Role of energy states and CART signalling in fear and extinction learning in rats

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled **'Role of energy states and CART signalling in fear and extinction learning in rats'** towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents work carried out by **Feba Chacko** at Indian Institute of Science Education and Research under the supervision of **Dr. Aurnab Ghose**, Associate Professor, Department of Biology, IISER Pune during the academic year 2019-2020.

Dr. Aurnab Ghose

Committee:

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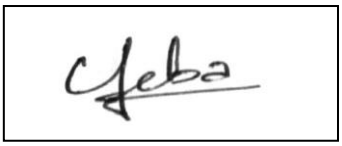
Declaration

I hereby declare that the matter embodied in the report entitled **Role of energy states and CART in fear and extinction learning in rats** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Aurnab Ghose and the same has not been submitted elsewhere for any other degree.



Feba Chacko

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Abstract

The body is a complex machine that consists of several subsystems working together to stay alive and reproduce. These subsystems and the resulting behaviours are regulated by the brain to best negotiate the changing environment. Survival circuits, as these circuits are called, involve the regulation of defence, feeding, reproductive behaviour, homeostasis etc. Under conditions of stress, such as starvation, the brain must regulate the use of its limited resources and decide which survival circuits should be prioritized. Fear and extinction memory is an interesting process to study the effect of energy states on survival circuits. It involves interactions between fear and memory circuits and their regulation under different energy states. The neural circuitry underlying fear and extinction memories is well studied, and there is evidence that energy states do influence these memories. But how the energy state is able to influence these memories is not known. One possibility is via energy state responsive neuropeptides. One such neuropeptide, CART, is upregulated in the brain under satiety conditions and is involved in both fear and memory, suggesting its possible involvement in fear and extinction memory. To investigate whether energy levels have an effect on fear and extinction memory, adult Sprague Dawley rats were subjected to fear training under fed and starved conditions and then to extinction training. Extinction learning was found to be higher in animals that had been starved before fear conditioning. To investigate the role of CART neuropeptide in the same, injections were given in the CeA of CART Ab (in fed animals) or aCSF (in fed and starved animals) at two points of the extinction protocol, one set before the fear conditioning and the other set before extinction training. No significant difference could be found in either the fear or extinction memory in these experiments. However, the sample size is not sufficient and further tests need to be done in order to ascertain the involvement of CART in fear and extinction memory. This study therefore concludes that starvation stress enhances extinction learning. However, CART neuropeptide signalling may not be involved in this cross-talk between survival circuits. A key observation is that starvation stress at the time of fear memory acquisition influences extinction learning later. This suggests that the memory engram varies depending on the energy state of the animal.

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(Feba Chacko)

1. INTRODUCTION:

1.1 Memory

In an ever changing environment, animals rely heavily on memory to evaluate novel stimuli to assess them for danger and also to better handle familiar situations. What feels like a seamless recollection, however, is not a single process. Multiple distinct neurobiological processes have to take place in an ordered fashion to make this apparent seamlessness possible. The different stages of a memory in the brain can be broadly categorised into encoding, storage and retrieval (Tulving, 1966). These processes are tightly regulated and result in normal memory. However, under acute, prolonged stress or when the regulation of such pathways is disrupted, neurological disorders can develop, such as post traumatic stress disorder (PTSD), anxiety, phobias, etc (Jovanovic and Ressler, 2010, Shin and Handwerger, 2009). To understand how these disorders develop, studying how memory and learning works is necessary.

1.2 Associative memory

Memory can be divided into different types, such as episodic, semantic, spatial, etc. All of these are complex processes involving multiple interactions. Hence, one common way to study memory is by looking at associative learning processes. An associative memory essentially enables the animal to anticipate events and we use this anticipation to score the association (memory) formed, provided the anticipation can be scored visually or mechanically (Curzon et al, 2009). Associative learning is essential to the animal in order to adapt its behavioural response to appetitive or aversive environmental cues.

Pavlovian (or classical) conditioning is a framework to study associative learning by pairing a stimulus that has biological relevance to the animal (unconditioned stimulus, US) (such as food or pain) with another stimulus that does not have any such relevance (conditioned stimulus, CS). Before conditioning, the presentation of the CS does not elicit any response from the animals. During conditioning the US elicits an unconditioned response that is innate (UR), and the CS and the US are

paired repeatedly. After conditioning, when the CS is presented alone, the animal exhibits a conditioned response (CR) in anticipation of the US as a result of the association between the CS and US (Pavlov, 1927).

1.3 Fear Conditioning and Extinction

Fear conditioning is a common form of classical conditioning used to study learning. Fear is a strong response that is very important for survival and so the animal gets conditioned to fear in a small number of trials .In fear (delay) conditioning, an animal is subjected to a harmless stimulus (CS) like a tone, in a particular context, and this is followed by a noxious stimulus (US) like an electric shock that causes pain. The US elicits a fear response (FR) from the animal, such as freezing and upon repeated pairings of the CS and US, the amygdala processes them to form an association and the animal makes the association between the two. Upon presenting the CS alone to the animal after conditioning, it recognises it as a predictor of the oncoming shock and expresses fear by freezing, which is now a conditioned response (CR)(Fendt and Fanselow, 1999).

Extinction is an interesting phenomenon that happens when after fear conditioning (or other forms of conditioning) the animal is presented with multiple CS alone trials and the association between the CS and US (by proxy of the CR) diminishes or extinguishes. This was first observed by Pavlov in 1927 and he also observed that although the CR extinguishes, it also made a spontaneous recovery at a later time. This later recovery suggested that the initial associative memory was not degraded and lost during extinction but that a new memory was being formed. It was proposed that extinction involves the development of a second inhibitory association that competes with the original excitatory CS-US association without erasing it (Konorski, 1967). The extinction memory was proposed to be encoded by an extinction engram that competes with the fear engram. A specific memory (of an experience) is thought to be stored and recalled by a sparce population of neurons, and the physical and biochemical changes in these neurons during the experience encode this memory and is called an engram or a trace (Sermon 1921). There have been studies that support this theory of competing fear and extinction engrams since (Lacagnina et al., 2019, Liu et al, 2012). The study of extinction and the mechanisms underlying it has

particular relevance as the impairment of the same has been shown in patients with PTSD (Milad 2009b). Also, exposure therapy, which is one of the main forms of therapy for PTSD, is based on the principles of extinction.

1.4 Neural circuitry

Amygdala refers to a set of two almond shaped set of nuclei set medially in the temporal lobes of the brain. It is part of the limbic system and is involved in behaviour, especially fear responses and fear memory (Davis, 1997). The sensory information about fearful stimuli is sent from the thalamus to the cerebral cortex to cognitively assess the danger posed (and then relayed to the amygdala for integration).But the same information is simultaneously sent to the amygdala, where a fear reaction can be initiated before the conscious appraisal of the danger can take place (Shi and Davis, 1999).The amygdala sends projections to the hypothalamus and the periaqueductal grey (PAG), which mediates fear responses such as the release of stress hormones, adjusting the cardiovascular activity, and freezing (LeDoux et al., 1988). Animals with lesions in the amygdala also showed impaired acquisition and expression of conditioned fear and much of the neural circuitry underlying fear conditioning has been elucidated since then (LeDoux et al., 1988, Maren and Quirk, 2004).

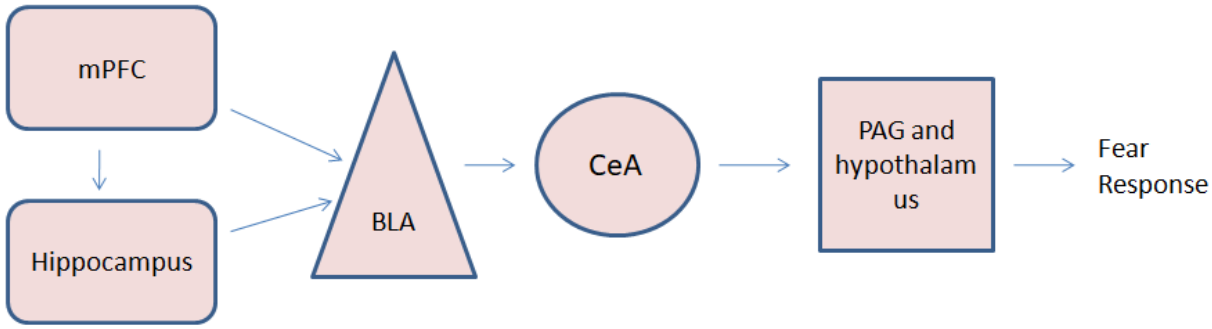


Fig 1. Fear conditioning neural pathway (representative: showing amygdala, PFC, PAG, hypothalamus, hippocampus)]

During fear conditioning, the sensory information about the CS and the US reaches the basolateral amygdala (BLA) via both cortical and subcortical pathways (Shi and Davis, 1999). BLA is the primary sensory interface of the amygdala and it also receives information from the hippocampus about the contextual cues involved in the conditioning. In the BLA, the information about the two stimuli are processed and integrated for an associative fear memory to form. The BLA then signals to the central amygdala (CeA) that acts as the output centre from where the fear response is initiated by signalling to the hypothalamus and PAG (freezing response) (Maren, 2001).If the CS is later presented to the animal without the shock, it is now trained to recognise the CS as a predictor of the oncoming shock and expresses fear because the amygdala responds to the cue as though it would to the shock, and signals to the periaqueductal grey (PAG) region, causing the animal to freeze, (Gross, 2012). The animal also shows freezing responses to the context in which it was trained, and the hippocampus is responsible for this contextual memory (Holt, 1999).

Decoupling the cue and the shock happens when the cue alone is presented to the animal multiple times without any aversive stimulus, and is called extinction. This is not due to erasure of the fear memory, but the formation of a new extinction memory (Rescorla, 1975), that appears to compete with the original fear memory for expression. While the mechanism of this extinction memory is not fully known, we do know that it depends heavily on prefrontal cortex (PFC) to amygdala connections (Trouche et al., 2013, Milad and Quirk, 2002).

1.5 Survival Circuits

While memory plays a crucial role in survival for animals, there are other survival circuits in the brain that interact with each other to optimise survival depending on the changing environment. This includes circuits responsible for defence, thermoregulation, reproduction, feeding, etc. Under a stress such as starvation, which causes a change in the energy state, the brain has to optimise the usage of these limited resources to best negotiate the environment and ensure survival. In ` , it has been shown that a mild starvation enhances appetitive conditioning and that the hunger provides motivation for the formation of memory (Colomb et al., 2009). The expression of appetitive memory is also enhanced by a mild starvation, with the

hunger being the motivation for the memory expression (Krashes et al., 2009). This is suggestive that satiety and hunger interact with memory process and that energy states play a role in memory formation and expression.

These learning and memory processes are due to electro chemical signalling within neuronal populations. This signalling includes chemicals such as neuropeptides or neuromodulators that are regulated by energy states. While we know a lot about the circuitry of fear conditioning and we have some information about that of extinction, not much is known about the role of such neuropeptides in learning and memory. Verma et al. (2016) have recently shown that the energy state regulates memory formation and extinction of fear via neuropeptide signalling pathways using the fear conditioning paradigm.This suggests that the energy states could play a role in learning and memory through neuropeptides.

1.6 CART

Cocaine-and-amphetamine-regulated-transcript (CART) is a neuropeptide that was discovered when its transcript was found to be expressed more in the rat brain when the animal was given cocaine or amphetamine (Douglass et al., 1995). It is an anorexic peptide that is involved in appetite and homeostasis control, and exogenous CART administration has been shown to inhibit food intake. The CART mRNA and peptide has been found to be robustly expressed in hypothalamic regions that are key regulators of food intake, and energy balance, such as the arcuate nucleus, paraventricular nucleus, dorsomedial nucleus, and ventromedial nucleus (Subhedar et al., 2014). Starvation was shown to lead to a decrease in CART levels in the arcuate, which was recovered after refeeding (Germano et al., 2007). While the expression of CART is regulated by energy levels, its function is not limited to modulating food intake and research has shown the peptide to play a role in fear response, reward and reinforcement, and regulation of motor activity (Subhedar et al., 2014).

CART is found in the limbic system, including the central amygdala, ventralbed nucleus of stria terminalis (vBNST) and the hypothalamus which are important in emotional responses. Recently, the peptidehas been shown to be a crucial

modulator for the innate fear response pathway in the CeA-vBNST pathway (Rale et al., 2017).The peptide was shown to be sufficient to increase Fos induction in the same pathway and it was suggested that the baseline excitatory glutamatergic drive is enhanced in the region. This suggests that CART peptide potentiates the fear response in the CeA-vBNST pathway. This is of interest to us as the neural circuit underlying both fear conditioning memory and the innate fear response pathway involves freezing response relayed via the CeA and its projections to the hypothalamus (Kalin et al., 2004, Maren, 2001). Unpublished data from the lab suggests that CART neurons of the arcuate project to the amygdala. This finding along with the finding that levels of CART in the arcuate is dependent on energy levels (Germano et al., 2007), suggests that CART levels in the amygdala could in turn be regulated by energy levels and lead to energy state dependent modulation of amygdalar responses, which merits further study.

In 2011, Upadhya et al. showed that exogenous administration of CART before a learning test (Morris Water Maze) led to an increase in spatial learning and memory in rats. In a different study, exogenous administration of the peptide in mice with memory deficits has also been shown to improve memory, and this effect was associated with improvements in synaptic ultrastructure and long-term potentiation (Jin et al., 2015). Therefore it was hypothesized that the energy state could affect learning and memory, particularly fear memory, in the expression of which the CeA plays a major role; with CART signaling as a possible mechanism by which this regulation of memory happens.

2. OBJECTIVES:

The aim of this study was:

1. to determine if energy states affect on the formation, consolidation, retrieval, or extinction of associative memory, within the fear conditioning and extinction paradigm
2. to determine whether CART signalling in the CeA couples energy states to fear and extinction memory

3. MATERIALS AND METHODS:

3.1.Subjects:

The subjects were 7-11 week old Sprague Dawley male rats, weighing between 230-260.They were maintained on a 12 hour dark/light cycle (lights on at 7 AM). The animals were group housed (three to four rats per cage) before surgery with free access to food and water. To reduce stress due to handling during the experimental procedure, the animals were handled for 3 days before the start of the experiment.

i: The subjects for the non cannulated experiments were 7-11 week old SD male rats, weighing between 210-260.

ii: The subjects for the experiments involving microinjections were cannulated at 8-9 weeks, within a weight range of 180-210. The rats were given 6-9 days to recover from the surgery and weighed around 240-270 during the time of the experiment.

All experimental protocols were approved by the by the Institutional Animal Ethical Committee (IAEC) constituted by the CPCSEA, Govt. of India.

3.2. Surgery (Cannulation):

Rats were anaesthetized by injecting a mixture of ketamine (60 ml/g) and xylazine (10 ml/g) intraperitonealy. Once lack of sensation was confirmed using a toe pinch, the hair above the skull was shaved using a trimmer to expose the scalp. Adrenaline was injected subcutaneously under the scalp to induce local vasoconstriction in order to prevent excessive bleeding. The animal was transferred to the stereotaxic frame and the head positioning adjusted to place the top incisors over the bite plate. The head was then secured using blunt ear bars while taking care that the head is level. Using a surgical blade, a mid-sagittal incision was made, exposing the skull. The incision was clipped open using bulldog forceps, and the blood and connective tissue cleared using Q-tips. Burr holes were drilled targeting the central amygdala CeA using the stereotaxic coordinates -1.9mm caudal, ± 4.0 mm lateral and – 7.8 mm ventral with respect to bregma and two stainless steel guide cannulae (made in house as described by Kokare *et al.*, 2011; internal diameter 0.36 mm, outer diameter 0.5 mm) were implanted and secured to the skull with anchoring screws

and dental cement. Dummy cannula was inserted into the guide cannula regularly after surgery to prevent occlusion. The rats were housed in individual cages after the surgery to prevent damage to the cannula. Only the rats showing quick recovery and no sign of infection were included in the experiments. The animals were divided randomly into different groups for each experiment.

Post-necropsy, the placement of the guide cannula was verified by sectioning the brain. Only data from animals with bilateral hits of the cannulae in the target region were considered for analysis.

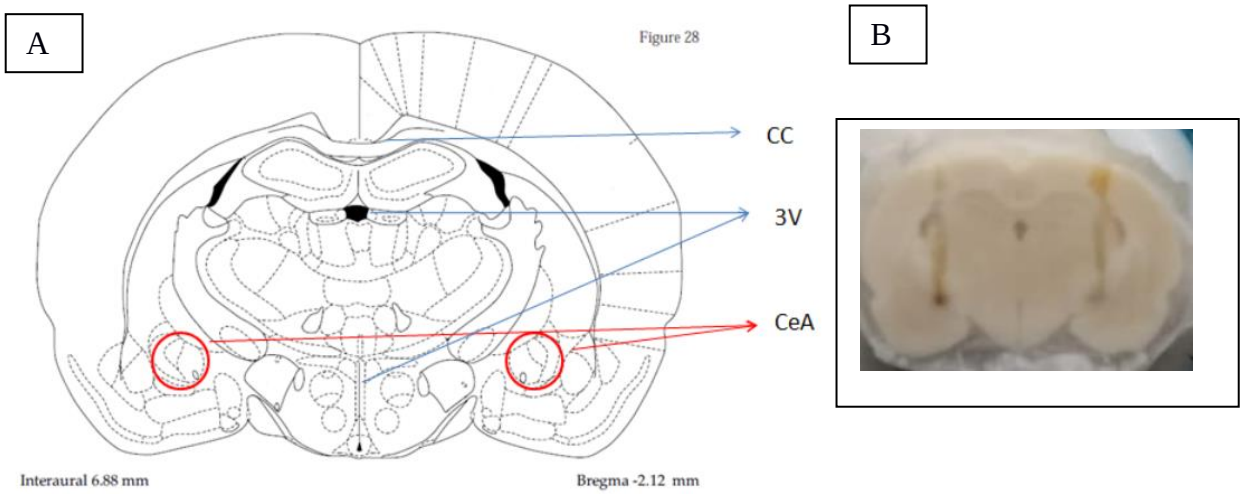


Fig. 2 A) Diagrammatic representation of the position of CeA at co-ordinates (-2.12 mm) with respect to bregma (Paxinos and Watson, 1998), B) Representative image of a successful bilateral stereotaxic cannulation

3.3. Microinjections (Drug infusion):

Microinjections were given into the guide cannulae using an injection cannulae (fabricated in house; internal diameter 0.16 mm, outer diameter 0.31 mm) connected via PE-10 polyethylene tubing to a microlitre syringe (10 µl, Hamilton, USA) and extending 0.5 mm beyond the guide cannulae to target the CeA. Rats were bilaterally administered different agents as assigned by their treatment group. The control group was bilaterally injected with total 0.25 µl/ side of artificial cerebrospinal fluid (aCSF; 119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose, 2.5 mM $CaCl_2$ (pH 7.4)) over a period of 5 minutes. Administration of the CART Ab (10 ng CART antibody dissolved in 0.25 µl

aCSF/side, Tocris Pharmaceuticals USA) was given in the same manner and the rats injected 15 minutes prior to behavioural testing.

3.4. Behavioural experiments:

3.4.1. Fear Conditioning apparatus:

Behavioural procedures were performed in an enclosed sound attenuated outer wooden compartment with a height of 60 cm, equipped with infrared and visible lights (14 lux), and an additional fitting of LED lights (84 lux). Within the compartment was a square Plexiglas chamber (30 x 30 x 30 cm, depth x width x height) placed above 20 stainless steel rods creating a metal grid (1.5 cm diameter, spaced 1 cm apart). The metal grid was connected to a shock generator to deliver a foot shock as the US and placed on a tray holder which holds a black tray as the background underneath and the Plexiglas chamber above the metal grid. A speaker was mounted overhead to provide a tone as the CS. The experiment was recorded using an overhead video camera (iBall face 2 face) and the entire system, including CS and US, was connected and automated by computer software (Fear Monitoring System, VJ Instruments).

3.4.2.Procedures:

The animals were handled for 3 days before the start of the experiment to reduce stress due to handling. The extinction training protocol used for the cannulated animals was modified from the the one used for the non cannulated animals as it was seen to be sufficient.

Freezing behaviour is defined as the cessation of all bodily movements other than that required for breathing for 2 seconds or more. The freezing of the animals during the experiment was used as a measure of fear and was analysed using Anymaze software. Analysis was done automatically using low sensitivity to match initial analysis done manually by two researchers. The videos were divided into first 600 seconds of exploration time, which represented the context memory recall, and

subsequent time was divided into trials of 120 seconds each (corresponding to each CS presentation and the ITI after it), which represented cue memory recall.

Graphs were plotted for freezing as context recall (freezing before onset of cue, while in context), cue recall (average freezing during the first five cues), and fear recall, extinction and extinction recall (freezing during fear recall, extinction, extinction recall respectively, with the context and cue wise freezing plotted separately and lines joining the points). The error bars given in the graph are SEM.

3.4.2.i. Protocol for non cannulated animals:

The animals were fear conditioned and extinction trained in the same chamber (context), with clear plexiglass, only infrared, and the black tray cleaned with 70% ethanol. On day 1, the rats were habituated to the conditioning chamber for 10 minutes to allow for encoding of contextual cues and to reduce anxiety due to a novel environment. On all subsequent days, the animals were given 3 minutes of exploration time before the onset of any cues and their freezing during this time indicated any fear associated with the context itself. On day 2, the rats were divided into the test and control groups. The test group received 5 trials of a tone (CS, 70 dB, 30 s) immediately followed by a footshock (US, 0.75mA, 0.5 s), separated by an intertrial interval (ITI) of 90 seconds. The control group received 5 trials of a tone (CS, 70 dB, 30 s) separated by an intertrial interval (ITI) of 90 seconds without the footshock. On day 3, the rats were presented with 45 CS alone presentations (with 90 s ITI) for extinction training and the freezing during the first 5 CS presentations was used to measure fear learning and it was called fear recall. On day 4, they were presented with 5 CS alone presentations(with 90 s ITI) to measure the extinction learning, and called extinction recall.

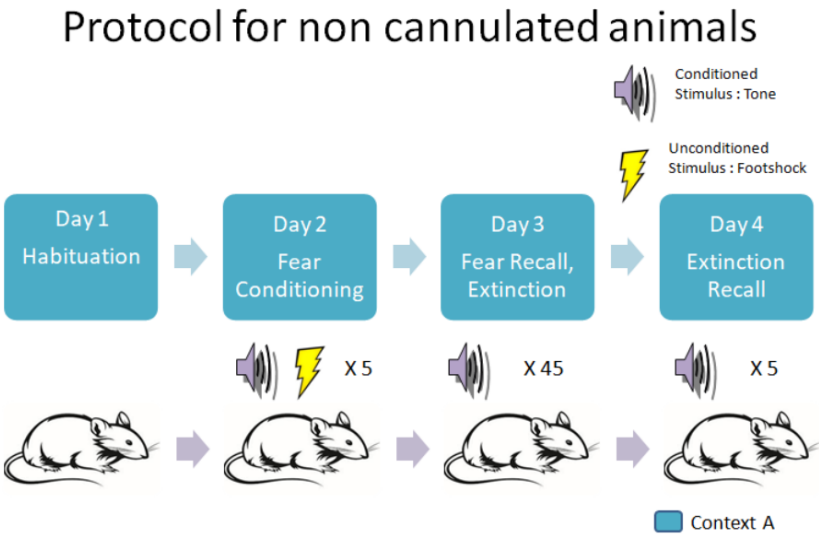


Fig. 3 Fear conditioning and extinction setup for non-cannulated animals

3.4.2.ii. Protocol for cannulated animals:

The animals were fear conditioned and extinction trained in different contexts.

- Context A: clear plexiglass, bright LED lights on, and the black tray cleaned with 70% ethanol
- Context B: plexiglass covered with a black paper, dim visible light on, and the black tray cleaned with 1% acetic acid

The different contexts were used to make the extinction training more efficient as the animals showed a sustained fear response to the context in which they were fear conditioned and this slowed the extinction learning process. Although the rats learned extinction even when both fear conditioning and extinction training was done in the same context, as in the non-cannulated experiments, it was faster (required lesser tone alone trials) when it was done in a context different from the fear conditioning context. The senses that allow the animal to differentiate the contexts are touch, sight and smell. Out of these, touch could not be changed as it was inherent to the apparatus. Although the visuals were altered (by changing the light intensity and the transparency of the enclosing box), it had to be taken into account that rats have rather poor vision. Given that rats have very good olfactory abilities,

the strongest way to change the context for the rat was to change the odour, which was done by cleaning the tray with 70% ethanol in one context and 1% acetic acid in the other context.

On day 1, the rats were habituated to the conditioning chamber (context A) for 10 minutes to allow for encoding of contextual cues. On all subsequent days, the animals were given 10 minutes of exploration time before the onset of any cues, and their freezing during this time indicated any fear associated with or due to the context itself. On day 2, the rats received 5 trials of a tone (CS, 70 dB, 30 s) immediately followed by a footshock (US, 0.75mA, 2 s), separated by an intertrial interval (ITI) of 90 seconds(5 CS-US pairings) in context A. On day 3, the rats were presented with 15 CS alone presentations (90 s ITI) in context B for extinction training and the freezing during the first 5 CS presentations was used to measure fear learning and called fear recall. On day 4, they were presented again with 15 CS alone presentations (90 s ITI) in context B for further extinction training and the freezing during the first 5 CS presentations was used to measure extiction learning. On day 5 the rats were again presented with 5 CS alone presentations (90 s ITI) in context B to measure the extinction learning, and called extinction recall. Two days of extinction training was done to ensure that the extinction training was sufficient to cause fear extinction.

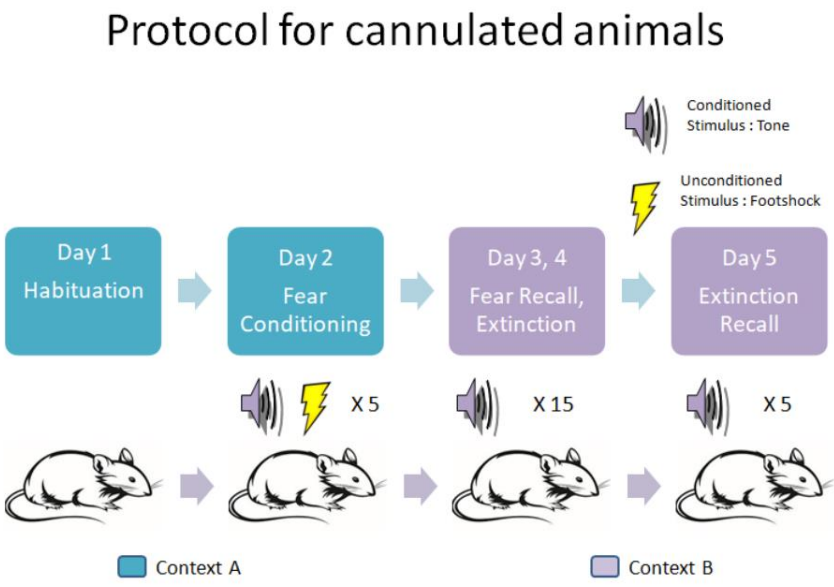


Fig. 4 Fear conditioning and extinction setup for cannulated animals

3.4.3. Experiments:

Experiment 1: Fear conditioning in starved (6 hour) vs fed animals

In this experiment, 24 rats were used. Twenty four hours after their last handling session, rats underwent only the fear conditioning protocol described in Fig. 3, without the extinction training or recall. Twelve animals were starved for 6 hours before conditioning (day 2), and the other twelve were fed *ad libitum* throughout. From both the fed and the starved groups,6 were control (no shock on day 2) and the other 6 were test (shock on day 2).

Experiment 2: Fear conditioning in starved (16 hour) vs fed animals followed by extinction training and recall

In this experiment, 30 rats were used. Twenty four hours after their last handling session, rats underwent the protocol described in Fig. 3. The animals were divided into 4 groups, test animals starved for 16 hours before conditioning (day 2, shock, n=16),control animals starved for 16 hours before conditioning (day 2, no shock, n=4), test animals fed *ad libitum* before conditioning (day 2, shock, n=6) and control animals fed *ad libitum* before conditioning (day 2, no shock, n=4).

*Experiment 3:*Fear conditioning followed by extinction training and recall. Intra CeA (cannulated) microinjection before conditioning

In this experiment, 11 rats were used. Twenty four hours after their last handling session, , rats underwent bilateral CeA cannulation surgery after which they rested for 6-9 days.After the recovery period, the rats underwent the protocol described in Fig. 4. The animals were divided into 3 groups, and received microinjections 15 minutes prior to the start of the conditioning (day 2).

i) Animals starved for 16 hours before conditioning, microinjected with aCSF (day 2, n=5),

ii) Animals fed *ad libitum* before conditioning, microinjected with aCSF (day 2, n=3), and

iii) Animals fed *ad libitum* before conditioning, microinjected with CART antibody (day 2, n=3).

Experiment 4: Fear conditioning followed by extinction training and recall. Intra CeA (cannulated) microinjection before first day of extinction

In this experiment, 9 rats were used. Twenty four hours after their last handling session, , rats underwent bilateral CeA cannulation surgery after which they rested for 6-9 days. After the recovery period, the rats underwent the protocol described in Fig. 4. The animals were divided into 3 groups, and received microinjections 15 minutes prior to the start of the first extinction session (day 3).

i) Animals starved for 16 hours before conditioning, microinjected with aCSF (day 3, n=3),

ii) Animals fed *ad libitum* before conditioning, microinjected with aCSF (day 3, n=3), and

iii) Animals fed *ad libitum* before conditioning, microinjected with CART antibody (day 3, n=3).

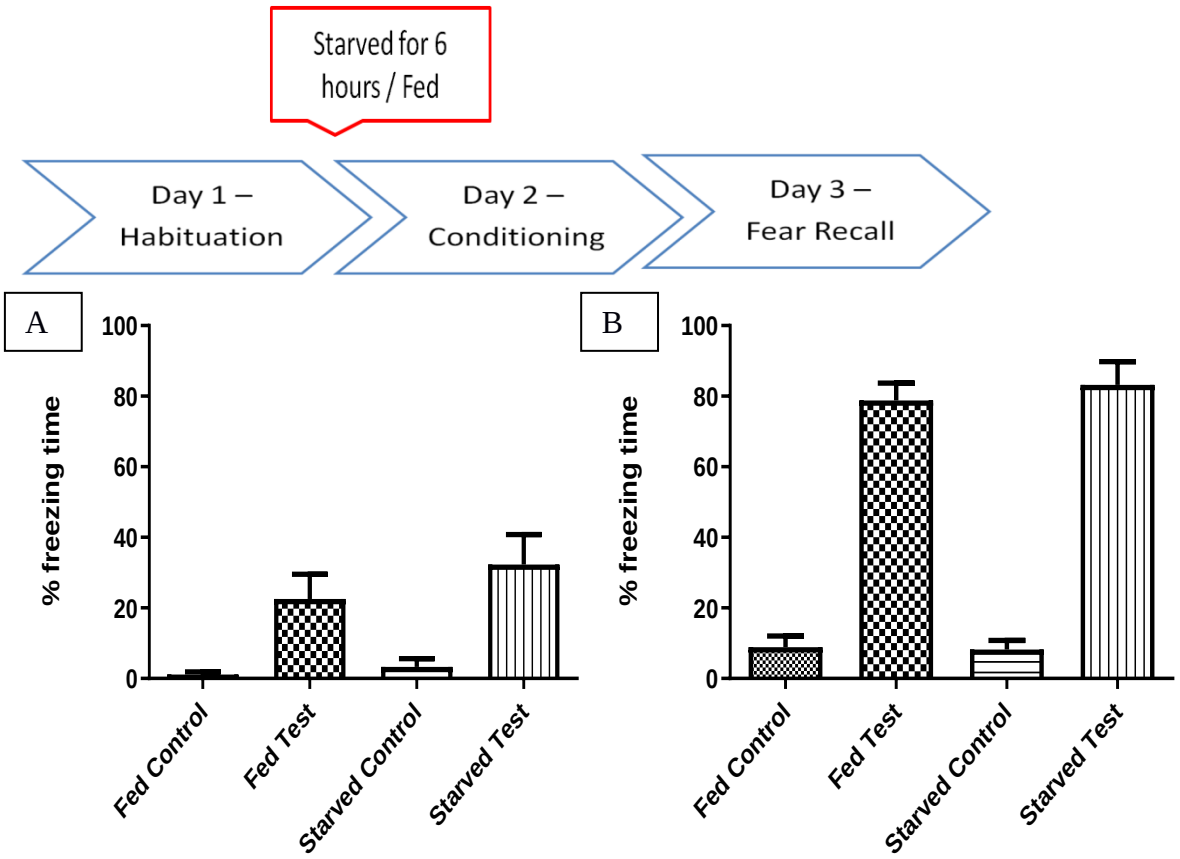
3.5. Statistical analysis:

The results were analysed and the graphs were plotted using GraphPad Prism 5.0 statistical software with the error bar showing SEM. Statistical significance was determined using Tukey one way ANOVA test for cue wise graphs and t-test for bar graphs. Differences were considered significant at $p<0.05$.

4. RESULTS:

4.1: Six hour starvation before fear conditioning does not affect fear recall

The fear conditioning experiment (Fig. 3) was completed for n = 6 (started during previous semester) to check whether a 6 hour starvation before fear conditioning has any effect on the acquisition of the fear association to the sound cue. Half of the rats were fed *ad libitum* and the other half were starved for 6 hours before the conditioning. Half the rats from both the groups received foot shocks (US) during conditioning while the other half were only presented with the tone (CS). On the third day the rats were presented with tone alone presentations and their freezing responses recorded to measure the fear recall (Fig 5). [Extinction was not studied for these animals and the protocol ended after 5 CS alone trials on day 3]



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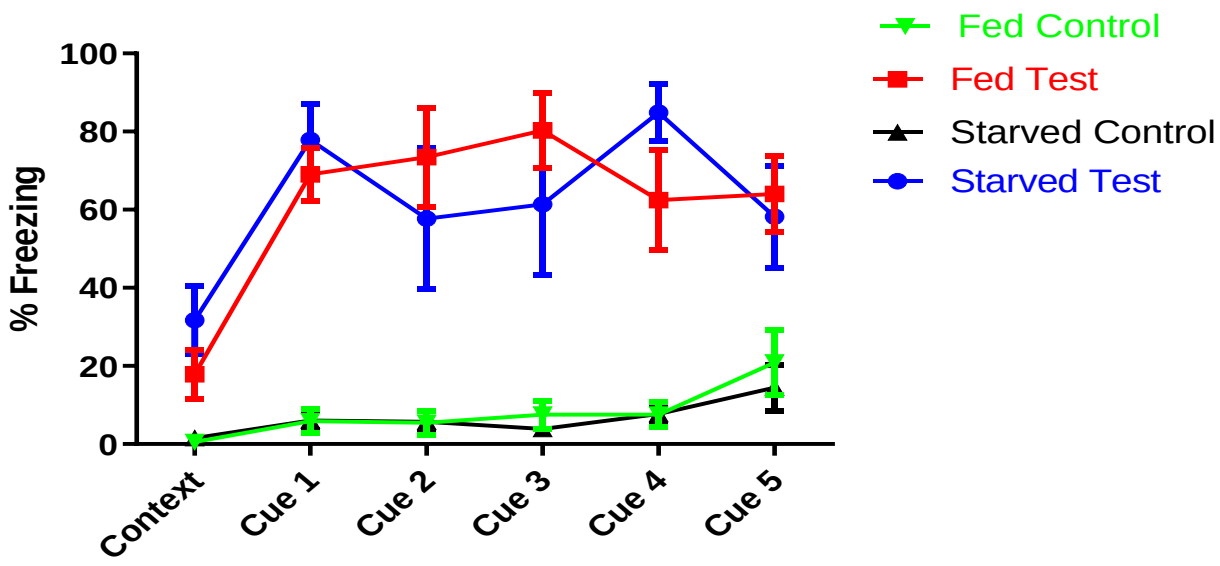


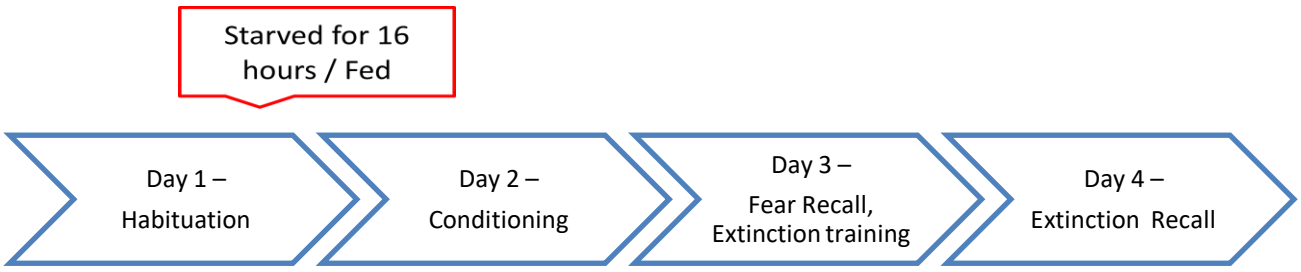
Fig. 5. Fear conditioning results with a 6 hour starvation period before fear conditioning. On the first day, rats were placed in the apparatus for 5 minutes to habituate. On the second day, half of the rats were fed *ad libitum* and the other half were starved for 6 hours before the conditioning. The animals were given 180 seconds of exploration, after which they were fear conditioned by presenting them with 5 trials of a tone (30 s, 70 dB) with an intertrial (ITI) period of 90 s. For half the rats from both the groups, each tone was immediately followed by a shock (0.75mA, 0.5s) (Test) while the other half were only presented with the tone (Control). On the third day the animals were again given an exploratory period followed by five trials of tone alone presentations (30 s, 70 dB) (ITI 90 s) and the freezing response was measured. The freezing during the exploratory period was counted as context freezing and the freezing during the tone presentations as cue freezing. (n=6 for all groups)
A) Context Recall (with SD): The freezing before the tone presentation to test for context recall,
B) Cue Recall (with SD): The total freezing during all the tone alone presentations,
C) Fear Recall (with SEM): The freezing during context recall and cue recall, with freezing during each of the cue presentations and during context recall given separately.

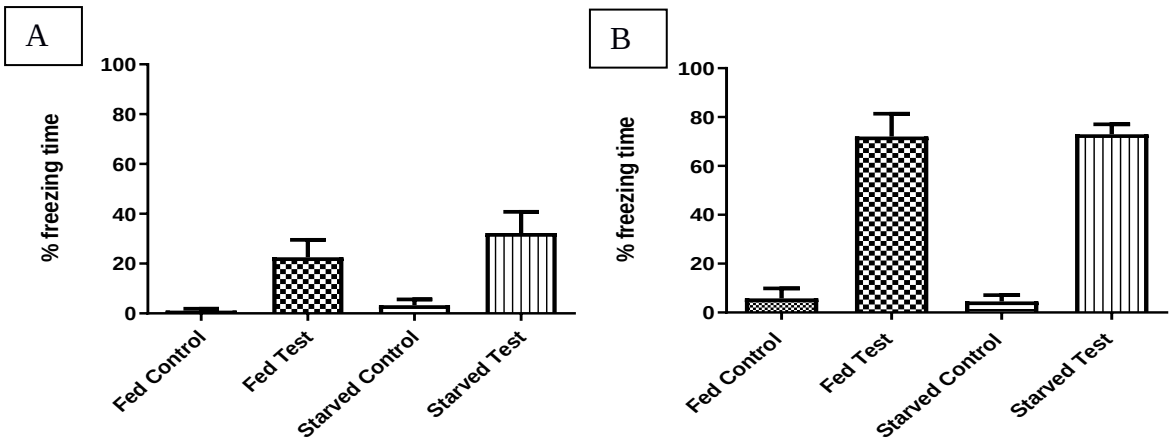
In Fig 5. A and B, there is a significant difference in the freezing between the control and test animals (Context $p=0.0019$, Cue $p<0.0001$, t-test), showing that only the animals that received the shock expressed fear and that the freezing wasn't due to just the tone alone. From the same figures (fig. 5 A, B, and C), it can be observed that the freezing in response to the cue is much higher than the freezing before the onset of cue presentation, showing that the association of the cue to the shock is strong, and the paradigm works to test the fear memory. There was no significant change in the freezing response to the cue of the rats that were subjected to a 6 hour starvation before fear conditioning from the ones that were fed ($p=0.9506$, t-test). The data is for n=6 in each group.

4.2 : Sixteen hour starvation before fear conditioning does not affect on fear recall, but facilitates extinction learning

A six hour starvation before fear conditioning did not show any effect on the acquisition of the fear association to the sound cue. So the experiment was repeated for a longer starvation period (of 16 hours) before conditioning to test whether a more intense starvation that causes a more intense enegry state difference would lead to a difference in fear and extinction memory. The animals were divided into 4 groups, test animals starved for 16 hours before conditioning (day 2, shock, n=16),control animals starved for 16 hours before conditioning (day 2, no shock, n=4), test animals fed *ad libitum* before conditioning (day 2, shock, n=6) and control animals fed *ad libitum* before conditioning (day 2, no shock, n=4). The control groups had only four subjects each as they only showed very minimal freezing which was consistent. Also, they did not receive a shock and cannot show any extinction.

Also, to check whether the starvation before conditioning would affect extinction learning afterwards, the rats were subjected to extinction learning along with fear recall. After the 180 s of context recall, the rats were given 45 trials of cue alone, the first 5 of which were taken as cue fear recall (Fig 6. B and C). [Following the protocol of Maren et al., 2006 and Ramanathan K. R., 2018]. The freezing during the exploratory time before tone presentation was measured as context fear recall (Fig. 6 A and C). On the 4th day, the animals were again given an exploratory period, the freezing during which was counted as context extinction recall (Fig. 6 D and F). This was followed by 5 tone alone presentations, the freezing during which was measured as cue extinction recall (Fig. 6 E and F).

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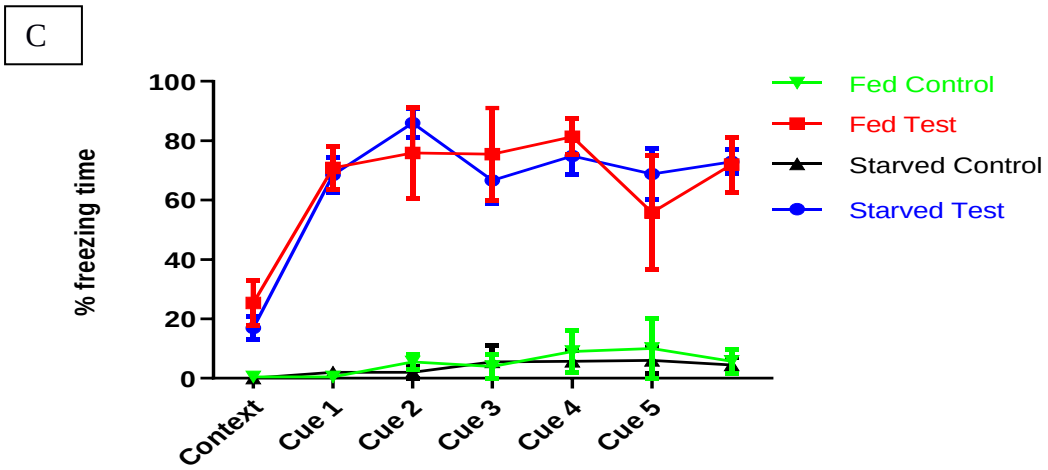
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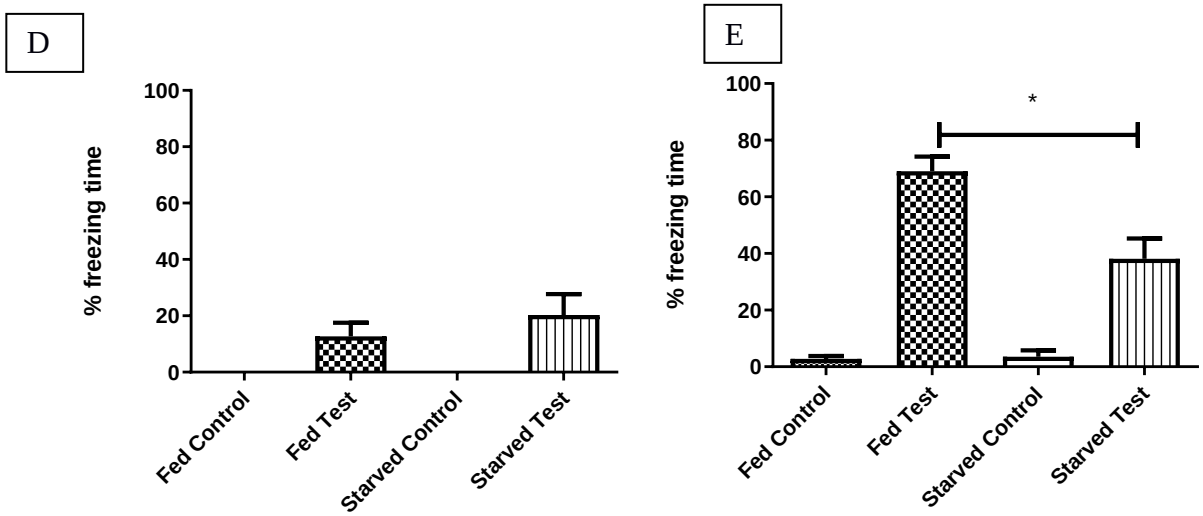
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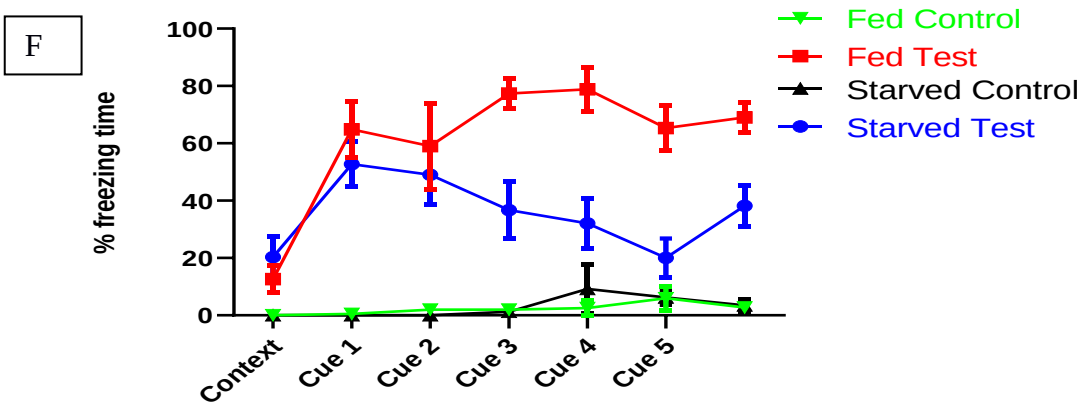


Fig. 6 Fear conditioning and extinction results with a 16 hour starvation period before fear conditioning. On the first day, rats were placed in the apparatus for 5 minutes to habituate. On the second day, the rats were either fed *ad libitum* or were starved for 16 hours before the conditioning. The animals were given 180 seconds of exploration, after which they were fear conditioned by presenting them with 5 trials of a tone (30 s, 70 dB) with an intertrial (ITI) period of 90 s. For test rats from both the groups, each tone was immediately followed by a shock (0.75mA, 0.5s) (Test) while the other half were only presented with the tone (Control). On the third day the animals were again given an exploratory period followed by 45 trials of tone alone presentations (30 s, 70 dB) (ITI 90 s) that led to extinction training. The freezing response to the first 5 tone alone trials was measured as cue fear recall and the freezing during the exploratory period as context fear recall. On the 4th day, the rats were again given 3 minutes of exploratory time, the freezing response during which was recorded as extinction context recall. This was followed by 5 tone alone presentations and the freezing during this time was recorded as extinction cue recall.

A) Context fear recall (with SD) Freezing during exploratory time on the 3rd day,
B) Cue fear recall (with SD) Freezing during cue presentation on the 3rd day,
C) Fear Recall (with SEM) The freezing during context fear recall and cue fear recall, with freezing during each of the first 5 (sequential) cue presentations and during context recall given separately,
(n for each group : Control Fed - 4, Test Fed – 6, Control Starved- 4, Test Starved- 16)
D) Context extinction recall (with SD) Freezing during exploratory time on the 4th day ,
E) Cue extinction recall(with SD) Freezing during cue presentation on the 4th day,
F) Extinction recall (with SEM) The freezing during context extinction recall and cue extinction recall, with freezing during each of the cue presentations (sequential) and during context recall given separately
(n for each group: Control Fed - 4, Test Fed – 6, Control Starved- 4, Test Starved- 13)

In Fig 6. A (context fear recall) and B (cue fear recall), there is a clear difference in the freezing between the control and test animals (Context $p=0.0186$, Cue $p<0.0001$, t-test). There was no significant change in the freezing response to the cue of the rats that were subjected to a 16 hour starvation before fear conditioning from the ones that were fed ($p=0.9313$, t-test) (Fig. 6. C). In Fig. 6 D (context extinction recall)

and E (cue extinction recall) , we can see that in extinction, the freezing values of the control animals do not fall as much as the test animals, mainly because it was very low to begin with. The extinction paradigm is not the most efficient as can be seen by the low amount of extinction. But a clear significant difference can be seen in cue extinction recall between the animals that were starved for 16 hours before conditioning and the ones that were fed throughout (Cue recall p=0.0139, t test) (Fig. 6 C).

4.3 : Administeration of CART Ab in the CeA before conditioning does significant affect on fear recall, extinction recall, or extinction learning

A sixteen hour starvation before fear conditioning showed an effect on the extinction recall, suggesting that starvation before conditioning enhances extinction learning. (The data from animals that were confirmed to have bilateral cannulation hits were taken.) The next step is to check whether this is influenced by CART levels. Since CART levels would decrease in the arcuate nucleus during starvation, its projections to the amygdala could also see a decrease in CART levels, that could play a role in the fear memory. To investigate whether CART plays any role in this effect, CART levels were abolished in the CeA by administering CART Ab in fed animals (before fear conditioning) and the memory was studied. CART antibody blocks CART activity by binding to it and preventing it from binding to its receptor. Animals were also starved and injected with aCSF in the CeA before conditioning. This was done to ascertain that the trend in the CART activity abolished animals and starved animals is similar. The animals were divided into 3 groups, and received microinjections 15 minutes prior to the start of the conditioning (day 2).

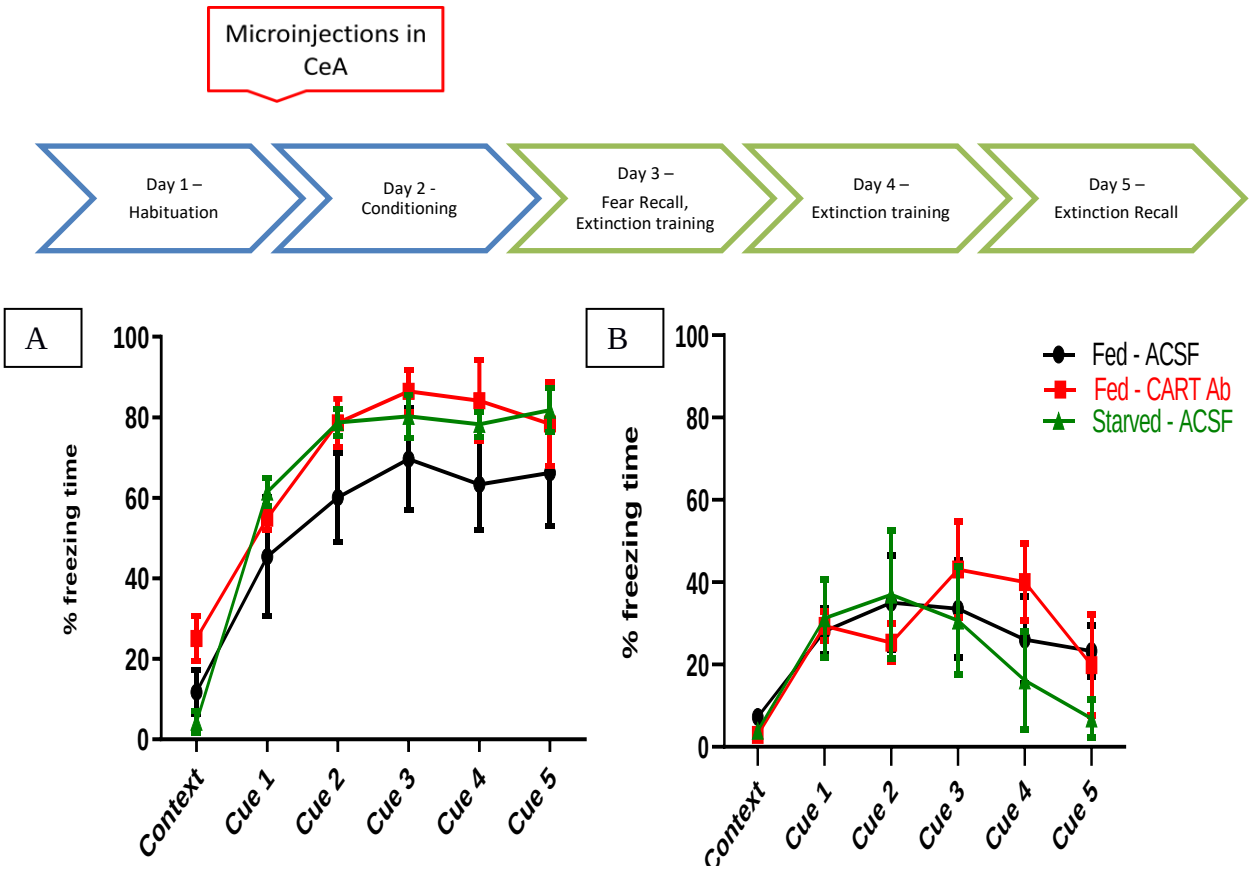
i) Animals starved for 16 hours before conditioning, microinjected with aCSF (day 2, n=5),

ii) Animals fed *ad libitum* before conditioning, microinjected with aCSF (day 2, n=3), and

iii) Animals fed *ad libitum* before conditioning, microinjected with CART antibody (day 2, n=3).

The animals were habituated to the conditioning context for 10 minutes on the first day. On the second day the animals were given microinjections and 15 minutes to

recover. They were placed in the conditioning context and given 10 minutes of exploratory time and then presented with 5 trials of the tone (30s, 70 dB) followed by footshock (0.75 mA, 2 s) with intertrial periods of 90 s. The next day (3rd) the animal was placed in a different context and given an exploratory period of ten minutes followed by 15 tone alone presentations (extinction training, Fig. 7 C) and the freezing during the exploratory period and the first 5 trials were measured as fear recall (Fig. 7 A). On the 4th day, the animals were again given 15 tone alone trials after a 10 minute exploratory period and served as further extinction training (Fig. 7 D). The freezing during this period measured extinction learning On the 5th day the animals were again given a 10 minute exploratory period, followed by 5 tone alone presentations and the freezing during this time was measured as extinction recall (Fig. 7 B)



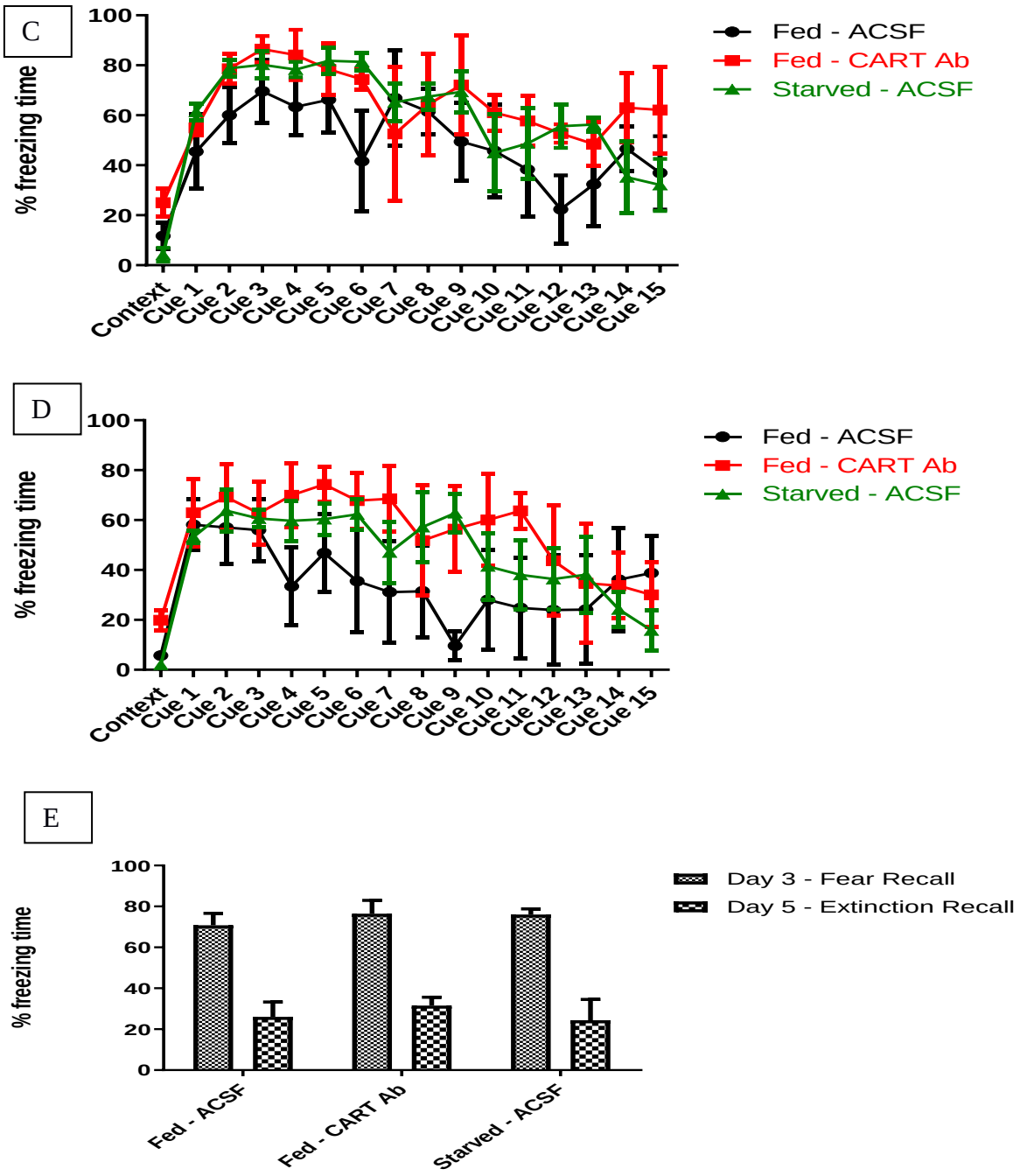


Fig. 7 Fear and extinction recall with microinjections in the CeA before conditioning
On the first day, rats were placed in the conditioning context for 10 minutes to habituate. On the second day, the rats were either fed *ad libitum* or were starved for 16 hours before the conditioning, given microinjections 15 minutes before the start of the behavioural experiment. The animals were given 10 minutes of exploration, after which they were fear conditioned by presenting them with 5 trials of a tone (30 s, 70 dB) immediately followed by a footshock (0.75 mA, 2 s) with an intertrial (ITI) period of 90 s. On the third and fourth day the animals were placed in a different context (from the conditioning context) and given an exploratory period followed by 15 trials of tone alone presentations (30 s, 70 dB) (ITI 90 s) that led to extinction training. The freezing response to the first 5 tone alone trials on the third

day was measured as cue fear recall and the freezing during the exploratory period as context fear recall. On the fifth day, the animals were given 10 minutes of exploratory time followed by 5 tone alone presentation and the freezing response during this period was recorded as extinction recall.
A) Fear recall (with SEM) Freezing on day 3, separately for context and the 5 sequential cues,
B) Extinction recall (with SEM) Freezing on day 5, separately for context and the 5 sequential cues,
C) Extinction learning - day 1 (with SEM) Freezing on day 3, separately for context and the 15 sequential cues,
D) Extinction learning - day 2 (with SEM) Freezing on day 4, separately for context and the 15 sequential cues,
E) Fear recall vs Extinction Recall (with SD)
(n=3 for Fed aCSF and Fed CART Ab, n=5 for Starved aCSF)

In Fig 7. E (as well as A and B), there is a significant difference in the freezing between the fear and extinction recall, suggesting that the extinction paradigm is efficient ($p<0.0001$, t-test). This increase in extinction is expected as the extinction training was extended to two days and was also done in a different context than the conditioning context. There was no significant difference in the fear or extinction recall to the cue between the different treatment groups (Fear recall $p=0.5756$, Extinction recall $p=0.7242$, one way ANOVA). In Fig. 7 C and D, we can see that extinction learning, however is different for the different treatment groups (Day 3 extinction $p=0.0454$, Day 4 extinction $p=0.0053$, one way ANOVA), with the fed aCSF animals showing higher extinction than the other two groups on both days of extinction. This is in direct contrast with the results of the earlier non cannulated experiments and merits further investigation.

*4.5 :*Administeration of CART Ab in the CeA before extinction training did not have any effect on fear recall, or extinction recall

The experiment to check whether abolishing CART levels by administering CART Ab in the CeA before fear conditioning did not show any significant effects on extinction recall. To further investigate whether CART activity is important for extinction training, CART levels were abolished in the CeA in fed animals before extinction training and fear and extinction memory studied.

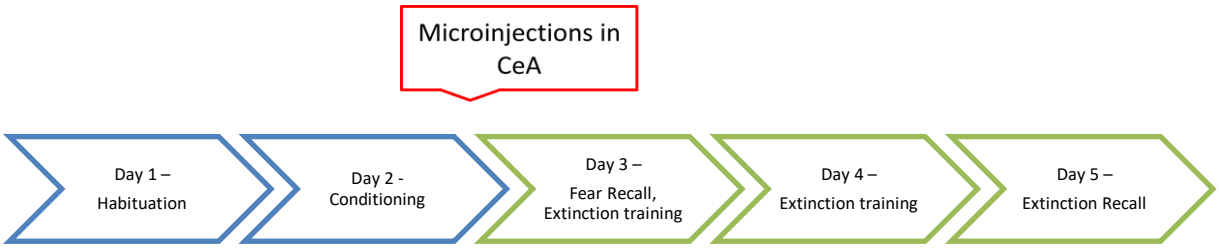
The animals were divided into 3 groups, and received microinjections 15 minutes prior to the start of the extinction training (day 3).

i) Animals starved for 16 hours before conditioning, microinjected with aCSF (day 3, n=3),

ii) Animals fed *ad libitum* before conditioning, microinjected with aCSF (day 3, n=3), and

iii) Animals fed *ad libitum* before conditioning, microinjected with CART antibody (day 3, n=3).

The animals were habituated to the conditioning context for 10 minutes on the first day. On the second day, they were placed in the conditioning context and given 10 minutes of exploratory time and then presented with 5 trials of the tone (30s, 70 dB) followed by footshock (0.75 mA, 2 s) with intertrial periods of 90 s. On the 3rd day the animals were given microinjections and 15 minutes to recover. Then the animal was placed in a different context and given an exploratory period of ten minutes followed by 15 tone alone presentations (extinction training) and the freezing during the exploratory period and the first 5 trials were measured as fear recall (Fig. 8 A). On the 4th day, the animals were again given 15 tone alone trials after a 10 minute exploratory period and served as further extinction training. The freezing during this period measured extinction learning. On the 5th day the animals were again given a 10 minute exploratory period, followed by 5 tone alone presentations and the freezing during this time was measured as extinction recall (Fig. 8 B)



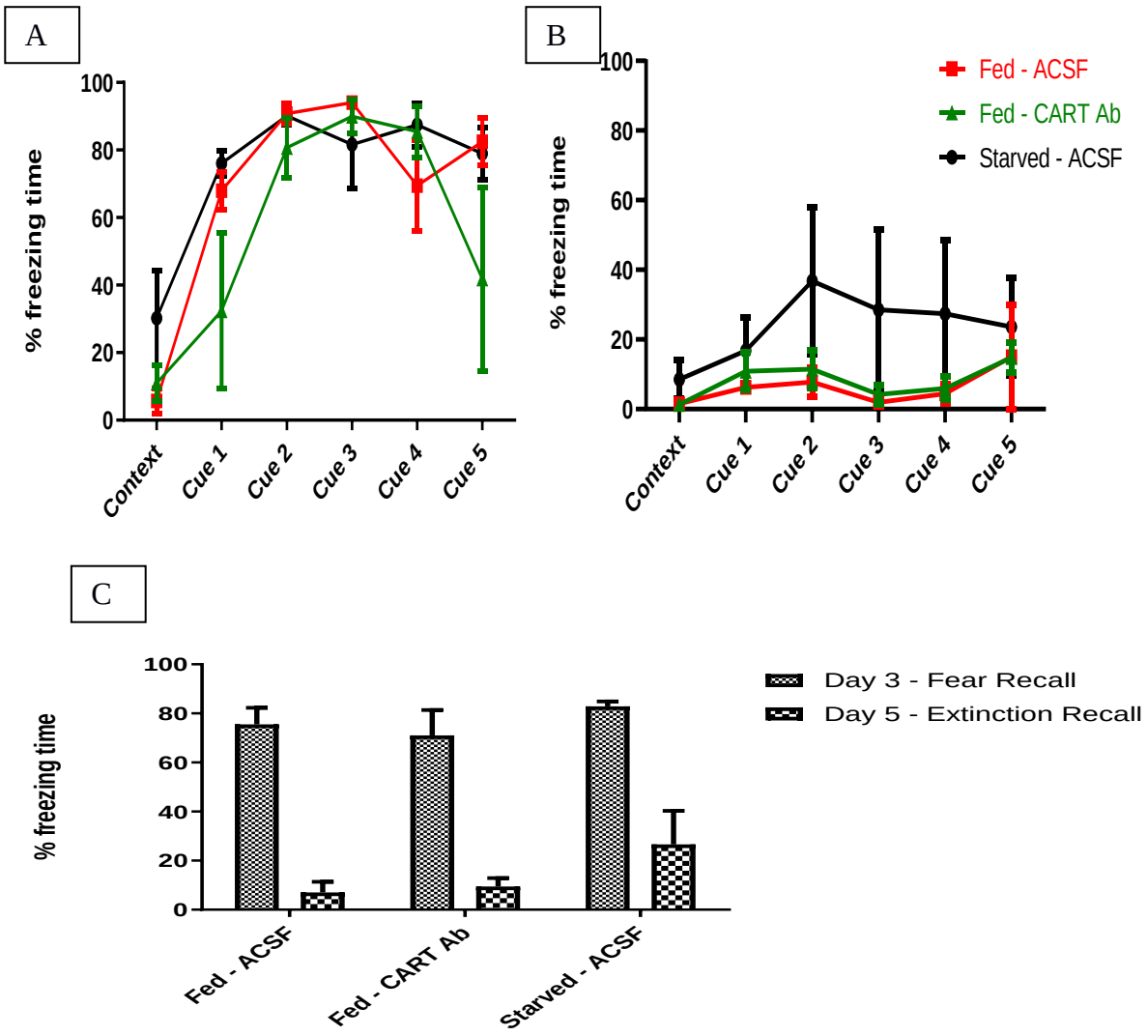


Fig. 8 Fear and extinction recall with microinjections before extinction training
On the first day, rats were placed in the conditioning context for 10 minutes to habituate. On the second day, the rats were given 10 minutes of exploration, after which they were fear conditioned by presenting them with 5 trials of a tone (30 s, 70 dB) immediately followed by a footshock (0.75 mA, 2 s) with an intertrial (ITI) period of 90 s. On the third day, they were either fed *ad libitum* or were starved for 16 hours before the conditioning and given microinjections 15 minutes before the start of the behavioural experiment. On the third (after the microinjection) and fourth day the animals were placed in a different context (from the conditioning context) and given an exploratory period followed by 15 trials of tone alone presentations (30 s, 70 dB) (ITI 90 s) that led to extinction training. The freezing response to the first 5 tone alone trials on the third day was measured as cue fear recall and the freezing during the exploratory period as context fear recall. On the fifth day, the animals were given 10 minutes of exploratory time followed by 5 tone alone presentations and the freezing response during this period was recorded as extinction recall.
A) Fear recall (with SEM) Freezing on day 3, separately for context and the 5 sequential cues,
B) Extinction recall (with SEM) Freezing on day 5, separately for context and the 5 sequential cues,

C) Fear recall vs Extinction Recall (with SD)
(n=3 for each: Fed aCSF, Fed CART Ab, Starved aCSF)

In Fig 8. C (as well as A and B), there is a significant difference in the freezing between the fear and extinction recall, suggesting that the extinction paradigm is efficient (p=0.0033, t test). There was no significant difference in the fear recall to the cue between the different treatment groups (Fear recall p=0.6024, one way ANOVA). The extinction recall however seems to have a significant difference between the treatment groups (Extinction recall p=0.0011, one way ANOVA). The extinction learning however has not been analysed. But none of this is conclusive for two reasons. One being the low sample size of 3 animals in each group. The second being that most of the cannulations have not been verified.

5. DISCUSSION:

Fear conditioning resulted in a significant increase in freezing response of both intact non cannulated rats as well as cannulated rats.There was no significant change in the freezing response of the rats that were subjected to either a 6 or 16 hour starvation before fear acquisition. The freezing during the cue alone presentations of extinction training was measured as extinction, but this is the same as the cue alone presentations of fear or extinction recall. This poses a problem as any cue alone presentation would inevitably lead to extinction training, even the cue alone presentations to measure fear recall.

Extinction learning or extinction retrieval appears to be higher in animals that were starved for 16 hours before fear conditioning. This is an interesting finding as it suggests that starvation at an earlier time point is able to affect learning and memory at a later time point. It seems to add evidence to the theory of fear and extinction memory being two separate engrams that compete for expression, which has recently been shown by Lacagnina et al. (2019).

Contextual fear memory is encoded in an ensemble of 'fear engram cells' that are activated during the memory formation and their reactivation is able to evoke fear in the animal. The extinction memory was proposed to be encoded by an extinction engram that competes with the fear engram. This was shown by Lacagnina et al when they used activity dependent neuronal tagging and saw the activation of different neuronal ensembles during fear and extinction training. Interestingly, they saw extinction training also led to the suppression of reactivation of the contextual fear engram. This result supports the competing engram theory and also provides a framework to hypothesise how the energy state during fear conditioning is able to influence extinction, which happens later. The energy state at the time of the formation of an engram could affect the strength or stability of the engram. This could explain the above results as the fear engram that is formed under starvation is unable to outcompete the later extinction engram formed under fed conditions. This energy dependency could be mediated by CART, the levels of which is regulated by the energy state and it could affect the engram strength or stability. To investigate whether CART has any role to play in this process of fear and extinction learning, CART manipulation experiments were carried out.

The Central amygdala (CeA) was chosen as the site of CART manipulation as it is a crucial interface that signals the freezing response, that also has a high abundance of CART positive fibres (Sharma et al., 2014). Bilateral cannulation was done on the rats, targeting the CeA. The surgery itself reduced the freezing response of the animal to the shock and the shock administered was increased to obtain similar levels of robust freezing that was seen in non cannulated animals. The reason for this decrease is not determined, but it was not considered a serious drawback as a small increase in the footshock was able to restore the freezing response. Recurring inconsistencies in the footshock given by the fear conditioning apparatus also could have been responsible for this drop in freezing, which also could be eliminated to a limit by increasing the footshock, ensuring the animal receives some amount of shock.

CART activity was obstructed by immunoneutralization using CART Ab (aCSF as control) in the CeAa) before fear conditioning and b) before extinction training. There was no significant difference between the CART Ab administered and aCSF administered fed animals in either of the experiments. This suggests that fear and extinction memory is not affected by CART levels. The CART Ab injected animal (fed) as well as the starved animal injected with aCSF before fear conditioning showed lower extinction learning (compared to fed animal injected with aCSF) on the second day of extinction training. However, the sample size is not sufficient to firmly conclude any of the above. Also, the immunoneutralization of CART by the CART Ab has to be verified by doing IHC of brains that received CART Ab. Also, to ascertain the role of CART in the results seen above, it has to be checked whether CART peptide injections in starved animals lead them to have behaviour similar to fed animals.

There is already evidence that CART levels in the arcuate is regulated by energy states, with starved animals showing a lower abundance of CART levels which is restored upon feeding. Based on unpublished data from the lab showing the presence of projections of CART positive from the arcuate to the amygdala, we hypothesised that CART levels in the brains, especially the CeA, is dependent on energy state. This has to be measured to evaluate if there is any difference in the CART peptide level due to energy states. Immunohistochemistry was done on one

fed and one starved brain. However, the data from a single set of animals is not sufficient to make conclusions and has not been included in the results.

Experiments need to be done to increase the sample size for all the cannulated experiments, which would decrease statistical errors. Further experiments also need to be done to study whether CART peptide injection in starved animals is able to mimic the extinction learning trend of the fed animals injected with aCSF, to ensure that the trend is not merely an artefact. Also important is increasing the number of IHCs and quantification. This would tell us about the regulation of CART peptide levels in the central nucleus of the amygdala and neuronal activity of CART cells at different energy levels.

Studying extinction is essential to understand the mechanisms underlying it and for the advancement of techniques used to treat PTSD. The increase in extinction recall in animals that were starved before conditioning is a promising result that deserves further study. While there are multiple pathways through which this could be happening, CART is one interesting candidate due to its role in modulating bodily functions depending on the energy levels. However, further experiments are required to conclude any involvement of CART in the same.

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