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Exploring the Impact of High Fat Diet on Anxiety and Memory: Insights from the Anterior Insular Cortex

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List of Abbreviations

aIC……..……..……..anterior Insular Cortex
BLA …..……Basolateral Amygdala
BMI…..……..Body Mass Index
CNO …..……N-Oxide Clozapine
DREADD…..……Designer Receptor Exclusively Activated by Designer Drugs
EPM…..……..……Elevated Plus Maze
fMRI…..……..….functional Magnetic Resonance Imaging
GFP…..……Green Fluorescent Protein
HDLC……..High Density Lipoprotein Cholesterol
HFD…..…….……..High Fat Diet
HFSD…..……High Fat and Sugar Diet
IC …..……Insular Cortex
MAD…..……..……Absolute Deviation from the Median
mCh ……..……...mCherry
mIC…….medial Insular Cortex
NA …..…….Numerical Aperture
NOR(M)…..……..……Novel Object Recognition (Memory)
NSFT …..…….……..Novelty-Suppressed Feeding Test
OFT …..……..……Open Field Test
OR(M)…..…..……Object Recognition (Memory)
PFA …..……..……Paraformaldehyde
pIC……..posterior Insular Cortex
RI…..……Recognition Index
SD …..……Standard Diet
WHO …..……..World Health Organization

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Acknowledgement

I would like to express my sincere gratitude to Dr. Anna Beyeler, Principal Investigator of her own Research Group at INSERM, the French National Institute of Health and Medical Research. Her invaluable support, guidance, and encouragement throughout my internship and the completion of this thesis have been pivotal to my Master's project. Anna Beyeler's mentorship not only provided me with essential skills and knowledge in neuroscience, but also inspired me with her dedication and commitment to conducting groundbreaking research in this field. Additionally, I am truly grateful for her professional guidance and trust at every stage of this project, including the unforgettable opportunity to present my poster at the institute's symposium in April 2023. Anna, it has been an honor to be part of your team and to learn from such an excellent scientist like you.

1. Abstract, research questions and objectives

Firstly, the abstract provides a brief overview of the research project, summarising the topic, methodology, findings, and implications. Further, the research questions and objectives define the aim and outline the relevance of this thesis.

1.1 Abstract

Obesity has emerged as a major public health concern globally, with the European Region experiencing a troubling rise in obesity rates over the past few decades. The detrimental effects of obesity extend beyond physical health, with associations found between overweight and emotional disorders, as well as deficits in memory function. The anterior insular cortex (aIC), a brain region implicated in various functions including interoception, taste, anxiety, and memory, is of particular interest in this study. Here, we investigate the impact of a high fat diet (HFD) initiated in adulthood on anxiety and object recognition memory (ORM) in a pre-clinical mouse model, focusing on the role of the aIC. Advanced neurobiological techniques such as chemogenetic manipulation and fiber photometry are employed to elucidate the neural mechanisms in the aIC underlying these effects. Interestingly, our findings reveal that HFD induces anxiety-like behavior and is significantly associated with glutamatergic neurons within the aIC. The inhibition of aIC neurons reversed the anxious phenotype of animals fed a HFD. The ORM remains largely unaffected in mice fed a HFD compared to lean mice on a standard diet (SD). However, chemogenetic results of mice fed a SD strongly support the relevance of neurons in the aIC in ORM, since aIC inhibition led to memory impairment in the ORM test. Although our research has implicated the role of the aIC in memory consolidation in animals fed with a SD, our chemogenetic experiment of animals fed a HFD did not result in alterations in ORM. This underscores the importance of considering the timing of diet onset in studies on diet-induced cognitive effects. Overall, these findings

provide valuable insights into the complex relationship between diet, brain function, and behavior, contributing to a deeper understanding of the impact of a HFD on anxiety and memory function. Moving forward, future research should delve deeper into the specific mechanisms through which a HFD influences neural circuits within the insular cortex. Additionally, clinical studies are warranted to investigate the translational relevance of our findings, potentially paving the way for targeted interventions to mitigate the cognitive and emotional consequences of HFD-induced obesity.

1.2 Research questions and objectives

Based on the bibliography and the results obtained in my host laboratory, we hypothesize that the activity of excitatory neurons in the anterior insular cortex (aIC) of mice under a high fat diet (HFD) is underlying HFD-induced anxiety. Additionally, we hypothesize that mice under HFD have impaired object recognition memory compared to those on a standard diet (SD). Furthermore, no studies have yet explored sex differences in the activity of aIC neurons. By examining the neural responses in this brain region in males and females, we aim to uncover how diet influences memory and anxiety-related behaviors, ultimately contributing to a more comprehensive understanding of the complex interplay between diet, brain function, sex, and behavior.

To test our hypotheses, the following objectives were defined:

A. Establish an animal model of HFD-induced anxiety-like behavior and HFD-induced memory impairment through the administration of a high caloric diet at adulthood.

B. Record of the activity of excitatory neurons of the aIC during anxiety and memory tests, using fiber photometry, which is a technique to monitor the activity of specific neuron populations *in vivo* during behavioral tests in pre-clinical models. The aim is to identify differences in neural activity in the aIC between HFD and SD mice.

C. Test the causal role of aIC excitatory neurons in anxiety and memory tests, utilizing chemogenetic DREADDs. We selectively inhibit glutamatergic neurons in the aIC, aiming to mitigate memory impairment and the anxious phenotype induced by the HFD. This approach is used to reveal a causal connection between behavior and diet within the aIC.

Altogether, this project aims to provide a neurobiological understanding of the effects of a HFD on anxiety and memory by examining alterations in the IC, based on behavioral analyses, advanced imaging techniques, and chemogenetic approaches. This study serves as the foundation for better understanding the link between obesity, memory impairments, and anxiety disorders.

2. Introduction

Researching the effects of HFD on brain function and behavior, particularly focusing on the aIC in memory as well as in anxiety, requires a multidisciplinary approach. Understanding how dietary factors can impact brain structures and functions is essential, as it has significant implications for understanding conditions such as cognitive decline and increased anxiety associated with obesity.

2.1 Obesity and the insular cortex

The World Health Organization (WHO) defines obesity as a distribution or abnormal accumulation of body fat, and is characterized by a body mass index (BMI) of 30,0 kg/m² or higher (1). Nowadays, obesity is one of the major health issues (2). Indeed, the WHO states that in 2016, around 13% of the world's adult population, those aged over 18, were obese. It is projected that the obesity epidemic in European countries will peak between 2026 and 2054, with the USA and UK reaching the highest levels first, followed by other European countries (2). Generally, the over-consumption of energy-dense food is also a major risk factor for developing lifestyle-related diseases such as diabetes mellitus type 2 and metabolic syndrome (3).

Furthermore, obesity has been linked to a higher prevalence of anxiety disorders (4)(5). It has been shown that obese individuals have a 25% greater chance of developing a psychiatric disorder, including anxiety (4) or depression (5), compared to those who are lean. Obesity is a significant risk factor for Alzheimer's disease and other neurodegenerative diseases, leading to cognitive decline according to human studies (6)(7)(8).

The anterior part of the insula (aIC) shows a higher response in obese subjects in response to food cues in obese individuals (9). The study conducted by Wu et al. (2020) demonstrated that optogenetic inactivation of aIC glutamatergic neurons leads to an increase in food intake (10). In humans, a decrease in the

activity of the posterior insular cortex (pIC) has been observed when consuming foods high in fat, leading to a reduction in body activity and affecting the hypothalamus, which is responsible for maintaining homeostasis (11). The granular and dysgranular layers of the pIC are particularly crucial in this process (12). Therefore, both aIC and pIC appear essential for maintaining a balanced energy state.

2.2 Anatomy of the insular cortex

The insular cortex (IC) is an area of the neocortex located deep within the lateral sulcus of each hemisphere in primates. The IC is not visible on an exterior view of the brain, as it is hidden beneath the frontal, parietal, and temporal lobes (13). However, in mice and rats, it can be found on the lateral surface of the hemisphere, mostly along the rhinal fissure. Despite structural differences between mice and humans, there are similarities in the cytoarchitecture, cell types, and connectivity (14). This makes mice a valuable tool, along with circuit mapping techniques, to understand the functional differences between different parts of the insula (15).

Two parts, anterior and posterior, are separated by the central sulcus (16). The insular cortex can be divided into three main areas: the dorsal granular zone, the central dysgranular area, and the ventral agranular area, as shown in ***Figure 1A*** and ***Figure 1B***. The dysgranular area is associated with gustatory modalities, while the granular area is linked to viscerosensory cortical regions. The posterior agranular area is responsible for multimodal and limbic-autonomic integration (17)(18)(19).

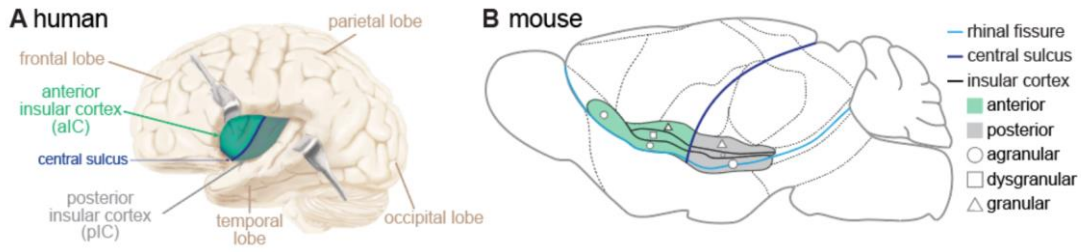

Figure 1. The insular cortex in humans and rodents.

Figure 1. The insular cortex in humans and rodents. A. Location of the insular cortex in the human brain. **B.** Location and sub-sections of the insular cortex in the rodent brain. *Adapted from Kang et al. 2016, Scientific Reports.*

2.3 Functions of the insular cortex

The anterior part of the insula (aIC) has been described as a sensory or gustatory cortex while the posterior insula (pIC) has historically been more involved in emotional processing (16). Studies suggest that the IC is involved in certain disorders like anxiety (20), obesity (21), and memory formation (22). Further, the insula plays multiple functional roles, as it is connected to several cortical and subcortical regions. Research has shown that around 88% of the neurons in the IC respond to the presence of food (23). The anterior part of the insula forms the primary gustatory cortex, responsible for taste perception (24). The insula is especially known to be involved in food processing due to its somatosensory, visceral, and visceromotor functions. The somatosensory functions of the insula relate to the body's processing of touch, pain, and temperature sensations. The visceral functions relate to the processing of sensations from internal organs, such as hunger and fullness. The visceromotor functions are responsible for controlling the body's internal organs, such as the digestive system (24).

For instance, a study using magnetic resonance imaging (fMRI) of 14 patients with social anxiety disorder showed hyperactivation of the insular cortex when exposed to aversive stimuli (25)(26). Similarly, hyperactivation of the anterior insular cortex has also been observed in overweight individuals during food-cue exposure (21). The study of Federico Bermúdez-Rattoni (2014) proposes that the IC and its related circuitry are highly involved in the conversion of novel to familiar stimuli for both object and taste recognition memory, where the release of

acetylcholine and dopamine in the IC is initiated by the experience of novel stimuli (22).

2.4 Role of the insula in anxiety

Anxiety is an essential emotion leading to a complex psychological and physiological state, evolutionary selected as it allows animals to prevent exposure to harmful situations. Anxiety is defined as the anticipation of a potential threat, with an uncertain probability of occurrence, leading to uneasiness, apprehension, and increased arousal (27)(28)(29).

A hyperactivation of the IC has been observed in humans suffering from anxiety disorders (25). The pharmacological study of Méndez-Ruette et al. (2019) examined the involvement of the IC in anxious behaviors in order to characterize functional differences between anterior and posterior sections in rats. The study found that the inactivation of the aIC through the injection of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a glutamatergic AMPA receptor antagonist, induced an anxiolytic effect, while the inactivation of the posterior insula cortex with the same compound induced an anxiogenic effect (15). The recent study of Nicolas et al. (2021) has shown that there is increased calcium activity in the glutamatergic neurons of the aIC in spaces that provoke anxiety, such as the open arms of the elevated plus maze (EPM) and the center of the open field (OFT) (30). Meanwhile, interestingly, the study by Gehrlach et al. (2019) conducted on rats has shown that deactivation of the aIC using a glutamatergic AMPA receptor antagonist leads to a reduction in anxiety, while deactivation of the pIC has the opposite effect. However, by the use of optogenetics and chemogenetics it has been suggested that activating the pIC may also induce anxiety (31).

2.5 Cognitive function of the insula after high fat diet

Previous research has associated memory impairment with the consumption of HFD during adolescence (32). Precisely adolescence is the period of life where the brain structure and memory acquisition develop, resulting in a phase where HFD consumption can be particularly detrimental (32)(33). HFD in adolescence has shown an impairment in spatial discrimination learning and decreased neurogenesis in terms of hippocampal function (34). The recent study of Glushchak et al., 2021 utilized three cohorts of rats (adolescent HFD, adult HFD, and adolescent HFD with recovery) to explore the effects of HFD at different ages and results show that HFD alone only impairs preference for novel objects during adolescence. The data affirm that the adolescent period is susceptible to long-lasting dysfunctions of hippocampal behavior by a HFD (35). Many other publications associate the effect of memory impairment with deficits in hippocampus-dependent memory (32)(33). However, there is extensive evidence of involvement of the insula in object recognition memory (36)(37)(38). Dietary habits can have a profound impact on cognitive function (39). High fat diets, particularly those rich in saturated fats, have been linked to various health issues, including obesity and cardiovascular problems, and may negatively affect cognitive processes, including memory and emotional regulation (40)(41)(42)(43). Several previous research studies have provided evidence that suggests a direct link between obesity and impairments in both episodic and spatial memory (44)(45). Episodic memory is a subtype of declarative memory which involves recalling personal experiences, events, and details, including time, place, and emotions (46)(47). The prefrontal cortex, the cerebellum, the hippocampus and surrounding cortical areas have been described in the formation of episodic memory (48)(49). The IC is involved in several cognitive functions, since it receives limbic inputs from the amygdala, dorsomedial nucleus of the thalamus, and the prefrontal cortex. This converge of primary sensory inputs is not found in other sensory areas of the cortex (50)(51)(19).

The novel object recognition task (NOR), also called object recognition memory test (ORM), reflects the subject's capacity to remember and recognize previously

encountered stimuli (52)(53). Mice were divided into groups and given a specific treatment for 30 days, followed by evaluation using the ORM test. Results show that saccharin and trans-fat caused memory impairment, while turmeric improves memory (54).

A 2018 study highlights that a HFD can lead to impairments in episodic, spatial, and contextual memory within a few days of starting the diet. However, the effect of a HFD was not sufficient for an impairment in the recognition of novel objects. Furthermore, the study has shown to reverse memory deficits by switching to a low fat diet (55).

Previous research especially highlights the role of glutamate in learning and plasticity in general (56). The perirhinal cortex is highly involved in the ability to recognize objects. It works together with the IC and needs glutamate receptor activity to remember, retrieve, and consolidate information (57). Another experiment investigated the role of the neurotransmitter dopamine using photostimulation and photoinhibition techniques. Dopamine has been shown to significantly impact the acquisition and consolidation of object recognition memory, as it is being released by the VTA (ventral tegmental area) into the IC. This process is mediated through D1-like receptors in the IC (43).

The object-place recognition task (OPR), testing spatial memory, involves recalling the specific spatial or environmental context in which an event occurred (53)(52). Research results indicate that individuals classified as obese may potentially experience difficulties in recalling past events and remembering locations or directions (44)(45)(58)(59). The hippocampus is a key brain area in the formation of episodic memory in rodents (60). For instance, lesion displays a deficit in the OPR task in rats (53)(52). Another animal study shows that long-term HFD consumption in aged mice leads to cognitive function decline, as observed in the OPR test (61).

Social recognition memory (SRM) is the cognitive ability that allows individuals to remember and recognize familiar faces and other social cues. Studies have demonstrated that the insula is particularly involved in SRM. This contrasts with the hippocampus and amygdala, which have not been found to play a significant role in the study of Cavalcante et al. (2017). The same study

has shown that the dopaminergic D1/D5, β-adrenergic, and serotonergic 5-HT1A receptors in the IC are involved in the consolidation of SRM (62). A recent study published in 2023 examines the role of the basolateral amygdala (BLA) and insular cortex in the social affective behavior of female rats. Inhibiting either the BLA or the insula, disrupts the rats' preference for stressed juveniles and naive adults, suggesting that these regions are crucial for normal social affective behavior (63). However, the NMDA-glutamatergic and H2-histaminergic receptors in the IC do not seem to participate in the consolidation of SRM (62).

3. Materials and methods

In this section the experimental procedures and techniques employed to investigate the effects of HFD on anxiety and object recognition memory will be outlined.

3.1 Animals

The animals used for the fiber photometry recordings consisted of 11 male and 11 female C57BL6J mice, meanwhile, for chemogenetic experiments, 20 male and 20 female mice were utilized. All mice were sourced from Charles River Laboratory (France) and were 8 weeks old at the start of the specific diet. Mice were kept in a 12-hour reversed light/dark cycle with an ambient temperature between 20 and 24 °C and a humidity between 45% and 65%. They were grouped in cages of 3 mice according to their sex and diet. Animals were given water and pellets ad libitum. Two diet groups were established: control mice, fed with a "standard diet" (SD), and high fat diet (HFD) mice, receiving "hypercaloric" food pellets rich in fats (D12451, Research Diet). The standard diet food pellets consisted of 3,3 kcal/g, with 8% lipids, 19% proteins, and 72% carbohydrates mostly from starch. The high fat pellets had an energy value of 4,7 kcal/g and consisted of 45% lipids mostly saturated fat from lard, 20% proteins, and 35% carbohydrates chiefly from sucrose. Mice were weighed once a week and marked on the tail twice a week to get used to the experimenter. At week 8, stereotaxic surgery was performed and in week 12, behavioral tests such as anxiety assays as well as memory tests were performed (***Figure 2***).

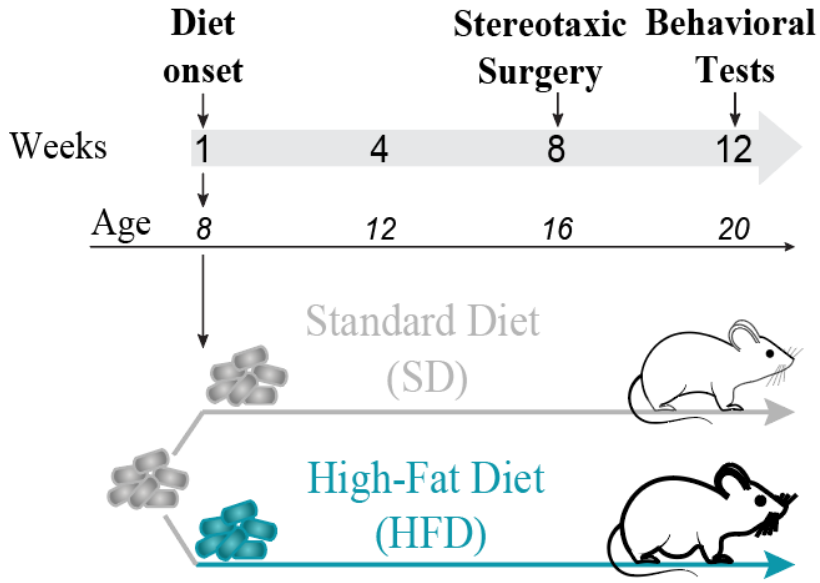

Figure 2. The experimental timeline for animals used in the photometry and chemogenetic experiment.

Figure 2. The experimental timeline for animals used in the photometry and chemogenetic experiment. At the age of 8 weeks, mice were fed either a standard diet or a high fat diet for a period of 12 weeks until the behavioral assays took place. In week 8 of the diet onset, animals underwent stereotaxic surgery.

3.2 Stereotaxic surgery

Stereotaxic surgery is a technique used in neuroscience that allows to precisely target specific regions of the brain. This method is used to perform various procedures such as intracranial injections and placement of implants to inject substances like viruses or drugs directly into targeted brain regions. This allows researchers to study the effects of various substances on neural circuits and behavior. Stereotaxic surgery also facilitates the placement of implants such as optical fibers or electrode arrays, which can be used for optogenetic or electrophysiological experiments, respectively. By accurately positioning these implants, researchers can manipulate or record neural activity in specific brain regions, providing insights into brain function and connectivity (64).

To begin the procedure, mice were first anesthetized with 5% Isoflurane (Virbac) for induction, and then the anesthesia was maintained at 1,5% isoflurane throughout the surgery. To minimize pain, a non-steroidal anti-inflammatory injection (Metacam, 5 mg/kg) was given. The eyes were protected with a lubricant (Ocrygel) and a heating pad was placed under the animal to prevent hypothermia.

After shaving the skull, a local anesthetic (Lidocaine) was applied, and the head was positioned on the stereotaxic frame. The skin was then disinfected using 10% povidone-iodine and 70% ethanol, followed by an incision of the skin and subcutaneous tissue.

In the fiber photometry experiment animals, 300 nanoliters of a viral vector AAV9-CaMKIIα-GCaMP6f from Addgene were injected into the aIC. This vector expresses the calcium reporter GCaMP6f (the 6th version of GCaMP with fast kinetics) specifically in glutamatergic neurons, thanks to the CaMKII promoter.
A craniotomy was performed at the level of the IC, at coordinates: antero-posterior: +1.70; medio-lateral: 3.10; dorso-ventral: -3.50, to from bregma. This viral vector was injected at a volume equal to 300nL (speed: 5-7 nL/s), thanks to a glass micropipette (3-000-203-G/X, Drummond) and a Nanoject III system (3-000-207, Drummond). After the viral injection, an optical fiber was implanted. This fiber, of a diameter of 400µm, and efficiency: >90%, was inserted 50 µm above the injection site. A biocompatible resin and dental cement were used to fix the implanted fiber to the brain.
After the surgery, the incision was closed with sutures, and a heating lamp was used to maintain the body temperature of the mice until they fully recovered from anesthesia. The weight of the animals was monitored for 5 days post-surgery, and they stayed in their home cages for 3 to 5 weeks to ensure proper viral expression.

For the chemogenetic experiments, 150 nanoliters of vector AAV9/2-mCaMKIIa-hM4D(Gi)-mCherry-WPRE-hGHp(A) were injected in the aIC in both hemispheres of 10 SD mice (5 males and 5 females) and 10 HFD mice (5 males and 5 females). This vector expresses an inhibitory Designer Receptor Exclusively Activated by Designer Drugs (DREADD) under the CAMKII promoter. As for the control group, 150 nanoliters of vector AAV-mCaMKII-mCherry-WPRE were injected into the aIC of 10 SD mice (5 males and 5 females) and 10 HFD mice (5 males and 5 females). This vector expresses mCherry under the promoter CaMKII. The DREADD ligand Clozapine-N-oxide (CNO) was injected to all diet groups intraperitoneally (2 mg/kg) diluted in sodium chloride, 45 minutes prior to the start of behavioral experiments.

3.2.1 Fiber photometry

Calcium imaging relies on the expression of the GCaMP protein. This protein gets excited by blue light at around 470 nm wavelength, and emits green fluorescence at around 510 nm only when calcium is present. (30). The GCaMP protein is made up of the fluorescent protein GFP (Green Fluorescent Protein), the calcium-sensitive protein Calmodulin, and the peptide M13. The amount of green fluorescence that GCaMP produces when exposed to blue light is proportional to the level of intracellular calcium, which provides a measure of neuronal activity *in vivo* in living animals.

To monitor neuronal activity, the optical fiber implanted above the population of neurons of interest receives light from two LEDs, blue at 470 nm and violet at 405 nm. The neurons of interest emit green light by the GCaMP sensor in the presence of Ca^{2+} to the detector. For detection, a 20x objective is connected to a cable connected to the optical fiber implanted in the brain and to a CMOS (Complementary Metal-Oxide Semiconductor) camera that detects the fluorescence emitted by GCaMP6f, as shown in ***Figure 3A.***

The optimal wavelength for the excitation of GCaMP6f is 470 nm, while the isosbestic wavelength is 405 nm. The isosbestic signal works as a negative control since its emitted fluorescence does not depend on the concentration of Ca^{2+}. This allows the elimination of artifacts related to movement and signals unrelated to neuronal activity during data processing. Thus, the signals obtained at 470 nm must be normalized to the signals obtained at 405 nm (65). To minimize the effect of sensor photobleaching, the light intensities at the end of the connector cable need to be adjusted to approximately 140 µW for the 470 nm channel and 58 µW for the 405 nm channel prior to the recordings. Matlab code is used to synchronize the GCaMP6f light excitation performed by the LEDs (470 nm and 405 nm pulsed alternately) with each image obtained by the second behavior-monitoring camera, placed 1,5 meters above the mouse.

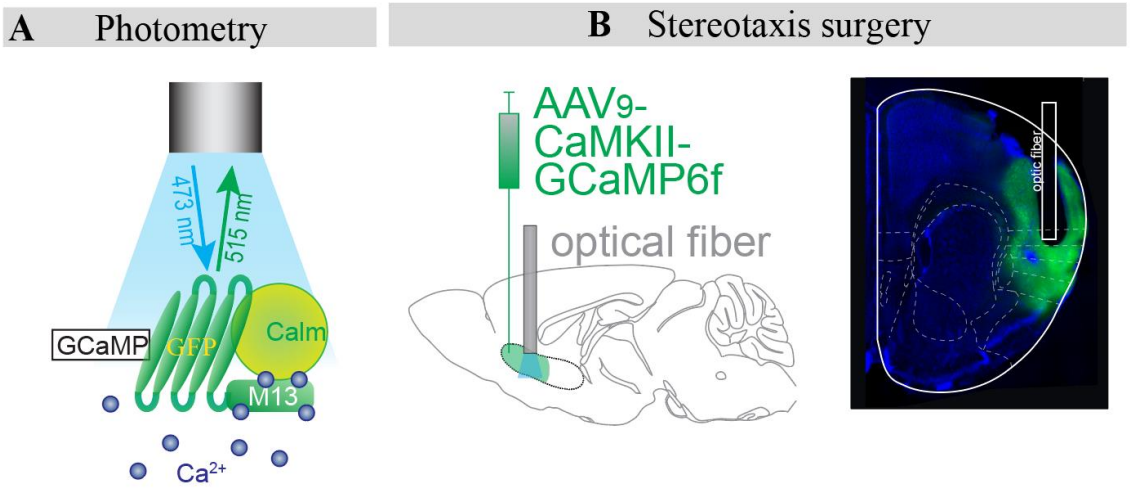

Figure 3. Calcium imaging experimental procedure.

Figure 3. Calcium imaging experimental procedure. A. Schematic of fiber photometry: The optical fiber implanted above the population of neurons of interest transmits the light emitted by two LEDs to the neurons of interest, and the green light emitted by the GCaMP sensor to the detector. **B**. Stereotaxic surgeries were performed during the eighth week of diet onset to inject a viral vector expressing the calcium reporter GCaMP6f into glutamatergic neurons of the anterior insular cortex and to implant an optical fiber.

3.2.2 Chemogenetic inhibition via DREADDs

Chemogenetic experiments involve using a Designer Receptor Exclusively Activated by Designer Drugs (DREADD) to manipulate neuronal activity. For this, at week 8 of the experimental timeline (***Figure 4A***), stereotaxic surgery has been conducted, as previously described, with viral vectors encoding for inhibitory DREADDs to inhibit glutamatergic excitatory neurons located in the aIC. A hM4Di receptor has been used, which is coupled with a Gi protein and is derived from the M4 muscarinic receptor. By administering the ligand CNO to the animal, the molecule binds to the hM4Di receptor, causing an inhibition of the targeted neuronal cell, leading to decreased signaling in the targeted neurons. However, recent studies indicate that CNO may not cross the blood-brain barrier *in vivo*, and instead converts to clozapine, which activates the DREADDs.

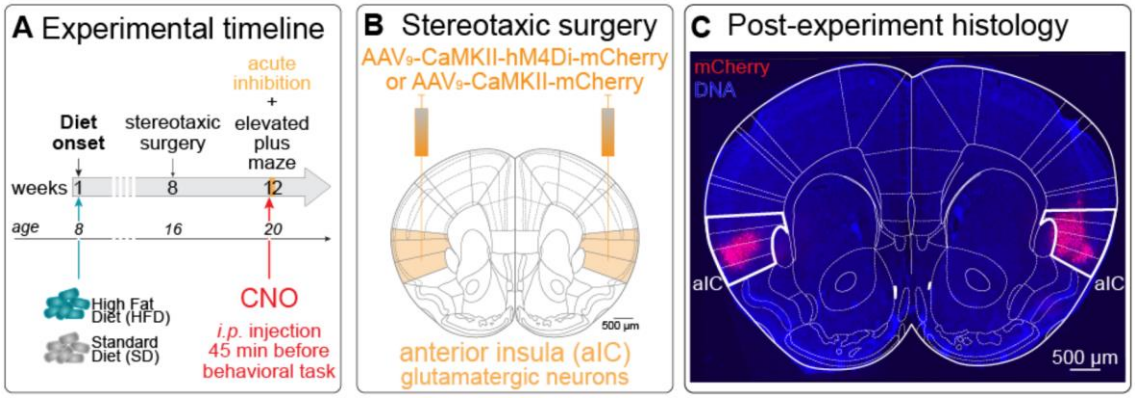

Figure 4. Chemogenetic inhibition of glutamatergic neurons in the aIC.

Figure 4. Chemogenetic inhibition of glutamatergic neurons in the aIC. A. Experimental timeline. Week 8 to 12 administration of either standard diet (SD) or high fat diet (HFD), week 8 performance of stereotaxic surgeries and week 12 start of behavioral tasks. Intraperitoneal injection of CNO 45 minutes before behavioral test. **B**. Stereotaxic surgery performed via bilateral injection of viral vectors expressing an inhibitory Designer Receptor Exclusively Activated by Designer Drugs under the CaMKII promoter into the aIC glutamatergic neurons. **C**. Post-experiment histology, images of coronal sections (100 µm) were acquired under a fluorescence microscope to observe the level of viral expression.

3.3 Behavioral testing procedures

Behavioral tests were conducted four weeks post stereotaxic surgeries, meaning after week 12 of the experiment, to allow for viral expression by neurons and animal recovery. Anxiety assays such as the elevated plus maze, open field test, and novelty-suppressed feeding test were conducted as well as the memory test called novel object recognition test (***Figure 5A-B***).

One week before the recordings, each animal was handled for ten minutes every day. For animals with an optical fiber implant, they were connected to the recording cable at least four times to ensure habituation to the experimenter and the system. Tests were performed during the dark phase of the cycle, using red light with an intensity of 15 ± 3 lux. The behavioral material (maze and arena) has been cleaned between each mouse using 2% acetic acid and then distilled water.

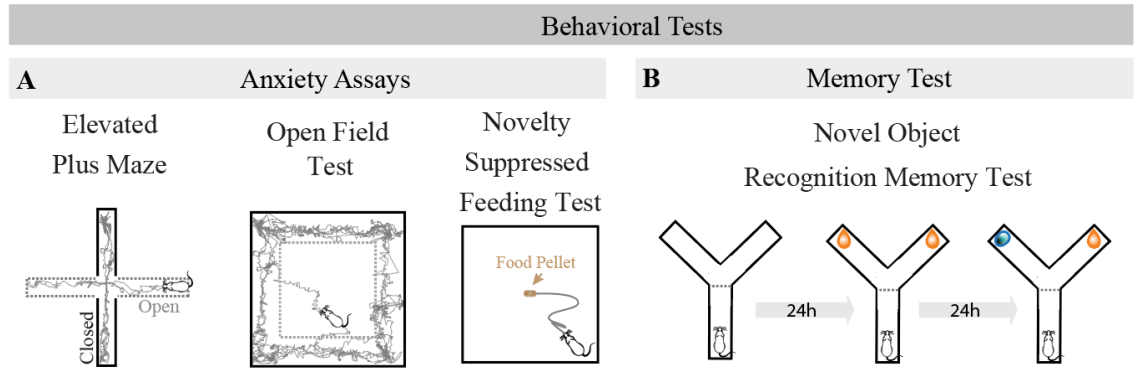

Figure 5. Behavioral tests.

Figure 5. Behavioral tests. A. Behavioral tests used to study anxious behavior using the elevated plus maze test, open field test, and novelty-suppressed feeding test. **B.** Memory performance was tested by using the novel object recognition memory test.

3.3.1 Elevated plus maze

To study anxious behavior, an elevated maze is used that is 60 cm high and shaped like a cross with arms with measurements of 75x75 cm and a width of 5 cm. Two of the arms of the EPM are open, while the other two are closed, as shown in ***Figure 5A***. The open arms are considered anxiety-provoking spaces, while the closed arms are protected spaces. Animals are placed in the center of the maze, facing an open arm, and are allowed to explore the maze for 15 minutes. As the open arms are known to provoke anxiety, it is known that normal mice tend to spend only about 20% of their time in the open arms, while they spend around 70% of their time in the closed arms, and 10% of their time in the center of the maze.

3.3.2 Open field test or open arena

In this experiment, mice are placed in a square-shaped arena that measures 60x60 cm. The purpose of this test is to measure the level of locomotion and anxious behavior in mice. The center of the arena, which represents 40% of the total area, is known to provoke anxiety in mice. Initially, mice are placed at the same angle in the area and are allowed to explore for 20 minutes, as shown in ***Figure 5A***. As the center is an anxiogenic space, in our experimental conditions,

mice spend about 80% of the time in the center and 20% in the borders of the arena. The more anxious the mouse, the less time it will spend in the anxiogenic spaces of the open arena.

3.3.3 Novelty-suppressed feeding test or hyponeophagia test

The NSFT is a test used to study anxious behavior via food intake latency in rodents. The arena used for this test is the same as the one used for the OFT. The type of pellet placed in the center of the arena depends on the diet of the mouse being tested — normal pellet for SD mice and high fat pellet for HFD mice. To ensure consistency, all animals are deprived of food for 20 hours prior to the start of the experiment. A camera is fixed on one edge of the arena to record the noises made by the mouse during food consumption. Animals are put in the same corner of the arena and are allowed to explore for 5 minutes (***Figure 5A***). This test measures the time it takes for the animal to consume the food, which is defined as latency. The assumption is that the more anxious an animal is, the more time it spends in the borders of the field, and the longer it may take for its first bite. Therefore, a higher latency in this test would translate to a more anxious behavior of an animal.

3.3.4 Object recognition memory test or novel object recognition test

The object recognition memory test (ORM) is a task based on the tendency of rodents to explore a novel object more than a familiar one. Naive rats typically explore the novel object, indicating recognition of the familiar one; a failure to do so suggests memory impairment (66)(67)(68).

The three-stage ORM test is performed as described in the current protocol in Neuroscience (69), with slight modifications explained as follows. A Y-maze is utilized as the assessment environment in this study for the photometry recordings and a V-maze is used in the chemogenetic experiment (***Figure 5B***).

Briefly, in the first stage, the habituation, mice are placed into the Y-maze (40 x 9 x 9 cm^3) and are allowed to freely explore the maze for 9 minutes prior to being

returned to their home cages. After 24 h, in the second stage, also called the training or sample phase, two identical objects (cylinder or lego) are placed at the end of two different arms of the maze. All objects used in this test vary in color and shape, but have similar height and width, and are counterbalanced. The animal is put into the maze and after a calibration time of 1 minute, the mouse is allowed to explore the two objects freely for 9 min. After this, animals are returned to their home cage again. After another 24 hours, in the third stage called the novel object recognition phase, the mouse is placed into the maze to explore the familiar and novel object (as shown in ***Figure 5B***) for 9 minutes, considering 1 minute of calibration time.

In the chemogenetic experiment, a completely new object (flask) gets introduced, and the same procedure as previously described (exploration of both objects for 9 min) takes place. The exploration time of the familiar and the novel object during the training and test phases gets registered. The recognition index is calculated as follows: the exploration time of either the familiar or novel object is divided by the total exploration time of both objects, then multiplied by 100. A recognition index of 50% indicates no preference for either object, while an index greater than 50% indicates a preference for the novel object. Mice need to explore objects (defined as nose pointed towards the object less than 1 cm away) for 40 seconds total during the sample phase of 10 minutes to be counted in the test trial.

3.4 Histology

After behavioral tests, animals were sacrificed using a mix of Exagon (300 mg/kg) and Lidor (30 mg/kg), and perfused with Ringer's solution (1L distilled water, 8,6 g of NaCl, 0,3 g KCl, 0,33 g CaCl2), followed by 4% PFA (paraformaldehyde). Brains were removed and left in PFA overnight at 4°C. Then, they were transferred to a 30% sucrose solution, in 1X PBS at 4°C, for cryoprotection. Then, coronal sections of 100 µm were made, with a freezing sliding microtome. Sections were incubated with a DNA-specific fluorescent probe Hoechst33342 and then mounted on gelatinized slides. The images were acquired under a fluorescence microscope (THUNDER Imager 3D Tissue, Leica) to observe the level of viral expression and the location of the implanted optical fiber.

3.5 Statistical analysis

The obtained data was tabulated, graphed, and analyzed with GraphPad Prism 8 and 9. Results are presented as means, and error bars represent the standard error of the mean (±SEM). Differences are considered significant if $p<0,05$*, $p<0,01$* or $p<0,001$**. Independent-sample Mann-Whitney nonparametric tests were used for comparison between the two groups. Wilcoxon non-parametric tests for dependent samples were performed for signal comparison in the same group of mice. A t-test was used to determine if there was a significant difference between the means of two groups and how they are related. One-way ANOVA was used for a single independent variable, or factor, and the aim was to investigate if variations or different levels of that factor have a measurable effect on a dependent variable. Two-way ANOVA was used to estimate how the mean of a quantitative variable changes according to the levels of two categorical variables; for instance, effect of sex and diet on body weight gain. Object recognition indexes were analyzed with a one-sample t-test with a hypothetical mean of 50% to assess object preference during the exploration time. Three-way ANOVA was used to determine if there was an interaction effect between three

independent variables on a continuous dependent variable (i.e., if a three-way interaction exists).

3.5.1 Behavioral analysis

Videos regarding anxiety-like behavior, recorded by the behavior camera, were analyzed to determine the location of the mice during the anxiety experiments (EPM, OFT and NSFT). Movement and locomotion of mice in the maze and the open arena was analyzed by using Bonsai software. This same software considers the simultaneous recording the reference LED lights of wavelength 470nm and 504nm transmitted to the optical fiber. Food consumption was detected by a Matlab code with the use of the soundtrack of the behavioral camera.

Regarding the object recognition memory test, the exploration time analysis for each object was made offline with the help of Boris software by one observer, blind to the treatment each mouse was receiving. Exploration was considered pointing the nose towards an object at 1 cm or less and/or touching it with the nose. Turning around, touching, sitting, or leaning on the objects were not considered exploration. Relative positions were counterbalanced. Furthermore, Boris software was used to capture the exact time frames of explorational behavior in the photometry recordings. Together with a Matlab code, the neural activity during the performance of the ORM test, was then analyzed.

3.5.2 Calcium imaging analysis

To analyze the calcium imaging recordings, a second custom Matlab code was used. The 470 and 405 nm signals were normalized, subtracting the average fluorescence over a one-minute window from the fluorescence recorded at each time point and step:

$$\frac{\Delta F}{F} = \frac{F - F\ (average\ 60s)}{F\ (average\ 60s)}$$

Then, the normalized signal independent of calcium (405 nm, isosbestic) was subtracted from the normalized signal GCaMP6 (470 nm, calcium), to eliminate the non-specific fluorescence:

$$\frac{\Delta F}{F} = \frac{\Delta F}{F\ (calcium)} - \frac{\Delta F}{F\ (isosbestic)}$$

The result obtained is the global signal (ΔF/F) which was used as an estimate of the tonic activity of the recorded neurons. This code is also used for the calculation of transient calcium signals, called transients. To do so, the global signal was filtered to retain only the values between 0,2 Hz and 6 Hz. The filtered peaks were detected from events with amplitudes greater than two absolute deviations from the median (MAD) over a one-minute window. Peaks above two MAD, are identified as transients and are used as an estimate of the phasic activity of recorded neurons (70).

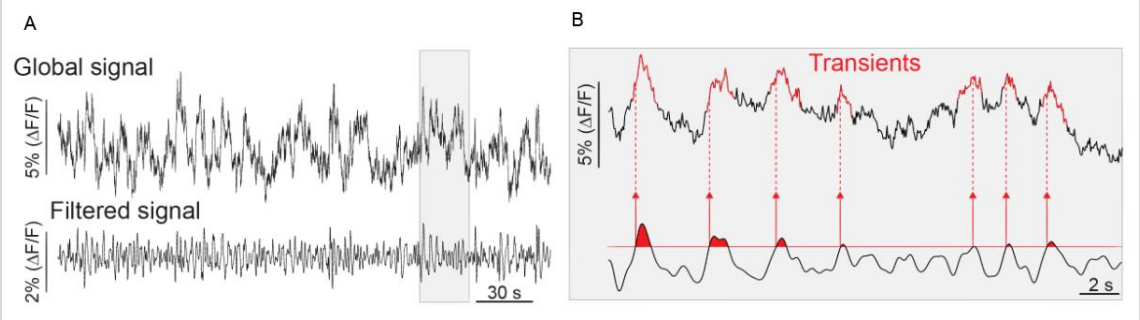

Figure 6. Descriptive examples of global signal and transients.

Figure 6. Descriptive examples of global signal and transients._A. Calcium imaging recording signal before and after subtraction of normalized isosbestic signal independent of calcium. **B**. Explanatory example of transient calculation. During the experiment there is a LED light blinking every 5 seconds, allowing the behavioral camera to be in frame with the photometry signal recording. The Matlab code analyses the calcium signal according to the position of the animal during the behavioral tests, or in relation to discrete stimulations such as the consumption of food of the NSFT.

3.5.3 Exclusion criteria

Mice were excluded from analysis if the virus was not expressed in the anatomical target or if the optical fiber location was outside the target area. During the EPM, animals having spent less than 3% of the time in open arms were excluded from the analysis.

In the object recognition memory task, the minimum exploration total for both objects was 40 seconds to be included in the test, as it cannot be confirmed mice spent enough time exploring to learn in the sample phase to discriminate the objects in the test phase.

4. Results

The following section presents the results of the study of the effects of a high fat diet on body weight, anxiety and object recognition memory in the anterior insular cortex.

4.1 Weight gain resulting from a standard diet or a high fat diet

The HFD mice were fed an obesogenic diet to induce chronic weight gain. ***Figure 7A*** shows the weight gain percentage between the two groups including male and female mice compared to the first week. The initial body weight of SD mice at day 0 was 23,39 g, while HFD mice weighed 23,99 g. There was no significant difference between both groups at this stage (***Figure 7B***). However, by week 12, the average weight of SD mice had increased to 27,24 g, while HFD mice had increased to 30,97 g. A two-way ANOVA test showed a significant difference in weight gain between the two groups over time ($F(_{1,49})$=7,37, **p=0,0091, Sidak post-hoc tests). Interestingly, SD mice showed a 16% increase in body weight, while HFD mice had a 26% increase (***Figure 7C***). The study also investigated whether there were any differences in body weight gain between male and female mice (***Figure 7C***). Results show no difference between the two sexes, but a significant difference between the diets (***p value<0,0001, as determined by a two-way ANOVA and Tukey's multiple comparisons test, ***Figure 7C***).

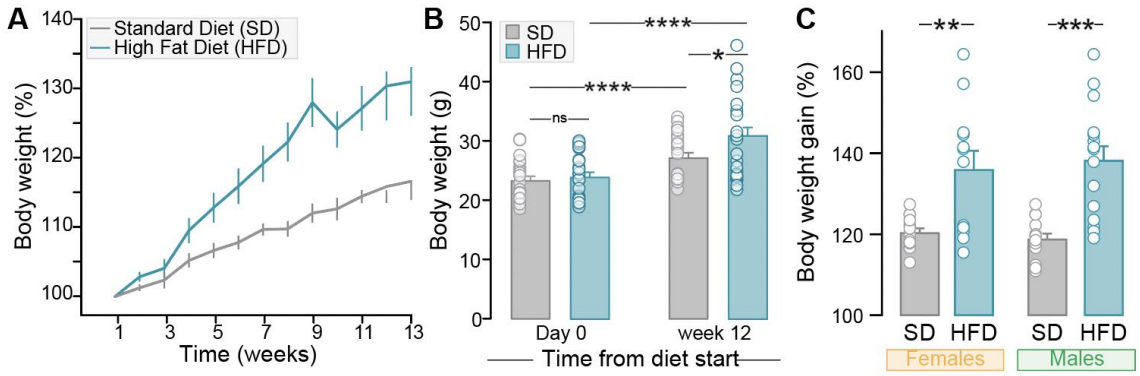

Figure 7. HFD impact on body weight of mice used for fiber photometry experiments.

Figure 7. HFD impact on body weight of mice used for fiber photometry experiments. A. Average body weight evolution (%) during 13 weeks of standard (SD, n= 25) and high fat diet (HFD, n=27). **B.** Average body weight (grams) comparison between first and last week of diet. HFD mice significantly gained weight during the 12 week experimental time comparing weight before and at the end of the experiment (Two-way ANOVA, time x diet: $F_{(1,49)}$=7,37, **p=0,0091, Sidak post-hoc tests). **C.** Average body weight gain (percentage) comparison between SD and HFD females and males at last week of diet. No effect of sex but a significant effect of diet was found. (Two-way ANOVA, diet $F_{(1,50)}$ = 33,72, ***p<0,0001; sex $F_{(1,50)}$ = 0,01352, p=0,9079, Tukey post- hoc multiple comparison tests).

For this project, 2 chemogenetic experiments have been conducted. The first experiment used male mice only counting 40 animals in total, called chemogenetic experiment 1. The same experimental design has been conducted on 40 more animals, consisting of females and males, named chemogenetic experiment 2. The following results have been obtained: ***Figure 8A,*** chemogenetic experiment 1 and ***Figure 8B***, chemogenetic experiment 2, represent the weight of animals measured at week 12 of the experiments. In chemogenetic experiment 1, a difference between animals of a SD and HFD group has been documented (***Figure 8A***). Surprisingly, in the chemogenetic experiment 2, when females and males were included, no difference in body weight between the diet groups was found. However, a main sex effect has been calculated via a two-way ANOVA, accounting for 83,84% of the total variation (***Figure 8B***). Regarding the fat mass of males measured via MRI in chemogenetic 1 animals, data show a highly significant diet effect with a p-value of 0,0001, but no effect regarding the injection type and no interaction between both factors (***Figure 8C***). Therefore, fat accumulation was more pronounced in HFD males. Looking at the body weight gain (%) over the 12 weeks between the two sexes and diet groups, no difference in body weight was found in chemogenetic experiment 2 animals, which meant that the animals on a HFD did not become

overweight. Therefore, this dataset couldn't be used to study the obesogenic effect of a HFD on the aIC in anxiety and memory tests (***Figure 8D***). However, it has been decided to only use chemogenetic experiment 1 data, meaning only data from male animals for this thesis. Possible reasons for no body weight gain in animals of the chemogenetic experiment 2 over the experimental timeline will be discussed in the discussion section.

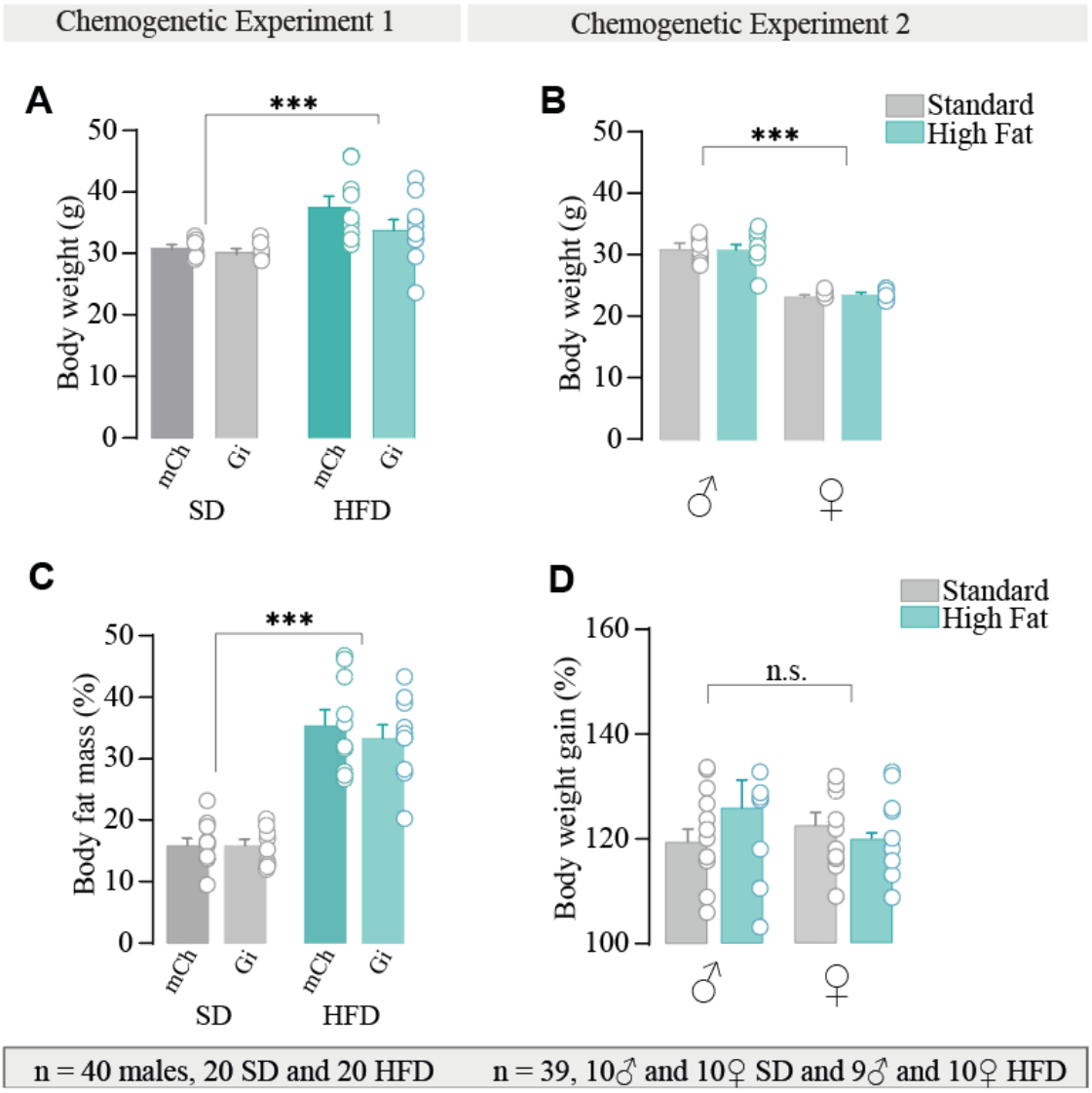

Figure 8. Effect of diet over the time of the chemogenetic experiments.

Figure 8. Effect of diet over the time of the chemogenetic experiments. A. Quantification of weight (g) in week 12 of the diet of male animals of chemogenetic experiment 1 (two-way ANOVA: injection (mCherry, Gi) and diet (SD n=20, HFD n=20) shows an effect of diet (***p=0,0002) but not injection (p=0,0785), with no interaction (p=0,2147). **B.** Weight (g) in week 12 of the diet of male and female mice (Two-way ANOVA: sex (male and female) has a major effect with ***p=0,0001; diet shows no effect (p=0,8132), nor interaction (p=0,7244). **C.** Percentage of fat mass of mice receiving a SD versus mice receiving a HFD shows a highly significant diet effect in male mice (Two-way ANOVA: diet effect ***p=0,0001, injection p=0,5678, interaction p=0,5670). **D.** Comparison of weight gain (in %) between males and females in week 12 of the diet (Two-way ANOVA, sex: $F_{(1,35)}$=0,1367, p=0,7138, diet: $F_{(1,35)}$=0,4011, p=0,5306, sex x diet: $F_{(1,35)}$=1,967, p=0,1696, n=20 SD with 10 males + 10 females and 19 HFD with 9 males + 10 females).

4.2 Effect of HFD on anxiety-like behaviors

Anxiety assays were conducted in week 12 of high fat diet administration. Three anxiety tests were used in this study, the EPM, OFT, and the NSFT, which led to the following results.

4.2.1 Elevated plus maze test

In week 12, after animals had recovered from surgeries and viral vector expression in the aIC glutamatergic neurons took place, behavior, and calcium activity were recorded in the EPM test. Interestingly, HFD mice spent less time in the open arms, the anxiety-provoking spaces of the maze, compared to SD mice with respective means of 8,37% versus 24,39% (***Figure 9A*** and ***Figure 9B***). Looking at the time spent in the open arms of the maze, no effect of sex was found between lean and HFD mice. However, within the female and the male mice groups, only male animals fed a HFD spent less time in the anxiogenic spaces of the EPM compared to lean mice (***Figure 9C)***.

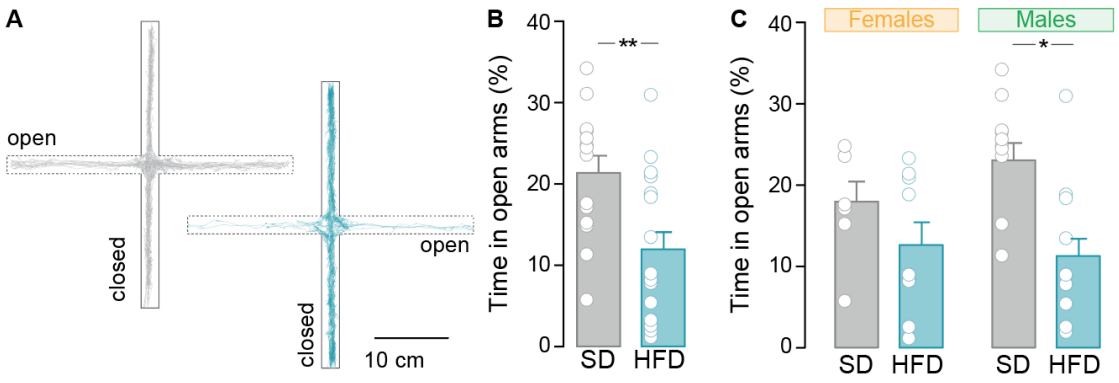

Figure 9. Behavior of SD and HFD mice in EPM anxiety test.

Figure 9. Behavior of SD and HFD mice in EPM anxiety test. A. Averaged tracking of SD (grey) and HFD (blue) in the EPM test. **B.** Time spent in open arms (%) of SD and HFD mice (Mann-Whitney U test, *p= 0,046, SD mice n=14, HFD mice n=17). **C.** Time spent in the open arms of the EPM between female and male mice (Mann-Whitney U test, p=0,4908 between female SD and HFD groups; *p=0,0152 between male SD and HFD groups).

Four weeks after viral injection, the activity of insula projection neurons (excitatory neurons) during the EPM test was recorded. In control mice, the activity of excitatory neurons was higher in the open arms of the EPM compared to the closed arms (***Figure 10A***). It also has been observed that this pattern of heightened activity in anxiety-inducing spaces has been present in mice fed a HFD and was even more pronounced compared to the control group (***Figure 10B***). The increased activity of the aIC excitatory neurons in anxiogenic spaces was evident in both, female and male HFD-fed mice, with no significant difference based on sex observed in the neurons' activity patterns (***Figure 10C***). Interestingly, a strong trend for an increase of transient frequency in the open arms of the EPM compared to the closed arms has only been found for HFD mice (***Figure 10D***). In the EPM, the time spent in open arms was shown to be inversely proportional to the animal's level of anxiety. Furthermore, a strong negative correlation was found between the calcium signal in the open arms and the time spent by the animals in the open arms in this experiment (***Figure 10E***).

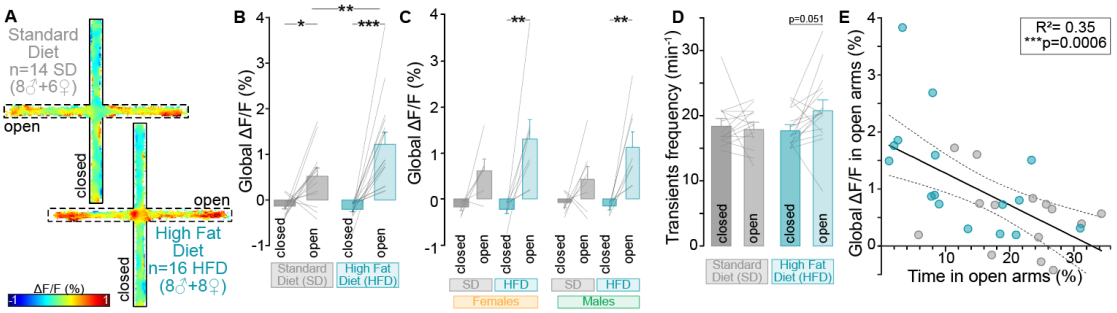

Figure 10. Encoding of anxiogenic spaces of EPM by excitatory neurons of the anterior insula is increased after HFD.

Figure 10. Encoding of anxiogenic spaces of EPM by excitatory neurons of the anterior insula is increased after HFD. A. Averaged heat maps of global signal recorded from aIC neurons during 15 minutes EPM test in SD and HFD mice. **B.** Global calcium signal is increased in open arms for SD mice and HFD mice, and the activity is higher in HFD mice (Two-way ANOVA diet x space interaction $F_{(1,28)}$=4,95, *p=0,0344, Sidak post-hoc tests). **C**. Global calcium signal is increased in open arms for both females and males HFD mice, when analyzed separately, but not for females and males SD mice, when analyzed separately (three-way ANOVA diet x space: $F_{(1,26)}$=4,409, *p=0,0456; diet x space x sex: $F_{(1,26)}$=0,00204, p=0,9643) **D.** Transient frequency tends to be higher in the open arm after HFD (Two-way ANOVA diet x space interaction $F_{(1,28)}$=3,507, p=0,0716, Sidak post-hoc tests). **E.** The calcium signal when mice are in the open arms is negatively correlated to the time mice spend in the open arms (Pearson correlation, R^2=0,35, ***p=0,0006).

During week 12 of the chemogenetic experiment 1, after animals had recovered from surgeries and viral vector expression in the aIC glutamatergic neurons had taken place, their behavior was recorded in the EPM test (***Figure 11A***).

In the EPM, results of a two-way ANOVA for the factors diet and DREADD show no effect for diet (p=0,0989) but DREADD (**p= 0,0075), explaining 17,11% of the total variance (***Figure 11B***). A one-way ANOVA with Sidak's multiple comparisons test reveals a difference in the time spent in the open arms of the EPM between the HFD mCherry and HFD Gi group (***Figure 11B***).

In conclusion, the inhibition of glutamatergic neurons of the aIC did influence the behavior in anxiogenic spaces of the EPM within the mice receiving a HFD making them more explorative towards the open arm of the EPM test.

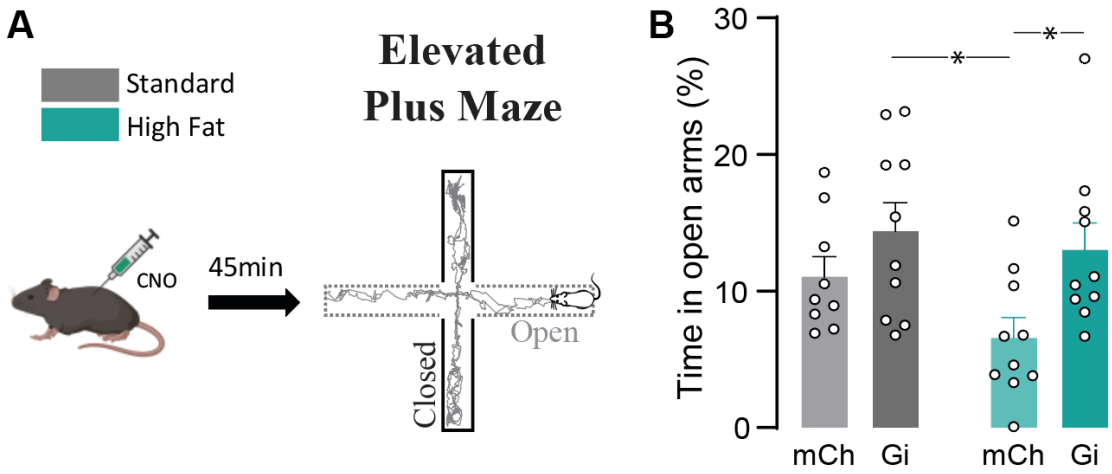

Figure 11. Behavior of SD and HFD mice in EPM anxiety test.

Figure 11. Behavior of SD and HFD mice in EPM anxiety test. **A.** To evaluate anxiety in mice, the EPM test was conducted. CNO was administered 45 minutes prior to the EPM test. **B.** SD-Gi mice spent more time exploring the open arms of the EPM, compared to the HFD-mCherry mice (Tukey's multiple comparisons test: SD-Gi vs. HFD-mCh adjusted *p-value=0,0130), showing a significant DREADD effect (Two-way ANOVA: diet p=0,0989, DREADD **p=0,0075, interaction p=0,3724). A one-way ANOVA with Sidak's multiple comparisons adjusted *p-value=0,0229 for HFD-mCh versus HFD-Gi.

Further, in a preliminary experiment, acute chemogenetic inhibition of anterior insula glutamatergic neurons has been performed to reverse anxiety-like behavior in HFD-fed mice (***Figure 12A***). Strikingly, acute bilateral inhibition of aIC excitatory neurons restored the increase of anxiety observed in the HFD-fed mice (***Figure 12A***), although the distance travelled remained unaffected by the CNO injection (***Figure 12B***).

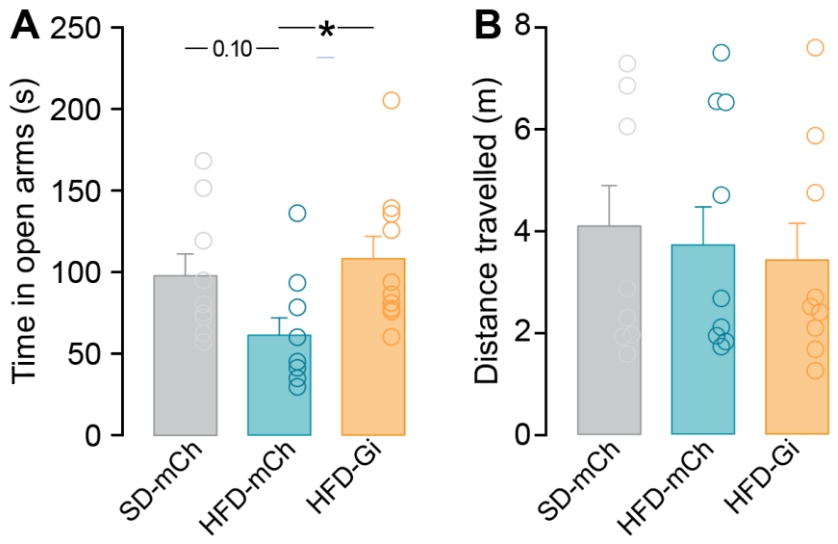

Figure 12. Preliminary data for acute chemogenetic manipulations of neural populations of the insula in HFD-fed mice.

Figure 12. Preliminary data for acute chemogenetic manipulations of neural populations of the insula in HFD-fed mice. A. Time spent in the open arms is decreased by HFD (mCh) and significantly rescued in hM4Di group (One-way ANOVA: $F_{(2,28)}$=3,895 *p=0,033, followed by post-hoc tests: SD-mCh vs. HFD-mCh p=0,10 and HFD-mCh vs. HFD-Gi *p=0,026). **B.** The distance traveled during the 15 minutes of the EPM is not different between the groups (distance x group interaction $F_{(2,25)}$=0,19, p=0,83) indicating that behavioral changes induced by HFD and chemogenetic manipulation are not related to motor effects.

4.2.2 Open field test or open arena test

The time animals spent in the center of the OFT was similar between SD and HFD mice, showing 23,53% and 24,05% respectively (***Figure 13B***). Nevertheless, the activity of aIC excitatory neurons was higher in the center compared to the border only for HFD animals, as shown in ***Figure 13A*** and ***Figure 13C.*** Besides the fact that the global calcium activity was higher for HFD animals in the center compared to SD in the center, no effect was observed in the transient frequency in HFD or SD mice.

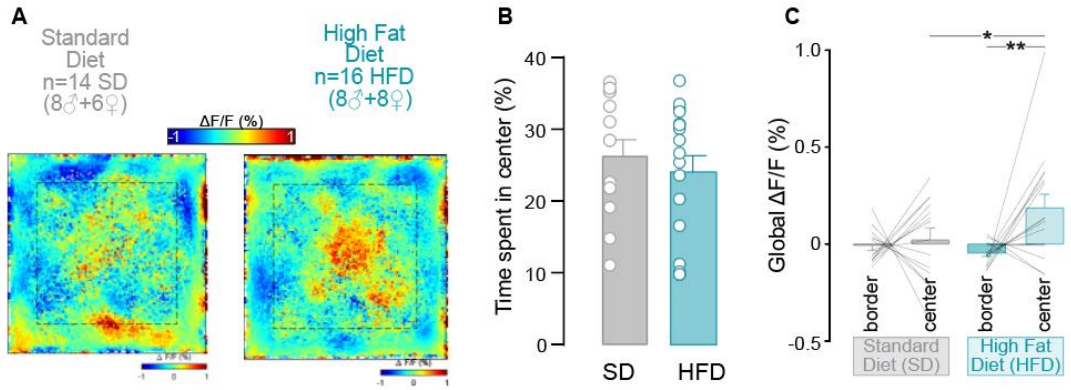

Figure 13. Encoding of anxiogenic spaces of OFT by excitatory neurons of the anterior insula is found after HFD administration.

Figure 13. Encoding of anxiogenic spaces of OFT by excitatory neurons of the anterior insula is found after HFD administration. A. Averaged heat maps of global signal recorded from aIC neurons during 20 minutes OFT in SD and HFD mice. **B.** Time spent in the center of the open field is not different between SD and HFD mice (Mann-Whitney U test, p-value =0,4683). **C.** Global calcium signal is increased in center for HFD mice but not for SD mice, and the activity in center is higher in HFD mice compared to SD mice (Two-way ANOVA diet x space interaction $F_{(1,29)}$=3,181, p=0,0850; diet interaction $F_{(1,29)}$=3,029, p=0,0924; space interaction $F_{(1,29)}$=5,759, p=0,0230, Sidak post-hoc tests).

Looking at the results of the OFT of the chemogenetic experiment consisting of 40 male mice in total, animals fed a standard diet were shown to spend more time in the center compared to the HFD animals (Two-way ANOVA shows a strong diet effect ***p=0,0001, $F_{(1,36)}$=18,01, ***Figure 14B***). Interestingly, the inhibition of aIC glutamatergic neurons in HFD mice reduced the time spent in the center of the OFT compared to SD control mice (Tukey's multiple comparisons test: mCh-SD vs. Gi-HFD **p=0,0058, ***Figure 14B***). Nevertheless, comparing the HFD control and HFD Gi group with each other, no difference between the exploration time of the center has been shown in post hoc tests.

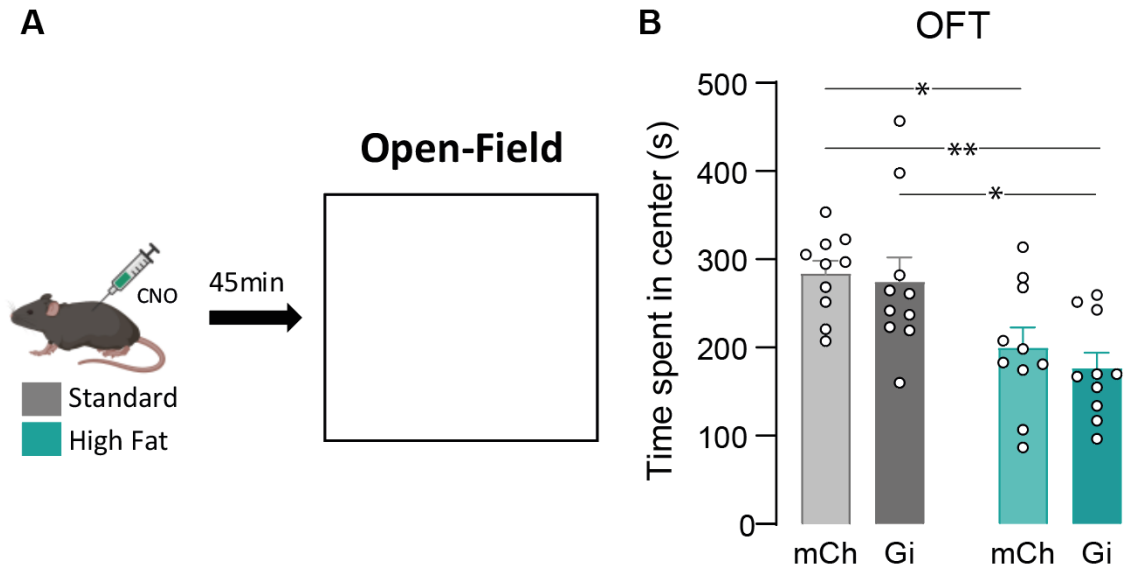

Figure 14. Behavior of SD and HFD mice in OFT anxiety test.

Figure 14. Behavior of SD and HFD mice in OFT anxiety test. A. CNO was administered intraperitoneally 45 minutes prior to the open field test in order to inhibit glutamatergic neurons of the aIC. **B.** A two-way ANOVA revealed a strong diet effect showing a p-value of ***p=0,0001, $F_{(1,36)}$=18,01. SD-control mice (SD-mCh) explored the center for a longer time when compared to HFD control mice (HFD mCh) and SD Gi were also found to explore the center more when compared to HFD-Gi mice (Tukey's multiple comparisons test mCh-SD vs. mCh-HFD *p=0,0421; Gi-SD vs. Gi-HFD *p=0,0133). HFD-Gi mice spent less time exploring the center of the OFT, compared to the SD-mCherry mice (Tukey's multiple comparisons test: mCh-SD vs. Gi-HFD **p=0,0058 for n(total)=40 mice consisting of SD: 20 males, HFD: 20 males).

4.2.3 Novelty-suppressed feeding test or hyponeophagy test

After 20 hours of food restriction, mice were given access to a food pellet, placed in the middle of a field. The activity of aIC excitatory neurons was recorded during the time animals ate. There is a trend towards a decrease in the global calcium activity of aIC neurons for both standard diet and high fat diet mice, but no significant difference was observed (see ***Figure 15B-E***).

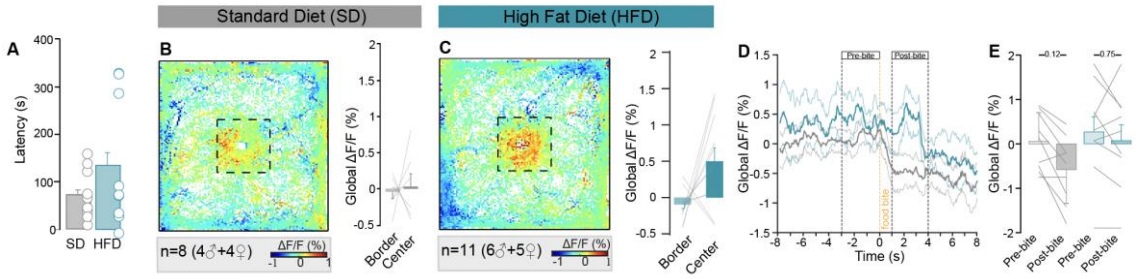

Figure 15. Results of HFD in NSFT.

Figure 15. Results of HFD in NSFT. A. The latency (sec) to consume the food located in the center of the arena is not different between the two groups (Mann-Whitney U test, p=0,0915, 61 s for n=9 SD and 101 s for n=12 HFD). **B**. Heatmap of global calcium signal recorded during NSFT for SD mice. The calcium signal is not different between the center and the edges of the arena (Wilcoxon, p=0,8203, n=9). **C**. Heatmap of global calcium signal for HFD mice. The calcium signal tends to increase in the center of the arena compared to the edges (Wilcoxon, p=0,0537, n=11). **D**. Averaged calcium signal before, during, and after food bites. **E.** Activity tends to be lower after a food bite in both groups (Two-way ANOVA: time effect $F_{(1,16)}$=7,46, *p=0,0148, n=9 SD, n=9 HFD, Sidak post-hoc tests).

Results of the chemogenetic experiment animals show that SD animals took les time to take the first bite compared to animals on a HFD, explaining 56,12% of the total variance. Interestingly, in the HFD group, the inhibition of aIC excitatory neurons lowered the latency to eat, compared to HFD control mice, suggesting an involvement of the anterior insular cortex in the hyponeophagy test in HFD-fed animals (***Figure 16B***).

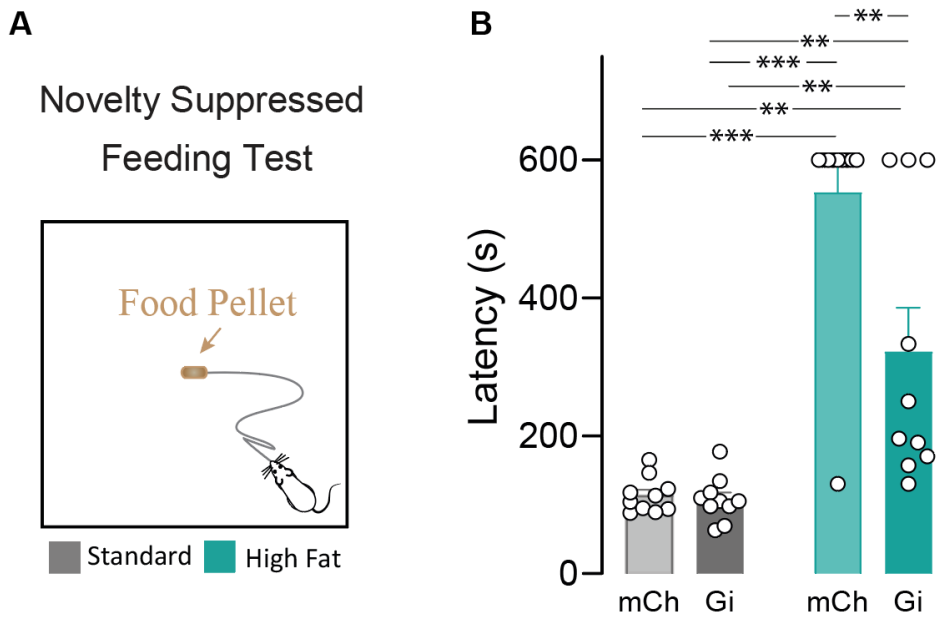

Figure 16. Comparison of behavior during the NSFT in SD and HFD mice of the chemogenetic experiment.

Figure 16. Comparison of behavior during the NSFT in SD and HFD mice of the chemogenetic experiment. A. During this test, a food pellet is placed in the center of the arena. The time until the first bite is monitored via a behavioral camera and audio tracking. **B.** The latency (sec) to consume the food pellet is different between the two diet groups, explaining 56,12% of the total variance (Two-way ANOVA: diet ***p<0,0001, injection **p=0,0054, interaction: **p=0,0078; latency 110,6 s for SD and 437,8 s for HFD). Tukey's multiple comparisons test: mCh-SD vs. mCh-HFD ***p <0,0001, mCh-SD vs. Gi-SD p= 0,9996, mCh-SD vs. Gi-HFD **p= 0,0037, mCh-HFD vs. Gi-SD ***p <0,0001, mCh-HFD vs. Gi-HFD **p= 0,0013, Gi-SD vs. Gi-HFD **p= 0,0028, n=40.

4.3 Effect of HFD on object recognition memory

The administration of a high fat diet given to animals from adulthood onwards resulted in the following outcomes in the object recognition test.

4.3.1 Behavior during the object recognition test

To explore the role of the anterior insula in the object recognition memory task in the photometry experiment animals, firstly, a habituation phase was conducted in order to minimize external factors that could influence the test performance. During the sample phase, animals have been presented with 2 identical objects; only females in the HFD group explored one object more than the other (one-sample t-test compared to chance 50%: HFD-F: n=5, t=7,303, **p=0,0019,

Figure 17A). Interestingly, only HFD mice show a higher interest for the left object compared to the time spent with the right one (Mann-Whitney U test HFD mice n=11: ***p= 0,0009, ***Figure 17B***). All mice of both diet groups spent over 40 seconds in total exploring both objects in the sample phase which is the minimum exploration time to be included in this study (***Figure 17C***).

After 24 hours, mice were tested to see if they could distinguish between a familiar object and a novel object. Male mice in both, the SD and HFD groups, showed a clear preference for the novel object. This was apparent from the recognition index, which indicated a significant difference from the hypothetical mean of 50% (t=3,767, *p=0,0327 for SD-males; t=3,500, *p=0,0173 for HFD-males), shown in ***Figure 17D***. In contrast, females in the SD and HFD groups showed impairment in object recognition memory (t=2,284, p=0,0712 for SD females and t=1,583, p=0,1887 for HFD females), failing to distinguish between the familiar and novel objects. Looking at the time spent to explore the familiar and novel objects, only the HFD group preferred to spend more time with the novel object in the test (SD mice (n=10), p=0,0499; HFD mice (n=11), **p=0,0062, ***Figure 17E***). Interestingly, the total exploration time for both objects has decreased in the test phase for the male SD group, revealing no difference to the initial exploration time of minimum 40 seconds only in the male SD group. On the other hand, the other groups (SD females, HFD males and females), explored both objects for more than 40 seconds (t=5,935, **p=0,0019 for SD-females; t=2,251, p=0,1098 for SD males; t=5,277, **p=0,0062 for HFD-females; t=6,665, **p=0,0011 for HFD males, ***Figure 17F***).

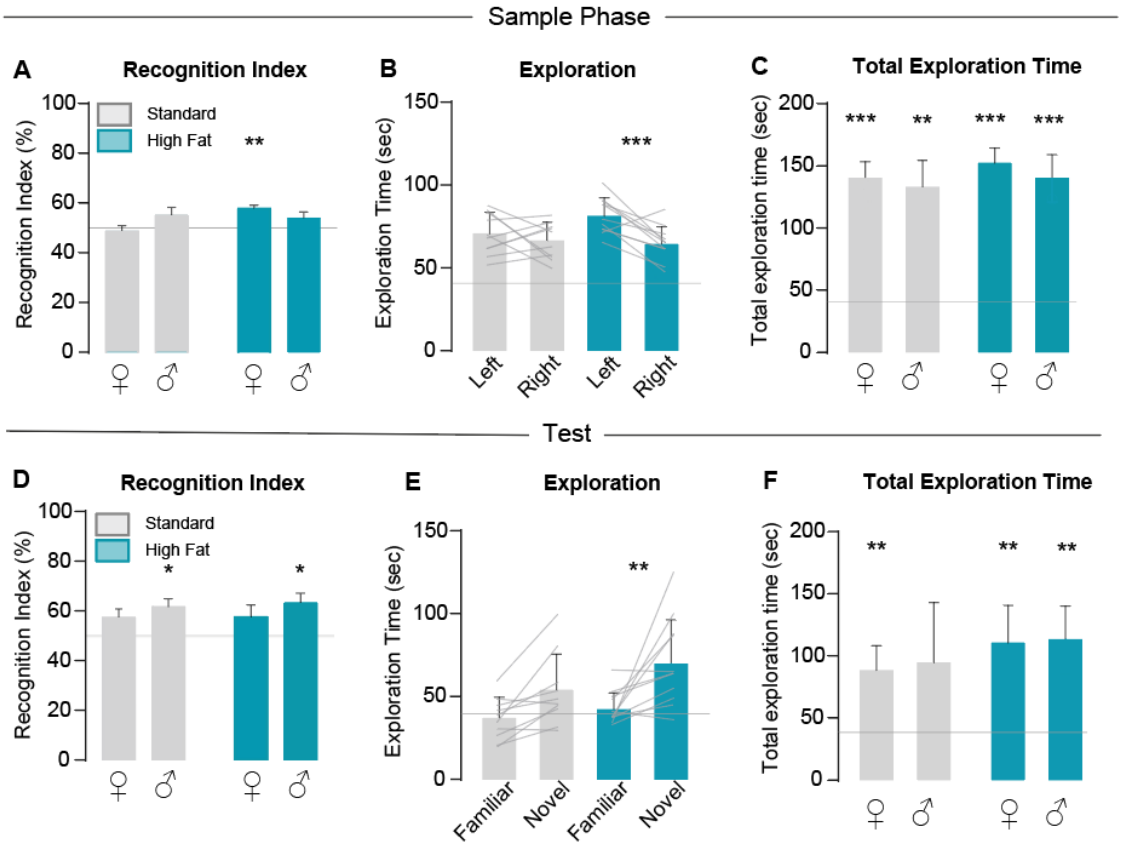

Figure 17. Effect of diet on recognition index and exploration time in adult mice during the NOR memory test.

Figure 17. Effect of diet on recognition index and exploration time in adult mice during the NOR memory test. A. Mice did not have any preference for either object in the sample phase except for female HFD mice compared to 50% (one-sample t-test: SD-F: n=6, t=0,5544, p=0,6032; SD-M: n=4, t=1,744, p=0,1795; HFD-F: n=5, t=7,303, p=0,0019**; HFD-M: n=6, t=1,834, p=0,1261). **B.** Exploration time of both objects separately, no difference between the exploration times of the objects in SD mice (Mann-Whitney U test SD mice (n=10): p=0,4466) but a significant difference in the exploration times of the objects between HFD mice (Mann-Whitney U test HFD mice (n=11): ***p= 0,0009) has been shown. **C.** Mice of both diet groups explored the objects in the sample phase for more than 40 seconds in total (one-sample t-test compared to 40 sec: SD-F: n=6, t=18, ***p=<0,0001; SD-M: n=4, t=8,710, **p=0,0032; HFD-F: n=5, t=20,81, ***p=<0,0001; HFD-M: n=6, t=12,85; ***p= <0,0001). **D.** Both male groups (SD-M and HFD-M) showed a higher preference for the novel object in the ORM test (One-sample t-test compared to chance 50%: SD-F: n=6, t=2,284, p=0,0712; SD-M: n=4, t=3,767, *p=0,0327; HFD-F: n=5, t=1,583, p=0,1887; HFD-M: n=6, t=3,500, *p=0,0173). **E.** Exploration time of both objects separately, SD mice explored both objects equally (Mann-Whitney U test: SD mice n=10, p=0,0499), while HFD mice preferred exploring the novel object in the test. (Mann-Whitney U test: HFD mice n=11, **p=0,0062). **F.** Mice of the groups SD-F, HFD-F, HFD-M explored the objects more than male SD mice, compared to chance 40sec. (one-sample t-test: SD-F: n=6, t=5,935, **p=0,0019, SD-M: n=4, t=2,251, p=0,1098, HFD-F: n=5, t=5,277, **p= 0,0062, HFD-M: n=6, t=6,665, **p=0,0011).

The performance in the ORM test at 24 hours of the chemogenetic mouse batch 1, both groups of animals with mCherry injections, the SD and HFD mice, as well as the HFD-Gi animals reached a discrimination index over 50%, suggesting the ability to discriminate between the familiar and the novel object. The inhibition of neurons of the aIC resulted in a lower discrimination index only in SD mice in the

24 hour test. Looking at the performance in the HFD groups, the injection type did not result in any difference in the performance at 24h test (One-sample t-test compared to 50%: SD-mCherry: t=5,077 ***p=0,0007; SD-Gi: t=2,072 p=0,0681, HFD-mCherry: t=5,928 ***p=0,0002, HFD-Gi: t=3,490 **p=0,0082, ***Figure 18A***).
SD-fed animals with inhibited aIC neurons were not able to distinguish between the novel and familiar objects in the 48 hour test, showing memory impairment. On the other hand, the inhibition of aIC neurons in the HFD group resulted in no memory impairment, but interestingly memory impairment was found in HFD control mice (One-sample t-test compared to 50%: SD-mCherry: t=2,776 p=0,0216; SD-Gi: t=1,419 p=0,1897, HFD-mCherry: t=0,2463 p=0,8110, HFD-Gi: t=3,422 **p=0,0076, ***Figure 18B***).
Results show that, irrespective of the injection type, animals of the HFD group highly prefer the novel object in the 24h test, while the inhibition of aIC neurons in SD mice results in an inability to distinguish between the novel and the familiar object.
Inhibition of the aIC resulted in memory impairment in SD animals but not in HFD animals in the 48h test. Interestingly, control animals of the HFD group showed worse recognition indexes compared to HFD males with inhibited anterior insula neurons.
Altogether, these findings suggest that hM4Di-mediated inactivation of the aIC does impair object recognition memory in animals on a SD in both, the 24h as well as in the 48h NOR test, suggesting an involvement of the aIC in object recognition memory. The assumption of an overactivation of the aIC in animals fed a HFD cannot be confirmed yet because the data of this thesis are too heterogeneous.

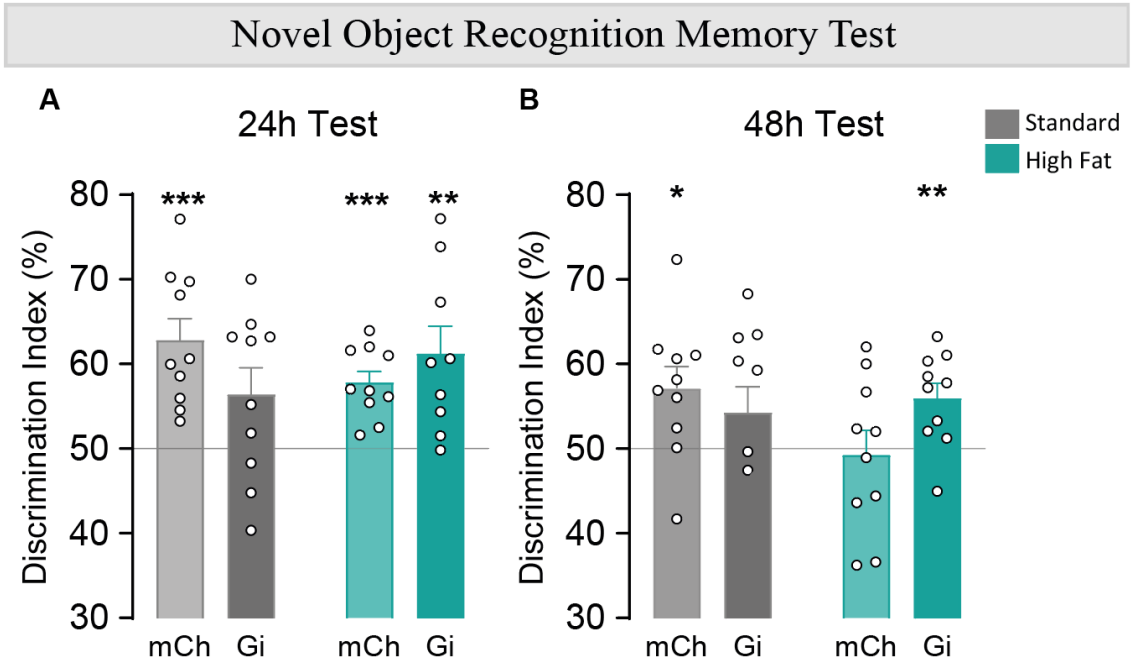

Figure 18. Chemogenetic inhibition of the aIC in 24h and 48h ORM test in mice with a SD or HFD exposure in adulthood.

Figure 18. Chemogenetic inhibition of the aIC in 24h and 48h ORM test in mice with a SD or HFD exposure in adulthood. A. Animals of all groups except the SD-Gi group discriminated the novel from the familiar object in the 24h test (One-sample t-test compared to 50%: SD-mCherry: t=5,077 ***p=0,0007; SD-Gi: t=2,072 p=0,0681, HFD-mCherry: t=5,928 ***p=0,0002, HFD-Gi: t=3,490 **p=0,0082). **B.** Mice of a SD and DREADD expression show a lower recognition index, compared to the SD-mCherry group in the 48h test. On the other hand, animals that received a HFD and had an inhibited anterior insular cortex during the ORM performance at 48h, were able to distinguish the novel from the familiar object, compared to the SD-mCherry mice (One-sample t-test compared to 50%: SD-mCherry: t=2,776 p=0,0216; SD-Gi: t=1,419 p=0,1897, HFD-mCherry: t=0,2463 p=0,8110, HFD-Gi: t=3,422 p=0,0076**). Data are represented as mean ± SEM and circles show individual data points. $p \leq 0.05$, *** $p < 0.001$ (one-sample t-test compared to chance 50%).

4.3.2 Neural activity during object recognition test

The activity of glutamatergic neurons in the anterior insular cortex was measured via fiber photometry recordings and showed a decrease in activity shortly before the start of the exploration of the left object in both diet groups. The same tendency has been shown for mice of the SD group while exploring the right object (***Figure 19B*** and ***Figure 19E***). Further, there is an immediate increase in calcium from the moment a mouse starts exploring an object in both diet groups. However, the signal from the right object in the HFD group does not show this trend as strongly (***Figure 19B*** and ***Figure 19E***). Additionally, during the exploration of the left object, there is an interaction between diet and sex (Two-

way ANOVA: sex x diet *p=0,0389, ***Figure 19C),*** which accounts for 20,75% of the total variance. The global calcium signal as well as the transient frequency per minute in the sample phase did not show any difference between the diet groups (Two-tailed paired t-test: SD n=10, p=0,2969; HFD n=11, p= 0,2415, ***Figure 19G***; SD-left object mean: 1,151; SD-right object mean: 1,048; HFD-left object mean: 1,236; HFD-right object mean: 1,147, ***Figure 19H).***

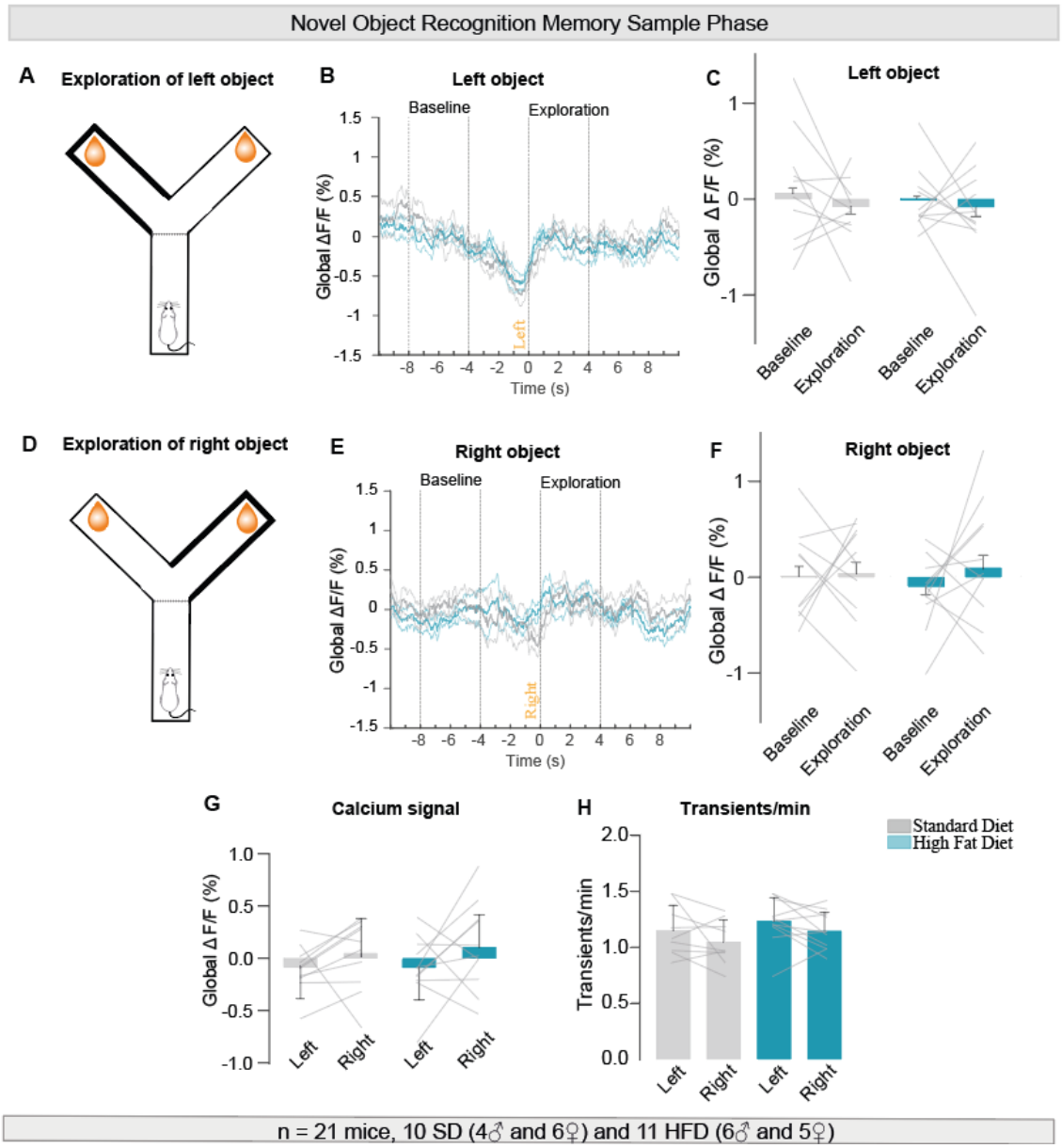

Figure 19. Neural activity in glutamatergic neurons of the aIC during sample phase of ORM task in SD and HFD mice.

Figure 19. Neural activity in glutamatergic neurons of the aIC during sample phase of ORM task in SD and HFD mice. A+D. Illustration of exploring the left and right objects in the maze. **B+E.** Temporary evolution of the calcium signal before, during and after exploration of the left and right object in the sample phase (SD n=10, HFD n=11). **C+F.** Calcium signal before (baseline) and after (exploration) the exploration of both identical objects, no difference in neuronal activity between baseline (-8 sec to -4 sec before the start of exploration and exploration (0 sec to 4 sec) in left (Two-tailed paired-t-test: SD mice n=10, p= 0,3455; HFD mice n=11, p=0,5299) and right object (Two-tailed paired-t-test: SD mice n=10, p= 0,8383; HFD mice n=11, p=0,2162) has been found. **G.** Global calcium signal during the exploration of familiar objects does not show any significant difference between SD and HFD mice (Two-tailed paired t-test: SD n=10, p=0,2969; HFD n=11, p= 0,2415). **H.** No difference in the transient frequency between the diet groups has been found (Two-tailed paired t-test: SD n=10, p=0,2268; HFD n=11, p=0,1626; SD-left object mean: 1,151; SD-right object mean: 1,048; HFD-left object mean: 1,236; HFD-right object mean: 1,147).

During the ORM test phase, the firing rate of glutamatergic neurons in the anterior insular cortex visually appears to be different when mice explore the novel, compared tp the familiar object (***Figure 20B*** *and* ***Figure 20E)***. The aIC activity while exploring the familiar object appears to be irregular over time, while a clear increase in activity can be found for animals exploring the novel object. There is no significant difference in the global calcium signal between mice fed with a SD or HFD while exploring both objects (***Figure 20C*** and ***Figure 20F***). Interestingly, the calcium signal in the SD group shows the tendency of an increase while exploring the novel object, while in the HFD group the same tendency in neural activity has been found as strongly (***Figure 20G).*** Interestingly, the calcium bulk signal during the exploration of the novel object shows an interaction between diet (SD+HFD) and sex (F+M) (Two-way ANOVA: sex x diet: 26,21% of the total variance: *p=0,0210, ***Figure 20G***). However, statistically, no significant difference has been found between baseline and exploration of both diet groups (***Figure 20C*** and ***Figure 20F***). Interestingly, the transient frequency per minute in the test did differ within the HFD group while exploring the familiar and novel object (Two-tailed paired t-test: SD n=10, p=0,6557; HFD n=11, *p=0,0318, ***Figure 20H)***.

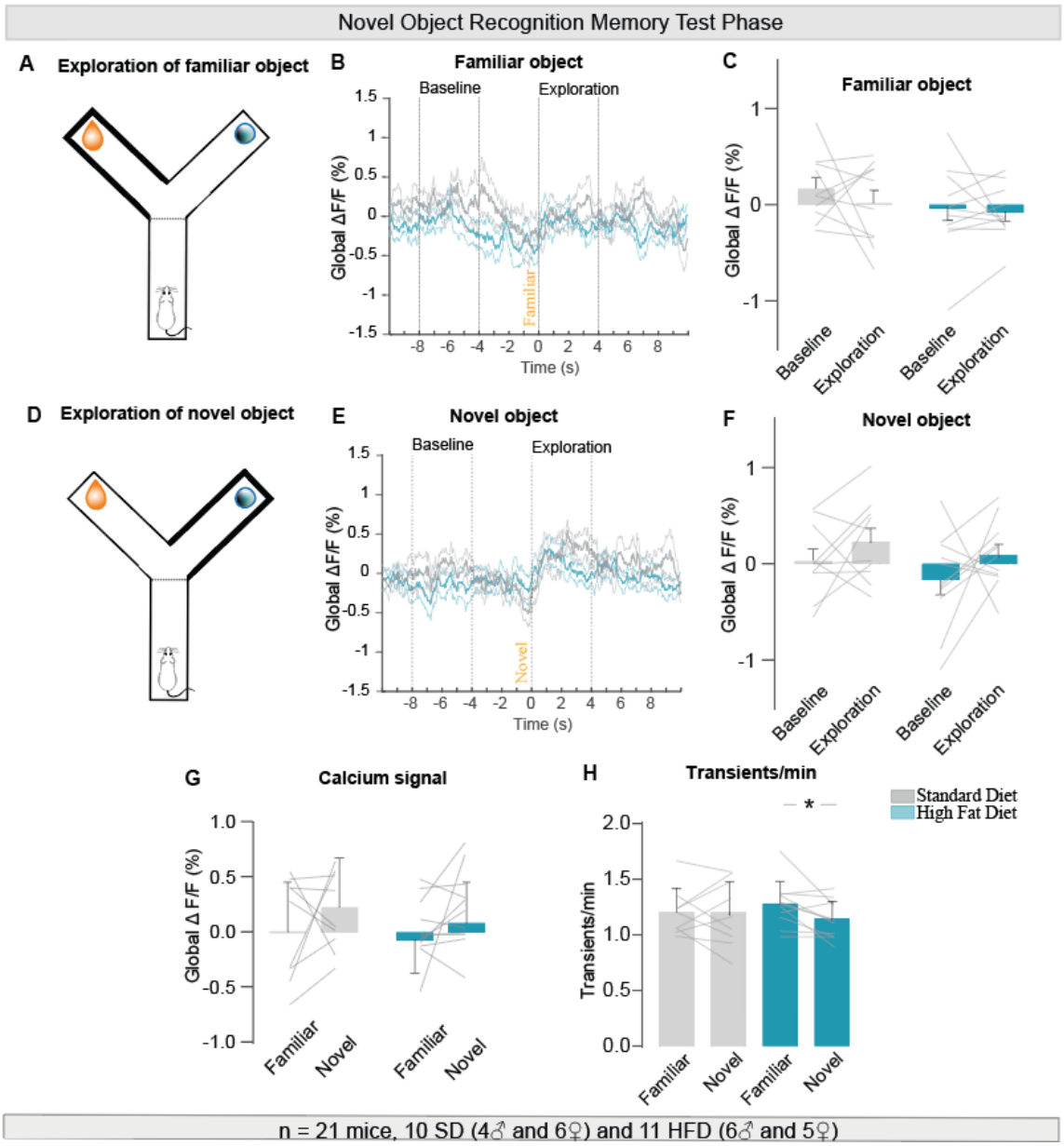

Figure 20. Neural activity in glutamatergic neurons of the aIC during the test phase of the ORM task in SD and HFD mice.

Figure 20. Neural activity in glutamatergic neurons of the aIC during the test phase of the ORM task in SD and HFD mice. A+D. Illustration of exploring the familiar and novel objects in the maze. **B+E.** Temporary evolution of the calcium signal before, during and after the exploration of the familiar and the novel object (SD n=10, HFD n=11). Baseline is -8 seconds to -4 seconds before exploring the object, exploration is from second 0 to 4 seconds. **C+F**. Calcium signal before (Baseline) and after (Exploration) the exploration of the two objects during the test; (Two-tailed paired t-test: SD n=10, p=0,4296; HFD: n=11, p=0,7380) shows no difference, when analyzed separately within each diet group for the familiar object. No difference between baseline and exploration is found for the novel object (Two-tailed paired t-test: SD n=10, p=0,2510; HFD: n=11, p=0,2244). **G.** Global calcium signal during the exploration of familiar and novel objects shows no statistically relevant difference in the neural activity in SD or HFD mice while exploring the novel object compared to the familiar object (Two-tailed paired t-test: SD n=10, p=0,2821; HFD n=11, p=0,1723). **H.** No difference in the transient frequency for the novel and familiar object in the SD group has been found, but a lower transient frequency while exploring the novel object, compared to the familiar object was calculated in HFD mice (Two-tailed t-test: SD n=10, p=0,6557; HFD n=11, *p=0,0318).

5. Discussion

In the discussion, the findings presented in the results section will be discussed and interpreted in the context of current literature, addressing potential mechanisms underlying the observed effects of a high fat diet on anxiety and object recognition memory.

5.1 Effect of high fat diet on body weight

In the experimental timeline, mice received a high fat diet for over 12 weeks and body weight (g) was measured weekly in the photometry experiment. For both, female and male mice of the photometry experiment, a diet effect was shown, meaning mice exposed to a HFD had higher percentage of body weight gain compared to mice on a standard diet (***Figure 7C***), which has statistically been proved by HFD mice having a higher body weight gain compared to the mice receiving a SD (***Figure 7B***). This same effect of diet on body weight has been described in the chemogenetic experiment 1 (***Figure 8A***) but interestingly, this diet effect has not been found in animals used for the chemogenetic experiment 2 (***Figure 8B***). However, in the chemogenetic experiment 2, sex of the animals affected body weight, meaning that males of the SD and HFD group had higher body weight in week 12, compared to females of both diet groups ***(Figure 8B***). Looking at the weight gain (%) over the experimental timeline, no difference between sexes, nor between SD- and HFD-fed mice has been found in animals of the chemogenetic experiment 2 (***Figure 8D***). Data of the collaborating team of the NutriNeuro laboratory that used only males for this experiment (chemogenetic experiment 1) found a highly different body weight **(*Figure 8A*)** and fat mass **(*Figure 8C*)** between the animals fed a standard diet compared to animals fed a high fat diet. The fat mass measurements of the male mice validate the impact of HFD on fat mass (***Figure 8C***). In the planned collaboration, the intention was to combine the data of this study with that of the collaborating laboratory. However, a notable discrepancy arose as both male and female mice were used in this

experiment named chemogenetic experiment 2 (***Figure 8B*** and ***Figure 8D***), whereas the collaborating team only employed male subjects (chemogenetic experiment 1, ***Figure 8A***). Due to the varied outcomes resulting from exposure to a high fat diet for various reasons, it was decided not to pool the data together and only utilize the collaboration's data, meaning the chemogenetic experiment 1 data set. Possible reasons for the discrepancy are outlined as follows.

Several studies have reported sex-specific differences in body weight gain between female and male animals that were fed either a SD or a HFD (71)(72). Young female mice have been described to be more protected against metabolic alterations induced by a HFD (73)(74). A recent study by Casimiro et al., 2021 found that male mice gained weight faster than females when fed a high fat diet, which is consistent with our own study's findings. While male mice are more vulnerable to weight gain and fat mass accumulation during DIO (diet induced obesity), compared to females (71), female animals were found to be more protected from the obesogenic effects of a HFD, such as diet-induced insulin resistance (72)(74). This may explain why female mice in our study, which were fed a HFD, had similar weight as SD females, as shown in the chemogenetic experiment 2 (***Figure 8B*** and ***Figure 8D)***.

Differences in obesity between sexes may be due to variations in gonadal hormone effects on metabolism. Estrogens or estradiol were found to prevent obesity, glucose intolerance, and insulin resistance in ovariectomized mice (75) and female C57BL/6 mice (76). The HFD intake has led to an increase in estrogen levels, which could be a protective mechanism, shown by the study of Shinoda et al. (2002) (77) and Fuente-Martin et al. (2013) (78) in women.

Androgens on the other hand, such as testosterone, have been found to enhance insulin sensitivity in males. HFD intake can lead to a decrease in testosterone levels in men, as documented by Cano et al. (2008) (79). This decrease can promote obesity in men, according to Seidell et al. (1990) (80). However, animal studies have shown that neither castration (81), nor androgen depletion (82) led to obesity.

Looking at the data of the fat mass of male mice (***Figure 8C***), measured via MRI of the chemogenetic experiment 1, this method is a more precise way to

determine the fat mass and obesity state of the animals. However, due to the high costs associated with the use of MRI, it is not a widely used tool in pre-clinical studies.

Instead, the measurement of leptin in the blood, could be a marker to take into account because it is closely correlated to the fat mass. Although there are differences in weight gain and fat mass of the animals used in this study over the experimental timeline, these variations will be used as a reason to continue research with both sexes, as the mechanisms involved in this sex disparity may be relevant for understanding weight gain and obesity-related diseases.

5.2 Effect of high fat diet on anxiety

This study aimed to investigate the impact of diet on the role of glutamatergic excitatory neurons of the aIC in three anxiety assays. To get closer insight, photometry recordings of 21 animals, as well as chemogenetic experiments conducted on 40 animals in total, were included in this study. Male and female animals were included, which were exposed to a certain diet (SD or HFD) for 12 weeks, beginning from adulthood starting at the age of 8 weeks. Mice were submitted to EPM, OFT, and NSFT at week 12 of diet onset.

5.2.1 Anxiety-related behavior in photometry animals

Mice assessed for photometry recordings presented higher anxiety-like behaviors in the EPM after HFD administration in adulthood which has been shown by a decrease in time spent in the open arms of the EPM (***Figure 9B***). Interestingly, being on a HFD statistically lowered the time spent in the anxiogenic spaces of the EPM only in male mice, not in females (***Figure 9C***).

Focusing on the difference found in the time spent in the center of the open field, data contradict previous bibliography, which has reported that animals on a HFD result in a higher anxiety-like behavior – tested in the open field test as well as in the Light/Dark box test – in young C57BL/6J mice (71). Another study in rats

shows that males on a HFD had increased anxiety-like behavior in the OFT, while no impact of the diet (SD or HFD) was monitored in females (83).

The novelty-suppressed feeding test has emerged as a validated method for evaluating anxiety-like behavior in rodents (84). This test involves food deprivation after which the animal is faced with the choice of approaching a piece of food placed in the center of a novel open arena, while the latency to take the first bite is monitored. Our study investigated the latency as the time in seconds of mice fed with a SD compared to a HFD in the NSFT. However, our findings revealed that there was no significant difference in the approach behavior between the two groups. These results are consistent with previous research that also found no differences in the NSFT between SD and HFD rodents after 12 weeks of HFD (85). Since there is no difference in anxiety-like behavior between the two diet groups in two out of three anxiety tests used in this study, it is difficult to conclude the significance of a trend of the HFD on anxiety-like behavior. Because the center of the open arena represents an anxiety-provoking space, HFD animals were expected to spend less time in the center, which has not been the case in our study.

This difference between results could be explained by the argument of an elevated age of the animals used in our study at the time of behavioral testing (86). Animals in our experiment were exposed to a HFD for 12 weeks before photometry recordings during the behavioral testing procedure took place. Therefore, the photometry recordings have been made of animals, 6 weeks older than animals in the study of Nicolas et al. (30). Further, it is plausible that the anxiety-like behavior observed in mice during experimentation was influenced by their exposure to stressful environments before testing. Notably, mice underwent the EPM prior to experiencing the OFT and other tests, which may have impacted the results of the OFT and NSFT.

Comparing the anxiety assays used in our study, previous research indicates that the most accurate method for evaluating anxiety-like behaviors might be through the use of the EPM. According to a comparative analysis performed in 2023, the EPM was found to be a more reliable method for quantifying anxiety levels, since this test creates a highly anxiogenic environment specifically designed for

anxiety-assays (87)(86). Moreover, previous research has tried to compare anxiety levels measured by different tests, however, no significant correlation has been discovered between the EPM and OFT. This indicates that high anxiety levels observed in one test may not necessarily indicate high anxiety levels in another test (88).

It is also important to note that sexual dimorphism has been reported in the behaviors of HFD mice in anxiety assays such as in the EPM and OFT (89). Indeed, in the study of Winberg et al., female mice receiving HFD from the age of 4 weeks for 8 weeks, exhibited increased anxiety-like behavior in the EPM, which has not been observed in male mice fed with a HFD. Furthermore, in the OFT, the HFD caused a reduction in the distance traveled and this only for male mice. Differences between male and female mice may account for the similar results in the NSF test between the SD and HFD groups. This suggests that there may be a link between obesity and anxiety that is affected by sex in rodents. However, we did not observe this difference in our study data of the photometry experiments possibly due to the small sample size of males and females in both the SD and HFD groups (11 male and 11 female mice, total n=22). However, we did find a sexual dimorphism in anxiety-like behavior in the EPM test, suggesting a higher impact of HFD on the male gender in anxiety-like behavior (males in chemogenetic experiment n=20 SD, n=20 HFD).

5.2.2 Anxiety-related behavior in chemogenetic animals

When the aIC was inhibited via chemogenetic DREADDs, results of a two-way ANOVA suggest the important role of injection type in the time spent in the open arms of the EPM, shown in ***Figure 11B***. Interestingly, post-hoc tests found that the inhibition of the aIC in HFD animals led the animals to spend more time in the open arms, the anxiogenic spaces of the maze. These findings align with the hypothesis, that an inhibition of aIC neurons would lead to a mitigation of anxiety-like behavior in animals on HFD. Here, our hypothesis is valuable only for male animals fed with a HFD from the start of adulthood. Our data is consistent with previous study results of our lab, showing that acute bilateral inhibition of the aIC

excitatory neurons restored the increase of anxiety observed in HFD-fed mice (***Figure 12A*** and ***Figure 12B)***. Other studies align with the results of this anxiety test, which found male mice were more vulnerable to the anxiogenic effects of the HFD, compared to females (83)(90).

Results of the inhibition of the anterior insular cortex in the OFT in our study found that diet changes the anxiety-related behavior in a two-way ANOVA, meaning animals on a HFD spent less time in the center of the open field, compared to animals fed a lean diet, shown in ***Figure 14B***. Our findings align with results of other studies using C57BL6 mice (91). In this test, the injection did not have any effect on the anxiety-like behavior. As previously mentioned, the EPM test is considered the most accurate method for monitoring anxiety-like behavior and should be prioritized for use in anxiety research.

Further, results of the NSFT, shown in ***Figure 16,*** found an interaction between diet and injection type on the latency to take the first bite. Interestingly, the hypothesis, that the aIC may be overactivated in animals fed with a high fat diet, has been confirmed since animals with inhibited aIC neuronal activity and on a HFD showed lower latency to eat compared to HFD control mice.

5.2.3 Photometry activity during anxiety-related behaviors

The aIC activity in the elevated plus maze test aligns with previous findings of our laboratory, showing higher activity in the anterior insular cortex in anxiogenic environments (30). Therefore, a higher activity of the aIC in the open arms of the EPM in both, SD and HFD animals, has been found (***Figure 10A***). The activity of neurons in the open arms of the maze differs between SD and HFD mice, indicating that diet affects how these anxiogenic environments are processed via the insular cortex (***Figure 10B***). These findings are very promising and confirm the idea that after HFD, mice exhibit higher activity in the anterior insular cortex in anxiogenic environments. Furthermore, a trend for a higher transient frequency has been found in the open arms of the EPM, compared to the transient frequency in the closed arms in animals on HFD (***Figure 10D***). Further, a negative correlation indicates that the more anxious an animal is, spending less

time in the open arms, the more excitatory neurons of the aIC are active in the open arms (***Figure 10E***). Data strongly suggests that the internal properties of the neurons in the aIC undergo significant changes following a HFD. Further, our findings support the hypothesis of an aIC hyperactivation after HFD exposure, presenting an alteration of tonic and phasic action potential firing. One of the researchers in the group specializes in electrophysiology and is already conducting single-unit electrophysiological recordings with the aIC samples from mice presented in this thesis, yielding promising results.

In contrast to the EPM, in the OFT, increased activity of the neurons in the center of the test in SD mice has not been found in our batch of animals, as previously found in the study of Nicolas et al. (30). However, we did observe this increased activity in HFD mice (***Figure 13C***). These findings suggest that after HFD, the aIC may be involved in the encoding of anxiogenic environments in the OFT.

Observations of this study are similar for the NSFT, as our results show no significant difference in approach behavior between the two diet groups. These results are consistent with previous research which also found no differences in the NSFT between SD and HFD rodents after 12 weeks of HFD (85) (***Figure 15A-E***).

5.3 Effect of high fat diet on object recognition memory

The aim of this study was also to investigate the impact of diet on the role of glutamatergic excitatory neurons of the aIC in object recognition memory. To investigate this, photometry recordings of 21 animals, as well as chemogenetic experiments conducted on 40 animals in total were included in this study. Male and female animals were considered which were exposed to a certain diet (SD or HFD) for 12 weeks, beginning from adulthood starting at the age of 8 weeks. Mice were submitted to novel object recognition test in Y- and V-mazes and tested in the 24h and/or 48h test.

5.3.1 Photometry mice behavior in the object recognition memory test

Results of the photometry experiment show that only the HFD group explored the novel object more than the SD group in the test (***Figure 17E***). Further, memory impairment has been found only in female mice of both, the SD and HFD groups, resulting in no discrimination between the novel and familiar objects in the test (***Figure 17D***). As an explanation of the discrepancy, first of all, studies considering both sexes are very limited by now which results in a low number of studies using female animals. This fact may explain why female animals have been described as not performing as well as males in memory tasks, particularly in studies examining spatial memory (92). Only 23,52% of articles in the preliminary search in object recognition memory tests included females (93). Further, the small sample size of a total of 21 animals, of which 11 were female in the photometry experiment, is limiting the significance of the results. For this reason, more studies including both sexes are required.

Our data are supported by studies on memory in males, which were fed a HFD for 23 weeks. Animals exhibited robust 24h ORM, but a marked impairment of the hippocampus-dependent object location memory has been reported (94). On the contrary, the study of Hayashi et al. demonstrates an impairment in the ORM test in male C57BL/6J mice which were administered to a HFD from the age of 6 weeks for 10 weeks (95). Besides the limited data on female performance in memory tasks, another possible explanation - as documented in the anxiety-assay discussion - may be the time of the diet onset and the length of diet administration. In our study, diet onset was at 8 weeks of age for 12 weeks. It has to be noted that mice used in our study were 4 weeks older and received a HFD for a longer period of time, compared to mice of Hayashi et al., which included adolescence HFD exposure. Another study on male mice fed an HFSD (high fat and sugar diet) from 3 weeks of age on, had intact short-term spatial memory in the Y-maze but impaired long-term ORM (96). These two factors may have an impact on memory since previous studies have shown that especially adolescence is a critical period for memory formation (32)(33).

5.3.2 Chemogenetic mice behavior in the object recognition memory test

This study hypothesized that the aIC may be involved in object recognition memory and may be overactivated in animals, fed with a HFD. The first point is supported by the chemogenetic results of the ORM test in animals fed a SD. Data show that mice with inhibited aIC neurons and on a SD performed worse in the ORM compared to the SD control mice without inhibition of the aIC neurons in both, the 24 hour and 48 hour test (***Figure 18A-B***). This strongly suggests an involvement of the aIC in object recognition memory.

Mice of the HFD group, with and without inhibited neuronal activity of the aIC, were able to discriminate the novel from the familiar object in the 24h test (***Figure 18A***). This is in line with the results of previous studies investigating the role of HFD exposure starting in adulthood (35). However, in the 48h test, inhibition of aIC neurons in the HFD group resulted in better performance in the NOR test compared to the HFD control group, which is not consistent with the hypothesis of this study (***Figure 18B***).

5.3.3 Photometry activity in the object recognition memory test

While no difference in neural activity while exploring the familiar objects in the sample phase was found (***Figure 19C*** and ***Figure 19F***) between SD and HFD groups, in the 24h test phase, a higher transient frequency per minute in HFD animals while exploring the familiar object, compared to the novel object was found (***Figure 20H***). These findings suggest an involvement of glutamatergic neurons in memory consolidation which is consistent with previous works in rats, showing that HFSD affects memory consolidation rather than memory encoding (97)(98)(99).

Results of a human study published in 2019 suggest that a high serum high density lipoprotein cholesterol (HDLC) level is not only associated with preserved memory function in elderly patients, but also with gyrification of the insular and frontal opercular cortex. Serum HDLC may be a biomarker for memory function and cortical structure (100). Since the high fat pellets consisted of 45% lipids consisting mostly of saturated fat from lard, including high cholesterol content could be another reason for the protection of animals fed a HFD in adulthood from memory impairments.

Further, studies show that one important plasma parameter moderately increases after the administration of a HFD – leptin concentration in the plasma (32). Interestingly, different parts of the insula do appear to be involved in different phases of processing information in recognition memory formation. A study compared the aIC, pIC and mIC and found that the mIC has been shown to send more outputs to memory-related areas, such as the entorhinal cortex and the ventral hippocampus (101). A human study on 14 patients suggest that the right insula appears to serve recognition memory by focusing on processing identical information, while the left anterior insula appears to serve conflict monitoring by focusing on processing different information, which could be explored more precisely in future works (102).

5.3.4 Impact of aIC inhibition on the object recognition test

In this study we found memory impairment in SD mice, when the aIC has been silenced, suggesting an involvement of the anterior part of the insular cortex in object recognition memory (***Figure 18A-B***). In this study, glutamatergic excitatory neurons were targeted by using specific viral vectors that had been injected into the aIC 4 weeks prior to the recordings. Interestingly, the neurotransmitter dopamine has also been described to be involved in the IC, but not in the dorsal hippocampus in object recognition memory acquisition. During the first presentation of the objects, dopamine activity was significantly reduced in old mice compared to young transgenic mice. This was correlated with enhanced beta-amyloid pathology in the IC in the study of Guzmán-Ramos et al., published in 2012 (103).

Functional interactions between the insula and the basolateral amygdala (BLA) have been described to mediate emotional arousal effects on different forms of recognition memory. Noradrenergic activation of the BLA was found to enhance the consolidation of ORM via a mechanism involving suppression of aIC activity, possibly enabling the activation of other brain networks and enhancement of higher-order cognitive processes, including long-term memory storage (104).

One part of this project is therefore to study the role of neurons of the aIC targeting the BLA in HFD-induced anxiety and object recognition memory. Data on this topic are still being analyzed.

Taken together, the findings suggest an involvement of aIC excitatory glutamatergic neurons in novel object recognition memory in animals on a SD.

5.4 Limitations

The rationale for utilising a high-fat diet (HFD) mouse model in this study was based on the comorbidity of obesity and anxiety disorders. It is important to note that although the elevated plus maze (EPM) and open field test (OFT) are the most commonly employed anxiety assays, they are not representative of pathological anxiety. Rather, they are designed to model the physiological range of anxiety. Nevertheless, to study models of pathological anxiety, different paradigms can be used, such as innate behaviors and responses to certain stimuli (mild electric foot shock) or learned responses including the Vogel conflict test. These models are mostly used to study the effects of anxiolytic drugs on learned fear responses such as psychological stress models based on stressors, which induce anxiety-like behavior by forced swimming, restraint, or exposure to elevated platforms (105). Since preclinical studies often rely on animal models, in this study on mice, the model using ORM provides valuable insights but may not fully capture the complexity of human cognition and memory processes. Thus, findings from this animal study may not always directly translate to humans.

6. Conclusion

In summary, this study provides valuable insights into the intricate relationship between obesity, anxiety disorders, and memory impairment, with a focus on the aIC. Experiments in this study uncover the involvement of excitatory glutamatergic neurons within the aIC in mediating HFD-induced anxiety. In the anxiogenic spaces of the EPM, a higher neural activity in the open arms of the maze has been shown in animals on a HFD. However, weight gain resulting from HFD has had a different impact on male and female animals, which should be further explored in future research on both sexes. Furthermore, results of this study indicate that the aIC may be involved in the consolidation of object recognition memory, with the aIC playing a particularly interesting role in animals fed with a SD. Our results suggest that the onset of HFD in adulthood may not profoundly affect object recognition memory, underscoring the complexity of diet-brain interactions regarding the timing of diet intervention studies. In closing, this research lays the groundwork for future studies aimed at unraveling the interplay between diet, brain function, and behavior, paving the way for novel therapeutic interventions targeting obesity-related cognitive impairments and anxiety disorders.

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Appendix

Abstract (German)

Fettleibigkeit hat sich weltweit zu einem großen Problem der öffentlichen Gesundheit entwickelt. Besonders in der Europäischen Region war in den letzten Jahrzehnten ein besorgniserregender Anstieg der Adipositasrate zu verzeichnen. Die schädlichen Auswirkungen von Fettleibigkeit gehen über die körperliche Gesundheit hinaus, wobei Zusammenhänge zwischen Übergewicht und emotionalen Störungen sowie Defiziten in der Gedächtnisfunktion festgestellt werden. Von besonderem Interesse in dieser Studie ist der vordere Inselcortex (aIC), eine Gehirnregion, welche an verschiedenen Funktionen wie Interozeption, Geschmack, Angst und Gedächtnis beteiligt ist. In dieser Forschungsarbeit werden die Auswirkungen einer im Erwachsenenalter begonnenen fettreichen Ernährung (HFD) auf Angstzustände und das Objekterkennungsgedächtnis untersucht. Fortschrittliche Techniken wie chemogenetische Manipulation und Faserphotometrie werden eingesetzt, um die zugrunde liegenden neuronalen Mechanismen in der vorderen Inselrinde zu untersuchen. Interessanterweise zeigen unsere Ergebnisse, dass eine fettreiche Ernährung angstähnliches Verhalten hervorruft und signifikant mit glutamatergen Neuronen innerhalb des vorderen Inselcortex assoziiert ist. Die Hemmung der aIC-Neuronen hebt den ängstlichen Phänotyp von Tieren auf, welche eine fettreiche Nahrung zu sich nehmen. Das Objekterkennungsgedächtnis bleibt bei Mäusen mit einer fettreichen Ernährung im Vergleich zu Mäusen einer Standarddiät (SD) weitgehend unbeeinflusst. Ergebnisse der chemogenetischen Untersuchungen von Tieren, welche eine Standarddiät bekamen, bestätigen die Relevanz des vorderen Inselcortex für das Objekterkennungsgedächtnis. Die Hemmung der neuronalen Aktivität dieser Gehirnregion führte in Mäusen mit einer Standarddiät im Objekterkennungstest zu Gedächtniseinschränkung. Dies unterstreicht, wie wichtig es ist, bei Studien zu ernährungsbedingten kognitiven Auswirkungen den Zeitpunkt der Ernährungsintervention zu berücksichtigen. Insgesamt liefern diese Ergebnisse wertvolle Einblicke in die komplexe Beziehung zwischen Ernährung,

Gehirnfunktion und Verhalten und tragen zu einem tieferen Verständnis der Auswirkungen einer fettreichen Ernährung auf Angstzustände und Gedächtnisfunktion bei. Zukünftige Forschungsarbeiten sollten die spezifischen Mechanismen, durch die Adipositas die neuronalen Schaltkreise in der Inselrinde beeinflusst, genauer untersuchen. Darüber hinaus sind klinische Studien erforderlich, um die translationale Relevanz unserer Ergebnisse zu untersuchen und möglicherweise den Weg für gezielte Interventionen zu ebnen, um die kognitiven und emotionalen Folgen von HFD-induzierter Adipositas zu lindern.