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Studying Fear and Reward in Bed Nucleus of the Stria Terminalis (BNST) Neural Circuits

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1. INTRODUCTION 1
   1.1 Emotions 1
   1.2 Fear and Reward 2
   1.3 Pavlovian Fear and Reward Conditioning 2
   1.4 Brain Structures of Interest 3
      1.4.1 The Amygdala 4
         1.4.1.1 The Basolateral Amygdala (BLA) 5
         1.4.1.2 The Central Amygdala (CeA) 5
      1.4.2 The Bed Nucleus of Stria Terminals (BNST) 5
      1.4.3 The Paraventricular Nucleus of Hypothalamus (PVH) 6
   1.5 General Question 6

2. MATERIALS AND METHODS 7
   2.1 Animals 7
   2.2 Pavlovian Conditioning 7
      2.2.1 Port Training 7
      2.2.2 Reward Conditioning 8
      2.2.3 Fear Conditioning 8
      2.3.4 Conditioning Test 8
   2.4 c-Fos Mapping 9
      2.4.1 General Procedure 9
      2.4.2 CTB — Retrograde Tracer 9
      2.4.3 Automated Cell Counting 9
      2.4.4 Statistical Analysis 10

3. RESULTS 11
   3.1 Fear and Reward Conditioning 11
      3.1.1 Establishment of the Conditioning Paradigm 11
      3.1.2 Reward Conditioning Phase 11
      3.1.3 Fear Conditioning Phase 13
      3.1.4 Testing for Successful Conditioning 13

4. DISCUSSION 19
   4.1 Fear and Reward Conditioning 19
   4.2 Early Gene Mapping (c-Fos) 19

5. CONCLUSION AND OUTLOOK 20
1. INTRODUCTION

1.1 Emotions
The sensation of feeling hungry, the shivering you experience during a scary movie, the buzz when you are head over heels in love or simply the glorious sentiment after you succeeded a tough exam: these are all emotions, but how can we define them?

As a set of psychological states, emotions comprise intuitive experience, behavior, altering thoughts and physical responses. Since emotions enable us to take the correct decisions depending on our external environment (unfavorable or beneficial) and adapt our behavior towards them, they are crucial for survival (LeDoux 2000, Gross and Barrett 2011, Damasio and Meyer 2013).

Even if it seems difficult to categorize emotions, we can roughly differentiate between basic and complex ones. Happiness, sadness, fear, anger, surprise and disgust describe the first category (Ekman 1972) whereas guilt, shame or embarrassment are assigned to the second class. Those require self-reflection and self-consciousness while basic emotions occur automatically without a cognitive evaluation (Weir 2012).

In the 1980s the American psychologist James A. Russell proposed his ‘Circumplex Model of Affect’ in which he hypothesized emotions being two-dimensional, composed of an axis for valence and one for arousal (Russell 1980). This map of emotions in the brain is currently one of the most appreciated theories.

![Figure 1: The Circumplex Model of Affect by James A. Russell.](image-url)
1.2 Fear and Reward
Interested in neural circuits associated with emotions we focused on basic emotions as fear and reward. Sharing the same arousal state but being differently valenced, we can assume that fear and reward share similar arousal circuits and specific valence circuits. Fear elicits avoidance while reward-seeking behavior provokes approach. As the avoidance and approach behaviors are so highly conserved and can even be observed in simple organisms, it can be concluded that they both promote survival (Darwin 1872, Anderson and Adolphs 2014).

Focused on a certain threat, fear aims to protect oneself from a threatening situation and promotes action to retain well-being (Sylvers et al. 2011). Animals, like mice we use as laboratory animals, soon learn to bypass situations where they were previously exposed to stress or pain (e.g. foot shock). If, however, situations seem escapable, fight-or-flight-behavior is most common. In case of an inevitable threat, freezing would be the ordinary response (Steimer 2002).

Reward however has not one but several definitions. To neuroscientists reward is referred as both an object or event interpreted as positive which evokes approach (for example water, food, sex). Reward has three components in itself: pleasure as the hedonic affect (‘liking’), the motivation to pursue the reward (‘wanting’) and the reward-related learning (Wise 2004, Berridge 2012, Salamone and Correa 2012).

Besides being essential for survival, learning means the obtainment of new skills, knowledge and behavioral changes. Describing virtually all forms of learning in the broadest sense, associative learning is a process in which a new response becomes associated with a particular stimulus. In animals these neural processes are practically investigated with tasks known in the field of associative learning like Pavlovian or operant conditioning (Salamone 1992).

1.3 Pavlovian Fear and Reward Conditioning
The Pavlovian Conditioning, often also referred to as Classical Conditioning, describes a form of associative learning for studying basic emotions as fear and reward in animals. For that, an unconditioned stimulus (US), which can be either positive (e.g. food) or negative (e.g. foot shock), is combined with a neutral conditioned stimulus (CS, i.e. sound). The US sets off an unconditioned response (UR, e.g. salivation or freezing) whereas the CS has no meaning and therefore no response at first. After several reinforcements or repeated temporal pairings of the US and the CS, the CS develops the quality of the US and is then by itself able to induce the feedback habitually caused by the US, the so called conditioned response (CR).
The Russian physician Ivan Petrovich Pavlov first observed this form of associative learning in his dog when he paired the ringing of a bell to the displaying of food. After several repetitions the dog had an increase in salivation even if the sound was no longer gone along with food distribution (Pavlov 1927). This study shows that the CS has then, due to the learning process the animal is going through, an impact not only on behavior but also on endocrine and autonomic functions such as the release of hormones and changes in the heart rate. Therefore Pavlovian fear conditioning (coupling of sound and foot shock) and reward conditioning (coupling of sound and e. g. sucrose drop), are by now well established protocols, of which we also make use of, to examine emotions or learning mechanisms in experimental animals.

1.4 Brain Structures of Interest

Emotions such as fear, anger as well as those related to sexual behavior and overall survival are processed in the limbic system (Fig 2). Previous studies have shown that the amygdala is the central hub of emotions in the limbic system. The amygdala is responsible for determining what memories are stored and where they are stored in the brain whereas the hippocampus sends out memories to the appropriate part of the cerebral hemisphere for long-term storage (Bailey 2016).
Comprising the thalamus and hypothalamus, the diencephalon is also part of the limbic system. While the thalamus plays a major role in sensory perception and motor functions such as movement, the hypothalamus acts as a very small but essential control unit in the endocrine system by regulating the pituitary and adrenal glands and body temperature. To summarize, the limbic system has, besides being responsible for emotional responses in the brain, a broad spectrum of tasks in the body (RajMohan and Mohandas 2007).

1.4.1 The Amygdala
As an almond-shaped group of nuclei, the amygdala is as an independent brain region located in the medial-temporal lobe. Well-known for its role in the processing of emotions, notably fear, it is also engaged in a broad range of cognitive tasks such as learning and memory.

Each nucleus, which is often subdivided into several sub-nuclei, has unique in- and outputs and therefore diverse functions. Three of those nuclei are notably important for Pavlovian fear conditioning, namely the lateral (LA), the basal (BA) and central amygdala (CeA) (Tsvetkov et al. 2015).

**Figure 3: Nuclei of the Amygdala.** Brain structures we were interested in as follows: The central amygdala comprising the centrolateral (CEl, in pink) and centromedial (CEm, in blue) nucleus, the basolateral amygdala consisting of the basal (BA, in turquoise) and lateral (LA, in green) nucleus of the amygdala. (Keifer et al. 2015)
1.4.1.1 The Basolateral Amygdala (BLA)
The basolateral complex of the amygdala, the BLA is divided into three sub-nuclei, namely the basal (BA), the basomedial (BM) and the lateral nuclei of the amygdala (LA) (Tovote et al. 2015). Functioning as the major gatekeeper of the amygdala, the LA receives sensory inputs from the auditory, somatosensory, olfactory and taste systems (LeDoux 2007). Neural plasticity in the LA which is induced by Pavlovian conditioning is essential for the acquirement of conditioned responses (Szinyei et al. 2007). The BA however plays an important role in controlling motor actions and receives mainly information from the hippocampus (LeDoux 2007).

1.4.1.2 The Central Amygdala (CeA)
The amygdala’s major output is the CeA which is further divided into the centromedial (CEm) and centrolateral (CEl) nuclei. Both parts are characterized by its neuron size and shape, though there is only one marker which allows the distinction between them, namely PKCδ (Haubensak et al. 2010). The CeA’s projection to a great number of structures in the brainstem makes it crucial in controlling emotional responses, especially freezing in a fear state as well as hormonal and autonomic control (LeDoux 2007). For the research group Keifer et al. “it is becoming increasingly clear that the CeA is involved in the acquisition, expression, and even consolidation of conditioned fear. Current evidence suggests that the role of the output station can be subscribed to the CEm, whereas the site of plasticity is the CEl” (Keifer et al. 2015).

1.4.2 The Bed Nucleus of Stria Terminals (BNST)
The BNST is one of the most complex brain regions, due to its large number of nuclei and sub-nuclei and manifold afferent and efferent projections (Sun and Cassell 1993). It monitors physical and social situations, processes the received information and initiates the proper behavior (homeostasis or fight-or-flight) by integrating the valence of stimuli with the mood, motivation and energy status via its connections with the respective brain areas (Lebow and Chen 2016). Due to its role in homeostasis maintenance, it is perhaps unsurprising that the BNST has been reported as a major center in anxiety- and addiction related behaviors (Avery et al. 2016). Important inputs we were interested in reach the BNST from the BLA and CeA (Stamatakis et al. 2014). In fact, the LA does not project to the BNST. The major efferent projections reach the BNST from the BA (Dong et al. 2001). Essential outputs of the BNST are the Paraventricular Nucleus of Hypothalamus (PVH), the Ventral Tegmental Area (VTA), the Periaqueductal Grey (PAG) and the Nucleus accumbens (NAc) which are involved in fear and/or reward (Lebow and Chen 2016). As it connects with important limbic structures and brainstem regions, it is speculated to play an important part in merging physiological responses with behavior (Crestani et al. 2013).
1.4.3 The Paraventricular Nucleus of Hypothalamus (PVH)
As a group of neurons located in the hypothalamus and lying adjacent to the third ventricle, the PVH can be activated by physiological changes including stress (Alheid and Heimer 1988). Many PVH neurons project directly to the posterior pituitary where oxytocin is released into the general circulation. While several PVH neurons control various anterior pituitary functions, still another group directly regulates appetite and autonomic functions in the brainstem and spinal cord (Ferguson and Renaud 1984).

1.5 General Question
Thus, only reciting the most relevant information about the anatomical structures that are involved in emotion circuits, the wealth of literature shows that a great number of studies are recently conducted on these brain circuits in the last decades. Yet little is known about how these brain areas differentially process emotional states such as fear and reward.

In the course of my bachelor internship we focused on the BA-BNST-PVH neural circuitry: Via c-Fos counting we were able to measure the amount of activated neurons in the BA and PVH after the animals had gone through the Pavlovian fear and reward conditioning protocol. Thereafter we were eager to know if there was a correlation between the contemporaneous activation of neurons in the BA and the PVH. Ultimately we statistically compared the data from the behavior protocol and the c-Fos counting to see if there is a correlation (e. g. between freezing and the activation of neurons in the BA).
2. MATERIALS AND METHODS

2.1 Animals
A total of 24 adult male C57BL/6J mice (Charles River Laboratories International Inc., Germany) aged a minimum of three months were used for all behavior experiments. Mice were organized by four to five animals per cage and maintained under a 14h/10h light/dark cycle at 21 °C. Mice were given water and food ad libitum, except during water deprivation.

2.2 Pavlovian Conditioning
Handling and weighing of the mice started several days prior to the experiment. Fear and reward conditioning was performed in four identical, sound isolated boxes (16.5 cm wide x 16.5 cm deep x 30.5 cm high, Coulbourn Instruments, Pennsylvania, USA). Mice were water deprived 20 hours before the beginning of the protocol to increase motivation to the sucrose response. At least 45 minutes after the end of conditioning, water was provided to the mice in their home cage for 5-15 minutes, depending on the weight and general physical condition. The body weight was measured each day before the conditioning sessions. Located in the back of the port on both sides of the drinking tube, a light beam sensor (Coulbourn Instruments H24-01M) measured port visits (i.e. nose pokes) during port training, conditioning and test sessions. Speakers above the cages provided the custom-designed sounds (Audacity). Video cameras monitored the animals' behavior during the whole experiment. Sounds, reward-delivery and shocks were controlled by a custom-made Matlab program (MathWorks, Inc., NM, USA).

All behavior experiments were performed in agreement with the Austrian (BGBl nr. 501/1988, idF BGBl I no. 162/2005) and European (Directive 86/609/EEC of 24 November 1986, European Community) legislation on animal experimentation and covered by the license MA58/002220/2011/9.

2.2.1 Port Training
On the first day prior to the reward conditioning protocol the mice were habituated to the new environment (i.e. the reward cage), described as context A (ethanol-scented, grid floor, house-light on) by training them how to visit the port in the center of the right box wall for reward collection. A syringe located outside of the cage provided 5 µl per trial of a 5 % (w/v) sucrose solution upon opening of an electronically regulated valve. In total, 36 sucrose drops were delivered during an overall session of 50 minutes at random inter-trial intervals (ITIs) of 100 sec ± 30 sec after a baseline of one minute.
2.2.2 Reward Conditioning
One day after the port training, the reward conditioning protocol followed in which the animals were presented a baseline period, lasting one minute, prior to a 10 sec sound (reward CS, white noise, 50 ms pips repeated 10 times at 0.9 Hz, 75 dB). A drop of sucrose solution was dispatched to the port directly afterwards through which the mice would learn the association of the reward CS with the positive stimulus. These CS-US pairings were repeated 24 times (ITIs of 110 sec ± 20 sec) throughout a session of 48 minutes and carried out on eight consecutive days. The house-light was switched on during reward conditioning sessions. Each day the learning progress was analyzed after conditioning. The number of port visits 10 seconds before (so called baseline) and during CS presentation were evaluated and expressed as the rate of port visits per minute.

2.2.3 Fear Conditioning
On day 9 the animals were put into a different cage (context B, lemon scented, metal rod floor, house-light off, no sucrose). After a baseline of two minutes they were presented a different sound (fear CS, 3 kHz tone, 2-s pips repeated at 0.4 Hz, 75 dB) followed by a moderate foot shock (1 sec, 0.5 mA, precision regulated animal shocker H13-15, Coulbourn Instruments) five times. Thereby mice should learn to associate the fear CS with an aversive stimulus. This protocol was only 12 minutes long and the sound-shock pairings were randomly presented with an interval of 100 sec ± 30 sec. The house-light were off the whole time. The software ANY-MAZE (ANY-MAZE software, Stoelting, IL, USA) measured fear behavior by counting the duration of freezing during the fear CS compared to baseline. Freezing is determined as ‘absence of movement except for respiration’ (Curzon et al. 2009). Results were represented as percentage of time spent frozen (% freezing) and analyzed using EthoVision XT software (Noldus Information Technology, Inc.).

2.3.4 Conditioning Test
After the learning protocol and to confirm the efficacy of the Pavlovian conditioning paradigm, the mice were submitted for a conditioning test in which the two sounds were displayed in context A. The test consisted of a 60 sec baseline prior to the presentation of one sound four times (counterbalanced fear or reward CS) with the house-light on. The sound demonstration was randomized with an interval of 60 sec ± 20 sec. On day 2 of the conditioning protocol the procedure was repeated but with the other CS. The same software mentioned above measured the correct behavioral outcome via visits/min and % freezing.
2.4 c-Fos Mapping

Since I have not conducted the c-Fos mapping by myself but other colleagues in my team, the procedure is only roughly described in order to give a little insight into the order of experiments.

2.4.1 General Procedure
The perfusion of the mice (n = 20) was carried out after day 2 of the test conditioning. This timing of perfusion was chosen because of the reported peak in c-Fos expression 90 minutes after stimulation (Chaudhuri et al. 2000). 20 mice were subjected to fear and reward conditioning. Half of the mice (n = 10) were perfused after the reward test session, the other half (n = 10) after the fear test session. One control group was used in which four mice were maintained in their home cage and did not take part in the Pavlovian conditioning experiment ('homecage' group, n = 4). This control group has been added to investigate c-Fos expression due to only being exposed to the home cage with no associative learning task.

2.4.2 CTB — Retrograde Tracer
With the c-Fos labelling we were only able to measure the activity of firing neurons but not if those were connected in the brain circuit we were interested in. Therefore we injected the recombinant cholera toxin subunit B (CTB) into the BNST. CTB is a retrograde tracer, thus is transported retrogradely from the axonal terminals to the somata of neurons. To design the tracer, the subunit B of the cholera toxin was conjugated with Alexa Fluor™ 647 (ThermoFisher Scientific, C34778). Only subunit B of the toxin is used, as it is responsible for the retrograde transport and subunit A is known to be toxic (Baldauf et al. 2015). Consequently we were able to identify the neurons that project to the BNST.

2.4.3 Automated Cell Counting
After cutting the brains into 20 µm coronal sections using a cryostat, proceeding with the immunohistochemistry and scanning the slides with a fluorescent microscope at x20 (done by other colleagues), areas of interest (BA and PVH) were circled in Pannoramic viewer, exported and submitted for automated cell counting (Definiens Architect XD) to count DAPI-stained and c-Fos positive nuclei. For this, DAPI nuclei were defined by setting a size threshold. C-Fos nuclei were defined setting a size and intensity threshold. Correct counting was verified before processing the data. At this point it should be mentioned that due to unsuccessful CTB-injections into the BNST we had to exclude some mice. Eventually we did experiments on a total of 20 animals.
2.4.4 Statistical Analysis

Data presented in graphs is shown as mean ± SEM. For statistical analysis GraphPad PRISM (GraphPad Software, Inc.) had been used. Variance analysis was accomplished by using 2-way ANOVA for two dependent factors (repeated or not) and 1-way ANOVA for one factor. Correlation analysis was performed to evaluate a linear correlation (co-variation) of cell counts merged with behavioral data. P-values < 0.05 were considered as significant.
3. RESULTS

3.1 Fear and Reward Conditioning

3.1.1 Establishment of the Conditioning Paradigm
We established a Pavlovian fear and reward conditioning protocol based on previously published studies (Shabel and Janak 2009, Kim et al. 2010) and adapted it to our requirements. Our protocol consisted of one day of habituation to the context and port training which were followed by eight days of reward conditioning, one day of fear conditioning and two days of testing the learning outcome.

3.1.2 Reward Conditioning Phase
First the mice were habituated to the context of the reward set up where they were trained to visit a port where a drop of sucrose was randomly distributed. All of the animals (n = 20) learned successfully that in order to collect the reward they had to visit the port, thus all of them could continue with the next step of the conditioning protocol.

Eight ensuing days of reward conditioning followed where the reward CS, a 10 sec sound, was paired with the delivery of sucrose as reward. A significant increase in port visits during the reward CS compared to 10 seconds prior to the CS were observable, consequently the mice seemed to expect the reward delivery after the display of the sound (2-way ANOVA, effect of session: F(7, 133) = 17.99, P < 0.0001, effect of sound: F(1, 19) = 85.41, P < 0.0001, interaction of session x sound: F(7, 133) = 34.54, P < 0.0001). Starting from day 2, the animals began to visit the port...
more during the CS (Holm-Sidak's multiple comparisons test, P < 0.0001 at days 2-8). At day 5 a plateau in improvement had been reached (Fig 5).

Figure 5: Learning Curve of Reward Conditioning. Mice (n = 20) learned the connection between reward CS and subsequent reward delivery (sucrose drop), shown by a significant increase in port visits during the CS and across sessions (Holm-Sidak's multiple comparisons test: Baseline vs. Sound, * P < 0.05 and *** P < 0.001).
3.1.3 Fear Conditioning Phase
Thereafter, fear conditioning followed where the animals learned that a moderate foot shock was given after the fear CS which had been a different 10 sec sound. A significant increase in freezing compared to baseline after every 10 sec sound was observable (1-way ANOVA, sound presentation effect, F(19, 95) = 4.155, P < 0.0001) (Fig 6).

![Learning Curve of Fear Conditioning](image)

**Figure 6: Learning Curve of Fear Conditioning.** Mice (n = 20) could successfully draw a conclusion between the fear CS and the foot shock, noticeable by the increase of freezing behavior after each session in comparison to the baseline (first 2 min of the session) (Holm-Sidak's multiple comparisons test: Baseline vs. CS: * P < 0.05, ** P < 0.01, *** P < 0.001; Trial vs. Previous Trial ## P < 0.01).

3.1.4 Testing for Successful Conditioning
Later, the animals were tested if they had learned to link the right associations (reward CS- sucrose, fear CS- foot shock) to the correct expression of behavior (port visits for reward collection, freezing as a sign of fear). On two consecutive days either the fear or the reward CS were played to the mice in a counterbalanced way in which no US reinforcement took place.

As expected, there was a significant increase in port visits during reward CS detectable whereas the mice did not visit more often when the fear CS was played (Fig 7a). It can be therefore concluded that the animals awaited the reward and consequently had been successfully conditioned (2-way ANOVA, affect of CS valence: F(1, 18) = 14.51, P = 0.0013; period effect: F(1, 18) = 15.3, P = 0.0010; interaction valence x period: F(1, 18) = 12.79, P = 0.0022). Further, a significant increase in freezing levels could be observed during the fear CS, but not during baseline or reward CS (2-way ANOVA, affect of CS valence F(1, 18) = 17.75, P = 0.0005; period effect...
F(1, 18) = 66.3, P < 0.0001; interaction valence x period: F(1, 18) = 47, P < 0.0001). This data shows that the mice expressed the correct behavior to the aversive stimulus (Fig 7b).

Figure 7: Test Session. Mice (n = 20) were presented either the fear or the reward CS and behavioral outcome (port visits or freezing) was measured. a. Significantly more animals visited the port for sucrose collection during the positively valenced CS but not during baseline or fear CS. b. Freezing was significantly elevated during the negatively valenced CS but not during the reward CS (Holm-Sidak’s multiple comparisons test: CS vs. Baseline during the same test *** P < 0.001, CS vs. other CS ### P < 0.001).
3.2 Immediate Early Gene Mapping (c-Fos)
A useful tool for investigating the activity of neurons is the application of immediate early genes, the most common one being c-Fos (Morgan et al. 1987). Since c-Fos is a protein that is shortly expressed after a strong activation of a neuron and has its peak in expression after 90 min, it is ideally used through immunohistochemistry (Kovacs 1998). As previously stated (see 2.4.3 Automated Cell Counting) we focused our analysis on the BA and PVH.

In both brain structures the c-Fos positive neurons were quantified and the ratio to the overall cell number (counted by stained nuclei, DAPI) was calculated, represented in % c-Fos/DAPI. As expected, we can see that the c-Fos positive neurons in the BA (Fig 9a) were elevated after both fear and reward sound reexposure, however no significance could be reached (one-way ANOVA, F (2, 10) = 0.8174, P = 0.4690). In the PVH (Fig 9b), the number of c-Fos positive cells is statistically different between the groups. In particular, c-Fos positive cells were significantly higher after the fear CS compared to homecage (posthoc test P < 0.01), while there is a trend for higher c-Fos cells in the reward CS compared to controls (P = 0.08) and lower c-Fos cells compared to the fear CS (P = 0.05).

Figure 8: Representative Pictures of c-Fos in the LA and BA. The pictures clearly show the c-Fos positive nuclei (bright green spots) rich regions after the fear and reward CS compared to mice kept in their home cages.
Figure 9: % c-Fos/DAPI in the (a) BA and (b) PVH. (a) No significance between the conditioning groups compared to homecage could be reached in the BA, although c-Fos looks higher in fear and reward CS groups. (b) c-Fos expression is higher after the fear CS (significantly) and reward CS (trend) compared to homecage.

Figure 10: (a) % CTB/DAPI and (b) % c-Fos+CTB/DAPI in the BA. (a) The graph shows that CTB is equally distributed throughout the fear CS, reward CS and homecage group. (b) Quantification of co-labeled activated and projecting neurons among DAPI. There is no difference between the groups (P = 0.9163).
We also measured both the activated neurons in the BA that also project to the BNST (i.e. co-labeled cells), represented in % c-Fos+CTB/DAPI (Fig 10b). However, there is no difference between the groups (one-way ANOVA, \( F(2, 10) = 0.08815, P = 0.9163 \)).

We expected an increase in co-labeled cells (c-Fos+CTB) in fear and reward, compared to homecage which would implicate that the activated neurons are also projecting to the BNST. However, this is not the case and consequently it seems as if there are two populations of cells which are involved in fear and reward responses, namely the activated and the projecting one. Further the values are very low (around 0.05 %), connoting very low c-Fos and CTB co-expression.

Moreover we were interested in the percentage of activated and projecting neurons among the activated neurons in the BA, represented in % c-Fos+CTB/c-Fos (Fig 11a). However the groups are not different (one-way ANOVA, \( F(2, 10) = 0.1302, P = 0.8794 \)). Only around 4 % of the activated neurons were also projecting to the BNST.

In Figure 11b we measured the percentage of activated and projecting neurons among the projecting neurons in the BA, represented in % c-Fos+CTB/CTB. Repeatedly there is no difference between the groups (one-way ANOVA, \( F(2, 10) = 0.9832, P < 0.05 \)). Additionally the values are relatively low (5-10 %), meaning that only a minority of projecting cells are also activated by fear and reward. It should be noted that the high variability of the reward group is due to only one sample in which 100 % of colocalized cells were found.

![Figure 11: % c-Fos+CTB/c-Fos and % c-Fos+CTB/CTB in the BA.](image)

(a) Quantification of the activated and projecting neurons among the activated neurons in the BA could not reach significance (\( P = 0.8794 \)). (b) Quantification of the activated and projecting neurons among the projecting neurons could not reach significance (\( P < 0.05 \)) either.
Ultimately we measured the correlation between the activated neurons in the BA and PVH (Fig 12). Although no significance could be reached, there is a certainly strong trend regarding the activation of the BA towards the activation of the PVH, suggesting that the more the neurons of the BA are activated, the more the neurons of the PVH are activated (Pearson $R^2 = 0.2302$, 95 % confidence interval, $P$ (two-tailed) = 0.0513).

![Correlation analysis of % c-Fos/DAPI in the BA and PVH.](image)

Figure 12: Correlation analysis of % c-Fos/DAPI in the BA and PVH.
4. DISCUSSION

‘Studying Fear and Reward in Bed Nucleus of the Stria Terminalis (BNST) Neural Circuits’ was the aim of my bachelor internship. Therefore we created a behavioral design to study both fear and reward in mice. Furthermore we used immediate early gene mapping as a tool to investigate the activity of neurons. CTB as a retrograde tracer helped us additionally to find crosslinks between the brain regions of interest, mainly the BA, BNST and PVH. Through statistical analysis and correlation tests we strove for better understanding by merging behavioral data with c-Fos staining and retrograde tracing.

4.1 Fear and Reward Conditioning

As expected and confirmed by literature the learning curve of both the reward and the fear conditioning showed significant results compared to the homecage control group. Furthermore the graph of the following test conditioning showed the awaited significant results compared to homecage.

4.2 Early Gene Mapping (c-Fos)

Although we couldn’t observe a significant increase in neural activation in response to a fear or reward CS in the BA, significance could be reached in the PVH. However, there is an apparent trend towards an increase in neural activation for both fear and reward in the BA compared to the homecage control group. Considering previous studies that demonstrated c-Fos expression in the amygdala upon fear CS presentation (e. g. Rosen et al. 1998) the lack of significance in the results seem incomprehensible. As for the PVH the literature is congruent with the results, namely that the PVH can be activated by physiological changes including stress (Alheid and Heimer 1988) which clearly is the case in fear and reward response. Consequently we hypothesize that the homecage control alone is not a sufficient comparison group, thus the number of control animals and experimental subjects overall are crucial to identify significant differences in c-Fos positive cells. Another more suitable control group could be a ‘CS only’ group in which mice undergo the whole conditioning procedure except that they don’t learn to link the unconditioned stimulus (sucrose drop and foot shock) to the conditioned stimulus (sound) which means that the US is not given shortly after the CS. However, our findings suggest that the more the BA is activated, measured by the percentage of c-Fos expression, the more the PVH is activated. Thus this could imply a probable crosslink of the BA over the BNST to the PVH.
5. CONCLUSION AND OUTLOOK

Taken together, our results conclude that the neurons that are activated by fear and reward conditioning are not the ones projecting to the BNST likewise (Fig 11). Solely around 0.05 % of c-Fos expressing cells are also projecting to the BNST in both fear and reward (Fig 10b). A promising approach could probably be the injection of CTB into the PVH to investigate the activity of PVH-projecting neurons in the BNST through which we could get a better understanding of the circuit BA-BNST-PVH and its involvement in fear and reward.
6. ABSTRACT

6.1 Abstract
Emotions are crucial for our survival and overall changes in behavior as well as in motivation. Fear, for instance, associates stimuli with threats and evokes defensive behaviors, whereas reward-related emotions induce the opposite. But how does the brain differentially encode emotions and how are the connections wired?

Understanding the mechanics of emotional processing is of great importance for basic research and for the development of potential clinical applications. The fact that emotions like fear and reward-related behavior are not generated in one single brain area alone, but often distributed among many, adds to the difficulty of this undertaking.

My internship project was focused on investigating basic emotions such as fear and reward in selected limbic hubs. We targeted the Bed Nucleus of the Stria Terminalis (BNST) and its major input, the basolateral amygdala (BA), and major output, the paraventricular nucleus of hypothalamus (PVH). We combined different approaches such as Pavlovian conditioning, immediate early gene (IEG) mapping in the BA and the PVH. Further we used Cholera Toxin B (CTB) retrograde tracing of BNST-projecting neurons in the BA.

First the mice were trained according to a behavioral protocol that combined Pavlovian fear- and reward conditioning. Then they were sacrificed for c-Fos mapping as we aimed to measure the neural activity in the BA and PVH induced by fear and reward stimuli conditioning. Correlation analysis affirmed a positive correlation concerning the neural activity between both the BA and PVH. CTB retrograde tracing revealed that the BA activated neurons are not projecting to the BNST as previously hypothesized. Collectively, these data suggest a crosslink formed by the BA and PVH which modulates the expression of both conditioned fear and reward.

6.2 Zusammenfassung
Emotionen sind überlebensnotwendig und beeinflussen maßgeblich unser Verhalten und unsere Motivation. Angst, zum Beispiel, welche mit bedrohlichen Reizen in Verbindung gebracht wird, löst defensives Verhalten aus, während belohnungsassoziierte Emotionen, wie Genuss das Gegenteil hervorrufen.
Das Verständnis der zugrundeliegenden Mechanismen emotionaler Verarbeitung auf neuronaler Ebene ist für die Grundlagenforschung, aber ebenso im klinischen Bereich von großem Interesse. Allerdings wird jenes Ziel durch den Umstand erschwert, dass Emotionen zumeist nicht in einem einzelnen Gehirnareal (beispielsweise der Amygdala) lokalisiert werden können, sondern aus dem Zusammenspiel verschiedener Regionen hervorgehen, die durch ihre respektive Aktivität in unterschiedlichem Ausmaß einen funktionellen Beitrag leisten.


Dazu wurden die Versuchstiere zuerst klassisch konditioniert, einem Verhaltensprotokoll folgend, das sowohl die Assoziation eines appetitiven Reizes (US) mit einem neutralen Stimulus (CS) als auch die Assoziation eines aversiven Reizes mit einem anderen neutralen Stimulus, umfasste. Danach wurden die Mäuse geopfert, um die Expression des unmittelbar frühen Gens c-Fos zu messen, welches nach 90 Minuten empirisch die höchste Aktivität aufweist. Die Injektion des CTB zeigte, anders als ursprünglich angenommen, eine verhältnismäßig geringe Projektion der basolateralen Amygdala zum Bed Nucleus der Stria Terminalis (BNST). Dieser Nucleus bildet einen Schnittpunkt zwischen Stress und belohnungsassozierten Emotionen und ist zugleich Output der BA. Input erhält die Stria Terminalis vom limbischen System.

Korrelationsanalysen ergaben eine positive Korrelation der neuronalen Aktivität zwischen basolateraler Amygdala und Nucleus paraventricularis.

Zusammenfassend deuten diese Daten auf eine Vernetzung zwischen basolateraler Amygdala und Nucleus paraventricularis hin, welche die Expression von konditionierter Angst und Belohnung moduliert.
# 7. INDEX

## 7.1 List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTB</td>
<td>Cholera toxin subunit B</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidin-2-phenylindol</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned Stimulus</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned Stimulus</td>
</tr>
<tr>
<td>UR</td>
<td>Unconditioned Response</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioned Response</td>
</tr>
<tr>
<td>CeA</td>
<td>Central Amygdala</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal Gray</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed Nucleus of Stria Terminalis</td>
</tr>
<tr>
<td>LA</td>
<td>Lateral Amygdala</td>
</tr>
<tr>
<td>BA</td>
<td>Basal Amygdala</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral Amygdala</td>
</tr>
<tr>
<td>PKCδ</td>
<td>Protein Kinase Cδ</td>
</tr>
<tr>
<td>CEI</td>
<td>Lateral Central Amygdala</td>
</tr>
<tr>
<td>CEm</td>
<td>Medial Central Amygdala</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
</tbody>
</table>
8. REFERENCES


9. APPENDIX

9.1 List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>The Circumplex Model of Affect by James A. Russel</td>
<td>1</td>
</tr>
<tr>
<td>Figure 2</td>
<td>The Limbic System (Sokolowski and Corbin 2012)</td>
<td>3</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Nuclei of the Amygdala (Keifer et al. 2015)</td>
<td>4</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Behavioral Paradigm for Pavlovian Fear and Reward Conditioning</td>
<td>11</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Learning Curve of Reward Conditioning</td>
<td>12</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Learning Curve of Fear Conditioning</td>
<td>13</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Test Session</td>
<td>14</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Representative Pictures of c-Fos in LA and BA</td>
<td>15</td>
</tr>
<tr>
<td>Figure 9</td>
<td>% c-Fos/DAPI in the a. BA and b. PVH</td>
<td>16</td>
</tr>
<tr>
<td>Figure 10</td>
<td>(a) % CTB/DAPI and (b) % c-Fos+CTB/DAPI in the BA</td>
<td>16</td>
</tr>
<tr>
<td>Figure 11</td>
<td>% c-Fos+CTB/c-Fos and % c-Fos+CTB/CTB in the BA</td>
<td>17</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Correlation analysis of % c-Fos/DAPI in the BA and PVH</td>
<td>18</td>
</tr>
</tbody>
</table>