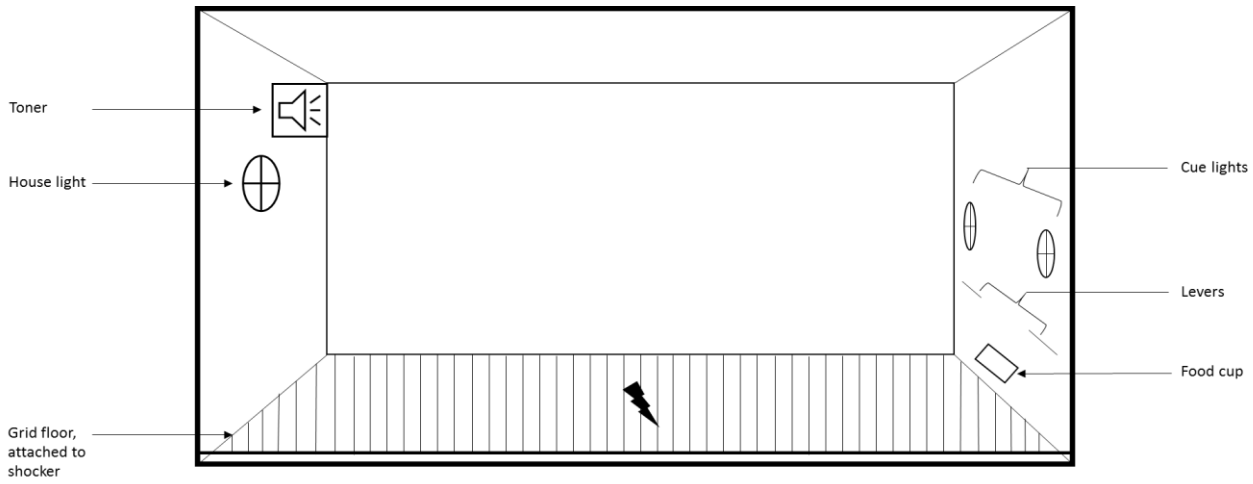

Figure 1. Schematic overview of operant conditioning chamber.

shocks. Recording experimental data was controlled by scripts written in MedState Notation using MED-PC for Windows.

Surgery

The rats were anesthetized with ketamine (75 mg/kg) and dexdorm (0.4 mg/kg) using KetDexdorm mix (i.m.). Carprofen (5 mg/ml) was used as analgesic. Next, the animals were placed in the stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). The coordinates used for placement of the bilateral cannulas above the BLA or PrL, relative to bregma (Watson & Paxinos, 2010), are shown in table 2.

Table 2. Coordinates relative to bregma of cannula placing in the rat brains. Shown are the coordinates in mm used for placement of the cannulas in the stereotact in the rat**.**

Direction	*BLA*	*PrL*
Anteroposterior (AP)	-3.4	+2.8
Mediolateral (ML)	±5.0	±1,8
Dorsoventral (DV)	-7.3 (angle 0°)	-2.7 (angle 20°)

Besides two cannulas, three miniature screws were also placed in the skull and a cement layer was placed on top, forming a cap that keeps the cannulas stable and in place. The animals were intraperitoneally injected with Atipamezol (0.6 mg/kg) to antagonize the anesthesia and NaCl was injected subcutaneously to keep the rats hydrated while waking up. Carprofen (5mg/kg, s.c.) was used as post-operative pain medication the 2 days after surgery. The rats were given approximately 1 week to recover prior to continuation of their behavioral training.

Training

Training was performed in the operant conditioning chambers as described above. The rats were first trained on a Fixed Ratio 1 (FR1) schedule, followed by a Random Interval 5 (RI5) schedule, in which every interval takes an average of 5 seconds. At the end of every interval a reward was earned if the rat pressed more than once. When the rats responded consequently, the time lock of the random interval was increased. The Random Interval 15 (RI15) followed. After they pressed well in this schedule too, consecutively RI30, RI60 and RI120 intervals were used. After the first active lever press the program would start an random interval in which active lever presses were recorded. One sucrose pellet was earned after the required presses in each session, the intervals lasts respectively an average of 5, 15, 30, 60 and 120 seconds. The animals underwent surgery in between the training sessions. In the training sessions no tone was presented and no foot shocks were used. Pressing the inactive lever was without consequences.

The STA-model

After training on the RI schedules, baseline sessions were introduced, which were largely similar to the RI120 sessions. The tone and the shock were not presented during the 30 minute baseline (BL) sessions. When the animals showed stable responding during the baseline sessions, i.e. when the mean of active lever presses of the last three baseline sessions of each animal did not exceed a difference of 25% of the overall mean of those three sessions, a test session was performed. Conditioned suppression test sessions using the STA-model were similar to the baseline sessions with the addition of a tone at the last 30 seconds of every interval. During the tone presentation, every active lever press could result in a shock with a probability of 25%, except for the first press which was never punished (figure 2). The shock intensity was 0.25 mA for the PrL animals and 0.30 mA for the BLA animals. The rats had to press at least once

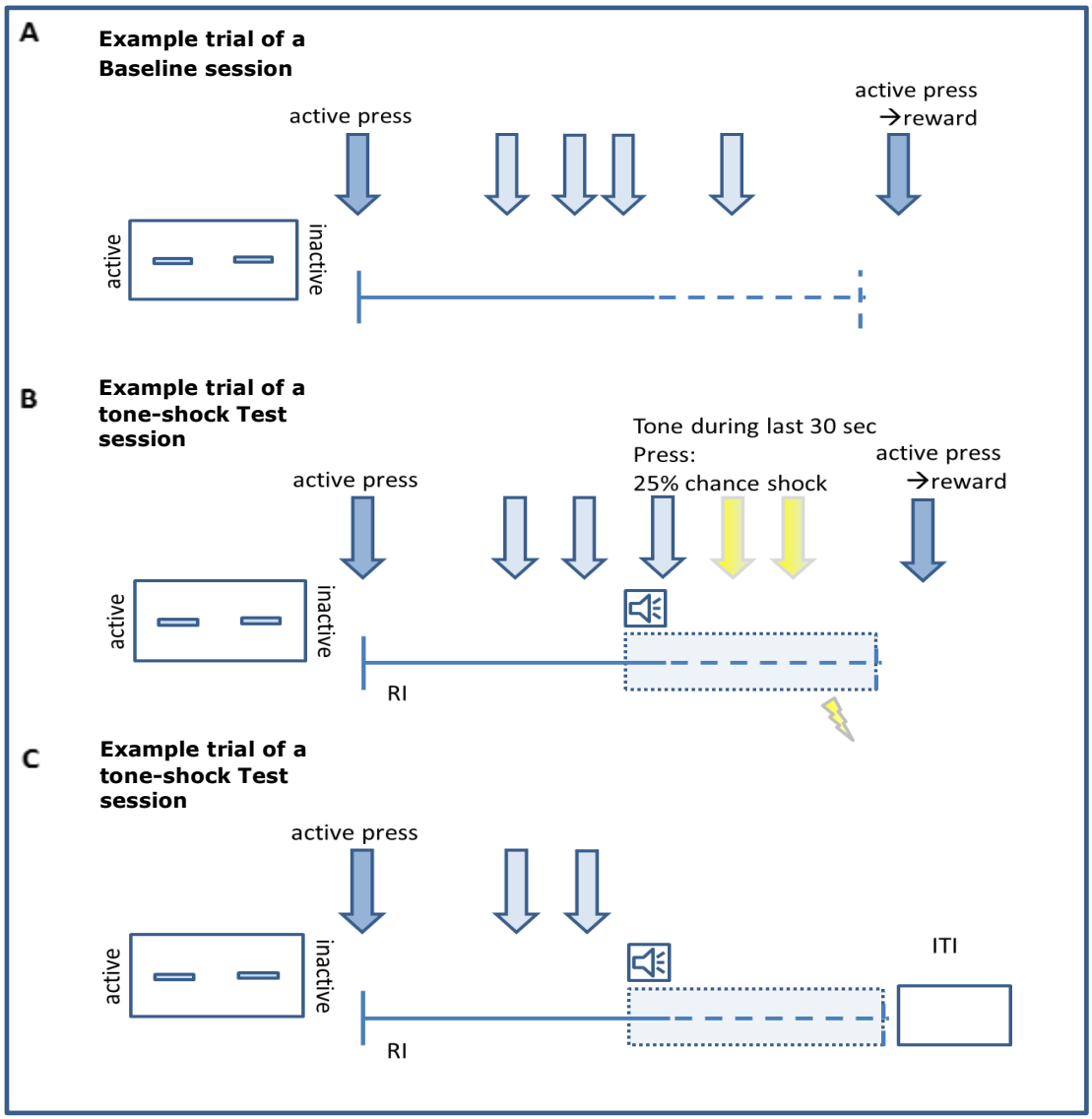

Figure 2. Schematic representation of model. (A) Baseline session, here no tone or shock is presented. (B) Test session where the requirement of at least one press during the tone is performed. (C) Test session were the required first active lever press during tone is not performed. Arrows indicate active lever presses.

during the tone (i.e. an active press in toneframe), otherwise the reward would not be presented and an inter trial interval (ITI) of 10 seconds would start. During the ITI the lights were off and both of the levers were retracted. The tone-shock sessions were always performed on consecutive days.

Microinfusion

Once an individual animal displayed a stable performance on the baseline sessions, it received a habituation infusion prior to a baseline session and a day later prior to a tone-shock session. This procedure consisted of wrapping the rat in a towel, removing the obturators, insertion of injectors in the cannulas, injecting 0.3 µl of the vehicle saline (0.9% HCl) bilateral in 1 min. using a Hamilton injector pump, leaving the injectors in place for an additional 60 sec to allow for diffusion of solution from cannula tip, removing the injector and placing the obturators back in the cannulas. Animals were placed back in their home cage and the session in the operant chambers started 10 minutes after the end of the micro infusions. On the test days, the same infusion procedure was followed, except for the infusion solution. First, we infused the PrL animals, a few weeks later we infused the BLA animals. Half of either the PrL of BLA group received 0.3 µl saline, the other half received 0.3 µl baclofen (1.0nmol)/muscimol (0.1nmol) solution, which are respectively GABA-B and GABA-A agonists. The order of the infusions was counterbalanced across animals (being their own controls), such that some rats received saline prior to B/M, while others received infusion in the opposite order. Prior to receiving their second infusion all animals were re-trained on baseline sessions for a minimum of two days.
The used dose and volume of micro infusions with BM has been used on a wide variety of behavioral experiments looking at the role of the BLA (Jean-Richards-Dit-Bressel & McNally, 2015; Millan et al., 2015) and at the role of the PrL (Limpens et al, 2015).

Testing

After the infusions were randomly and blind performed in the PrL or the BLA group each animal was housed in its home cage for 10 minutes to calm down. After this period of rest the rats were placed in the operant chamber and the STA-model script with tone and shock as explained above was started. Before testing, the shockers and the tone were always checked. After 30 minutes the session ended and the rats were placed back in their home cages.

Histology

After the completion of behavioral testing the rats were killed with CO_2. The brains were injected with ink (to check the placement of the cannulas), removed and fixed and immediately fresh frozen in -80°C on dry ice. Unfortunately, because of a lack of time, the histology of the frozen brains of the PrL animals was not executed yet. Coronal sections of 20 µm are meant to be sliced on a cryostat and analysed for correct placement of the cannulas using the injected ink spots under a light microscope. Rats

with cannula placements out of the target regions should be excluded from the experiment and data analysis.
The BLA rats were still alive, and used for further testing. Therefore, histological assessment of infusion sites was not done yet. Upon completion of all behavioral experiments, the rats will be sacrificed and the brains will be sliced and analysed to verify the infusion sites.

Data analysis

Statistical analyses were conducted using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, NY, USA). A repeated measures ANOVA within subjects was used to analyze data with the factors infusion (BM or saline) and session (baseline or tone-shock). Mauchly's test of Sphericity is not conducted because only 2 levels of repeated measures are used. There is only one set of varying scores, so there is nothing to compare those different scores to, to indicate a violation of sphericity. The Friedman Test was used to analyze the suppression ratio, which corrects for individual variation. The suppression ratio is calculated as [(number of presses during BL session - number of presses during tone-shock session)/(number of presses during BL session + number of presses during tone-shock session)]. Total suppression is reached when the suppression ratio is 1. No suppression is found when the suppression ratio is 0 or lower. The threshold of significance is $p<0.05$. The graphs are made using Excel.

Results

Pharmacological inactivation of the PrL

One rat was excluded of the experiment due to broken cannulas. The effect of PrL inactivation under threat of adversity on the total active lever presses during BL and tone-shock sessions is shown in figure 3. This is done for the two variables, namely BL and tone-shock sessions after a control infusion with saline and BL and tone-shock sessions after baclofen/muscimol (BM) infusions inactivating the PrL. A repeated measures ANOVA within subjects was executed. A significant effect of the factor session was observed ($F(2,12)=7.97$, $p=0.02$), meaning that the amount of total active lever presses is significantly higher in BL sessions compared to tone-shock sessions. A significant effect of the factor infusion was not found, meaning that the amount of total active lever presses is not significantly higher infusing saline compared to infusing BM. Also, there was no significant interaction effect found between the factors infusion and session ($p>0.05$). No post-hoc tests were conducted, due to the fact that there are only two levels of repeated measures.

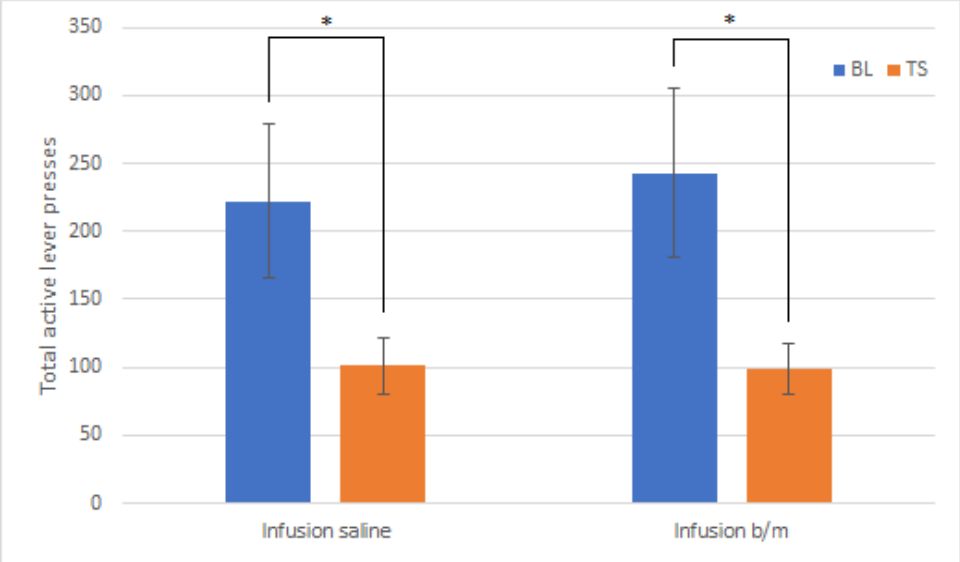

Figure 3. The active lever presses (ALP) of the PrL cannulated rats infused with saline or baclofen/muscimol (b/m) during baseline (BL) and tone-shock (TS) sessions. Showed is the mean of ALPs ± SEM during baseline and tone-shock sessions after infusions with saline and baclofen muscimol. *. P<0.05. *n*=11

The effect of PrL inactivation under threat of adversity on the active lever presses made only during the presentation of the tone in tone-shock sessions or a comparable timeframe in BL sessions is shown in figure 4. A repeated measures ANOVA within subjects was executed. A significant effect of the factor session was observed ($F(2,12)=5.95$, $p=0.04$), meaning that the amount of active lever presses in toneframe is significantly higher in BL sessions compared to tone-shock sessions. No significant effect of the factor infusion was found, meaning that the amount of active lever presses in toneframe is not significantly higher infusing saline compared to infusing BM. There was no significant interaction effect between the factors infusion and session ($p>0.05$).

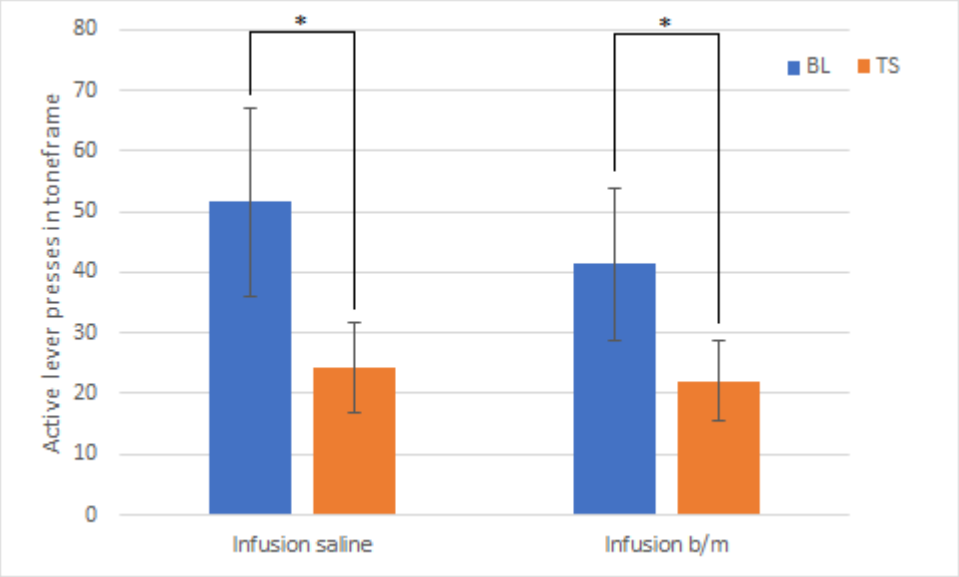

Figure 4. The active lever presses in the toneframe of the PrL cannulated rats infused with saline or baclofen/muscimol (b/m) during baseline (BL) and tone-shock (TS) sessions. Showed is the mean of ALPs during toneframe ± SEM during baseline and tone-shock sessions after infusions with saline and baclofen muscimol. *, P<0.05. *n*=11

Pharmacological inactivation of the BLA

The effect of BLA inactivation under the threat of adversity on the total active lever presses during BL and tone-shock sessions is shown in figure 5. This is done for the two variables, namely BL and tone-shock sessions with an intensity of 0.30 mA after a control infusion with saline and BL and tone-shock sessions after baclofen/muscimol (BM) infusions inactivating the BLA. A repeated measures ANOVA within subjects was executed. A significant effect of the factor session was observed ($F(2,12)=6.74$, $p=0.03$), meaning that the amount of total active lever presses is significantly higher in BL sessions compared to tone-shock sessions. A significant effect of the factor infusion was not found, meaning that the amount of total active lever presses is not significantly higher infusing saline compared to infusing BM. There was no significant interaction effect between the factors infusion and session ($p>0.05$). The rats pressed more on the active lever during the tone-shock session after the BM infusions compared to the tone-shock session after the saline infusions, although this was not a significant finding.

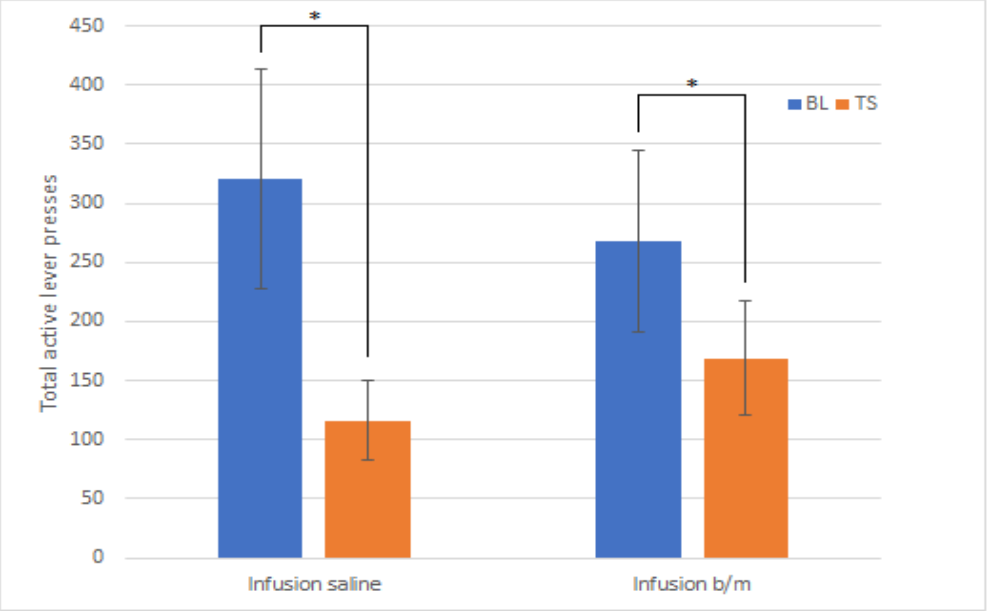

Figure 5. The active lever presses (ALP) of the BLA cannulated rats infused with saline or baclofen/muscimol (b/m) during baseline (BL) and tone-shock (TS) sessions. Showed is the mean of ALPs ± SEM during baseline and tone-shock sessions after infusions with saline and baclofen muscimol. *, P<0.05. *n*=12

The effect of BLA inactivation under threat of adversity on the active lever presses in toneframe during BL and tone-shock sessions is shown in figure 6. A repeated measures ANOVA within subjects was executed. No significant effect of the factor session was found ($F(2,12)=3.55$, $p=0.086$), meaning that the amount of active lever presses is not significantly higher in BL sessions compared to tone-shock sessions. Likewise, no significant effect of the factor infusion was found, meaning that the amount of active

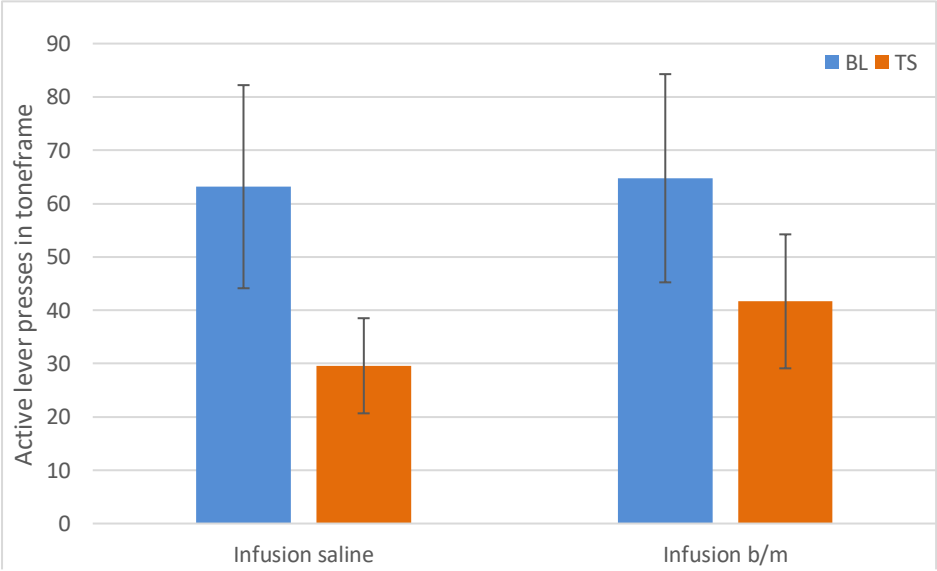

Figure 6. The active lever presses in the toneframe of the BLA cannulated rats infused with saline or baclofen/muscimol (b/m) during baseline (BL) and tone-shock (TS) sessions. Showed is the mean of ALPs during toneframe ± SEM during baseline and tone-shock sessions after infusions with saline and baclofen muscimol. *n*=12

lever presses in toneframe is not significantly higher infusing saline compared to infusing BM. Also, there was no significant interaction effect found between the factors infusion and session ($p>0.05$).

Suppression Ratio

The effect of inactivating the PrL under threat of adversity during sucrose seeking is presented in figure 7a. There is no significant effect of PrL inactivation on the suppression ratio: $\chi^2(1)=0.818$, $p>0.05$. Meaning that infusing BM into the PrL animals had no effect on suppression compared to infusing saline (vehicle) into the PrL animals. The effect of inactivating the BLA under threat of adversity during sucrose seeking is presented in figure 7b. There is no significant effect of BLA inactivation on the suppression ratio: $\chi^2(1)=0.333$, $p>0.05$. Meaning that infusing BM into the BLA animals had no effect on suppression compared to infusing saline (vehicle) into the BLA animals.

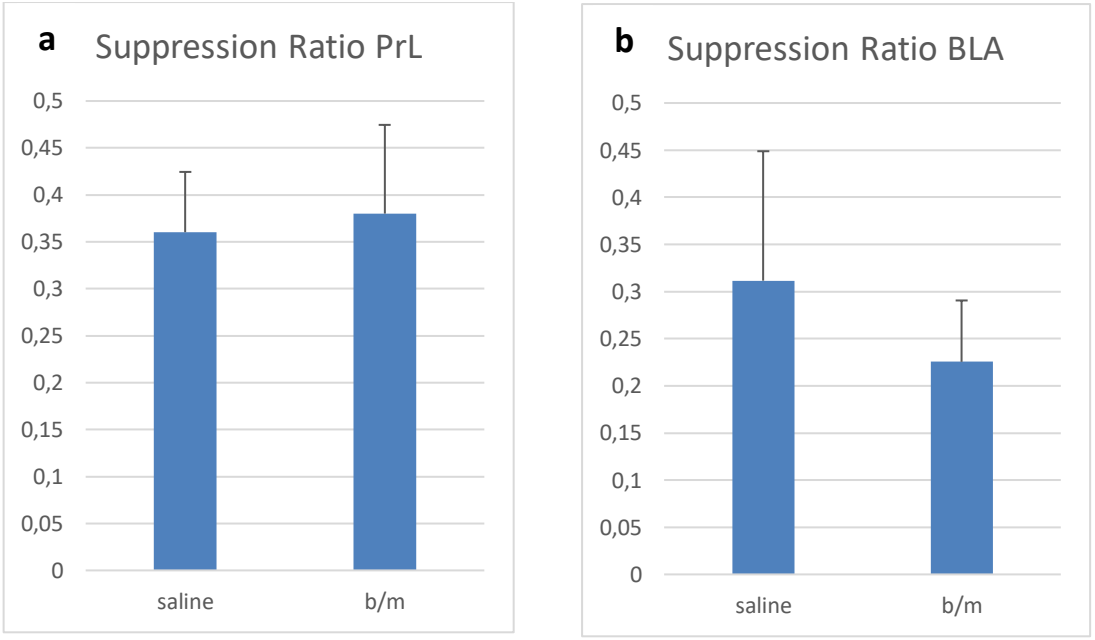

Figure 7. The suppression ratio of the inactivated PrL (7a) and of the inactivated BLA (7b). Showed is the mean of the suppression ratio ± SEM during baseline and tone-shock sessions after infusions with saline and baclofen muscimol. PrL: *n*=11, BLA: *n*=12

Discussion

In the present study, the hypothesis that inactivation of either the PrL and the BLA will increase loss of control over the task, was tested. We investigated the effect of pharmacological inactivation in these brain areas, using a mixture of the GABA agonists baclofen and muscimol, which was used before by others to inactivate comparative brain areas (McFarland and Kalivas, 2001; Van Kerkhof et al., 2013; Zeeb et al., 2015; Piantadosi et al., 2017) under threat of adversity during sucrose seeking using a novel model. We observed no loss of control (i.e. no increased sucrose seeking during tone-shock sessions), evident from a lack of an effect on the number of active lever presses over the entire session nor within the tone phases of the session. Similarly, we observed no effect of PrL or BLA inactivation on the suppression ratio, i.e. we observed no increased sucrose seeking during tone-shock sessions when either brain region was inactivated compared to control conditions. These data indicate that inhibition of neural activity in either the PrL and the BLA, using the STA-model, does not lead to loss of control over sucrose seeking. We will discuss our findings and the STA-model below.

The role of the affected brain areas

The BLA and the PrL were inactivated by the GABA agonists baclofen and muscimol before the test sessions. These brain areas are both important for the mesolimbic and dopaminergic reward system (Koob & Le Moal, 2005). Previous studies found a role of the BLA and the Prl in the loss of control over drug seeking behavior. To start with the PrL, the breakdown of prefrontal cortical control is thought to be essential for the loss of control characterizing addiction (Koob & Le Moal, 2005). The Prl has been associated with the reinstatement of drug seeking (Marín-García et al., 2013; McLaughlin and See, 2003; Pelloux et al., 2014, reviewed by Bossert et al., 2013). The PrL has also been involved in goal-directed behavior (Balleine & O'Doherty, 2010) and its role in control over compulsive aspects of cocaine seeking behavior has been revealed (Chen et al., 2013; Kasanetz et al., 2013; Mihindou et al., 2013; Seif et al., 2013). Additionally, it has been shown that lesions of the mPFC result in operant responding for both sucrose and cocaine, causing insensitivity to potential punishment (Resstel et al., 2008) and that inactivation of the PrL results in impaired decision-making (Zeeb et al., 2015). Taken together and including the findings of Limpens et al., 2015, indicating that inactivation of the PrL reduced conditioned suppression of cocaine and sucrose seeking, these data broadly implicate the role of the PrL in the loss of control over drug seeking behavior. By contrast, our results show no reduction of sucrose seeking despite negative consequences after inactivating the PrL pharmacologically, which is in line with Pelloux et al. (2013) who did not find an suppressive effect of PrL lesions on punished cocaine seeking. However, possibly our findings could be attributed to some limitations of the study, which are elaborated later on in this discussion.

Then, the BLA is thought to influence behavioral responses to aversive or threatening stimuli (Adolphs, 2013), which is in line with the finding in both humans and rats that BLA lesions eliminate conditioned fear responses (Adolphs et al., 1995; Erlich, Bush & Ledoux, 2012). Orsini et al. (2015) showed that BLA lesions shift preference away from smaller, unpunished rewards towards larger rewards that may also be punished. Furthermore, inactivation of the BLA decreased the suppression of punished responding (Jean-Richard-Dit-Bressel & McNally, 2015; Piantadosi et al., 2017). Taken together, this suggest a role of the BLA in recalling and utilizing the memory of an aversive event, and subsequently modifying responding behavior in situations where actions are punished. As expected, our results of BLA inactivation show an increase of punished sucrose seeking behavior compared to the control infusions, but this increase was not significant. McDannald and Galarce (2011) studied fear learning and memory by lesioning the BLA, they found a critical role for the BLA in fear acquisition, but a diminished role in conditioned suppression, which could be an explanation of our results.

The STA-model in comparison to other models for loss of control over substance seeking

Possibly, we observed no loss of control after inactivating the PrL and the BLA, because of the type of punishment model we used. In numerous addiction studies using punishment, every drug seeking behavior leading to substance taking was directly punished (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). In the STA-model we introduced, which is a new probabilistic punishment model for addictive behavior, not every instance of substance taking was punished (see figure 2) Due to the warning tone and unpredictable probabilistic punishment during the tone interval in this model, we have simulated punishment in drug seeking behavior as accurate as possible to addiction in human life. While human drug seeking or taking is not inevitable and directly punished (lose your job, fall into debts or social isolation), and addicts are often warned for the consequences of their addiction. By using this model, this study hopefully contributes to a better understanding of and to the quest for a possible treatment of the psychiatric disorder addiction.

Another relevant difference between our study using foot shocks as punishment and other punishment studies needs to be mentioned. Namely, foot shocks as punishment may be less comparable to punishment in the human addictive situation than for example quinine with its bitter taste (Wolffgramm & Heyne, 1995) or malaise inducing lithium chloride (Dickinson et al, 2002; Miles et al. 2003). Although, Ersche et al. (2016) found that human cocaine addicts are less proficient in the avoidance of electric shocks, suggesting that addiction could lead to reduced sensitivity of physical punishment (Vanderschuren et al., 2017), even in humans.

Compulsive drug seeking under the threat of adversity (i.e. despite the anticipation of aversive events) is a characteristic of (drug) addiction (Everit, 2014; Feil et al., 2010; Figee et al., 2016). In this study, we used mild foot shock punishment, the most widely

used punisher in addiction research (Jenkins et al., 1926; Deroche-Gamonet et al., 2004; Belin et al., 2008, 2009, 2011). The shock intensities used were 0.25 mA (PrL) and 0.30 mA (BLA). Also, a tone was used as a warning signal before a probabilistic punishment. The ability of animals tolerating mild foot shocks in order to obtain cocaine or alcohol has been indicated (Belin et al., 2008, 2009, 2011; Chen et al., 2013; Pelloux et al., 2007, 2013). On the other hand, it has been shown that a foot shock-associated conditioned stimulus inhibits sucrose seeking in rats (Limpens et al., 2015; Vanderschuren and Everitt, 2004), which endorse our findings in the sucrose motivation STA-model we used. The foot shocks could be too intense, causing a decline in motivation to press for sucrose. This could be attributed to the fact that sucrose was used as a substance of abuse (instead of alcohol or cocaine) or to the intensity of the foot shocks. Indeed, Limpens et al. (2014) found an shock intensity of 0.35 mV to suppress sucrose seeking behavior. Although, Minnaard and Smeets et al. (unpublished findings) found that 0.25 mA is the lowest shock intensity at which suppression for sucrose and alcohol seeking started. In our experiment we used a shock intensity of 0.25 mA for the PrL animals and we started with 0.25 mA shock intensity for the BLA animals, due to a lack of suppression we decided to test them on 0.30 mA, on which they showed suppression. Besides, Resstel et al. (2008) and Limpens et al. (2015) found that inactivating the PrL could lead to operant responding for sucrose, insensitive to potential punishment. Another possible explanation of our findings could be that the STA-model uses direct punishments (tone + shock), instead of using conditioned suppression: a conditioned stimulus (tone) associated with a shock (Limpens, 2015).

Limitations of the study

As mentioned before, some limitations of this study need to be acknowledged. First of all, there was not enough time in the three months internship to slice the frozen brains from the PrL rats, while the BLA rats were still alive at the end of the experiment, so we could not check if the bilateral cannulas ended exactly at the intended brain areas. Which is regularly done in experiments using cannulas to affect the neuroactivity in the brain (Jean-Richard-Dit-Bressel & McNally, 2015; Limpens et al., 2015; Piantadosi et al., 2017; Zeeb et al., 2015). Because of the uncertainty about the placement of the cannulas, we assumed the cannulas were placed in the right areas and we only exclude an animal with broken, unusable cannulas out of the data. Determining the coordinates for placing the cannulas was based on Watson & Paxinos (2010), The Rat Brain in Stereotaxic Coordinates. Second, a relatively small number of animals is used. Although Limpens et al. (2014) found that a sample size of 6 could be reliable to demonstrate conditioned suppression. However, we did not find a statistically effect of inactivating the brain areas on conditioned suppression under the threat of adversity using respectively 11 and 12 animals in the PrL and the BLA group. Thirdly, we investigated the role of the PrL and the BLA in punishment-induced behavioral suppression of compulsive drug seeking. Of course, this does not preclude the involvement of other (cortical) subregions part of the mesolimbic or mesocortical system in compulsive drug behavior. The possibility that other brain regions are involved is clear. For example the DLS is thought to affect goal-directed behavior and

supports habitual instrumental performance (Corbit et al., 2012; Yin & Knowlton, 2004; Zapata, 2010) or the insular cortex and the NAc, involved in modulating control over behavior (Millan et al., 2015; Seif et al., 2013).

In line with this, a recommendation for future studies would be to repeat this experiment with inactivation of other brain areas that could play a role in motivation behavior and conditioned suppression on addiction, like the DLS, NAc and the insula above-mentioned. Since reciprocal projections between the mentioned brain areas are linked to response-inhibition in animals and humans (Morein-Zamir & Robbins, 2015; Heilbronner et al., 2016; Wood & Ahmari, 2015), it would also be interesting to inspect connections between these structures in drug seeking tasks, which may help determine the directionality of communication between them underlying the punishment-induced behavioral suppression. Focusing on a treatment of addictive behavior, overtraining with positive reinforcers instead of using punishment is recommended. Since Ersche et al. (2016), found that cocaine-addicted patients are less sensitive to the outcome of their actions when overtrained with positive reinforcement such as rewards. While a punishment paradigm of overtraining had no effect. As long as there are no medically proven pharmacological treatments, changing addictive behavior mainly relies on psychosocial approaches. In the future transcranial magnetic stimulation or deep brain stimulation may be a viable treatment strategy for drug addiction (Li et al., 2013; Luigjes et al., 2012).

Conclusion

In conclusion, the present study shows no significant increase of sucrose seeking behavior under threat of adversity using the STA-model after pharmacological inactivation of the PrL and the BLA in rats. Although there was a non-significant increase seen in sucrose seeking behavior while the BLA was inactivated compared to the control condition. Along with past and future research, this study may contribute to the need to understand the underpinnings of addiction and develop effective treatment strategies for this chronic and relapsing disorder.

Acknowledgements

Upon completing my Master research thesis, I would like to sincerely thank Maryse Minnaard and Heidi Lesscher for being my supervisors, helping me and for everything they taught me in the past three months. My thanks also go to lab analysts Anne-Marie, Lisa and José for their help, patience and sociability. In addition, I want to thank Miranda, my high school friend and fellow student, and the other students too for their help and bringing a lot of fun (and good memories) into my internship. And of course thanks to my friends and family who supported me 'behind the scenes'.

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