CART in Modulating Memory and Risk-taking Behaviours

A Thesis

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Under the guidance of

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Certificate

This is to certify that this dissertation entitled '**CART in Modulating Memory and Risk-taking Behaviours**' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the study/work carried out by Nived AP at Indian Institute of Science Education and Research Pune under the supervision of Dr. Aurnab Ghose, Professor, Department of Biology, during the academic year 2023-2024.

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Declaration

I hereby declare that the matter embodied in the report entitled '**CART in Modulating Memory and Risk-taking Behaviours**' are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education & Research (IISER) Pune, under the supervision of Dr. Aurnab Ghose and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

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Abstract

Evolution demanded not only the acute ability to detect and respond to threats but also the capability to learn from these encounters. The interactions between survival circuits and energy states in the brain, including those that are less relevant to immediate survival needs, can vary depending on the energy state of the organism. CART (cocaine and amphetamine-regulated transcript) is a neuropeptide that is mainly studied in the context of appetite regulation and energy homeostasis; it is anorexic in nature. CART is robustly expressed in hypothalamic regions, which are key regulators of food intake and energy balance, such as the arcuate nucleus (Arc), paraventricular nucleus, dorsomedial nucleus, and ventromedial nucleus. It is also found in the limbic system, including the central amygdala (CeA), ventral bed nucleus of stria terminalis (vBNST); which play important roles in emotional responses, and the hippocampal regions; modulating higher cognitive functions like learning and memory. Here, we show that CART plays a role in learning and memory formation in mice using Novel object recognition test (NOR) as CART-/- mice show recognition memory impairment. Additionally, we also show that fasting has no effect on recognition memory in NOR before training and before testing. Further, we investigated the role of CART in energy state-dependent adaptive prioritisation of behaviours by optimising multiple behavioural paradigms assessing risk-taking behaviours along with chemogenetics to selectively modulate the activity of Arc^{CART} neurons in dissecting out the role energy states have on different survival circuits.

Contributions

Contributor name	Contributor role
Nived AP, Usashi Guha Roy and Aurnab Ghose	Conceptualization Ideas
Nived AP, Usashi Guha Roy, Nishikant K Subhedar and Aurnab Ghose	Methodology
Nived AP	Software
Nived AP	Validation
Nived AP	Formal analysis
Nived AP Usashi Guha Roy (Chemogenetics, PORT) Rushikesh Chavan (Chemogenetics, risk-taking assays) Vihang Vaidya (Light induced risk taking test)	Investigation
Aurnab Ghose	Resources
Nived AP	Data Curation
Nived AP	Writing - original draft preparation
Nived AP and Aurnab Ghose	Writing - review and editing
Nived AP	Visualization
Aurnab Ghose	Supervision
Aurnab Ghose	Project administration
Aurnab Ghose	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

1. Introduction

1.1 Energy state dependent adaptive prioritisation of behaviours

Neural circuits and mechanisms in the brain for detecting, responding, and adapting to threats and opportunities are essential for an organism's survival. They include mechanisms responsible for energy homeostasis, fear and threat perception, social behaviours and reproductive behaviours. Survival circuits can't be localised as they need to incorporate multiple information to ensure survival, hence they are interconnected with various brain regions and are modulated by numerous factors, including emotional states, environmental cues, and the organism's internal physiological status. The balance between risk and reward is a fundamental aspect that influences decision-making in organisms when foraging for sustenance in environments with potential threats (Sternson, 2013)[1]. Avoiding threats, obtaining rewards – mostly, studied separately, hence, there is a need to study these two combined along with linking it with the internal states of the organism for better understanding of the adaptive responses made by the animal. Emotion encompasses various aspects, one crucial element being the environment stimuli, even for those which are trained or associated using pavlovian conditioning (Cardinal et al., 2002)[2]. For an organism, in its natural habitat, they have to constantly assess the risk of encountering predators, while they forage for food. Fear is the internal state of an organism evoked by danger, thereby resulting in adaptive defensive responses ensuring survival. Depending upon the origin of the response, fear can be classified into innate and conditioned. Innate fear responses are from birth and doesn't require any prior experience for eliciting the response (Blanchard & Blanchard, 1971)[3], whereas conditioned fear requires association of the stimuli to fear responses through experience. In rodents, odours of predators are shown to elicit innate fear responses, one such is the TMT (2,4,5- trimethyl-3-thiazoline) a compound extracted from fox faeces, elicits freezing behaviour when exposed. Anxiety is a state of anticipatory apprehension preparing an organism to face potential threats. Fear requires an imminent stimulus, whereas anxiety can be elicited by anticipatory stimuli, even at times when there is no threat at all. All these defensive behaviours are closely coupled with the energy state of the organism. Hunger is shown to decrease anxiety like

behaviours and increase exploration (Li et al., 2019)[4], altered social behaviour and dampened somatosensory perception (Smith and Grueter, 2022)[5] (***Figure 1***)[5]. Behavioural studies using rats have shown specific adaptive responses in context of predators threat stimuli – moving robots (Choi and Kim, 2010)[6]. The motivation to obtain the reward avoiding imminent threat and pain is a cost vs benefit trade off that the organism has to make choosing appropriate behaviours (Hayden and Walton, 2014; Rangel et al., 2008)[7,8]. Understanding how these circuits work in comprehending the complex behaviours animals exhibit in order to survive based on their internal energy status is important.

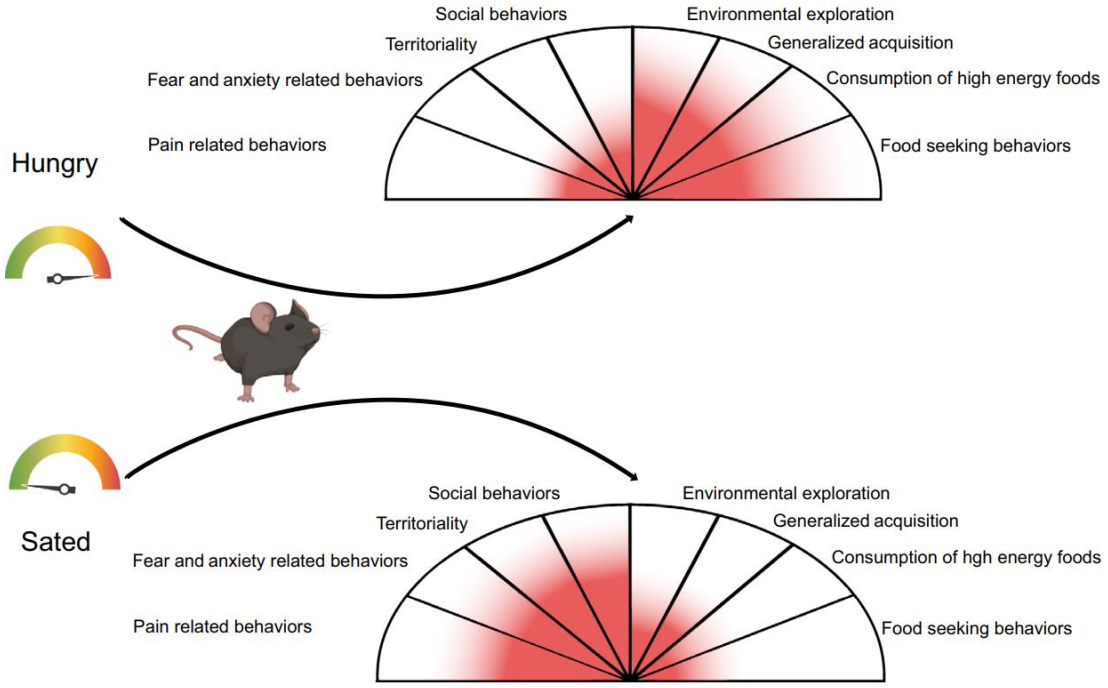

Figure 1 | Energy state dependent behavioural adaptation

1.2 Memory

Memory is a fundamental aspect of cognition, enabling organisms to encode, store, and retrieve information about past experiences. It plays an important role in shaping behaviour, decision-making, and learning. Memory processes are complex, involving multiple systems and neural mechanisms. From an evolutionary perspective, memory evolved as an adaptive mechanism, allowing organisms to learn from past experiences and make informed and appropriate decisions in the future. Memory can be broadly classified into short-term and long-term memory, based on the retention

times of information. The hippocampus, amygdala and the prefrontal cortex are the major regions involved in memory processes.

1.2.1 Object recognition memory

In rodents object recognition memory is assessed using novel object recognition (NOR) test (Ennaceur and Delacour, 1988)[9]. It is a simple and robust behavioural assay for memory that rely primarily on the animal's innate nature to explore things. It only requires a habituation session to the testing arena and training session using two same objects and finally testing using a novel object. There is no need for external motivation here for the animals to explore. By modulating the retention time between training and testing NOR can be used to study memories varying from short-term to long-term. Moojen et al., 2012[10] has reported successful novel object discrimination after a retention interval of 1.5 hours, 1 day and 7 days. This interval between training and testing sessions has a significant role in which brain structure is involved. Object exploration of 30 seconds each or 38s on one of the objects during training session enables the memory to be more hippocampal dependent from perirhinal dependent weak memory that would be observed when the criteria is not reached (Cohen & Stackman Jr., 2015; Cinalli Jr. et al., 2020)[11,12].

1.2.2 Neural circuitry underlying novel object recognition test

The primary brain regions involved in the NOR task include the perirhinal cortex (PRC), the hippocampus, and the prefrontal cortex (PFC). PRC encodes and processes the information related to object recognition, the hippocampus integrates spatial and contextual information along with consolidation of stronger representations depending on the training threshold, and the PFC helps in the maintenance and manipulation of object representations during the task. PRC receives input from the visual cortex, which has the information about the visual features of objects, and integrates this information to create a representation of the object that can be used for later recognition (Aggleton & Brown, 1999; Aggleton et al., 2010)[13,14]. The hippocampus receives input from the PRC and integrate this information with contextual and spatial information to form a representation of the object and its location in space (Eichenbaum, 2000)[15]. PFC receives input from both the PRC and the

hippocampus and is believed to integrate this information to create a more complex representation of the object and its context (Ranganath & D'Esposito, 2001)[16].

1.3 CART

CART (cocaine and amphetamine regulated transcript) is a neuropeptide that is involved in appetite and homeostasis control, it is anorexic in nature. CART was discovered when its mRNA was elevated upon treatment with cocaine or amphetamine (Douglass et al., 1995)[17]. CART is robustly expressed in hypothalamic regions; which 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)[18]. It is also found in the limbic system, including the central amygdala, ventral bed nucleus of stria terminalis (vBNST), hypothalamus and the hippocampal region; which play important roles in emotional responses. Starvation was shown to cause a decrease in CART levels in the arcuate nucleus, which was shown to recover after refeeding in rodents (Germano et al., 2007)[19]. While the expression of CART is regulated by energy levels, its function is not limited to modulating food intake but also plays role in fear response, reward and reinforcement, stress processing, regulation of motor activity etc. (Subhedar et al., 2014)[18]. CART is also a crucial modulator of innate fear response in the CeA-vBNST pathway (Rale et al., 2017)[20]. Central administration of CART is shown to enhance cognitive functions in Morris water maze and object recognition memory tests (Upadhya et al. 2011; Bharne et al. 2016)[21,22].

1.3.1 CART in energy state dependent adaptive prioritisation of behaviours

Exogeneous intracerebroventricular (i.c.v) administration of CART peptide was shown to decreases food intake (Kristensen et al., 1998)[23]. Whereas region specific role of CART was reported by exogenous administration of the peptide into specific hypothalamic nuclei, resulting in orexigenic effects of CART (Abbott et al., 2001)[24]. Reintroduction of CART in the arcuate nucleus of CART deficient CART-/- mice is shown to have an increase in energy expenditure, physical activity and goal directed behaviours (Lau et al., 2018; Somalwar et al., 2017)[25,26] highlighting the differential response that is shown by CART. DREADD (Alexander et al., 2009)[27] based activation

of Arc^{CART} neurons in CART-/- mice resulted in an increase in feeding, which was not seen when activated in CART+/- mice, pointing out to a inhibitory role of arcuate CART in inhibiting the orexigenic neuromodulators (Farzi et al., 2018)[28]. Previous studies have also shown prioritisation of behaviours by AGRP neurons in starved state in mice (Padilla et al., 2016)[29]. Previous findings from the lab suggests CART neuronal projections from arcuate nucleus of the hypothalamus to central amygdala, this connection potentially could facilitate the transfer of energy state related information to regulate amygdalar responses of fear, anxiety and threat perception. Therefore, understanding this circuit studying the function of Arc^{CART} neurons in energy state dependent adaptive prioritisation of behaviours is important.

1.3.2 CART in novel object recognition test

Studies using exogenous administration of CART peptide and antibody has shown insights into acute roles of CART in modulating object recognition memory, Bharne et al., 2016[22] showed dose dependent increase in discrimination indices upon administration of CART peptide both i.c.v and intrahippocampal (i.h) and a decrease in discrimination indices upon CART Ab administration in rats. Immunohistochemical analysis from the same study show significantly increased CART positive neurons in different regions of the hippocampus (CA3, DG, PRH and ENT) after training session in NOR. Previous studies from the lab, have shown energy state dependent memory components in fear conditioning in rats. Fasting before fear extinction training enhances extinction learning. Short-term fasting before fear acquisition affected long term fear memory and fasting before extinction training enhanced extinction learning in mice (Verma et al., 2016)[30]. Hence, studies looking at endogenous levels of CART, using CART knockout animals are crucial for providing insights into the role of systemic CART in modulating memory.

1.4 Objectives

i. To study the role of CART in modulating learning and memory formation.
    a. To assess different genotypes of CART for novel object recognition test.
    b. To evaluate whether fasting affects object recognition memory in NOR.

ii. To investigate the role of CART in modulating risk-taking behaviours under different energy states. *(Done together with Usashi Guha Roy, Rushikesh Chavan and Vihang Vaidya)*
    a. To investigate the role of Arc^{CART} neurons in modulating anxiety like behaviours and innate fear under different energy states in mice.
    b. To establish a behavioural paradigm for assessing risk taking behaviour with respect to energy states in mice.

2. Materials and Methods

2.1 Animals

All mice were on a C57BL/6J background. $CART^{cre/+}$ (+/- or Het) and $CART^{cre/cre}$ (-/- or KO or null) (Lau et al., 2016)[31] was a gift from Herbert Herzog (Garvan Institute of Medical Research, Sydney). All the genotypes were PCR verified after the completion of behavioural experiments. 8-14 weeks of aged mice were used for all behavioural experiments, were bred, housed under controlled humidity, temperature and maintained on a 12h light/dark cycle (7 am-7 pm) with *ad libitum* access to water and prescribed diet at the National Facility for Gene Function in Health and Disease, IISER Pune. All experimental procedures were performed as per the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India and were approved by the Institutional Animal Ethics Committee (IAEC), IISER Pune.

2.2 Surgery and Drug administration

6 weeks old mice were anaesthetised, administering a cocktail mix of ketamine (100mg/kg) and xylazine (10mg/kg) intraperitoneally (i.p). After fixing the mice onto the stereotaxic frame (Stoelting Co.), a mid-sagittal incision was made to expose the skull. Burr hole was made at the required injection site, and dura was removed. This was followed by an injection of 200 - 400nl of the Adeno-associated virus; cre-dependent hM3Dq (AAV8- hSyn-DIO-hM3D(Gq)-mCherry) with a titre value of $1x\ 10^{13}$ viral particles/ml, (Addgene #44361 Bryan Roth) or saline (PBS), neuronal tracer- DiI (Invitrogen, #C7001) (tracing experiments) into either the arcuate nucleus of the hypothalamus or dentate gyrus of the hippocampus using Nanoject III (Drummond Scientific) at an infusion rate of 60nl/min. or manually using a Hamilton syringe. Injection coordinates for arcuate nucleus (unilateral) are: AP, -1.7mm; ML, 0.2mm; from bregma and DV, -5.8mm; from the skull surface and for dentate gyrus (bilateral) are: AP, -1.9mm; ML, ±1.0mm; from bregma and DV, -2.0mm; from the skull surface. After the infusion, before lifting the needle, the system was left undisturbed for 5 minutes. to avoid tracing back, and then retracted slowly. The hole was then sealed using dental cement and secured using screws to the skull. Mice were housed individually post-surgery to prevent any potential damage and were monitored for post-

operative care. After a recovery period of 1 week, AAV injected mice were administered tamoxifen (Sigma-Aldrich #T5648) (150mg/kg) i.p for 5 days to induce recombination and were given a period of 2 weeks for DREADD expression. Mice received an i.p injection of either clozapine N-oxide (CNO) (Sigma-Aldrich #C0832) (1mg/kg) 30 mins. prior to the start of behavioural experiments.

2.3 Behavioural assays

Novel Object Recognition (NOR) test

Object recognition memory was evaluated using novel object recognition test. The test is based on the natural tendency of mice to preferentially explore novel objects over familiar ones. The test apparatus consists of a plexiglass box (30x 30x 30cm) with uniform illumination. Mice were habituated to the apparatus for 7.5 mins on day 1, followed by familiarisation training using two similar objects (glass bottle) next day and on test day a novel object (I- Pyraminx) and a familiar object (randomly chosen from the two) (***Figure 2***) was kept in the box and exploration times for these two objects, distance covered, time spent immobile, and discrimination indices; DI = (Novel – Familiar) / (Novel + Familiar) were determined. Only those animals with an exploration cut-off of 30s for each object or 38s for one object during training day was included for analysis (Cohen and Stackman Jr., 2015)[11]. Exploration was defined when the head of the animal was in the near vicinity (2.5cm) of the objects (Ennaceur and Delacour, 1988, Cohen and Stackman Jr., 2015)[9,11]. Place preference was eliminated by placing the objects interchangeably in the four quadrants of the box. For experiments involving object recognition memory after 3 and 5 days, new novel object (II- Coplin jar) was introduced. For experiments studying the effects of fasting on NOR, the interventions were made before training (T1) and before testing (T2) for a period of 22 hours. The objects and apparatus were cleaned with ethanol between each animal.

Innate preference test for objects

For innate preference test, two objects are kept in opposite quadrants of the open field (30 x 30 x 30cm) and the animal was allowed to explore the objects for 10 min. Only the object pair which have almost equal exploration time for both the objects, as validated by discrimination index is selected for NOR test.

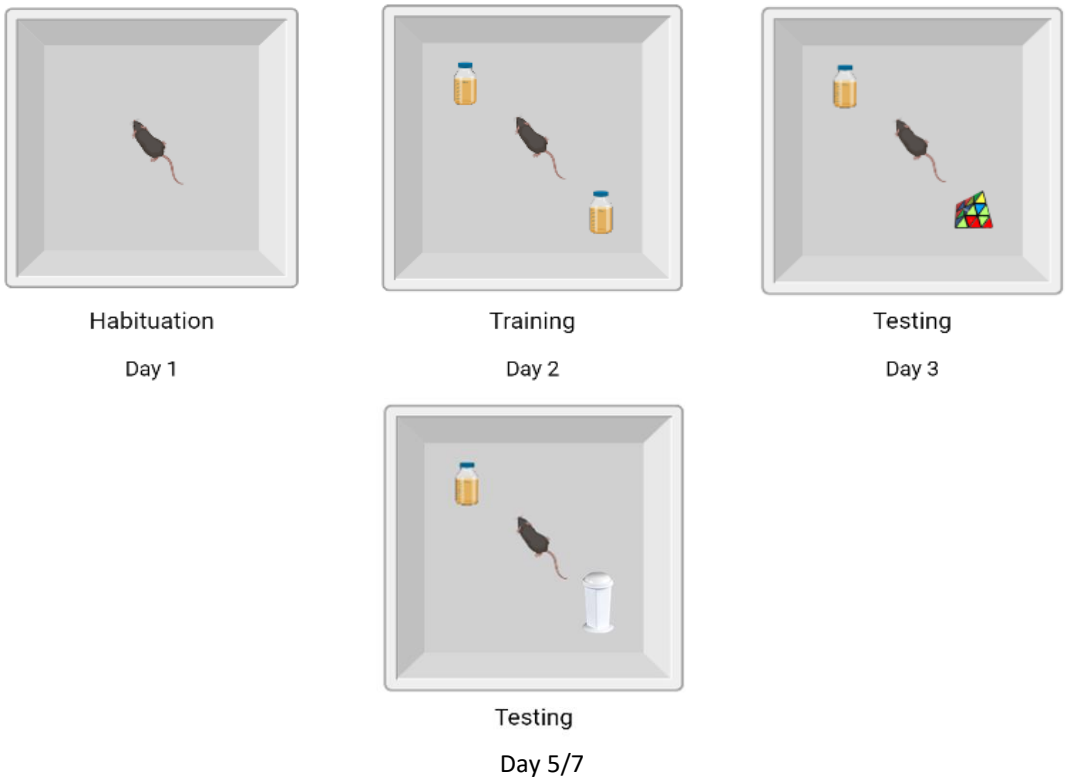

Figure 2 | Protocol for novel object recognition test.

Predator Odour Risk Taking (PORT) assay

The assay was performed as described by Dent et al., 2014.[32] The PORT apparatus is a plexiglass arena (90x 30x 30cm) divided into 3 chambers of equal size of length 30cm (***Figure 3***). Mice were released into one chamber and had to cross the middle chamber which had bedding material in it, in order to reach the chamber with food pellets. After a day of habituation to the apparatus (with bedding and food pellets), mice underwent a fasting period of 1h before habituation to increase the motivation to forage for food. Mice were tested with bedding mixed with 10% TMT (2,4,5- trimethyl-

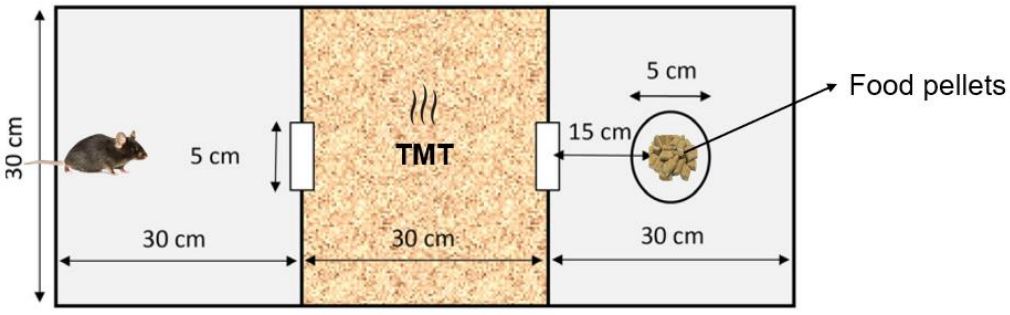

Figure 3 | Apparatus for PORT assay.

3-thiazoline) after 16h of fasting and finally for appropriate controls. The latency to acquire food, enter the food chamber and the first interaction with food were determined for three consecutive trials with a gap of 2 mins. between each.

Two chamber risk taking assay

Shuttle cage box for avoidance training was used as apparatus for this test. It consists of two chambers (30 x 30 x 30cm each)- one dark and one light, connected by a small door in the middle, which was only raised after 30s of placing the animal in the apparatus. Both the chambers had metallic rods as flooring, and only the light chamber's rods were electrified for this assay. Food (3-4 regular chow pellets) with dark chocolate shredded on top to increase the reward value of the food was kept on top of a shock-free platform in the light chamber and the dark chamber was kept empty. Mice were habituated to the apparatus for 2 days with food and without any

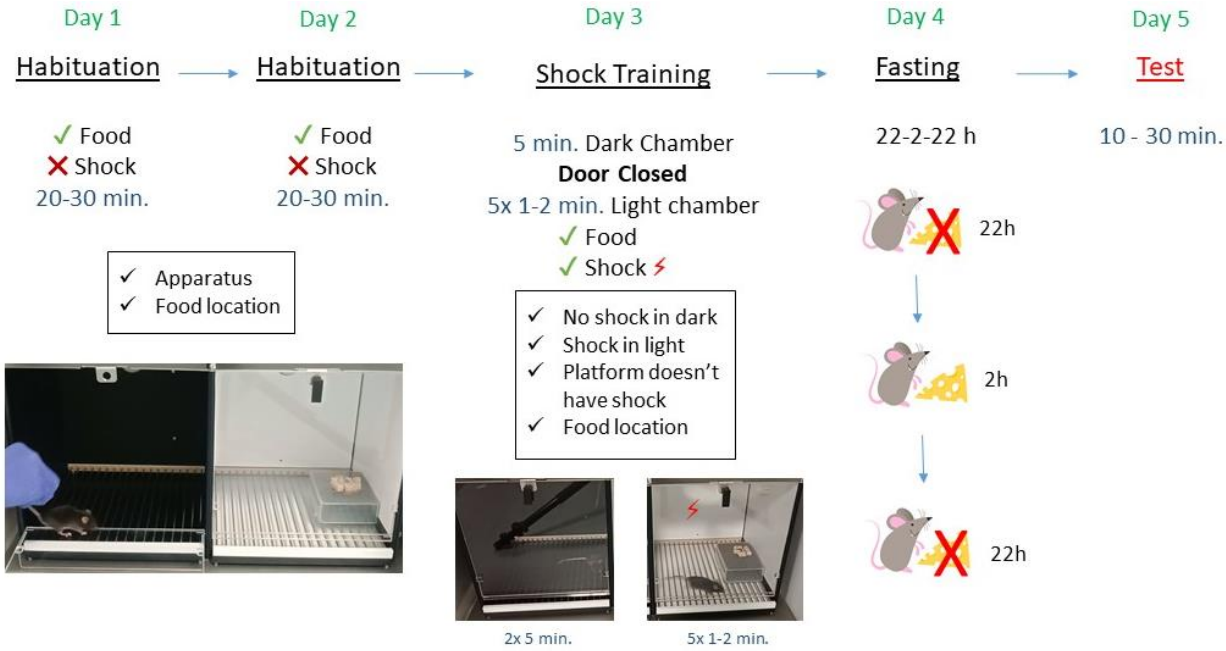

Figure 4 | Protocol for Two chamber risk taking assay with 22h-2h-22h

shock and received a shock training session on day 3 (***Figure 4***). Later they were food restricted and on test day mice were released into the dark chamber and had to go to the light chamber in order to access the food on the platform. Mice were monitored continuously to see whether they went to the food zone crossing the shock and were manually tallied for the number of attempts (paws on shock grill, nose pokes through

door) made to reach the food zone. The apparatus was thoroughly cleaned with ethanol between each animal.

Light induced risk taking test

Mice were released into the centre of the open field arena (60 x 45 x 45cm) for 10 mins. in a dark room, where all the lights were completely turned off. The apparatus consists of two zones; light and dark, the light zone (15 x 18cm) at the centre of the box was illuminated using a projector (Sony MP CD1) with a light intensity of 1500 lx (***Figure 5***). 3 Food pellets were kept in the centre of the light zone and ¼ of a pellet was kept at each corner depending upon the two scenarios that were tried. The time spent, number of entries into the light zone and the latency for the first entry into the light zone were determined. The apparatus was cleaned with ethanol and the food pellets were changed for each animal.

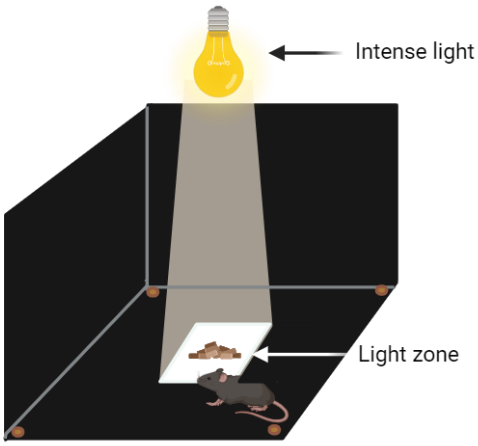

Figure 5 | Apparatus for Light induced risk taking test

Open Field Test (OFT)

Mice were released into the centre of the open field arena (60 x 45 x 45cm) with bedding material in it and were allowed to explore for 5 mins. The total distance covered in the arena, the time spent, the distance covered, and the number of entries to the centre (8cm away from all the four walls; 50% of total area) and periphery were determined. The apparatus was cleaned with ethanol and the bedding material was changed between each animal.

Elevated Plus Maze (EPM)

Mice were released into the centre of the plus maze. The elevated plus maze have the two open arms and two closed arms (55 x 5 cm each) elevated at a height of 50 cm

from the ground. The walls of the closed arms were 15 cm high. The total distance covered in the maze, the time spent, distance covered and the number of entries in the open and closed arms were determined. The apparatus was cleaned with ethanol between each animal.

TMT induced predator threat assay

2,4,5- trimethyl-3-thiazoline (TMT), compound extracted from fox faeces, an odour shown to elicit predator-like threat response in rodents. A rectangular plexiglass box (21x 9.5x 10cm) with two doors at its ends, each having an opening of 6x 6cm, which were covered with filter papers was used as the test chamber. Mice received 10 mins. of habituation to the apparatus for 4 days (***Figure 6***). On test day (5th day), 5 µl of TMT of desired concentration was applied to the filter papers on both of the doors before keeping the mice inside for a test duration of 20 mins. Time spent freezing was determined. Mice were said to be showing freezing behaviour, when it lacked all kinds of movements except breathing for a threshold of 2s. After each animal the filter papers were discarded and the apparatus was cleaned with alcohol.

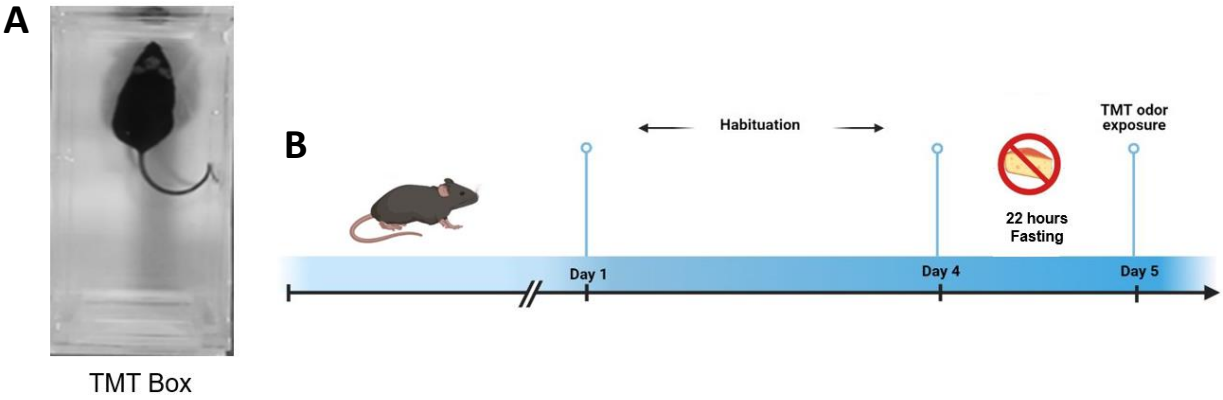

Figure 6 | (**A**) TMT box. (**B**) Protocol for TMT assay

2.4 Perfusion, sectioning and immunohistochemistry

Following chemogenetic experiments, mice were perfused with 4% PFA, brain tissue was kept in PFA overnight and transferred to 30% sucrose for cryopreservation. Brain tissues were serially sectioned (30 µm) on cryostat (Cryostar NX70, Thermofisher), Sections were permeabilised with 0.5% Triton X-100 (PBST) for 20 mins. Followed by blocking for 40 mins. using 5% Bovine Serum Albumin BSA. Sections were then incubated with primary antibody (anti-c-Fos) diluted in BSA (1:2000) overnight at 4°C. These sections were then rinsed again in PBS and then incubated with secondary antibody (anti-Rabbit Alexa Fluor 488) for 2 hours. After a subsequent PBS wash,

sections were then mounted on glass slide using a glycerol based mounting media containing 4,6-diamidino-2-phenylindole (DAPI) and then imaged using an epifluorescence microscope (Leica DM6 / Zeiss Apotome).

2.5 Behavioural analysis

Analysis for OFT, EPM, TMT assay and NOR were done using ANY-maze video tracking software (Stoelting). All of the readouts for the rest of the assays were manually scored.

2.6 Statistical analysis

Graphs were plotted using GraphPad Prism 9.5.0 and all the statistical analysis (except, DI for NOR) was done using the same. Animal wise break down of PORT data for WT animals was done using a custom written python script. All the analyses have been broken down in the results section. Data are expressed as mean ± SEM, and statistical significance was set at $p < 0.05$ (*p<0.05, **p<0.01, ***p<0.001). Estimation statistics for Discrimination index in NOR test: https://www.estimationstats.com (Ho et al., 2019)[33].

3. Results

3.1 Optimisation of objects for Novel object recognition test

Innate preference test for objects was done to choose the object pair best suited for NOR test. 5 object choices were tested for innate preference from mice; glass bottle, black cylinder, green box, pyraminx and Coplin jar (***Figure 7*A**). Black cylinder and green box were eliminated from object choices as mice showed very high preference for these objects, which were manually observed and no analytical data were measured for these. Both glass bottle & pyraminx (pair 1) and glass bottle & Coplin jar (pair 2) didn't show any innate preference as mice explored both the object pairs equally as represented by their discrimination indices (mean; 0.025 and 0.101 respectively, ***Figure 7*B**). This is also clearly evident from the exploration time for each animal in both the scenarios with pair 1 and pair 2 (***Figure 7* C&D**).

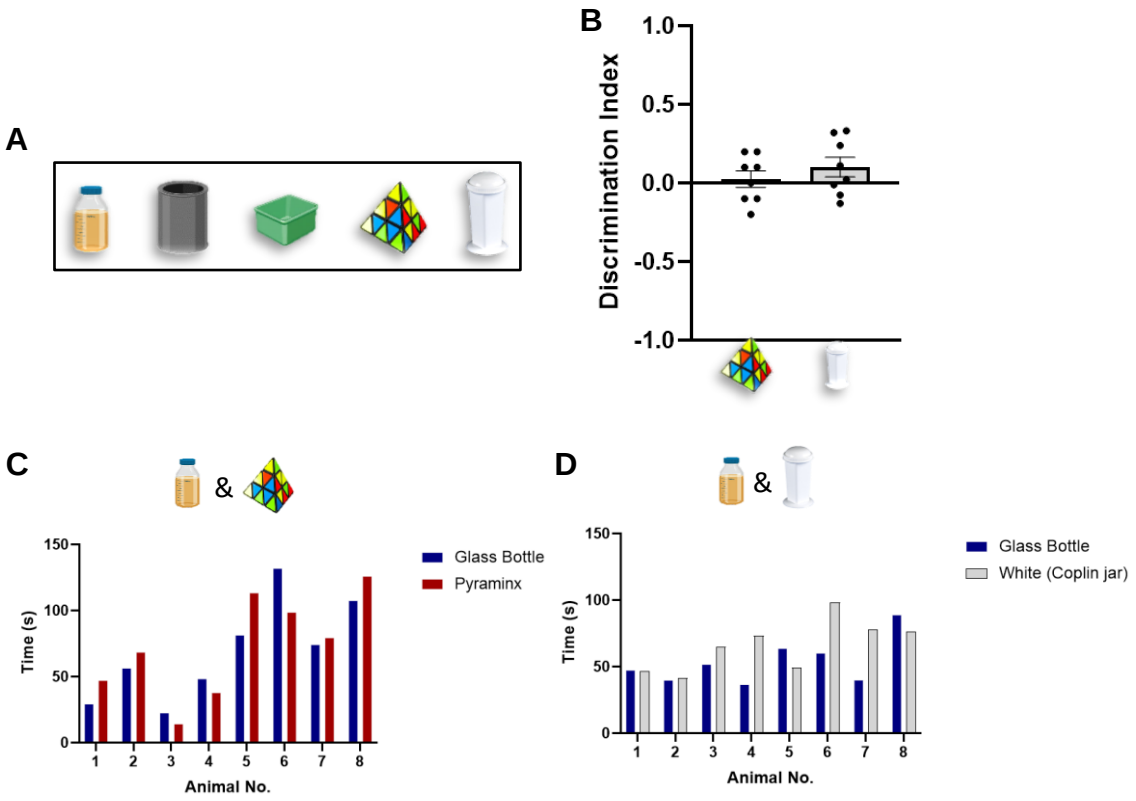

Figure 7 | Innate preference test for objects. (**A**) Representative images of the objects used for the study. (**B**) Discrimination index for pyraminx and coplin jar in innate preference test with glass bottle for WT animals (n=8 for each, represented as mean ± SEM). (**C**) Animal wise break down of exploration time for glass bottle vs pyraminx and (**D**) glass bottle vs coplin jar.

3.2 Endogenous knockout of CART impairs recognition memory in Novel object recognition test

Novel object recognition test was carried out using WT, CART$^{cre/+}$ and CART$^{cre/cre}$ mice 24 hours after training. Complete deletion of the CART gene in CART$^{cre/cre}$ mice impaired recognition memory, as these mice equally explored both the novel and familiar object (p=0.4316, Paired t-test, ***Figure 8*A**). Both the WT as well as the CART$^{cre/+}$ mice were able to discriminate between the two objects as they explored the novel object for a significantly longer duration compared to the familiar object (p=0.0067 and p=0.0025, Paired t-test, ***Figure 8*A**). It is to be noted that the difference observed here in terms of discriminating between the two objects is a direct readout of memory as revealed by the comparable levels of total distance travelled (***Figure 8*B**)

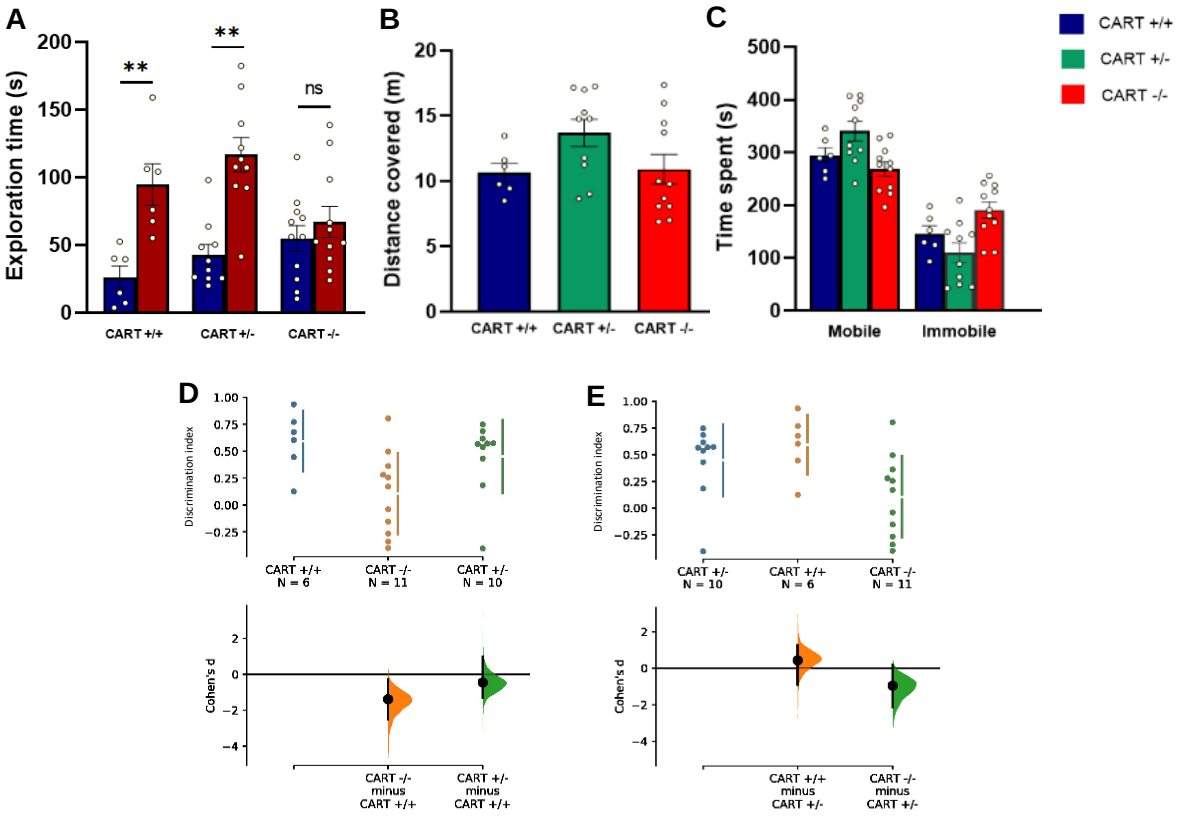

Figure 8 | CART deletion impairs recognition memory. (**A**) Exploration times for novel and familiar objects for CART$^{+/+}$, CART$^{cre/+}$ and CART$^{cre/cre}$ animals on testing day, (* p<0.05, ** p<0.01, paired t-test). (**B**) Plot showing the total distance covered by CART$^{+/+}$, CART$^{cre/+}$ and CART$^{cre/cre}$ mice. (**C**) Plot showing time spent mobile and immobile by CART$^{+/+}$, CART$^{cre/+}$ and CART$^{cre/cre}$ mice. (**D**), (**E**) The Cohen's d for two comparisons against the shared control CART +/+ and CART +/- respectively are shown in the above Cumming estimation plot. The raw data is plotted on the upper axes. On the lower axes, mean differences are plotted as bootstrap sampling distributions. **D**. The unpaired Cohen's d between CART +/+ and CART -/- is -1.39. The P value of the two-sided permutation t-test is 0.017. The unpaired Cohen's d between CART +/+ and CART +/- is -0.446. The P value of the two-sided permutation t-test is 0.43. **E**. The unpaired Cohen's d between CART +/- and CART +/+ is 0.446. The P value of the two-sided permutation t-test is 0.436. The unpaired Cohen's d between CART +/- and CART -/- is -0.956. The P value of the two-sided permutation t-test is 0.0416. (A, B, C and D); n=6 for CART$^{+/+}$, n=10 for CART$^{cre/+}$ and n=11 for CART$^{cre/cre}$.

and the time spent mobile and immobile (***Figure 8*C**) during the test between the three genotypes. Since exploration time for objects can vary with respect to overall exploration, Discrimination index is defined; which is exploration time for novel (N) – exploration time for familiar (F) / total exploration time (N+F), this represents the difference in exploration time for the objects as a proportion of overall time allocated for exploring the two objects. Estimation statistics was performed on discrimination indices, with shared control CART+/+ (***Figure 8*D**) and CART+/- (***Figure 8*E**). Cohen's d values are calculated and it shows a large effect on comparing the discrimination indices for CART+/+ and CART-/- (-1.39), CART+/- and CART-/- (-0.956). There is also a small to medium effect for CART+/+ and CART+/- (0.446).

3.3 Novel object recognition test: 3 days and 5 days after training

To investigate novel object recognition memory after 3 days and 5 days of training in WT animals, a new novel object (coplin jar) was introduced. Mice were trained with glass bottle and were tested after 1 day, 3days and 5 days. Testing after 1 day was done using pyraminx and 3day/5day was done using coplin jar, it is to be noted that these two experiments are independent of each other (separate sets of animals were used for day 3 and day 5). Mice explored the novel object (coplin jar) for a significantly longer duration than the familiar object 3 days after training (p=0.0245, Paired t-test, ***Figure 9*A**), however there was no significant difference in exploration times between

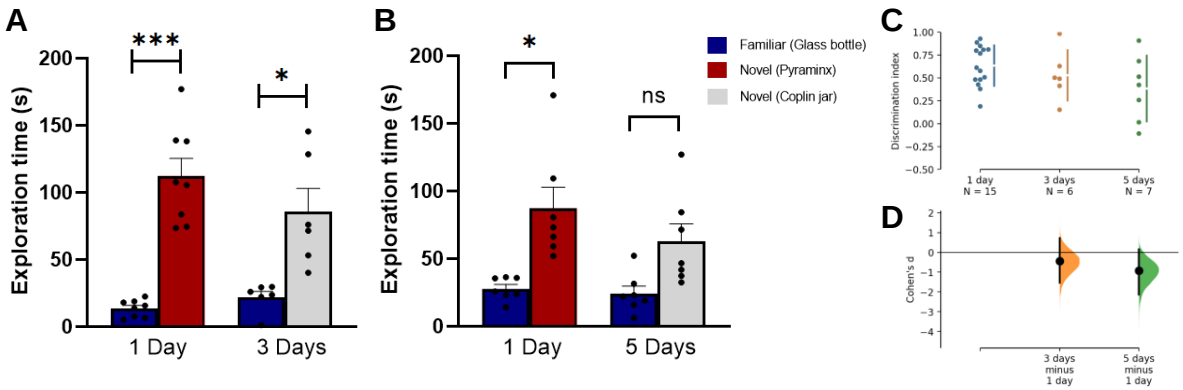

Figure 9 | Novel object recognition memory: 3 days and 5 days after training. (**A**) Exploration times for novel and familiar objects for CART$^{+/+}$ animals 1 day and 3 days after training, (n=8; 1 day and n=6; 3 days, * p<0.05, *** p<0.001, paired t-test). (**B**) Exploration times for novel and familiar objects for CART$^{+/+}$ animals 1 day and 5 days after training, (n=7, * p<0.05, paired t-test). (**C**) Discrimination indices for novel objects after 1 day, 3 days and 5 days after training (n=15; 1 day, n=6; 3 days and n=7; 5 days). (**D**) Cohen's d comparison comparisons for 3 days and 5 days after training vs a shared control 1 day after training. The unpaired Cohen's d between 1 day and 3 days is -0.448, the P value of the two-sided permutation t-test is 0.368. The unpaired Cohen's d between 1 day and 5 days is -0.925, the P value of the two-sided permutation t-test is 0.0506.

the two objects 5 days after training (p=0.063, paired t-test, ***Figure 9*B**). There is also a time dependent decrease in discrimination of the novel object as shown by the discrimination indices (0.632; 1day, 0.527; 3days, 0.383; 5days, mean value, ***Figure 9*C**). Cohen's d values of comparison between the shared control; 1 day also shows a large effect vs 5 days (-0.925) and small to moderate effect vs 3 days (-0.448).

3.4 Effect of fasting on novel object recognition test

3.4.1 Fasting on 1 Day novel object recognition test

To investigate the effect of fasting on novel object recognition test, mice were subjected to 22 hours of fasting before training (T1) (memory consolidation) or before testing (T2) (memory retrieval); it is to be noted that these two are two independent

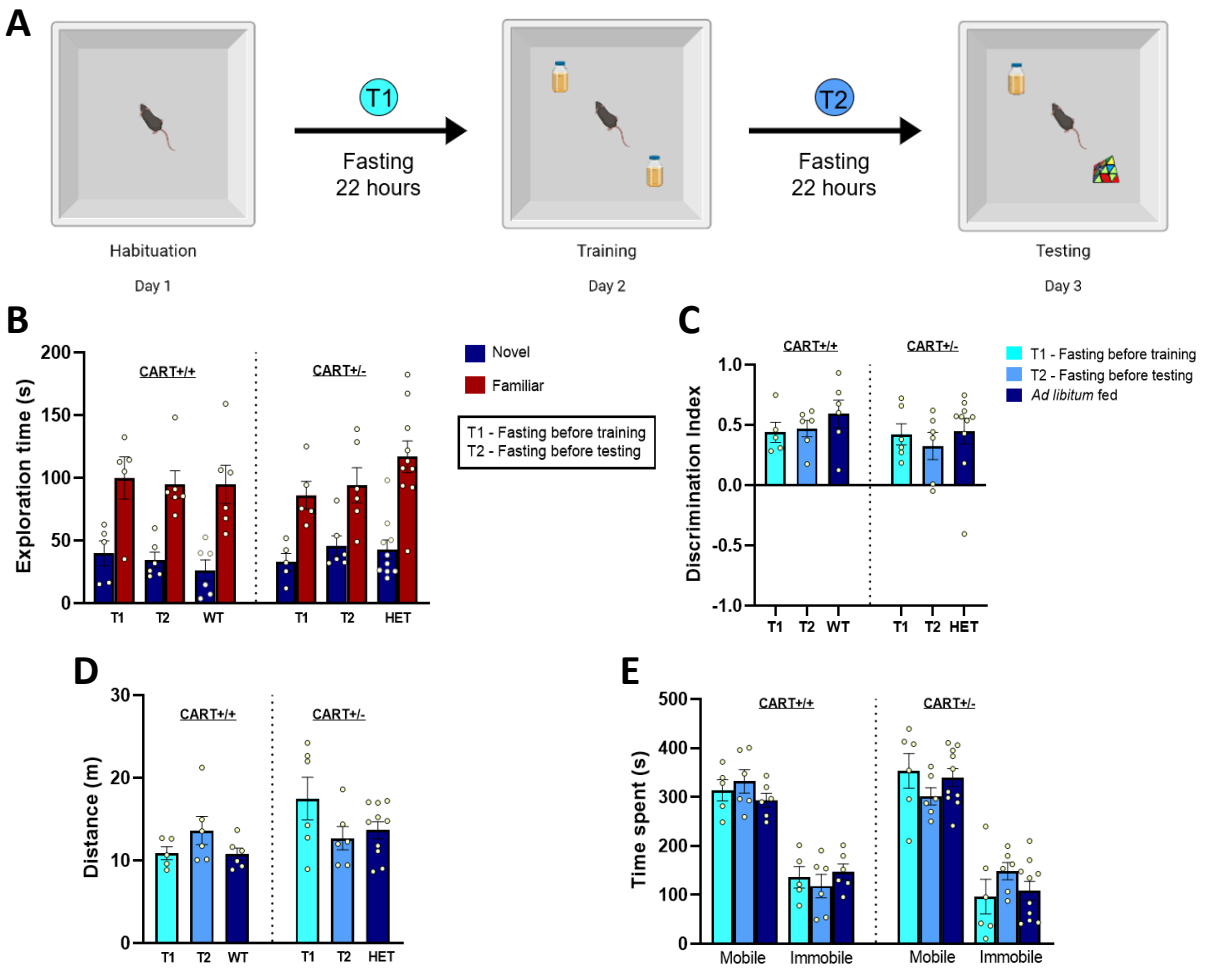

Figure 10 | Effect of fasting on 1 day novel object recognition test. (**A**) Experimental timeline showing fasting interventions. (**B**) Exploration times for novel and familiar objects on testing day, (**C**) Discrimination indices, (**D**) Distance covered and (**E**) Time spent mobile and immobile; for CART+/+ (WT) and CART+/- (HET) animals which are subjected to T1, T2 fasting and for control *ad libitum* fed ones. n=5; T1, n=6; T2, n=6; WT, n=6; T1, n=6; T2 and n=10; HET.

groups. Testing 1 day after training, WT animals upon fasting for 22h, before training and before testing didn't affect the ability to discriminate between novel and familiar objects, as all the 3 groups (T1, T2 and WT (*ad libitum*)) explored the novel object for a significantly longer duration of time (***Figure 10*B**). Similarly, for CART+/- (HET) animals, fasting didn't affect the ability to discriminate between novel and familiar object for all the 3 groups (T1, T2 and HET (*ad libitum*)) (***Figure 10*B**). The discrimination indices for both the fasting groups and *ad libitum* fed one for both the CART+/+ and CART+/- are comparable (***Figure 10*C**). There is also no difference observed in terms of distance covered (***Figure 10*D**) and time spent mobile and immobile (***Figure 10*E**) for CART+/+ and CART+/- animals which are subjected to T1, T2 and for control *ad libitum* fed ones.

3.4.2 Fasting on 3 Day novel object recognition test

Mice were tested for the effect of fasting in 3 day novel object recognition test, after subjecting to 22 hours of fasting before training (T1) and before testing on the 3rd day (T2). As observed for 1 day novel object recognition, there was no effect of fasting on novel object recognition test 3 days after training for both T1 and T2, as all the 3 groups (T1, T2 and WT (*ad libitum*)) explored the novel object for a significantly longer duration of time (***Figure 11*B**). The discrimination indices (***Figure 11*C**) were also similar for the 3 groups and the distance covered (***Figure 11*D**) and time spent mobile and immobile (***Figure 11*E**) were also comparable.

3.5 Standardisation of dentate gyrus coordinates for chemogenetics on novel object recognition test

To investigate the role of CART neurons in the NOR circuitry, targeted modulation utilising chemogenetics is to be done. Dorsal dentate gyrus (DG) (Jiang et al., 2023)[34] is shown to involve in NOR and is also known to have CART positive cells present (Koylu et al., 1998; Seress et al., 2004; Abrahám et al., 2005)[35–37], Bharne et al., 2016[22] also revealed a significant increase in CART immunoreactive cells in the DG after training in NOR, hence DG was chosen as the target for chemogenetic experiments. Standardisation for DG coordinates were done using neuronal tracer DiI and the finalised coordinates are: -2.0mm; AP, ±1.0mm; ML, -2.0mm; DV (***Figure 12***).

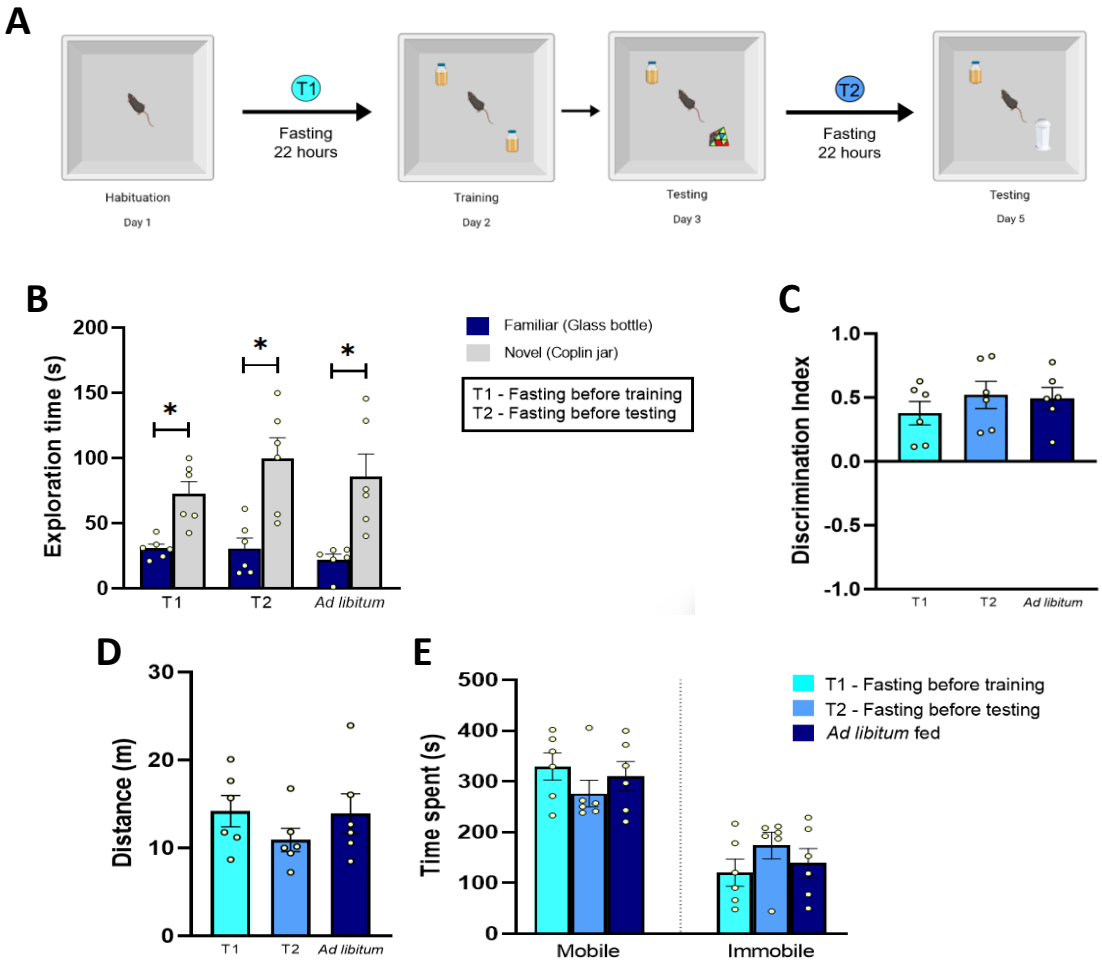

Figure 11 | Effect of fasting on 3 day novel object recognition test. (**A**) Experimental timeline showing fasting interventions in 3 day NOR. (**B**) Exploration times for novel and familiar objects, (**C**) Discrimination indices, (**D**) Distance covered and (**E**) Time spent mobile and immobile for WT animals which are subjected to T1, T2 fasting and for control *ad libitum* fed ones in 3 day NOR. n=6; for each group.

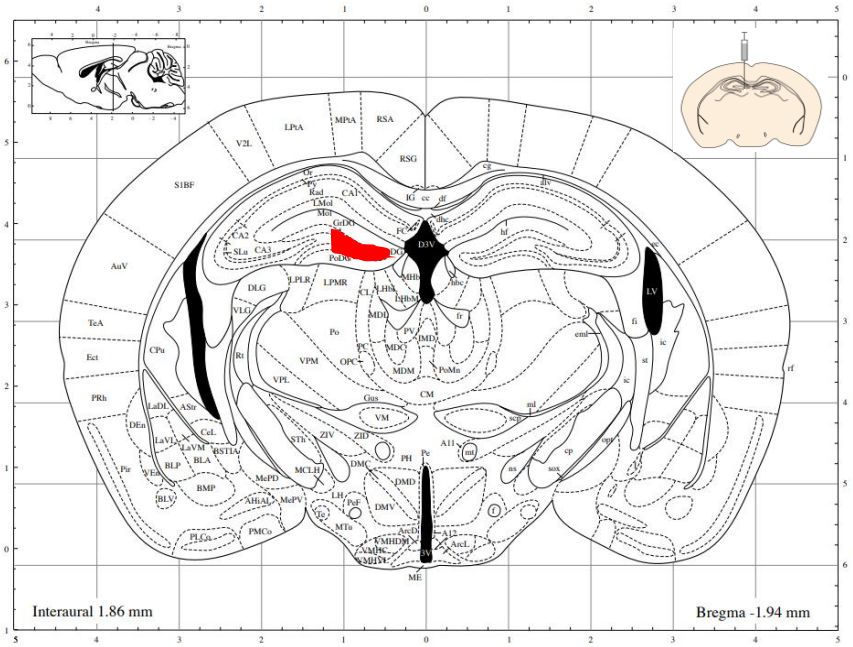

Figure 12 | Chosen stereotaxic coordinates for dentate gyrus, marked in red.

3.6 hM3Dq mediated activation of Arc^{CART} neurons enhances anxiety like behaviour upon fasting *(Done together with Usashi Guha Roy and Rushikesh Chavan)*

DREADD mediated activation of arcuate CART neurons were performed to investigate the role of CART expressing neurons in the arcuate nucleus of the hypothalamus in modulating anxiety like behaviour and innate fear responses upon fasting (22 hours). Mice were fasted for a period of 22 hours before each behavioural experiment and were injected with CNO for 30 mins. prior to the start of each behavioural experiment. CNO induced CART activation in CART$^{cre/+}$ mice after fasting resulted in a significant decrease in the distance covered in central zone in open field test (p=0.0152, Mann-

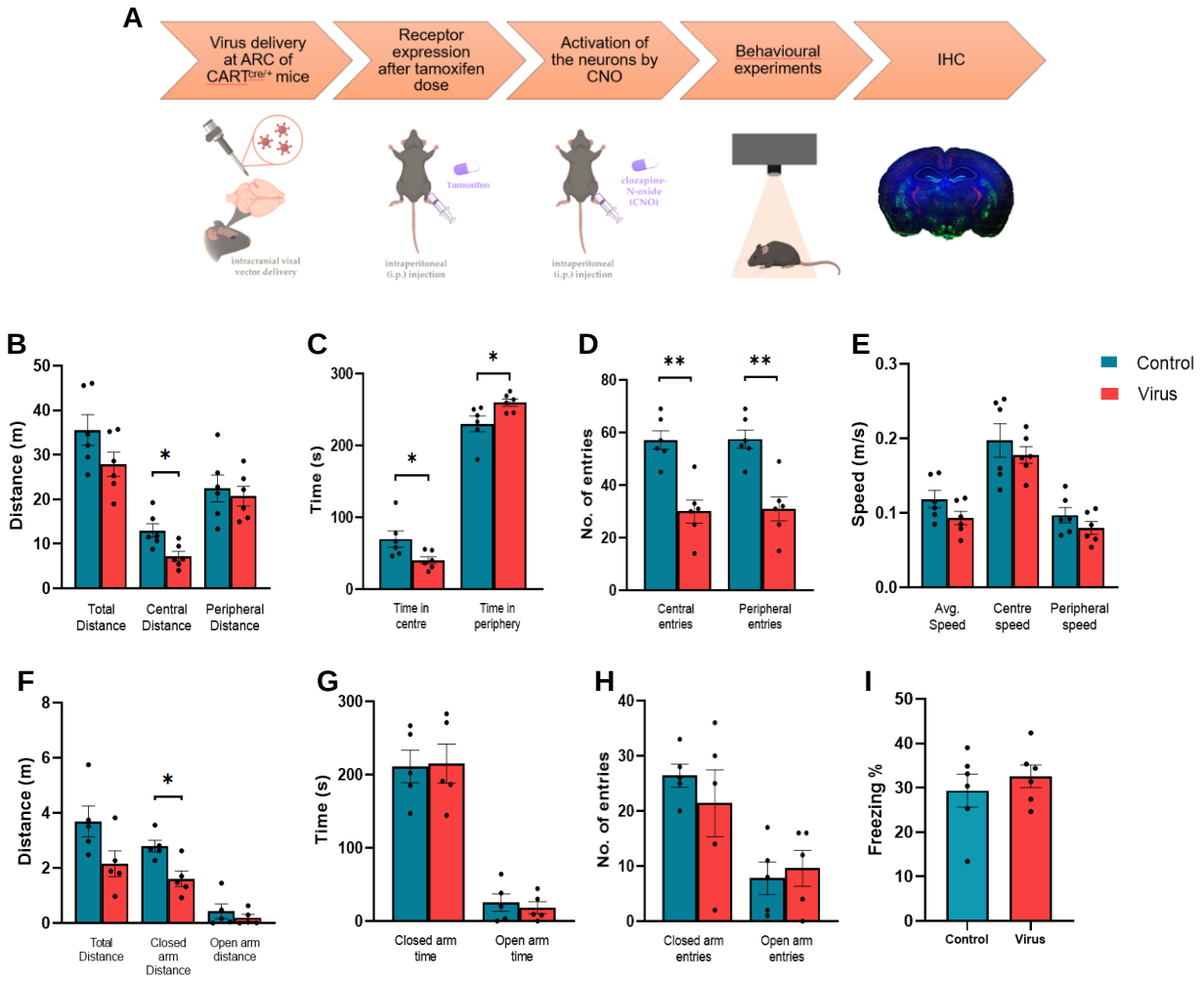

Figure 13 | Activation of arcuate CART neurons increases anxiety like behaviour upon fasting. (**A**) Representative timeline of chemogenetic experiments. (**B**) Distance covered, (**C**) time spent, (**D**) number of entries and (**E**) average speed; by control PBS injected (n=6) and hM3Dq (n=6) expressing animals in central and peripheral zones in open field test, (* p<0.05, ** p<0.01, Mann-Whitney test). (**F**) Distance covered, (**G**) time spent and (**H**) number of entries; by control PBS injected (n=5) and hM3Dq (n=5) expressing animals in closed and open arms in elevated plus maze, (* p<0.05, Mann-Whitney test). (**I**) Plot showing the time spent freezing in TMT induced predator threat assay for control PBS injected (n=6) and hM3Dq (n=6) injected animals.

Whitney test, ***Figure 13*B**). Time spent in centre and periphery were also significantly different between virus injected and control PBS injected mice (p=0.0260, Mann-Whitney test, ***Figure 13*C**). Number of central and peripheral entries were also significantly lesser for virus injected animals (p=0.0043, Mann-Whitney test, ***Figure 13*D**). There was no difference in terms of speed in both the zones for both virus and control groups (***Figure 13*E**). In elevated plus maze, virus injected mice covered significantly lesser distance in closed arm compared to control PBS injected ones (p=0.0159, Mann-Whitney test, ***Figure 13*F**), while total distance covered was insignificant (p=0.0952) between the two groups. There was no difference in terms of time spent and number of entries in both the arms. In TMT induced predator threat assay, there was no difference in terms of percentage time spent freezing in both control and virus injected mice (***Figure 13*I**). It should be taken into consideration that, these results are with low numbers and appropriate control experiments are further required for functional validation of CART in mediating energy state dependent adaptive prioritisation of behaviours, especially those which are more concerned towards survival. The lack of a proper behavioural paradigm linking energy states with risk-taking behaviours is also one crucial element that needs to considered. In the following section we tried to address this by optimising multiple assays linking internal states with risk-taking tendencies.

3.7 Optimisation of a conflict (risk-taking) paradigm in pursuit of food

Different behavioural assays were tried out to study risk-taking behaviours with respect to the energy state of an animal. The underlying rationale is that the animal has to overcome a threat (aversive stimuli) to reach to the food. This approach takes into account the foraging mediated decision-making process employed by the animal in its ecological niche. The aversive stimuli used in the following paradigms were selectively chosen to mimic and to produce different types of aversion like the direct pain-inducing (shock) and the ones which were able to produce innate threat-like aversive responses in mice (predator odour and strong light), which weren't pain inducing. All the optimisation experiments were done on wild type (WT) animals following fasting as described for each assay in the materials and methods section. The primary criteria kept for optimisation were robustness and reproducibility of the assays.

3.7.1 Predator Odour Risk Taking (PORT) assay *(Done together with Usashi Guha Roy and Rushikesh Chavan)*

To examine the impact of energy state on risk-taking behaviours in the context of a predator threat, induced by an odour extracted from fox faeces, TMT, mice were subjected to a fasting period of 16h and were tested for the latency to acquire the food in the presence and absence of TMT. Fasted WT mice spent the same amount of time as compared to *ad libitum* fed ones to acquire the food in presence of TMT, contradicting the difference that is observed in the absence of TMT (p= 0.0039, Mann-Whitney test, ***Figure 14A***). *Ad libitum* fed mice shows a significant difference in latency to acquire food in presence and absence of TMT (p=0.0039, Mann-Whitney test, ***Figure 14A***). Independent replicate; N=1 (***Figure 14B***) shows a significant difference in latency to acquire the food for fasted and fed mice in the presence of TMT (p=0.0079, Mann-Whitney test) and no difference in the absence of TMT, Contradictorily N=2 (***Figure 14C***) shows a difference in absence of TMT (p=0.0079, Mann-Whitney test) and similar latencies in presence of TMT. Both fasted and fed mice shows significant difference in food acquiring latencies in the presence and absence of TMT (N=1, ***Figure 14B***), whereas this difference is only seen in fasted mice and not in fed in N=2 (***Figure 14C***).

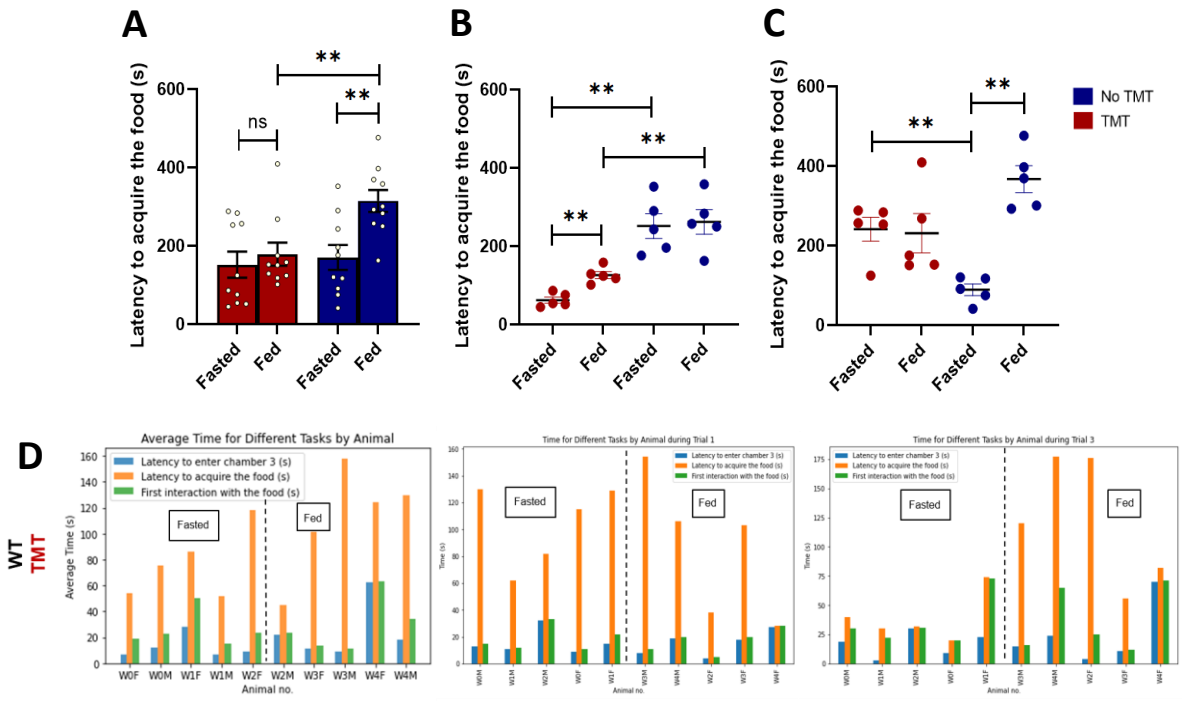

Figure 14 | Influence of TMT on latency to acquire food in PORT assay. (**A**) Latency to acquire food for WT animals in the presence and absence of TMT (n=10 for each group, N=2 combined) (* p<0.05, ** p<0.01, Mann-Whitney test). Separately shown for N=1 (**B**) and N=2 (**C**). (**D**) Bar plots showing animal wise break down of the parameters (latency to enter chamber 3, latency to acquire the food and the first interaction with the food) between trial 1 and 3 and the average across 3 trials.

Fasted mice follow a decreasing gradation in latency to acquire the food when they progress from trial 1 to trial 3 (n=5 for fasted and fed, ***Figure 14D***) in presence of TMT. Due to the significant variability in results and the necessity of controlling numerous variables within the paradigm, this assay wasn't taken further.

3.7.2 Two chamber risk taking assay *(Done together with Usashi Guha Roy and Rushikesh Chavan)*

Mice were tested in a two chamber setup which consists of a high-risk environment with food, with shock as the aversive stimuli. Mice were subjected to habituation and shock training sessions for acquainting themselves with the apparatus, associate the shock with light chamber and for recognising the platform as a shock free zone. For optimising the assay, different parameters were tested and standardised. Firstly, current was set at the bare minimum the apparatus can go, which is 25µA. Various current settings with varying inter stimulus intervals (ISI) were tried out: 1s ↯ + 0.5s/1s/2s ISI's, 0.5s ↯ + 0.5s/1s ISI's, finally 0.5s ↯ + 0.5s ISI was fixed as the current settings. Fasting periods of 16h, 18h, 22h, 48h were tried out and a mixed food restriction paradigm of 22h of fasting followed by 2h of refeeding and again 22h of fasting was chosen as the fasting protocol. Mice explored the dark chamber and had to cross the shock to get to the platform which contains food, primary readout of risk-taking for the experiment was whether the animal went to the platform taking the risk to acquire the food. Secondary readout was the number of attempts made by the animal in terms of nose pokes through door and paws on the shock grill while trying to reach the platform. It is to be noted that, secondary readouts were only calculated if the primary criterion of crossing the shock for food acquisition – was not met. Only 2 out of 10 fasted mice and 1 out of 7 fed mice took the risk of crossing the shock and having the food. Fasted mice made significantly higher number of nose pokes through door compared to *ad libitum* fed ones (p=0.0293, n= 8 for fasted and n=6 for fed, Mann-Whitney test, ***Figure 15B***). The number of times the animals kept their paws on shock grill were comparable for both the groups. For further optimisation a revised version of the same assay was done - reverse step-down assay (***Figure 15C***). Animals were habituated accordingly, to the platform as the home zone and food placed in the dark chamber, here the animal had to step down from the platform in order to go to the dark chamber to have food crossing the shock zone. 2 out of 4 fasted mice and 1 out of 4 fed crossed the shock and had food in the dark chamber, noticeably both mice

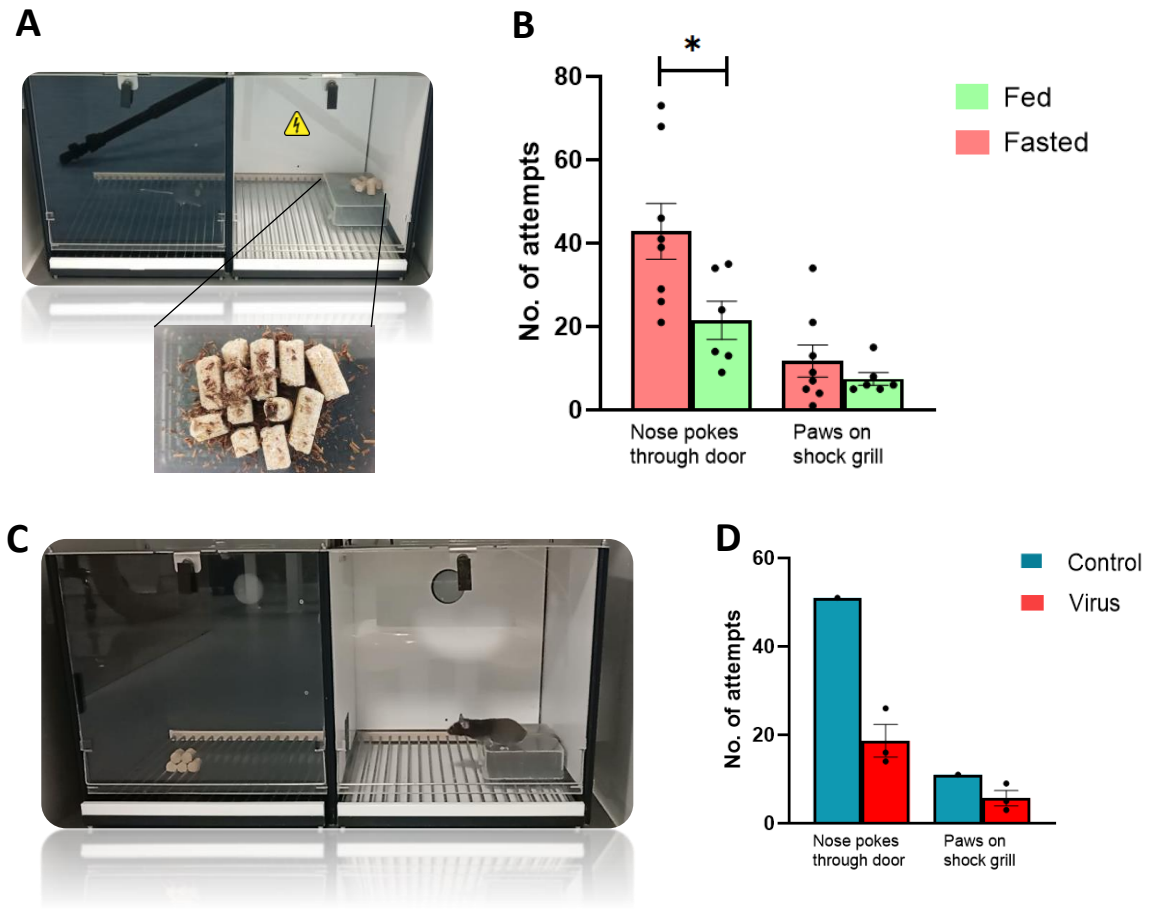

Figure 15 | Two chamber risk taking assay. (**A**) Representative image of the two chamber apparatus, alongside an enlarged picture of food pellets topped with dark chocolate. (**B**) Number of attempts in terms of nose pokes through door and paws kept on shock grill for fasted and *ad libitum* fed mice (n=8 and n=6 for fasted and fed mice respectively, *p<0.05, Mann-Whitney test). (**C**) Representative image of the apparatus for reverse step-down strategy. (**D**) Number of attempts in two chamber risk taking assay after hM3Dq mediated activation of Arc^{CART} neurons after fasting for control PBS injected (n=1) and hM3Dq (n=3) injected animals.

took about 90s before starting to have the food after entering the dark chamber. Few animals were also tested in the chemogenetics protocol for hM3Dq mediated activation of Arc^{CART} neurons in the two chamber risk taking setup following a fasting protocol of 22h-2h-22h (n=3 for virus, n=1 for PBS). CNO induced activation resulted in decreased number of attempts to cross the shock, for acquiring the food (***Figure 15*D**), none of the 4 animals crossed the shock. Even though the primary readouts showed inconsistency, the secondary readouts in the assay were robust and reproducible. To enhance the assay's effectiveness and reliability, taking into consideration for the existing limitations further refinement is required.

Table 1 | Optimisation of Two chamber risk taking assay

Strategy	Inference
Fasting: 16h, 18h, 22h, 48h and 22h-2h-22h	Increased number of risk taking attempts.

Current settings: 1s ⚡ + 0.5s/1s/2s ISI's, 0.5s ⚡ + 0.5s/1s ISI's and 0.5s ⚡ + 0.5s	Reduced unnecessary crossings during ISI's.
Increased habituation period (1 to 3 days, 30 mins.) and introduction of shock training sessions	Robust identification of the platform as a shock free zone and improved learning of the apparatus.
Platform brought to centre of the light chamber	No observable changes upon reducing the crossing distance between animal and food.
Reverse step-down assay	Mice tend to slip off the platform, consequently crossing the chamber.

3.7.3 Light induced risk taking test *(Done together with Rushikesh Chavan and Vihang Vaidya)*

Mice were tested in a light induced conflict assay, in which the animal had to go and forage for food in the centre of the open field lit with a high intensity light, which in itself is a region the animal innately avoids if it's anxious. Mice were subjected to 22 hours of fasting before testing. During optimisation two scenarios were tried; food pellets placed in centre (S1) and food pellets placed in centre as well as in corner (S2). In S1, both fasted and fed mice spent comparable amount of time in light zone (***Figure 16*A**). There was no difference in terms of the number of entries and the latency for first entry into the light zone for fed and fasted groups. Where as in S2, there was an increase in amount of time spent in the light zone, though insignificant (p=0.1636, Mann-Whitey test). The number of entries into the light zone was significantly higher for fasted mice in S2 (p=0.0242, Mann-Whitney test) (***Figure 16*B**). While the latency for first entry was similar even in S2. Also, it is to be noted that, the amount of time spent in the light zone for fed mice decreases in S2 compared to S1 (p=0.0857, Mann-Whitney test). Additional optimisation is required for further improving the assay, taking into account for the difference that is observed.

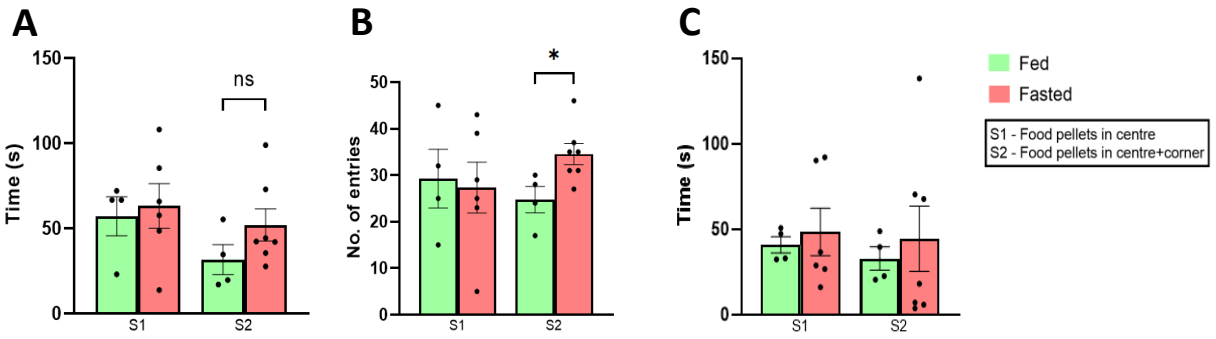

Figure 16 | Light induced risk taking test. (**A**) Time spent in light zone, (**B**) Number of entries into the light zone and (**C**) Latency for first entry into the light zone for scenarios S1 and S2 for fasted and *ad libitum* fed mice in light induced risk taking assay. n=6; S1, n=7; S2 and n=6; for both fed controls.

4. Discussion

4.1 CART in novel object recognition test

Bharne et al., 2016[22] revealed the role of CART in learning and memory formation using novel object recognition test, where they show how exogenous administration of CART peptide and CART antibody influence recognition memory in novel object recognition test in rats. i.c.v and i.h administration of CART peptide showed a dose dependent change in discrimination index. Through this study we showed that endogenous levels of CART play a role in recognition memory in NOR. For studying the role of endogenous levels of a protein, chronic loss of function through global deletion of the gene is essential to determine the function of the gene, but it needs to be taken into consideration that certain information can also be hindered by compensatory mechanisms that happen during development. We unveiled that genetic knockout of CART gene in CART-/- mice causes memory deficits in NOR. These mice explore the novel and familiar object for similar amounts of time, showing their inability to remember the familiar object. This is not observed for WT and CART+/- mice, as they are able to discriminate between the objects and shows a significant difference in their discrimination indices on comparison with CART-/- using estimation statistics. However, there is no difference in terms of distance covered, time spent mobile and immobile and average speed throughout the assay for WT, CART+/- and CART-/- mice. This also highlights that there is no energy state dependent effects for CART-/- animals in the memory circuitry, pointing towards a energy state independent role for CART signalling in recognition memory. The perirhinal cortex (PRh) and CA1 of the hippocampus plays complementary roles in recognition memory in NOR (Cinalli Jr. et al., 2020)[12], weak object recognition memory is governed by PRh and strong recognition memory by CA1 of the hippocampus. Evaluation of changes in object recognition memory after 3, 5 days showed the retention of memory after 3 and 5 days, however there wasn't a significant result ($p=0.063$) for memory 5 days after training, due to the low numbers. Testing for CART+/- and CART-/- 3 and 5 days after training needs to be done. Further we show that there is no influence of fasting in NOR for WT and CART+/- mice in 1 day NOR, and for just WT mice in 3 day NOR, when fasted before training during the memory consolidation process and before testing during

memory retrieval. Fasting experiments for CART-/- is to be done to complete the set. Hunger doesn't affect object recognition memory in our NOR assay, as it was shown for modulating long term fear memory and enhanced extinction learning in pavlovian fear conditioning (Verma et al., 2016)[30]. Since we see an energy state independent memory component in our NOR assay which is getting affected by endogenous levels of CART, further studies aimed at figuring the role of CART in NOR circuitry using chemogenetics needs to be done to understand the underlying circuitry and the significance of CART signalling in object recognition memory.

4.2 CART enhances anxiety like behaviour upon fasting

CART as an anxiogenic agent was previously shown by exogenous intracerebroventricular (i.c.v) administration in rats (Kask et al., 2000)[38], resulting in a dose dependent increase in anxiety like behaviours. While exogenous administrations give us information about acute responses, it also results in widespread, disorganised signalling which doesn't happens as part of normal physiology. Hence, in order to dissect out the function of CART neurons at the arcuate nucleus of the hypothalamus in energy state dependent adaptive prioritisation of behaviours DREADD's were utilised. Previous findings from the lab, shows that hM3Dq mediated activation of CART neurons at the arcuate nucleus on *ad libitum* fed animals enhance anxiety like behaviours and increases freezing levels in TMT induced predator threat assay. We show that hM3Dq mediated activation of CART neurons at the arcuate nucleus after fasting enhanced anxiety like behaviours whereas didn't affected the fear responses in TMT assay. Virus injected animals upon activation using CNO show elevated levels of anxiety as observed with decreased exploration in the centre compared to periphery in open field test and decreased exploration in terms of distance covered in elevated plus maze compared to control PBS injected animals. As shown by previous studies in the lab, WT animals when fasted results in increased exploration and decreased levels of anxiety in open field test. Consistent with this, activation of CART neurons at the arcuate nucleus after fasting mimics the responses as observed in WT animals when fasted. It also needs to be noted that, elevated plus maze doesn't show consistent difference with respect to open field test mainly because of the amount of time spent in the centre of the maze which is not accounted in the analysis, hence different strategies to assess anxiety like behaviours is essential. One alternative is

the elevated zero maze, which basically allows us to eliminate the concern with the central zone in elevated plus maze. Previously reported results using exogeneous administration of CART peptide and CART antibody (Rale et al., 2017)[20], show CART signalling at CeA-vBNST as a crucial modulator of innate fear processing. This doesn't get affected when CART neurons are activated in the arcuate after fasting (***Figure 13*I**), stating that CART signalling at the arcuate after fasting doesn't modulate innate fear processing elicited by TMT. The constrain of low sample size in the study has to be considered along with the requirement of appropriate control experiments for chemogenetics for drawing concrete conclusions on the role of CART signalling in the arcuate nucleus in adaptive prioritisation of behaviours.

4.3 Risk taking in pursuit of food

Starvation enables animals to engage in risky behaviours, that would typically be ineffective. Through PORT assay Dent et al.[32], proposed a risk taking paradigm naturalistic to rodents for obtaining reward considering the cost vs benefit trade-off. However, we modified the assay to take into account of energy state dependent risk-taking behaviours and showed that there is no as such risk-taking element within the assay as we contrastingly observed decrease in latency to acquire food in presence of predator odour -TMT as compared to control no TMT scenario for *ad libitum* fed mice (**Figure *14*A**). There were also too much variabilities in trends, even for controls and noticeably, there was change in latencies across trials; from 1 to 3, that mimic learning as a consequence of carrying out 3 trials (**Figure *14*D**) this is in line as previously reported by (Francesconi et al., 2020)[39]. Their study using 4 to 5 times larger dataset showed that a novel non-predator odour can elicit the same responses as observed in presence of a predator odour, and the latency that is observed is just a mere coincidence of novelty and has nothing to do with predator odour within the context of the assay. This study also unveiled sex specific differential risk-taking tendencies. Spread of TMT fumes into adjacent chambers can't be regulated, compromising the localisation of threat in one zone, additionally this needs to be taken into consideration that the time spent to acquire food can depend on the quantity of food consumed in the previous trial(s). These results point out to establish a paradigm

with a more suited controllable threat (aversive) stimuli in pursuit of food to evaluate risk-taking behaviours.

In the two chamber risk taking test, mice were tested for foraging mediated risk taking in a high risk environment for obtaining food. Fasted mice made significantly higher number of attempts than *ad libitum* fed mice (**Figure *15*B**), even though these were robust and reproducible, the primary criterion of animals taking the risk to cross and acquire the food wasn't satisfied by the majority of the animals tested. Various optimisation strategies did improve the assay, but didn't result in a solid paradigm to assess risk taking in mice. Chemogenetic activation also results in decreased number of attempts made in two chamber risk taking assay, same trend as seen in WT fed animals, suggesting that activation after fasting at the arcuate makes fasted animal behave like fed ones. Previously, similar assays were also put forward in rats to study conflicting decision making in pursuit of reward (Illescas-Huerta et al., 2021)[40]. Through their 3 conflict assays, they show that animal supress their defensive behaviours elicited by threats whether it's innate or conditioned, to obtain reward in conflicting scenarios. The motivation to forage for food in a high-risk environment with shock as the aversive stimuli makes the animal so overwhelmed that it even considers a significant duration of starvation to be not resulting in a motivational state that enables it to take the risk to acquire food. New optimisation strategies need to be incorporated for refining the assay further.

In the light induced risk taking test, the innate aversiveness towards the centre of the open field was paired up with the aversion to brightly lit regions. During optimisation, trying different scenarios, we found that placing pieces of food pellets in the corner along with placing them (concentrated) in the centre significantly increased the number of entries into the light zone and also increased the amount of time spent in light zone for fasted mice, stating that the motivation to forage for food in the corners reduced the tendency to forage in the light zone for *ad libitum* fed mice, as previously reported by (Illescas-Huerta et al., 2021)[40]. Further this assay could be even modified by associating sound (loud tone) when the animal enters the light zone, thereby increasing the anxiogenic stimuli present which may result in producing a significant difference with respect to the internal state of the animals.

Neuropeptide CART plays a prominent role in maintaining energy homeostasis and orchestrating adaptive behavioural responses to varying physiological needs and environmental cues. Through this thesis, we investigated the role of endogenous levels of CART in learning and memory using novel object recognition test and also revealed that fasting doesn't affect recognition memory in NOR. Using hM3Dq mediated activation we also examined the role of CART neurons in the arcuate nucleus of the hypothalamus in modulating anxiety like behaviours, innate fear and risk-taking tendencies upon fasting. Furthermore, we tried to address the lack of a robust behavioural paradigm for rodents, for studying risk taking behaviours with respect to energy state.

4.4 Future directions

4.4.1 CART in novel object recognition test

- 3 day NOR for CART+/- and CART-/-
- Effect of fasting on NOR using CART-/-
- Effect of refeeding after fasting in NOR
- Chemogenetics on CART neurons at the dentate gyrus in NOR.

4.4.2 Arc^{CART} neurons in anxiety, fear and risk-taking behaviours

- Chemogenetics on CART-/- animals.
- Retrograde virus at CeA to validate Arc to CeA projection and associated behaviours.
- Local activation using CNO at Arc and CeA to validate the projection.

4.4.3 Further optimisation for risk taking assays in pursuit of food

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