Mapping of hypothalamic defensive survival circuits using optogenetics and designer receptors

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This project was conducted at the Program for Physiology and Neurobiology at the Department of Biosciences, University of Oslo, between January 2014 and December 2015, under the supervision of associate professor Marianne Fyhn, associate professor Torkel Hafting, and postdoctoral fellow Mattis B. Wigestrand.

Firstly, I would like to express my deepest gratitude to Marianne for allowing me to be a part of this very unique and progressive research group and never failing to inspire me with her genuine devotion and passion for neuroscience. I would like to thank all the members of the group for creating a great work environment and always being around to answer questions, help, or share humorous stories about laboratory bloopers.

A special thanks to the dependable Mattis B. Wigestrand for putting up with what must be at least one centillion questions and for being my main go-to for these past two years.

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I would like to thank my father, Michael Tully, for lending me his Illustrator and Photoshop skills and helping me create beautiful illustrations for my thesis. My feeble attempts using Microsoft Paint were absolutely horrendous.

Lastly, I would like to thank my partner, Magnus, for being my rock and supporting me ever since I started my bachelor.

INTRODUCTION: Defensive behaviours are vital for survival. Animals portray a range of context specific defensive behaviours when faced with a threat. Previous studies have shown that electrical stimulation of the hypothalamus induces aggressive and defensive behaviours, but the exact cell type or neural connections that mediate these effects are unknown. In particular, electrical stimulation and lesion based approaches may activate or destroy axonal projections through the hypothalamus, thus complicating interpretation of these previous experiments. To further investigate the role of the hypothalamic regions in orchestrating defensive behaviours, we have attempted to use targeted and temporarily restricted manipulations in the lateral hypothalamic area (LHA).

METHODS: To investigate whether activation of LHA itself is *sufficient* to elicit defensive behaviours, we first activated distinct sub-regions of the LHA with optogenetics. Next, a chemogenetic approach using designer receptors exclusively activated by designer drugs (DREADD) was used to silence the LHA in order to investigate if LHA activity is *necessary* to elicit defensive responses in a looming shadow experiment. This setup is designed to induce visually stimulated innate fear specifically. Furthermore, to investigate whether the LHA is involved in learned fear, we performed DREADD silencing of the LHA during contextual fear memory expression.

RESULTS: The experiments showed that optogenetic activation of neurons in the LHA was sufficient to elicit a range of behaviours, including feeding, escape and freezing. Activation of the ventral area of the LHA elicited robust freezing, and activation of this sub-region did not elicit feeding or escape. DREADD silencing of LHA during looming shadow stimuli resulted in a drastic reduction of sustained, but not immediate freezing. DREADD silencing of LHA during contextual fear memory expression did not result in any significant difference in freezing.

CONCLUSIONS: Our experiments showed that LHA activation is sufficient to elicit innate fear responses. Further, silencing of the LHA during looming shadow exposure resulted in a significant decrease in sustained freezing, suggesting that the LHA regulates fear responses during exposure to a visual threat. Surprisingly, DREADD experiments showed that silencing of the LHA did not alter learned fear response, suggesting that separate circuits govern innate and learned fear.

Preface

This project is in collaboration with postdoctoral fellow Mattis B. Wigestrand who currently uses optogenetics to evoke defensive behaviours via activation of distinct anatomical outputs from the LHA. In order to maintain consistency during this project we shared the duties between us. I conducted all surgical procedures and injections of viral vectors, as well as euthanasia and perfusion of the animals. Wigestrand did the behavioural testing of the animals using optic and looming stimulation, fear conditioning, and most of the cryostatic brain sectioning. Both of us were involved in analyzing data, including scoring of behaviours, image analyses, figures and statistical analyses.

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1 Introduction

1.1 Behaviour and survival

Successful survival is closely related to the ability to express appropriate behaviour in certain situations. Defensive and aggressive behaviours, some of which are referred to as the fight-or-flight response, a term first described by Walter Bradford Cannon (1916), is an acute stress response induced by potential harmful events or threats. The theory states that threat to an animal will induce an activation of the sympathetic nervous system, resulting in the secretion of catecholamines such as adrenaline and noradrenaline (Jansen et al., 1995; Landis, 1930). This response is extremely fast acting and swiftly induces behavioural and physiological changes which may increase the likelihood of survival. Rats portray a series of observable defensive behaviours associated with threat, including freezing, defensive upright positions, backing, and defensive alerting (Dielenberg et al., 2001). Freezing is a defensive response seen in prey animals when confronted with a predator, feigning death as an attempt to stop the predatory attack.

In some prey animals, the fight or flight response can more appropriately be referred to as the fight, flight or freeze response (Figure 1).

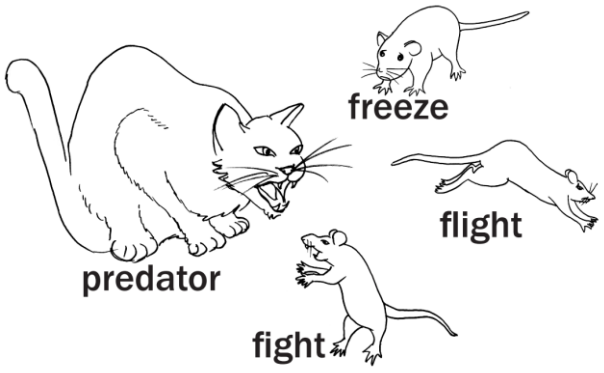

Figure 1: The defensive trio: The three main defensive responses seen in rodents when approached by a predator (Inspired by an illustration from the book *"Anxious"* by LeDoux (2015))

These three defensive behaviours are context dependent and initiated in accordance with predatory imminence; meaning proximity to the predator.

1.1.1 Predatory imminence continuum

The predatory imminence continuum describes how certain behaviour will occur as a predator approaches the prey (Figure 2).

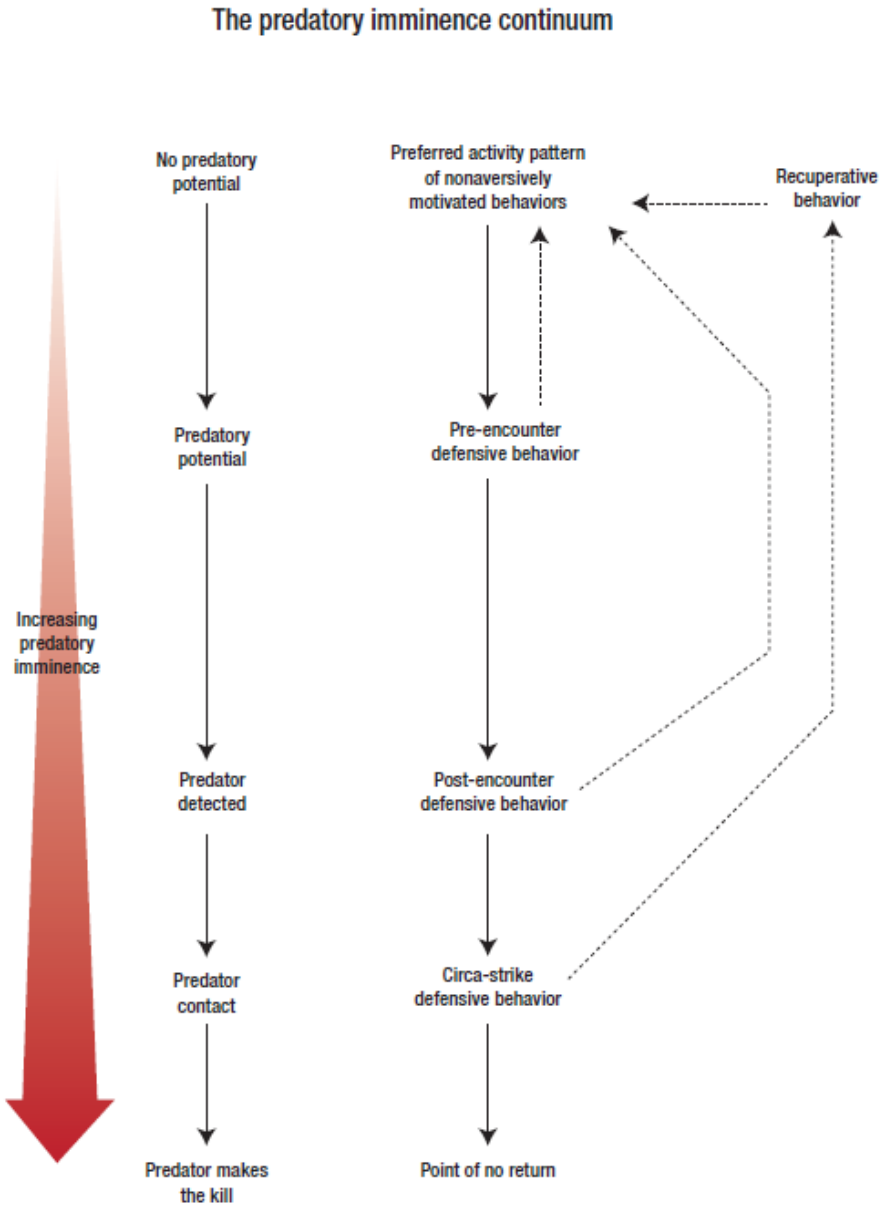

Figure 2: A schematic representation of the predatory imminence continuum (Adapted from Fanselow et al. (1988a)).

At low predatory potential and thus low threat, animals will engage in foraging, eating, mating, and nursing; activities collectively referred to as the **preferred activity pattern** (Fanselow et al., 1988a). As the potential for a predatory occurrence increases, animals express pre-encounter defensive behaviour, behaviour ultimately aimed at returning to the preferred activity pattern. These defensive behaviours have likely developed through evolutionary selection, which keeps the prey as close to no predatory potential on the predatory imminence continuum as possible, ensuring sufficient eating and mating and thus increasing overall fitness.

Each step down the continuum has different levels of threat and different environmental cues. The hypothesis states that each step on the scale has a selection of defensive behaviour patterns adapted to handle the particular level of threat. Any time spent away from the nest increases the likelihood of encountering a predator. Animals may change foraging patterns; e.g. time of day, frequency and area of foraging, to reduce the likelihood of encountering a predator. This is an example of pre-encounter defensive behaviour, where the degree of foraging modification is proportional to the likelihood of encountering a predator (Fanselow et al., 1988a).

Studies on pre-encounter defensive behaviour have been conducted using a foot shock obstacle to obtain food (Fanselow et al., 1988b). This showed decreased foraging frequency, while total caloric intake was almost unchanged. The increase in food intake for each session in response to the foot shock session is an example of pre-encounter defensive behaviour.

Post-encounter defensive behaviours may be more pronounced. Placing a cat in vicinity of a food deprived rat drinking a sweet sucrose solution caused the rat to immediately cease drinking, and engage in freezing behaviour; a response eliminated upon removal of the olfactory bulb (Mollenauer et al., 1974). Ultimately, rodents engaging in the freezing response are possibly more likely to survive predatory encounters than those who do not freeze. The freezing response is easy to measure experimentally, and is used to measure the strength of fear memories in the Pavlovian fear conditioning paradigm (Maren, 2001).

1.2 The brain and circuitry

The vertebrate brain consists of two main kinds of cells; neurons and glial cells.

While all cells in the body have means of communication, mostly through endocrine and paracrine signalling, neurons communicate by an action potential being initiated at the cell somata and travels down the axons, resulting in release of neurotransmitters from the nerve terminal and into the synaptic cleft. In the synapse, the electrical signal of the action potential is transformed to a chemical signal of the neurotransmitter. The neurotransmitter binds to receptors on the post-synaptic membrane and induces a change of membrane potential. (Figure 3).

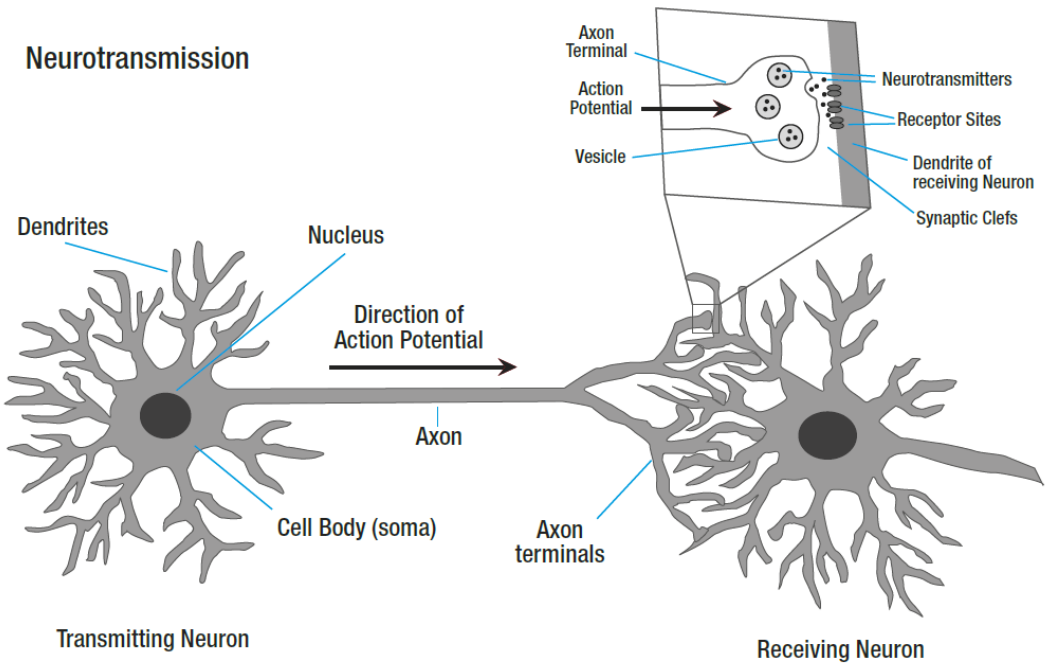

Figure 3: Two connecting neurons, and a closeup of the synaptic cleft between the transmitting neuron and receiving neuron (Adapted from LeDoux (2015)).

Neurotransmitter release in the synaptic cleft, between the pre-synaptic and the post-synaptic neuron, results in opening and activation of channels and receptors on the post-synaptic side. Several types of neurons exist with distinct morphological traits, and they can function as either excitatory or inhibitory cells. Excitatory and inhibitory neurons are mainly glutamatergic or GABAergic (Gamma-Aminobutyric acid) neurons, respectively. Two classes of GABA receptors are found on GABAergic neurons; $GABA_A$ receptors function as ligand-

gated ion channels, causing a rapid efflux of negative chloride ions and subsequent hyperpolarization of the cell, while $GABA_B$ receptors are metabotropic receptors linked via G-proteins to potassium channels (Kandel et al., 2000). The potassium efflux also causes a hyperpolarization. These different cell types collectively form a complex network of cells with distinct firing patterns.

Interconnections between cells in specific areas or subregions of the brain form local circuits or networks. These local circuits, in turn, can interconnect between other circuits in other subareas or areas of the brain, forming large complex neural networks in the brain (Figure 4).

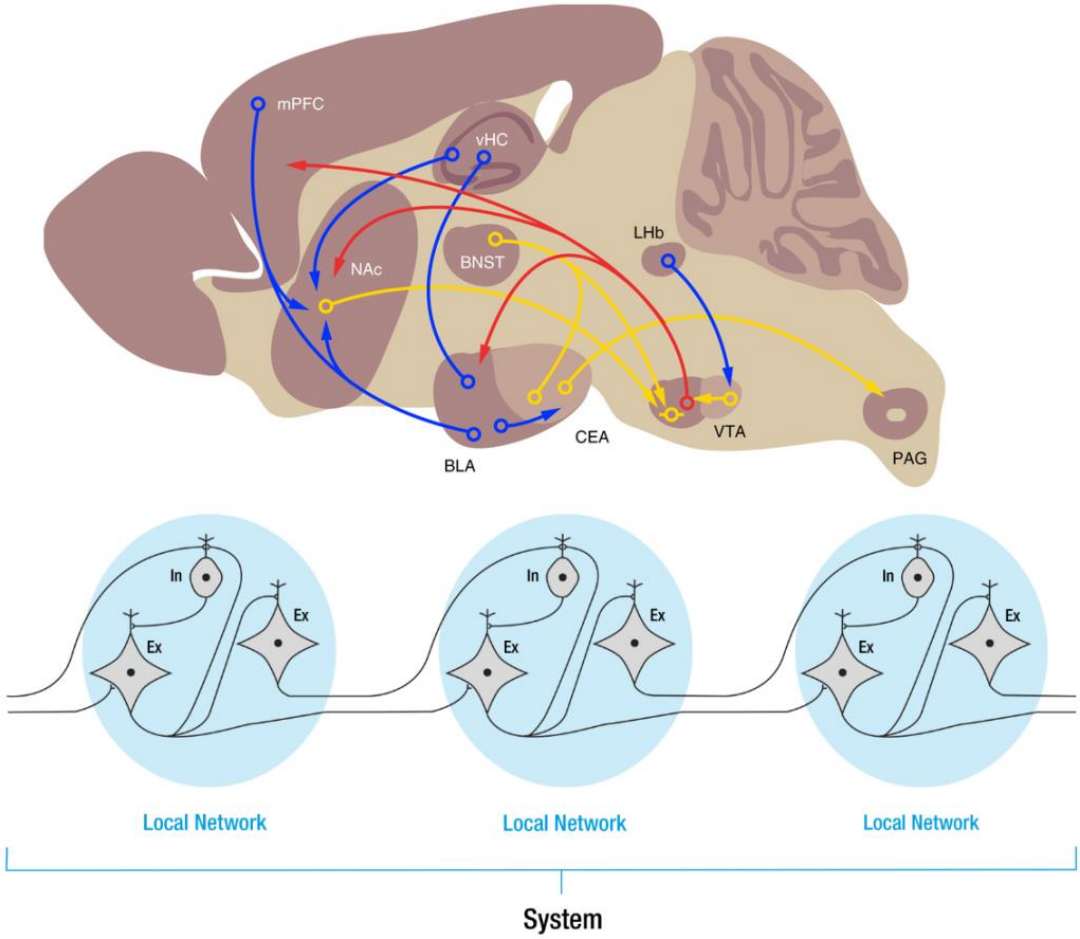

Figure 4: An example of networks within the rat brain, from a sagittal view. This shows known interconnections between the medial prefrontal cortex (mPFC) the basolateral amygdala (BLA) and nucleus accumbens (NAc). We can also see interconnections between the bed nucleus of the stria terminalis (BNST) interconnecting with both the central amygdala (CEA) and ventral tegmental area (VTA), which in turn is interconnected with the lateral habenula (Lhb). CEA also shows a pathway to the periaqueductal gray (PAG). Although not shown in this image, both the BLA, CEA, BNST, LHb and PAG have strong interconnections to the hypothalamus (Luthi et al., 2014). Below is a simplified illustration of what networks look like at a cellular level (Modified from LeDoux (2015)).

The vertebrate brain consists of three major zones, the forebrain, midbrain and hindbrain. The hindbrain is vital for primary functions such as breathing and maintaining a stable heartbeat, and damage to this region often lethal.

The midbrain, which along with the hindbrain is considered a part of the brainstem, is important for wakefulness, motor function and sleep pattern.

Between mammalian species, the largest variation lies in the forebrain. The forebrain consists of cortical areas, namely the neocortex and allocortex. These cortices process information from structures residing in the deeper and evolutionary older regions of the brain. Advanced species, such as primates, have a neocortex with a larger volume and surface area, resulting in grooves and wrinkles on the outer layer of the brain. This large neocortex likely accounts for the superior cognitive abilities observed in primate species.

Below the cortical areas lie the subcortical areas, consisting of the amygdala, basal ganglia, thalamus and hypothalamus. Within the subcortical midbrain resides the periaqueductal gray (PAG), a control centre for descending pain modulation in addition to being known to elicit certain defensive behaviours, and a group of nuclei involved in arousal (LeDoux, 2015).

While anatomical tracing has found several of these regions to be highly interconnected, the function of these interconnections as well as the cell types involved remains largely unresolved.

1.2.1 Brain circuits for defensive behaviours

It was early recognized that defensive behaviours and rage depend on the subcortical areas and function after extensive damage to the neocortex. Walter Cannon (1925) believed that conscious rage could not be experienced without the neocortex, and coined the term “sham rage” to describe the defensive and aggressive behaviours often towards non-threatening stimuli observed in animals with damage to the neocortex, suggesting the neocortex acts as an inhibitor for these subcortical areas.

Sympathetic activation such as increased heartbeat, increased blood pressure, sweating, and adrenaline release also occurs during sham rage (Kennard et al., 1947).

In the early 20th century, a similar sympathetic activation was seen during crude and explorative electrical stimulation in the brain. Electrical stimulation was the earliest attempt to control brain function, the rationale being that the brain functions by sending electrical impulses (Karplus et al., 1909).

By using this method of electrical stimulation in anaesthetized animals, the hypothalamus was identified as a key area involved in the activation of the sympathetic nervous system (Karplus et al., 1909). Furthermore, lesions that disconnected the hypothalamus from the lower areas in the midbrain and hindbrain removed defensive rage behaviours, confirming the hypothesis that the hypothalamus is vital for defensive and rage behaviours during threat (Bard et al., 1937). Based on these findings, it was hypothesized that the hypothalamus is an important area responsible for defensive behaviour and physiological responses during danger and threat. Later studies have also found that small lesions in the hypothalamus reduce sham rage (Savard et al., 2003).
Today, it is now known that the hypothalamus is a complex structure controlling many different behaviours (Canteras, 2012). Still, the underlying mechanisms of this circuitry remain elusive.

1.2.2 The hypothalamus

Structure and function

The hypothalamus is an ancient forebrain region that contains multiple molecularly distinct cell types with unique anatomical projection patterns, but the mechanisms of these distinct hypothalamic circuit elements, is largely unknown (Swanson, 2000).

The hypothalamic nuclei are also involved in regulation of hormones and is an important part of the endocrine system (Armstrong, 2004). In Figure 5 the anatomy of the hypothalamus in a rat brain is depicted.

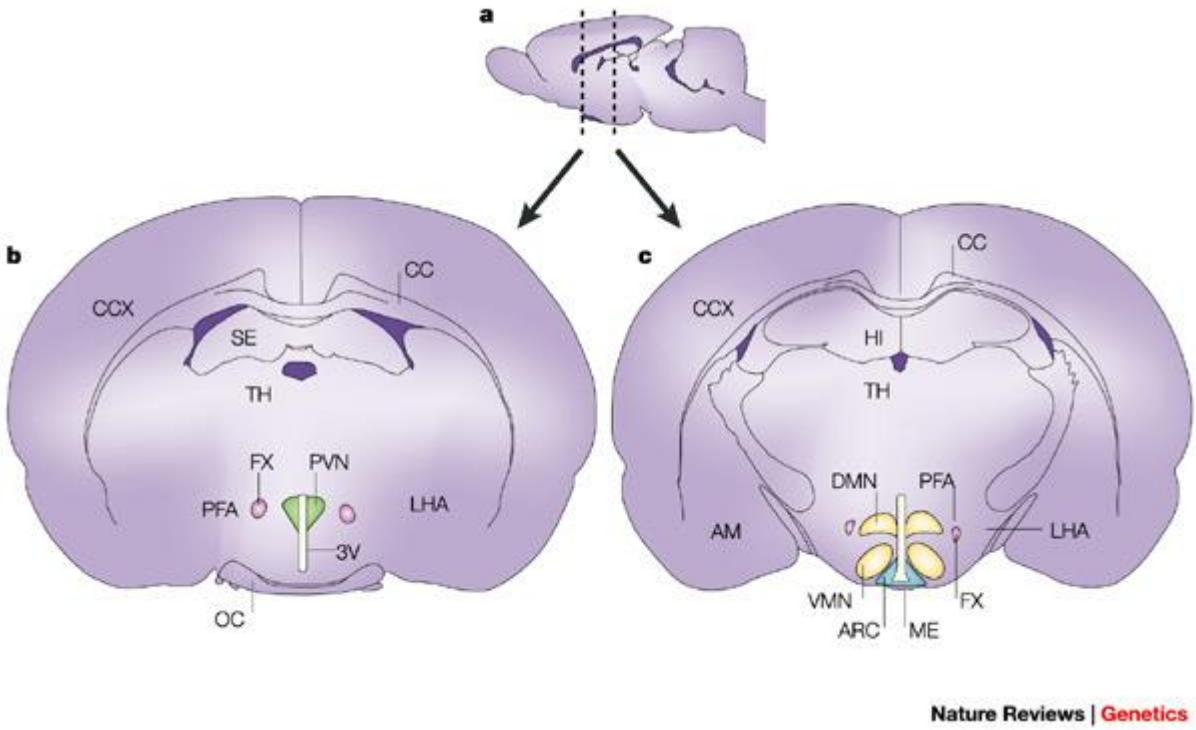

Figure 5: A) Sagittal view of a rat brain with lines indicating section areas for b) and c). B) A coronal section showing the hypothalamus and some of its regions; the fornix (FX), the perifornical area (PFA) and the lateral hypothalamic area (LHA). C) A more posterior coronal section showing the hypothalamus and some of its regions; the ventromedial hypothalamic nuclei (VMN), the dorsomedial hypothalamic nuclei (DMN), the LHA and the PFA (Barsh et al., 2002).

The hypothalamus consists of four main rostrocaudal regions; the preoptic, anterior, tuberal and mammillary regions. It is further divided in to three longitudinal zones; the periventricular, medial and lateral zones.

The lack of knowledge about form and behavioural function in the hypothalamus is in part related to technical limitations when aiming to target specific cell types and methodological problems that arise due to diffuse fibre systems that traverse this region. Electrical stimulation of the hypothalamus induces a range of defensive and offensive behaviours also observed when an animal is presented with a threat (Lammers et al., 1988; Yardley et al., 1986). However, electrical stimulation it is difficult to confine to small regions. Thus it has not previously been possible to locate the exact hypothalamic region or cell type involved in distinct behaviours.

Importantly, different cell groups in the hypothalamus have neural pathways that connect to different parts of the brain, including habenula and PAG (Oh et al. (2014); http://connectivity.brain-map.org/). The exact function of the different outputs from the hypothalamus and how they interconnect is largely unknown.

1.2.3 The lateral hypothalamic area and defensive behaviours

The medial defence zone of the hypothalamus has long been considered to be important for eliciting defensive behaviours. Following neuron depolarization, c-Fos, a proto-oncogene, is often expressed. The c-Fos protein can be immunohistochemically stained, and is a reliable marker for neuronal activity (Bullitt, 1990; Dragunow et al., 1989). Due to its rapid expression following stimulation it is referred to as an immediate early gene (Hu et al., 1994). Exposure to predator odours is known to result in an increase in c-Fos markers in the VMH and DMH as well as the PAG (Dielenberg et al., 2001).
However, some studies have found the perifornical region of the LHA (LHApf) to be important for initiating defensive behaviour during electrical stimulation (Hess et al., 1943). Hunsperger (1956) supported these findings when attempting to electrically stimulate a pathway including the amygdala, *stria terminalis* (ST), bed nucleus of the *stria terminalis* (BNST), LHApf and PAG; all of which elicit defensive behaviours upon electrical stimulation. Furthermore, actual exposure to a predator results in a significant c-Fos increase in the subfornical region of the LHA, confirming the importance of this region in regard to defensive behaviours (Motta et al., 2009).

Despite the evidence that the LHA orchestrates defensive behaviours, most studies related to defensive behaviours are limited to the medial defence zone.

The hypothalamus coordinates a range of behaviours, including ingesting behaviours. The LHA has been of interest regarding ingesting behaviour, perhaps governing feelings of hunger and satiety (Jennings et al., 2013).

The LHA is highly interconnected with the PAG, amygdala and habenula; all of which are known to be areas important in defensive behaviours (Hahn et al., 2015) (Brandão et al., 2008; Pobbe et al., 2010). In addition, the LHA has robust connections with the hypothalamic medial zone nuclei (Anterior hypothalamic nucleus (AHN), ventromedial hypothalamic nucleus (VMHdm), dorsal premammillary nucleus (PMd)), all of which have reported to be involved in fight-flight-freeze behaviour (Canteras, 2002; Canteras et al., 1997; Risold, 2004).

The evidence from electrical stimulation, c-Fos and anatomical tracing experiments suggests that the LHA is indeed important for the control of defensive behaviours. However, which cell types, regions or inputs/outputs from the LHA are important remain largely unknown, and much remains unresolved regarding the complex interconnectivity between regions.

Within the last 10 years, techniques like optogenetics have been developed which allow cell specific and regional and temporarily restricted manipulations, enabling the elucidation of the function of specific sub regions (Zemelman et al., 2002). Anatomical tracing has also shown to be useful for tracking neural pathways interconnecting different regions of the brain.

1.3 Optogenetics

A notion throughout the history of neuroscience has been that if we could record the activity of *all* neurons, we could understand the brain (Miesenböck, 2010).

However, even if we did record every single cell activity in the entire brain, we do not know how to entirely understand its pattern.

By manipulating and controlling the function of the brain rather than recording it, we may be able to further understand its functions by using a reverse engineering approach, simply by activating circuits and cells and observing their specific outcome.

Optogenetics, pioneered by Zemelman and Miesenböck (2002) and further developed by Boyden and co-workers (2005), is a technique that allows precise temporal and spatial dissection of cell function in the brain using direct manipulation of targeted neuronal activity.

1.3.1 The role of rhodopsins in optogenetics

Optogenetics was possible due to the discovery and cloning of channelrhodopsins (ChR) and halorhodopsins (HR) (Figure 6), proteins that function as light-gated ion channels. ChR is a light activated protein naturally found in green algae, where it enables movement in response to and towards light in a process called *phototaxis* (Sineshchekov et al., 2002).
HR on the other hand, is a microbial light-driven pump used by halobacteria to maintain the osmotic balance by pumping chloride in to the cytoplasm, maintaining osmotic balance during cell growth. This creates a hyperpolarizing current when introduced in to a neuron, inhibiting neuron activity.

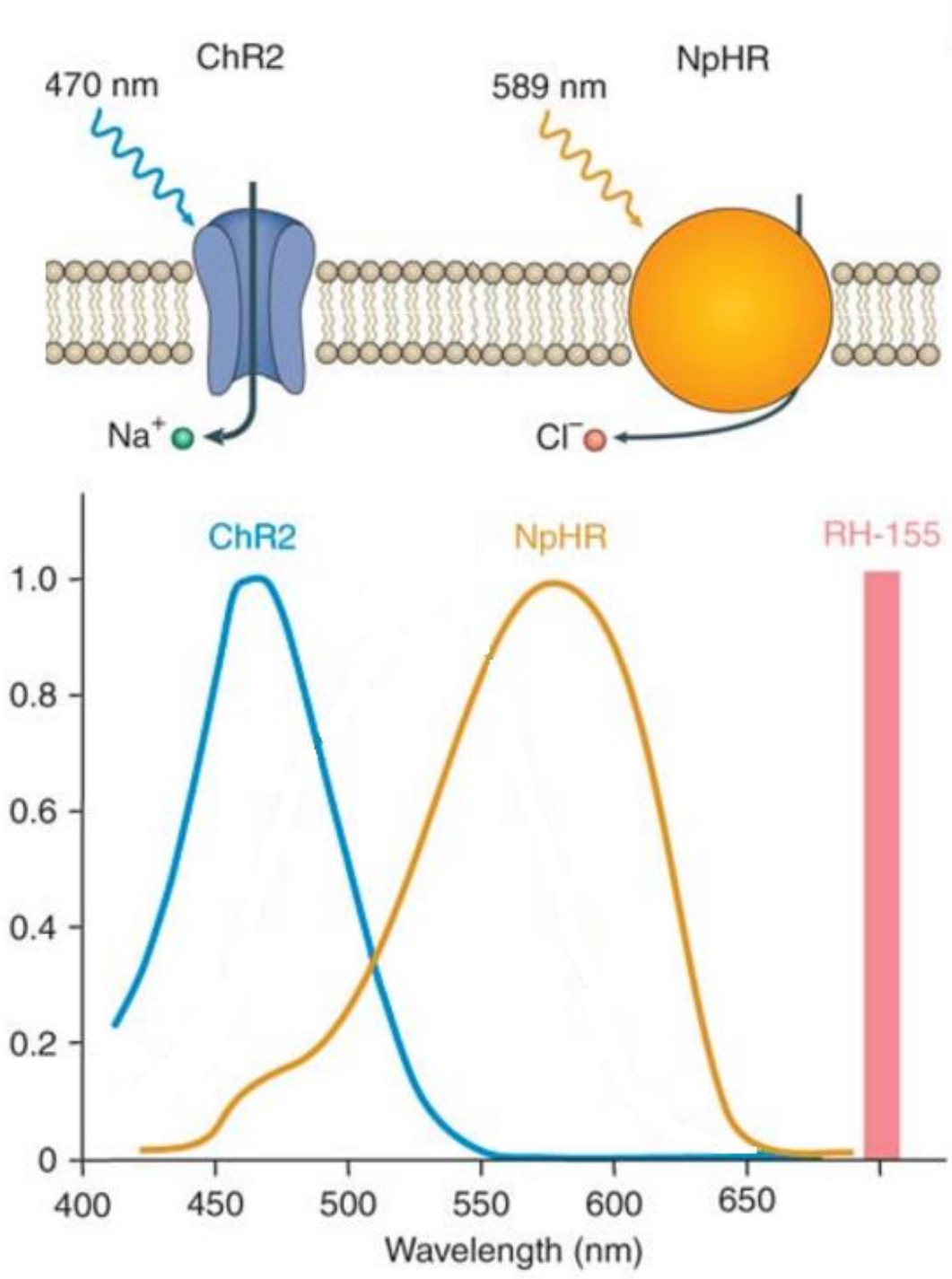

Figure 6: ChR2 embedded in the cell membrane. Illumination of blue light on the protein causes a conformational change which allows a flow of sodium ions through the membrane. Natronomonas pharaonis Halorhodopsin (NpHR) functions as light activated pump, actively causing an influx of chloride ions in to the cell and results in hyperpolarization (Modified from Zhang et al. (2010)).

By incorporating these light-activated proteins in to the neurons, their activity can be controlled using light pulses of specific wave lengths. Several designer channelrhodopsins and halorhodopsins exist today, allowing for both inhibition and activation, as well as using custom wavelengths (Hooks et al., 2015; Lin et al., 2013).

To express these proteins in neurons, one approach is to use viral vectors e.g. adeno associated viral vectors (AAV) carrying genes for ChR/HR.

1.4 Designer Receptors Exclusively Activated by Designer Drugs (DREADD)

Designer Receptors Exclusively Activated by Designer Drugs (DREADD), developed by Armbruster and co-workers (2007), is a novel and relatively non-invasive pharmacogenetic technique to control the activity of neurons in the brain. While optogenetics allow an instant on/off switch for neurons, these sudden and often synchronous changes in cell activity produce non-physiological activity patterns. Pharmacogenetics allows for more suble manipulations of neural activity.

The DREADD system relies on engineered G-protein coupled receptors. The receptors are modified to react solely to a molecule of choice which is otherwise inert and non-reactive. A commonplace molecule of choice has been Clozapine-N-oxide (CNO), an inactive metabolite from clozapine (an antipsychotic drug). Several modified receptors have been developed, allowing for both silencing and activation of neurons (Figure 7).

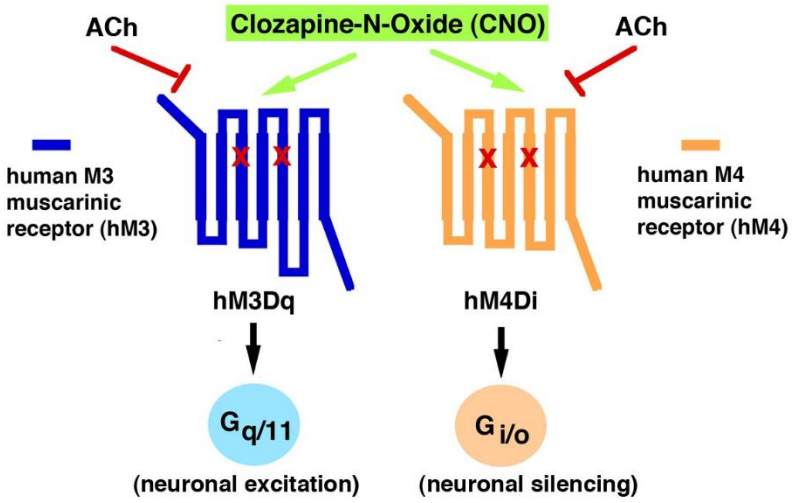

Figure 7: A human M3 and M4 muscarinic receptor (hM3 and hM4 respectively), resulting in neuronal excitation and silencing. These muscarinic receptors have Y3.33C and A5.46G point mutations in TM3 and TM5, leaving them unbound and unaffected by acetylcholine (niddk.nih.gov).

All receptors have Y3.33C and A5.46G point mutations in TM3 and TM5, leaving them unbound and unaffected by acetylcholine, and subsequently allowing for the binding of CNO.

The effects of activating G-protein coupled receptors are slow; mimicking the endogenous neural modulations of modulatory neurotransmitter systems acting on G-protein coupled receptors. DREADD also allows targeting of larger brain regions, compared to optogenetics that relies on illumination through tissue.

The chemogenetic method has shown similar reliability in its ability to silence and activate transduced neurons (Alexander et al., 2009; Armbruster et al., 2007; Dong et al., 2010; Rogan et al., 2011) although the effect is usually not complete. Just as for optogenetics, viral vectors can be used for efficient and targeted expression of the modified receptors.

1.5 Aims of the study

The main objective of this study is to reveal the role of LHA in defensive behaviour. In particular, we addressed the following questions:

a) Does optogenetic stimulation of specific LHA sub regions elicit distinct defensive behaviours?

b) Does chemogenetic silencing of LHA disrupt defensive behaviours to a perceived innate predatory threat?

c) Does chemogenetic silencing of LHA disrupt defensive behaviours to learned threat stimuli?

2 Materials and methods

2.1 Animals

All experiments were approved by the Norwegian Animal Research Committee (FDU) prior to project initiation. All experiments were approved by the Norwegian Animal Research Committee (FDU) prior to initiation. The housing and treatment of animals satisfy the criteria set by the European Union and the FDU. All participants in this project hold an animal researcher certificate, as required by the Norwegian Food Safety Authority (Mattilsynet).

All experiments were conducted using adult male Sprague Dawley rats, ordered through the Norwegian Animal Research Centre (NFS) at the Norwegian Institute of Public Health and Taconic Biosciences, Inc. (Denmark). Upon arrival their weight was approximately 250 grams. The animals were acclimated to their new environment for one week before any experiments were started. A total of 54 adult male Sprague Dawley rats were used for this project. All animals were housed on a 12-hour light/dark cycle, with lights on from 20.00. Housing temperature was kept at 21 °C, with a humidity level of 55 ± 10 %. The rooms have a ventilation rate of 5-20 times per hour. All animals were housed four individuals in a Plexiglas cage (35x55x30 cm) with woodchip bedding from Scanbur A/S. They were given ad libitum access to water and food and plastic tubes were placed in the cage as toys.

2.2 Surgical procedures

2.2.1 Anaesthesia and preparation

Rats were temporarily sedated using isoflurane to prevent distress during an intraperitoneal injection of a mixture of Ketalar (ketamine, a dissociative analgesic, 75 mg/kg) and Domitor (dexmedetomidine, a sedative α_2-adrenoreceptor agonist, 0.75 mg/kg). To ensure full anaesthesia, hind leg reflexes were tested using the toe pinching method, and cornea reflexes were tested by gently touching the eye area with a sterile autoclaved Q-tip.

Hair was removed using an electric hair shaver starting from just posterior to the upper eyelids to the anterior insertion of the auricles. Eyes and corneas were protected from drying out during surgery by applying ViscoTears (Thea Laboratories, Oslo, Norway) over the eyes.

All surgeries are performed stereotactically, where the head is fixed in a stereotaxic apparatus (World Precision Instruments Ltd, Hertfordshire, UK) to prevent movement during surgery, and to ensure accurate stereotaxic coordinates for injections.

The left ear pin was consistently fixed and placed in the left external auditory meatus, while the right pin was gently placed in the right auditory meatus and adjusted to stably fix the head to the apparatus. To ensure the flat skull position, the height-adjustable nose-clamp was adjusted accordingly. Stereotaxic measurement of coordinates was made in accordance to the atlas of the rat brain by Watson and Paxinos (Paxinos, 2007) and verified by histological examinations of brain sections post-mortem.

The skin was cleaned with 70 % ethanol, and a wedge shaped incision with a length of approximately 1.5 cm was made across the midsection using tweezers and blunt Mayo scissors. The periosteum enclosing the skull was grabbed using tweezers, and a small incision was made using iris scissors. The iris scissors were inserted in to the periosteum incision, and expanded to detach the periosteum from the skull. The loosened periosteum was subsequently cut away using iris scissors. Remaining residues of periosteum was gently rubbed away using sterile autoclaved Q-tips until the coronal, sagittal and lambdoid sutures of the skull were visible (bregma, midline and lambda, respectively).

For optogenetic animals, holes were drilled in the skull using an OmniDrill 35 (World Precision Instruments Ltd, Hertfordshire, UK) mounted to a holder attached to the stereotaxic apparatus, allowing for a total of four micro screws to be inserted anteriorly to bregma, and three screws to be inserted posterior to lambda, and one hole in accordance with injection coordinates to allow the passage of the syringe needle. The micro screws function as grounding for the dental cement keeping the implant in place (Figure 8).

For DREADD animals, where no implant was needed, only bilateral holes in accordance with injection coordinates were made.

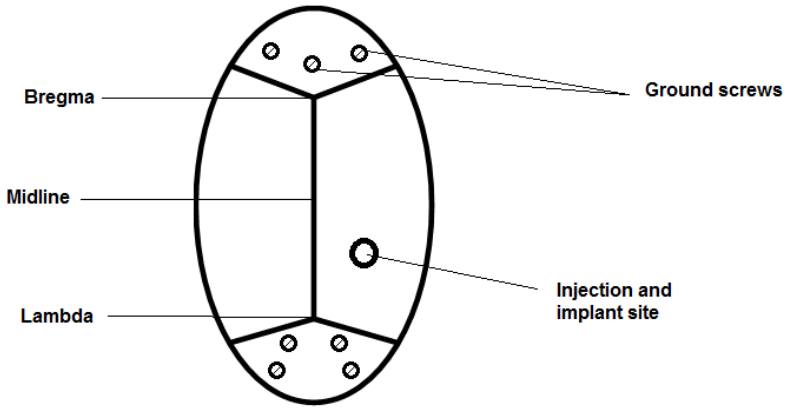

Figure 8: A top view of the skull depicted with three inserted screws anterior to bregma, and four inserted screws posterior to lambda, as well as injection and implant site. For DEADD animals there were bilateral injection sites.

Coodrinates used to target the various regions of the LHA are indicated in Table 1.

AP Inj.	ML Inj.	DV Inj.	AP Opto fiber	ML Opto fiber	DV Opto fiber	Virus serotype
-2.7	1.6	8.8	-2.7	1.6	8.1	AAV5
-2.0	1.6	8.8	-2.0	1.6	8.2	AAV5
-3.3	1.7	8.9	-3.3	1.5	8.3	AAV5
-3.0	1.6	8.8	-3.0	1.6	8.2	AAV5
-1.9	1.6	8.8	-1.9	1.6	8.2	AAV5
-3.0	1.1	8.9	3.0	1.2	8.4	AAV5
-2.7	1.6	8.8	2.7	1.6	8.2	AAV5
-2.7	1.6	8.8	2.7	1.6	8.1	AAV5
-2.7	1.6	8.8	N/A	N/A	N/A	AAV8

Table 1: Injection coordinates based on Paxinos atlas of the rat brain. From anteroposterior (AP) relative to bregma, mediolateral (ML) relative to midline, and dorsoventral (DV) relative to bregma. This table shows both injection coordinates, as well as coordinates for the optogenetic fiber. All optogenetic animals were injected using the AAV5 virus serotype AAV5-hSyn-hChR2(H134R)-EYFP, while DREADD animals were injected using the AAV8 virus serotype AAV8-hSyn-HA-hM4D(Gi)-IRES-mCitrine.

After completion of the surgical procedures, the animals were awakened by administering a subcutaneous injection of Antisedan (atipamezole hydrochloride, 0.25 mg), a synthetic α_2-adrenergic receptor antagonist, to reverse the anaesthetic effects of Domitor. Animals received a subcutaneous injection of Rimadyl (carprofen 5 mg/kg), a non-steroidal anti-inflammatory drug (NSAID), to reduce post-operative pain

2.2.2 Virus injections

Optogenetic animals

Optogenetic animals were unilaterally injected with AAV5-hSyn-hChR2(H134R)-EYFP (UNC Vector Core, North Carolina, USA). All injections were administered in the right hemisphere using a Hamilton syringe (33 gauge Hamilton Neuros Syringe, VWR, USA) mounted to a holder attached to the stereotaxic tower. The needle was slowly inserted and lowered to 0.1 mm below target depth, and immediately elevated to target depth to create a "pocket" for the injected liquid to prevent tissue strain during the injection. Every 60 seconds 0.05-0.1 µL of viral liquid was injected, for a total of 0.3-0.5 µL viral liquid. After the final injection, the needle was left for at least 8 minutes to allow a sufficient diffusion of liquid before the needle was very slowly elevated. Slow needle movement is crucial to avoid unnecessary tissue damage.

DREADD animals

AAV8-hSyn-HA-hM4D(Gi)-IRES-mCitrine was stereotaxically injected bilaterally using the same procedure as described above. Every 60 seconds for a total of 5 minutes, 0.2 µL of viral liquid was injected, for a total of 1 µL.

After the injections, the skull was cleansed using NaCl and sutured shut and covered with Fucidin (LEO Pharma, Ballerup, Denmark), an antibacterial ointment.

2.2.3 Optic fiber implantation

The optic fiber (2.5 mm diameter Stainless Ferrule, 200 µm diameter Core, 0.39 numerical aperture, Thor labs, USA) was mounted to a holder and attached to the stereotaxic tower, and carefully inserted using the same posterior and lateral coordinates as the virus injections.

However, dorsoventral coordinates were adjusted to be 0.6 mm above the injection point to illuminate transduced cells from above. To fix the fiber to the skull of the animal, Meliodent Rapid Repair dental cement (Heraeus Kulzer, Hanau, Germany) was applied to the entire open area of the skull, and carefully ensured to surround the inserted screws as well as creating a stable fix for the fiber. First layer of cement was mixed to low viscosity to ensure an even spread and most importantly ensuring adequate fix to the inserted screws. Primary layer was left until completely cured. Subsequent layers were placed using a high viscosity blend of dental cement and allowed for a gradual build in height. To avoid skin being trapped under the cement and causing infection, the surrounding skin was gently tugged to ensure a glitch between the skin surrounding the dental cement.

After the cement was completely cured, the optic fiber holder was gently elevated, and the surrounding area of the implant was treated with Fucidin ointment.

2.2.4 Post-operative care

Every day for three subsequent days after the operation, each animal was given a subcutaneous injection of Rimadyl (carprofen 5 mg/kg). All animals were carefully observed to ensure normal behaviour and eating habits in the days following surgery. Minor sores around the implant or sutured wounds were treated with Fucidin.

2.3 Lateral hypothalamic manipulation and behavioural analysis

2.3.1 Optogenetic animals

Prior to optic stimulation, the surface of the optic fiber was cleaned using 70 % ethanol to prevent any dirt from interfering with light pulses. All animals were left in solitude in clean individual cages in a quiet room different from the optogenetic stimulation room for at least 30 minutes prior to optical stimulation. The testing room was completely silenced and darkened, with the exception of a red LED ceiling light, to avoid unnecessary stimuli and stress for the animal. The setup box was cleaned with 70 % ethanol prior to every new animal to remove odours which may bias behaviour.

The behavioural effects of LHA stimulation was examined in three different contexts in the following order: open field analysis, shuttle box analysis and home cage analysis. The animals were given a minimum of two days of rest in between tests in different contexts.

For all optogenetic stimulation, we used a 473 nm DPSS laser (100 mW, Shanghai laser, China) to deliver 10 ms light pulses at 20Hz with a peak light intensity of 10 mW at the tip of the fiber. This expected to result in a light intensity of about 6 mW/mm^2 at the viral injection site located 0.6 mm below the fiber tip, and should therefore be sufficient to result in ChR2 activation (Berndt et al., 2009). Immediately prior to behavioural experiments, an optic patch cable (MFP_200/220/900-0.37_3.5 m_SMA-MF2.5, Doric lenses, Canada) was used to connect the laser to the optic implant attached to the rat skull. All behavioural sessions were videotaped and behaviour was scored offline.

Open field setup

An open field box (1 x 1 m) was used to examine whether LHA stimulation would trigger undirected defensive behaviours like freezing or activity bursts (Figure 9).

The rat was placed in the open field and allowed to explore the box for five minutes before we started the stimulation. We then initiated optogenetic stimulation for 60 seconds (10ms pulses, 20 Hz) followed by a 60 second period without stimulation. This process was repeated three times.

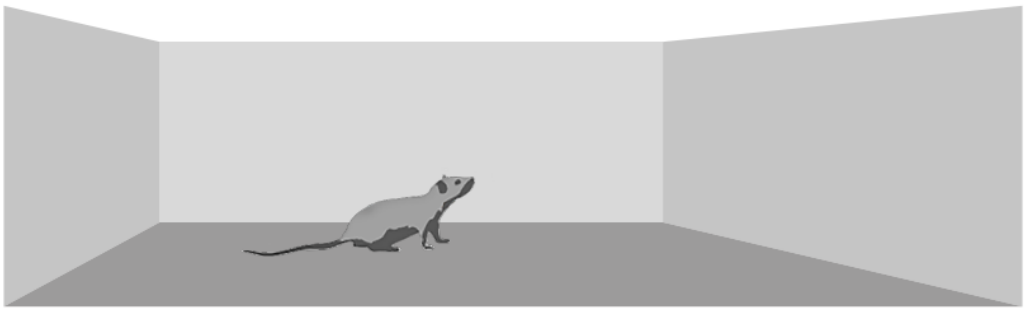

Figure 9: Open field setup. The rat was placed in an open field box and allowed to move freely within it.

On two opposing sides, two D-link DSC-932L IP cameras were placed to cover a full view of the box during data acquisition. During each recording, a baseline of approximately seven minutes was recorded, followed by four stimulation bins of one minute each. Each stimulation period lasted for no more than one minute to avoid heat build-up and tissue damage.

In addition to standard video recordings, all open field behaviour was monitored using a ceiling mounted Abus Euseo 520 TVL camera (Abus Security Tech, Germany) hooked up to daqUSB, a recording system provided by Axona (Herts, UK). During recording, the animals wore a small LED lamp attached to their skull mounted optic implants targeted upward at the tracking camera, giving a continuous tracking of animal location, which was later used for offline measurement of the running speed. In addition, we performed offline measurements of time spent freezing.

Shuttle box setup

We used a shuttle box (50 cm wide × 25 cm deep × 30 cm high) to examine whether LHA stimulation could trigger directed defensive behaviours like avoidance or withdrawal. The shuttle box consists of two identical chambers the rat may freely move back and forth from (Figure 10). A D-link DSC-932L IP camera was placed at an angle from above for a full view of both chambers.

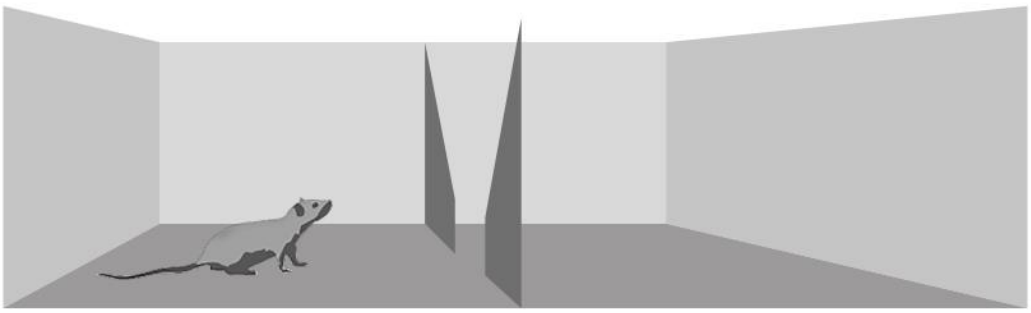

Figure 10: Shuttle box setup. The rat was placed in one of the compartments and allowed to move freely between them. Stimulation was consistently elicited in the starting compartment, and time spent in each compartment was recorded.

During each recording session, the rat was first allowed to move freely between the chambers to detect any baseline chamber preference. After baseline measurement, the rat received manually controlled optic stimulation every time it entered the chamber that had been randomly selected as the stimulation chamber. If the rat left the stimulation chamber, the optogenetic stimulation was immediately terminated. To avoid heat build-up and consequential tissue damage in the brain, optic stimulation was only performed for 60 seconds at a time. If the rat had not left the chamber after the 60 second stimulation, it was given a 30 second pause before 60 new seconds of stimulation and so on. We performed offline measurement of time spent in each chamber.

Home cage recording setup

Given that LHA is believed to be important for ingesting behaviour, we finally examined whether optic stimulation of LHA sub regions could elicit feeding behaviour. For this purpose, well-fed rats were optogenetically stimulated in their home cage where both food pellets and water were readily available (Figure 11).

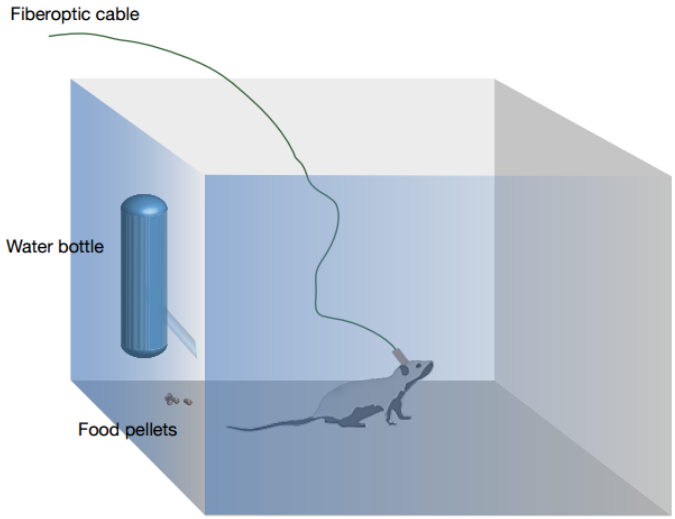

Figure 11: Stimulation and recording in a home cage environment. Food and water were readily accessible to monitor feeding behaviour during stimulation.

Food pellets and water were available at the same locations as during the normal housing conditions of the rat. The rat was connected to the optic cable, placed in its home cage, and allowed to move freely for three minutes before receiving 60 seconds of optogenetic stimulation.

The latency from stimulation onset to the rat picked up a food pellet and started chewing the food was measured.

A side-view D-link DSC-932L IP camera was placed facing the transparent side window of the home cage recording all behaviour. All data sent from the D-link IP camera was monitored using the iSpy application, creating detailed time stamped videos for further analysis.

2.3.2 DREADD animals

At the day of the experiment, we dissolved 25 mg CNO (Toronto Research Chemicals Inc., Canada) in a 10 mL sterile 0.9 % NaCl with 5 % dimethyl sulphoxide (DMSO) to aid dissolution. Rats were injected intraperitoneally with either CNO (5 mg/kg) or vehicle (0.9 % NaCl with 5 % DMSO) 45 minutes prior to behavioural testing.

Looming stimuli setup

The looming setup was built by placing an LCD monitor above the home cage of the rats (Figure 12).

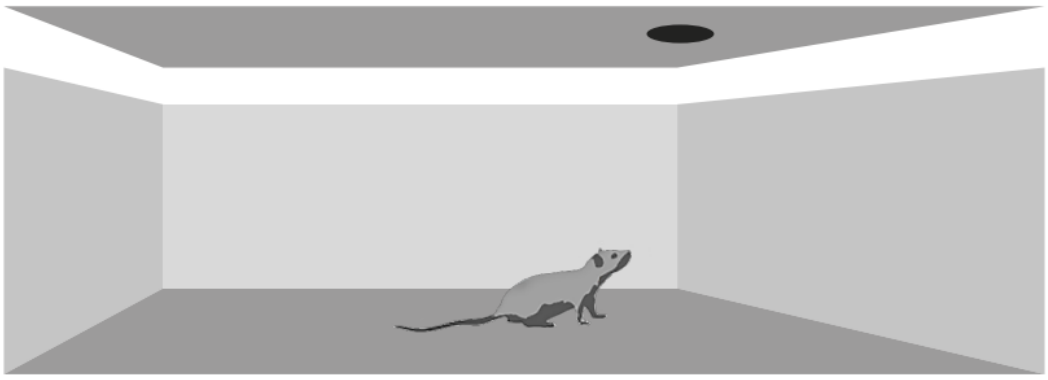

Figure 12: The looming stimulation setup, consisting of an LCD monitor placed above the home cage of the rats.

In one corner of the LCD monitor, a dot gradually increasing in size appears, simulating avian predators as viewed from the rats' upper visual field. This looming simulation consistently results in freezing behaviour in rodents (Yilmaz et al., 2013).

The rat was allowed three minutes of acclimation in the arena with a plain gray monitor. The "looming stimulus" was then started when the animal entered the zone below the planned stimulus. On a gray background, a black disc appeared directly above the animal at a diameter of 2 degrees of visual angle, expanded to 20 degrees in 250 ms, and remained at that size for 250 ms. The stimulation was repeated 15 times with 500 ms pauses.

Contextual fear conditioning setup

Four fear conditioning chambers (interior: 30.5 cm x 24.1 cm x 21.0 cm; MED-Associates, St. Albans, VT, US) were used. The chamber is made of Plexiglas (door, back wall and ceiling) and aluminium (two opposing side walls). It was placed inside a sound attenuating cubicle (1 x ENV-018MD Deep, extra tall, MDF; MED-Associates). The floor was made up of 19 steel rods (4 mm in diameter) that were spaced 1.5 cm apart. The rods were connected to an electric pulse generator (MED-Associates) that delivers the foot shock.

On day one, the animals were conditioned to fear the conditioning chamber. The rats were given five minutes to explore the chamber before receiving three foot shocks (0.4 mA) over the course of 15 minutes and 30 seconds (first shock at five minutes, next at 14 minutes, final shock at 17 minutes and 30 seconds). After the final shock, we waited two minutes before removing the rat from the chamber.

On day two, we injected the rats with CNO or vehicle before placing the rats in the conditioning chamber for ten minutes. During this time, the rats did not receive any footshocks (Figure 13).

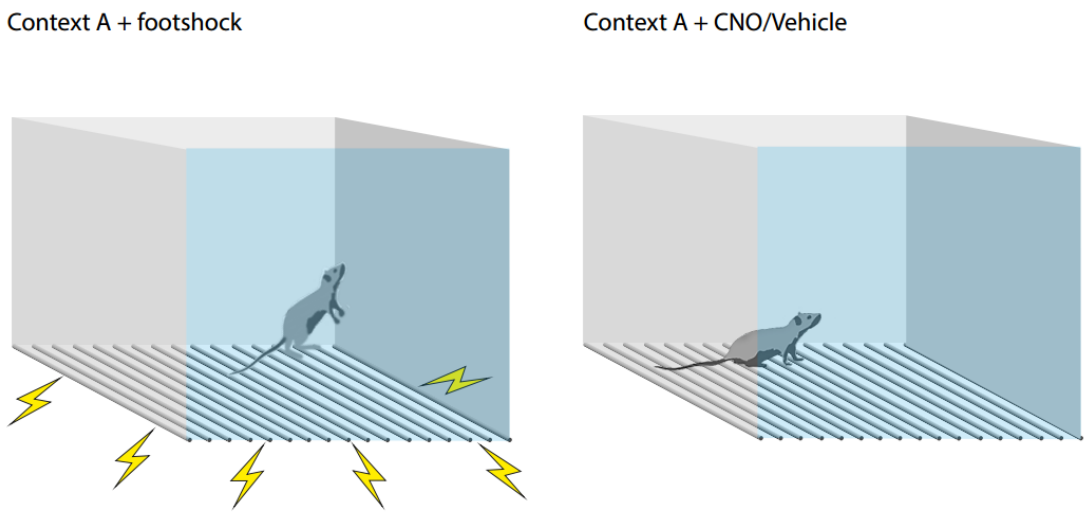

Figure 13: Contextual fear conditioning showing day one, where the animal is exposed to mild foot shocks, and day two, where no foot shock is present and animals were injected with vehicle or CNO prior to the experiment.

Movements in the chambers were filmed using infrared cameras placed above the transparent Plexiglas ceiling. The videos were recorded and saved on DVD.

2.4 Histology

All animals, with the exception of DREADD animals, were optogenetically stimulated 90 minutes prior to euthanasia to initiate c-Fos expression for immunohistochemical analysis.

2.4.1 Perfusion

The animals were sedated using isoflurane to prevent distress and potential pain during an intraperitoneal overdose of 200 mg pentobarbital (NAF, Oslo, Norway). Proper anaesthesia was confirmed by lack of hind leg reflexes during toe pinching, as well as lack of cornea reflexes.

Following anaesthesia and respiratory arrest, rats were transcardially perfused with 0.9 % NaCl until flow ran clear, followed by 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS). Harvested brains were post-fixated in a 4 % PFA solution overnight, before a three to four-day incubation period in a 30 % sucrose solution at 4 °C. Samples were frozen and sectioned in to 50 µm thick coronal sections using a Leica cryostat, gathering four sections, and discarding five sections at a time in the areas of interest. Sections were immediately collected in a 0.01 M PBS solution for immunohistochemistry staining.

2.4.2 Immunohistochemistry

Staining of c-FOS and GFP

The sections were gathered in nets and placed in wells filled with approximately 3 mL 0.01 M PBS, to wash the samples during gentle shaking. This process was repeated three times in a fresh 0.01 M PBS solution. Following the wash, the sample net was placed in 3 mL blocked with 1 % bovine serum albumine (BSA) (Sigma-Aldrich, USA), 0.2 % Triton 100-X (Sigma-Aldrich, USA) in 0.01 M PBS for one hour at room temperature during gentle shaking, before incubating overnight in a 2.5 mL 1:2000 0.01 M PBS/primary antibody solution with anti-c-Fos rabbit (Merck Millipore, Darmstadt, Germany) and anti-GFP chicken (Thermo Fisher Scientific, Oslo, Norway).

After an overnight incubation, the sections were washed 3x5 minutes in 0.01M PBS prior to a one-hour incubation in 2.5 mL 1:200 0.01 M PBS/secondary antibody solution with Alexa

Fluor® 488 goat anti-chicken and Alexa Fluor® 594 goat anti-rabbit (Thermo Fisher Scientific, Oslo, Norway). After the one-hour incubation, the sections were subsequently washed for 3x5 minutes in 0.01 M PBS before mounting on glass slides (Thermo Fisher Scientific, Oslo, Norway) and left to dry for approximately 30 minutes. After the slides were dry, each slide was dipped three times in distilled water (dH_2O), before placing three evenly spaced drops of ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Oslo, Norway) across the slide and cover slipped with Entellan.

Staining GFP

To see the extent of the spread of the virus, the GFP signal was intensified using anti-GFP. The sections were gathered nets and placed in wells filled with approximately 3 mL 0.01 M PBS, to wash the samples during gentle shaking. This process was repeated three times in a fresh 0.01 M PBS solution. Following the wash, the sample net was placed in 3 mL blocked with 1 % BSA (Sigma-Aldrich, USA), 0.2 % Triton 100-X (Sigma-Aldrich, USA) in 0.01 M PBS for one hour at room temperature during gentle shaking, before incubating overnight in a 2.5 mL 1:2000 0.01 M PBS/primary antibody solution with anti-GFP chicken (Thermo Fisher Scientific, Oslo, Norway).

After an overnight incubation, the sections were washed 3x5 minutes in 0.01 M PBS prior to a one-hour incubation in 2.5 mL 1:200 0.01 M PBS/secondary antibody solution with Alexa Fluor® 488 goat anti-chicken (Thermo Fisher Scientific, Oslo, Norway). After the one-hour incubation, the sections were subsequently washed for 3x5 minutes in 0.01 M PBS before mounting on glass slides and left to dry for approximately 30 minutes. After the slides were dry, each slide was dipped three times in dH2O, before placing three evenly spaced drops of ProLong Gold Antifade Mountant with DAPI across the slide and cover slipped with Entellan.

Nissl staining

To determine the injection site post-mortem, Nissl staining of nucleic acid was used as a more reliable visual method to determine tissue damage at the injection point.

The sections were incubated in 0.1 % Triton X-100 in 0.01 M PBS for one hour, followed by 2x5 minutes of washing in 0.01 M PBS at room temperature.

After washing, the sections were incubated in a 1:100 solution of NeuroTrace® Fluorescent Nissl Stain (Thermo Fisher Scientific, Oslo, Norway) diluted in 0.01 M PBS for thirty minutes at room temperature.

Sections were then incubated in 0.1 % Triton X-100 in 0.01 M PBS for ten minutes at room temperature and subsequently washed for 2x5 minutes in 0.01 M PBS at room temperature.

Sections were mounted on glass slides.

2.5 Data analysis

2.5.1 Microscopy

Histological images were acquired at the Norbrain Slidescanning Facility at the Institute of Basic Medical Sciences, University of Oslo. High-resolution images of histological sections were acquired using an automated slide scanner system (Axio Scan Z1, Carl Zeiss Microscopy, Munich, Germany). Images were inspected using the Zen Lite Blue software (Carl Zeiss Microscopy).

DREADD localized intensity

To confirm that our DREADD viral injections successfully injected LHA neurons, we examined the spread of mCitrine, a marker of DREADD infection, in LHA and the surrounding regions.

Each relevant sub region in the hypothalamus, namely the anterior LHA (LHAa), anterior hypothalamic area (ACH), internal capsule (IC), zona incerta (ZI), dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), tuberal LHA (LHAt), LHApf, was individually analysed for mean fluorescent intensity using Zen 2 Lite. By measuring

mean background fluorescence intensity at multiple locations without viral expression, we found that different brain regions and different animals contained a relatively constant amount of background fluorescence (The exception was the IC, which due to its low cell number contained a lower background than other regions). As a result, we subtracted the measured viral fluorescence in all regions with the same estimated background fluorescence, with the exception of the IC, where we used a lower baseline subtraction based on specific IC measurements.

2.5.2 Behaviour analysis

Open field behaviour

Freezing behaviour was defined as cessation of all movement except respiratory movement for any given time, and was scored using a digital stopwatch, counting how many seconds the animals were observed freezing during each stimulation period. This measure was used to calculate time spent freezing for each individual rat.

We used the animal location data obtained with Axona to calculate movement speed. Running behaviour was defined as running speed above 50 cm/s. In this way, we could calculate the time spent running for each rat. As intended, this measure appeared to correlate well with the visually observed presence or lack of running bouts in animals.

Shuttle box behaviour

Time spent in each compartment was noted both during the ten-minute baseline period, and during optical stimulation using a digital stopwatch. The optogenetic stimulation was defined as aversive if rats spent less than 25 % of their total time spent in the stimulation chamber. Similarly, the optogenetic stimulation was defined as rewarding if the rat spent more than 75 % of the total time in the stimulation chamber. If animal showed strong baseline preference for a chamber (above 75 % time spent in one chamber), it was excluded from the analysis.

Home cage behaviour

Evoked feeding behaviour, defined as gnawing or chewing on food or objects, was quantified by measuring the latency from optogenetic stimulation onset until the rat picked up a food pellet and started chewing the food. By default, latency was set to 120 seconds if no feeding commenced.

DREADD looming behaviour

Total freezing was scored in five second bins for a total of 75 seconds using a digital stopwatch. After 30 seconds, a 15 second looming shadow session was initiated. Freezing was scored for an additional 30 seconds after the looming shadow session ended to observe sustained freezing behaviour.

DREADD fear conditioning behaviour

Freezing was scored for the first ten minutes after placing the rat in the chamber. A total of ten minutes of each video was scored for freezing behaviour using a digital stopwatch. Time was separated in to two minute bins.

2.5.3 Statistical analysis

Statistical tests were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The freezing response over time to a looming visual stimulus was analysed using a two-way analysis of variance (ANOVA) with CNO and time as main factors. Sidak multiple comparisons was used when a significant interaction effect was detected. For comparison of two experimental groups, we used the non-parametric Mann-Whitney test. Statistical difference was considered significant at a P-value less than 0.05. Results were presented as mean ± standard error of mean (SEM).

3 Results

A total of 30 and 24 male Sprague Dawley rats were used for the optogenetics and DREADD experiments, respectively.

3.1 Methodological assessment

We chose several approaches to address the hypothesis that LHA is important for defensive behaviours.

The optogenetic approach with ChR2 reliably activates transduced neurons (Boyden et al., 2005). In addition, the rapid depolarization of neurons expressing ChR2 in response to light pulses allows observation of behavioural changes within milliseconds of activation.

To determine whether or not neural activity in the LHA is *necessary* for visually induced innate defensive behaviours and expression of learned fear, we used the chemogenetic DREADD approach to silence the LHA during visual innate fear as well as fear conditioning

3.2 Optogenetic stimulation

To verify our hypothesis that activation of LHA is sufficient to elicit defensive behaviours, we attempted to selectively activate several regions in this area using optogenetics while recording behaviour.

3.2.1 Behavioural effects based on targeting

Optogenetic stimulation in various areas of the LHA initiated different behaviours, such as escape/running, feeding and freezing. To anatomically correlate behaviours with specific regions, Nissl staining was used to reveal placement of optical fibers, pairing these findings with observed behaviour during optogenetic stimulation (Figure 14).

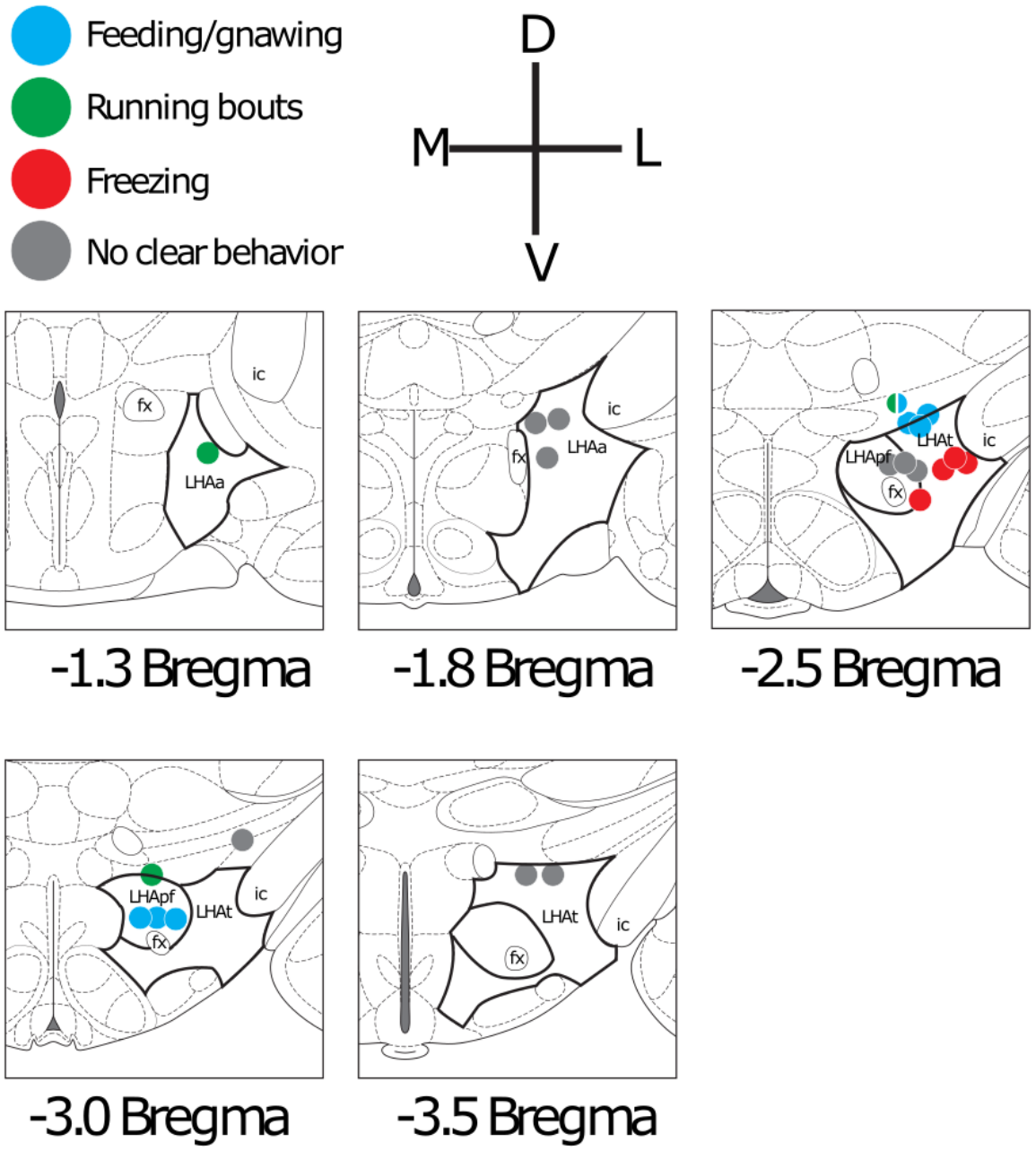

Figure 14: Each behaviour is colour coded, where feeding, running, freezing or no clear behaviour is shown as blue, green, red and grey respectively. Each circle represents one animal. Each hypothalamic region is outlined at various distances from bregma comparing the brain sections with Paxinos atlas. The anterior LHA (LHAa), the perifornical LHA (LHApf), and the tuberal LHA (LHAt) are outlined.

All four animals that were stimulated in the ventral part of the LHAt showed robust freezing. On average, these four animals spent 67.5 ± 8.1 % of the total stimulation time freezing. By contrast, animals stimulated in areas outside of the ventral LHAt showed little to no freezing, and froze on average 4.3 ± 6.0 % of the total stimulation time.

Stimulation of the LHAa in one animal resulted in running bouts (green) (movement > 50 cm/s) for 33 % of the total stimulation time. Stimulation in the dorsal regions of LHApf in one animal also resulted in running bouts 20.8 % of the total stimulation time. In addition, in one animal stimulated near the zona incerta (ZI) we observed feeding/gnawing (blue) (9.4 s latency to feed) alternating with running bouts (17.8 % of the total stimulation time).
In three animals we observed feeding/gnawing upon stimulation within the LHApf, where the mean latency to feed was 13.47 ± 6.3 s. Feeding was also observed in the dorsal areas of LHAt, with a mean latency to feed of 20.4 ± 12.1 s. In the shuttle box experiments, where no food was available, the animals that displayed home cage feeding often showed intense gnawing on the dividing walls of the shuttle box.

Interestingly, our results indicated that freezing behaviour could be elicited by stimulating a specific ventral region of the LHAt. To further analyse freezing behaviour, we investigated whether this freezing behaviour correlated with other behaviours. Our data shows that freezing behaviour did not overlap with running or feeding (Figure 15).

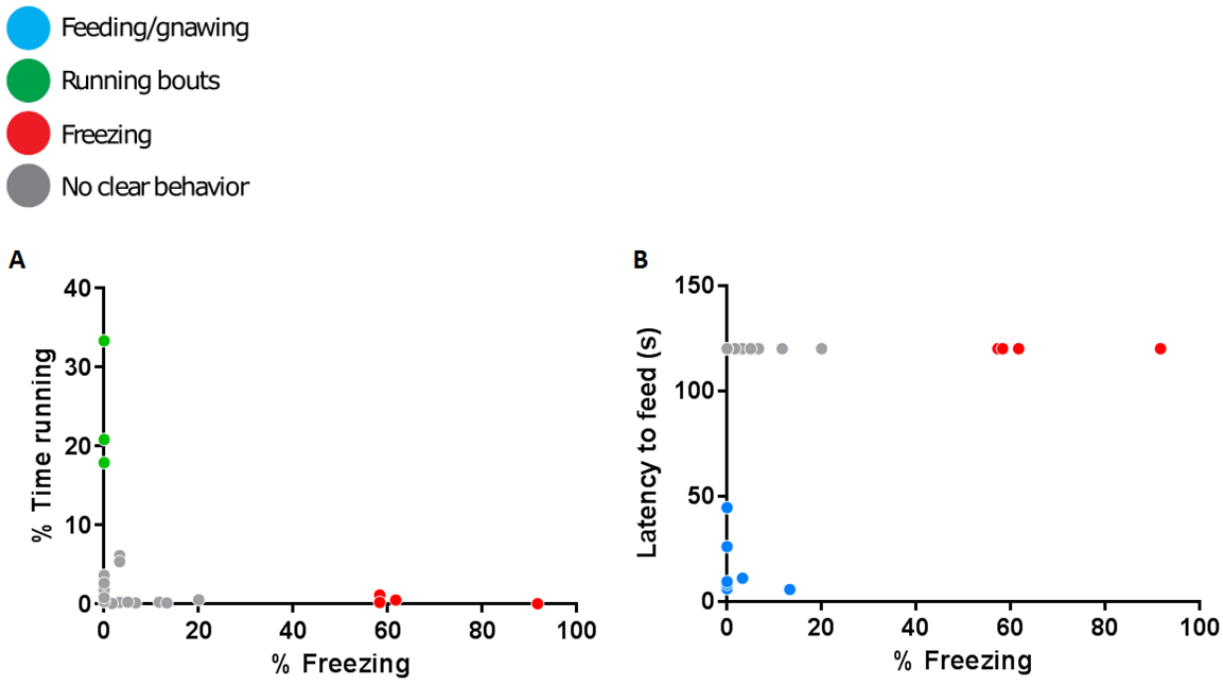

Figure 15: Freezing behaviour plotted against running (graph A) or against feeding behaviour (graph B). Each dot represents the behavioural effects of one animal. Movement above 50 cm/s was considered running. A latency to feed below 120 s after stimulation onset was defined as feeding behaviour.

As there is no overlap in freezing behaviours with other behaviours upon stimulation on various regions in the LHA, this further supports that ventral LHA is involved in eliciting freezing behaviour.

Activation of a defensive survival circuit is expected to yield aversive behaviour, that is, something the animal would avoid if given the choice. Aversive and rewarding behaviour was also analysed during the shuttle box experiment (Figure 16). Aversive behaviour was defined as less than 25 % of the total time spent in the stimulation chamber in the ten minute stimulation period, while rewarding behaviour defined as more than 75 % of the total time spent in the stimulation chamber. Three animals were excluded from these results as they showed no movement between chambers during the baseline trial.

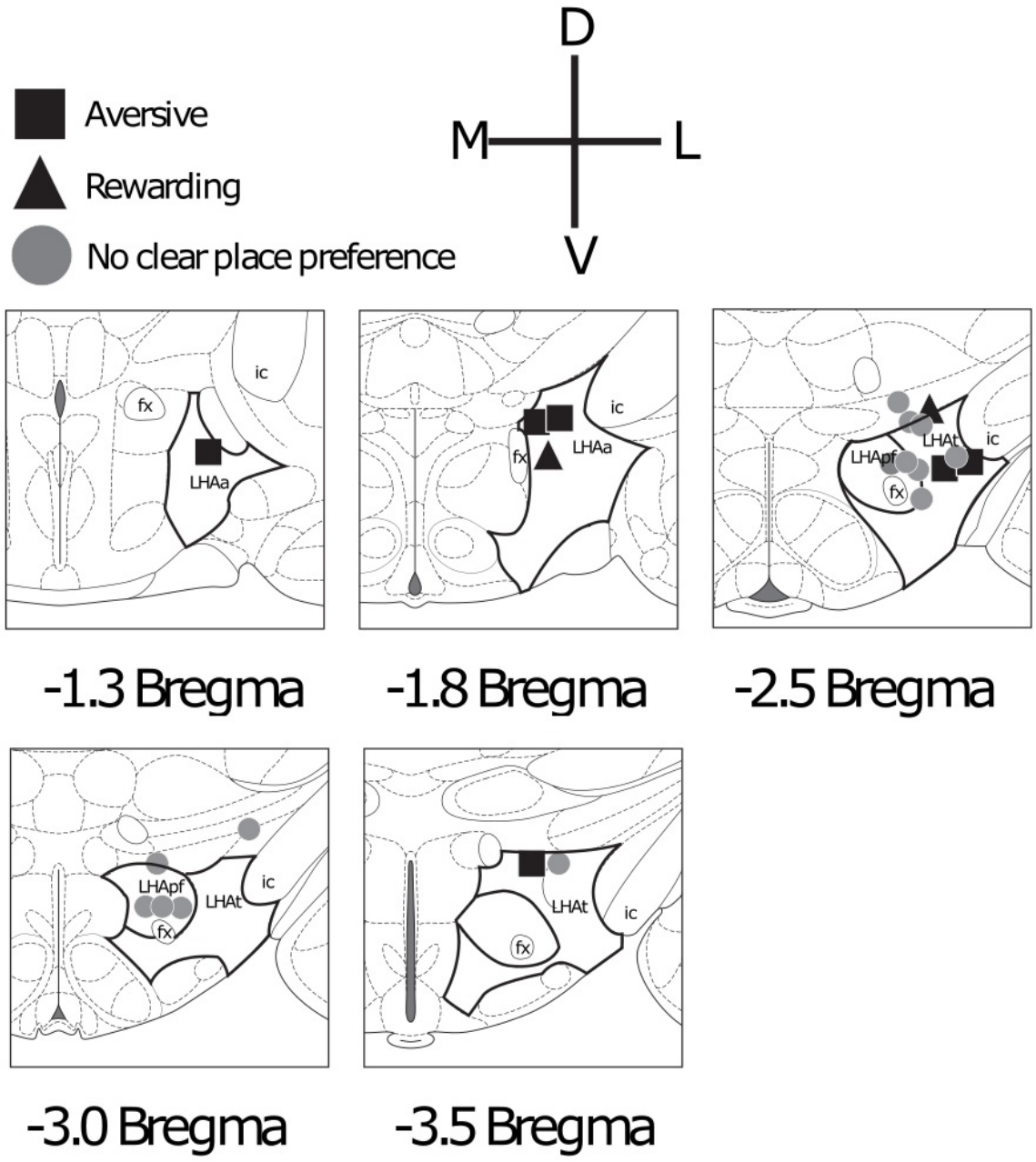

Figure 16: Aversive and rewarding behaviour were shape coded as squares or triangles. No clear preference was colour coded as a grey circle. Each shape represents one animal. Each region is outlined, showing the anterior LHA (LHAa), perifornical LHA (LHApf), and tuberal LHA (LHAt).

Stimulation of the LHAa in three animals resulted in aversive behaviour towards the stimulation chamber, with a mean of 6.4 ± 4.8 % time spent in the stimulation chamber during the stimulation period. However, in one animal, stimulating the more ventral parts of LHAa resulted in rewarding behaviour with 88.2 % time spent in the stimulation chamber. Aversive behaviour was also observed in one animal after stimulation in the ventral parts of the LHAt, with 12.16 % time spent in the stimulation chamber. Stimulation of LHApf resulted in no

clear place preference, with a mean total time of 48.6 ± 6.0 % spent in the stimulation chamber.

Importantly, aversive behaviour during the shuttle box experiment was seen in several of the same animals who engaged defensive related behaviour in the open field (running and freezing). Further, aversive behaviour was not observed in animals that engaged in feeding behaviour during optogenetic stimulation. Although it is difficult to interpret the shuttle box results given that many of the animals showed locomotor effects (i.e. freezing or running) that could confound the shuttle box behaviour, observations indicate that the stimulation resulted in an aversive motivation rather than a pure locomotor effect. In particular, the animals that showed aversion not only withdrew quickly from the stimulation chamber, but also displayed increased time to approach the stimulation chamber ($p = 0.03$, Figure 17), indicating that an association between the stimulation chamber and a negative outcome had been formed.

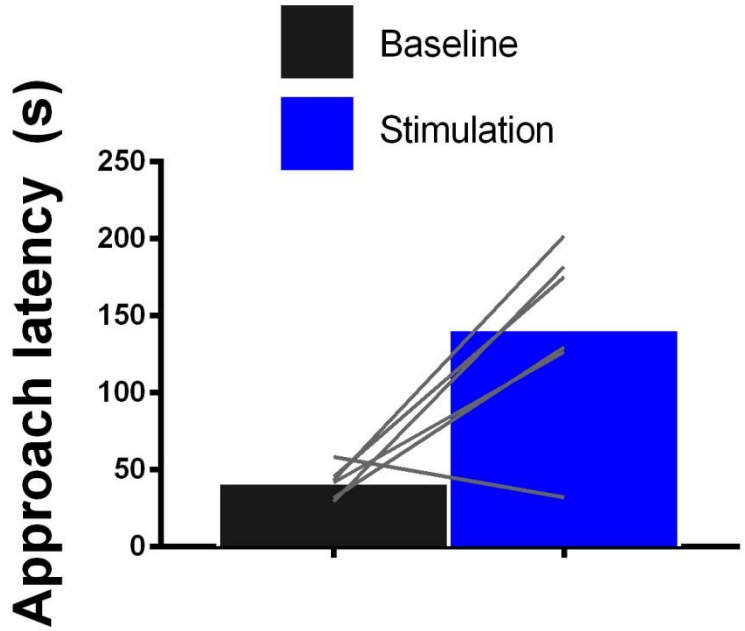

Figure 17: Latency to approach the stimulation chamber during the 20 minute recording session. The baseline period and stimulation period were ten minutes long each. Each line represents one animal, all of which have been classified as having aversive behaviour. The data was analysed with the Mann-Whitney test.

Further, in many animals (data not shown) the stimulation initiated a directed U-turn that allowed the animal to rapidly escape the stimulation, as opposed to the seemingly undirected running behaviour observed in these animals in the open field.

3.2.2 Localized c-FOS

In order to verify that we selectively activated the LHA rather than surrounding regions in animals displaying defensive behaviour, we optogenetically stimulated the animals 90 minutes prior to euthanasia to initiate the c-Fos activity marker. After euthanasia, the brains were harvested and immunohistochemically treated to reveal c-Fos activity and viral GFP.

The brain section in Figure 18 from a rat that was optogenetically stimulated in the LHA shows a considerable increase in c-Fos activity in the LHAt and ZI.

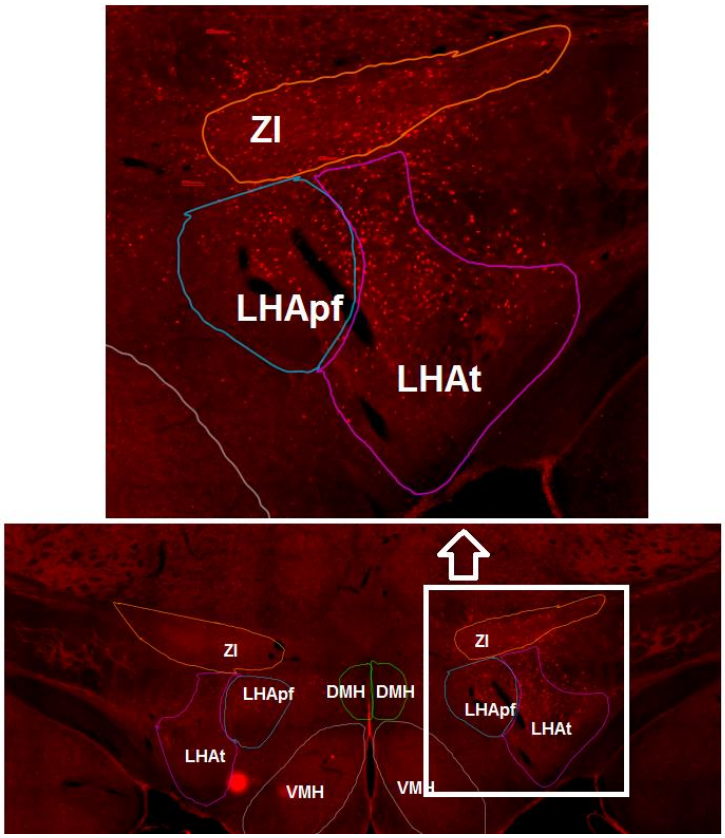

Figure 18: A c-Fos stained section with outlined regions (DMD outlined green, VMH outlined grey, LHApf outlined blue, LHAt outlined purple, ZI outlined orange), including a magnified section of LHA. C-Fos mostly confined within the LHAt and ZI.

Consistently, there was little observed c-Fos activation outside of the LHA, and no activation in the medial hypothalamic areas. An extensive analysis of c-Fos activity based on region was to be performed, but due to lack of time such an analysis was not completed at the time of writing this thesis.

3.3 DREADD

3.3.1 The effects of DREADD in the looming stimuli group

Our optogenetic results suggest that activation of certain parts of the LHA is sufficient to elicit defensive behaviour. With optogenetic experiments alone, it is difficult to distinguish whether LHA activity is important for responses to visual, olfactory, auditory or contextual threats, which likely involve different circuits. If LHA activity is *necessary* for initiating freezing behaviour in response to visual threat, we would expect to see that silencing this area would cause a significant decrease in freezing behaviour in response to an innately threatening visual stimulus. We therefore used chemogenetic silencing of the LHA during exposure to a looming shadow.

A total of eight animals were used in this particular experiment. Two groups of AAV-HM4Di-mCitrine injected animals were given either an intraperitoneal vehicle or CNO injection before exposure to a looming shadow. The intraperitoneal injection of CNO should silence transduced cells in the injected region. To confirm that we were able to specifically transduce the LHA with our virus, we examined the spread of mCitrine in the LHA and its surrounding regions. The mCitrine signal was restricted to the tuberal LHA, the perifornical LHA and the zona incerta (ZI) (Figure 19), confirming that we were able to specifically infect the LHA/ZI without affecting the medical hypothalamic defence zone and other surrounding areas.

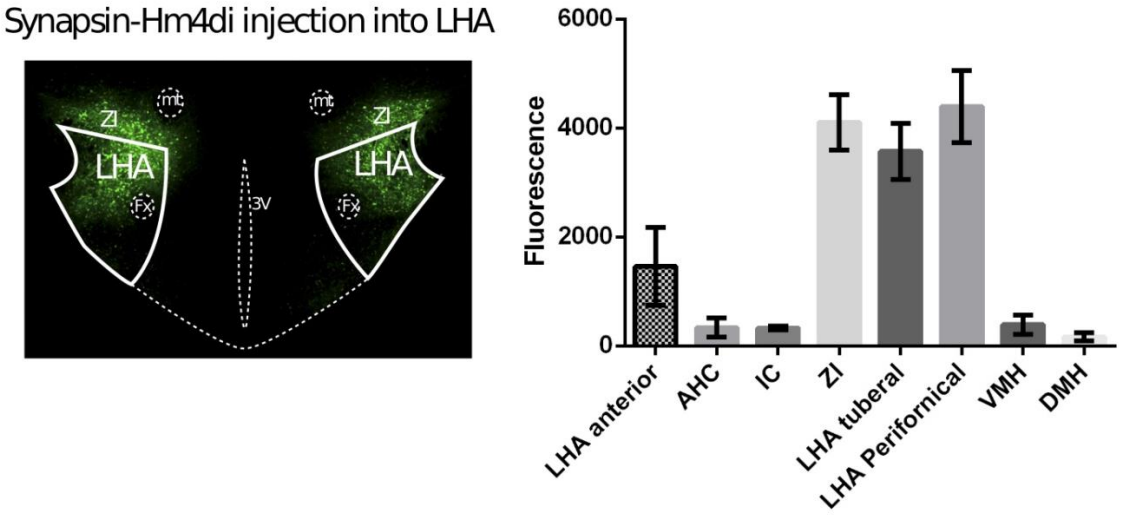

Figure 19: A microscopy sample depicting the spread of mCitrine. Fluorescent intensity for all subjects was evaluated and separated by hypothalamic areas (anterior lateral hypothalamic area (LHA anterior), anterior hypothalamic area (ACH), internal capsule (IC), zona incerta (ZI), dorsomedial hypothalamic nucleus (DMD/DMH), ventromedial hypothalamic nucleus (VMHVL/VMH), tuberal lateral hypothalamic area (LHA tuberal), perifornical lateral hypothalamic area (LHA perifornical)). Shown with SEM error bars.

While the initial freezing response the first five seconds after looming shadow presentation was not significantly different between the vehicle group (7.91 %) and the CNO group (7.8 %), there was a significant decrease in **sustained freezing** following looming stimulation in the CNO group compared to the vehicle control group ($p<0.05$) (Figure 20 and Figure 21).

In detail, a two-way ANOVA revealed a significant main effect of CNO infusion ($F(1,6) = 16.35$, $p = 0.007$), a significant main effect of time ($F(14,84) = 15.36$, $p < 0.0001$, and a significant interaction ($F(14,84) = 4.01$, $p < 0.0001$). A Sidak post hoc test revealed that there was no significant freezing difference between the vehicle group and the CNO group during the first five seconds after looming shadow onset ($p > 0.05$). After this initial period however, the CNO rats showed drastically reduced freezing levels at most time points ($p < 0.05$).

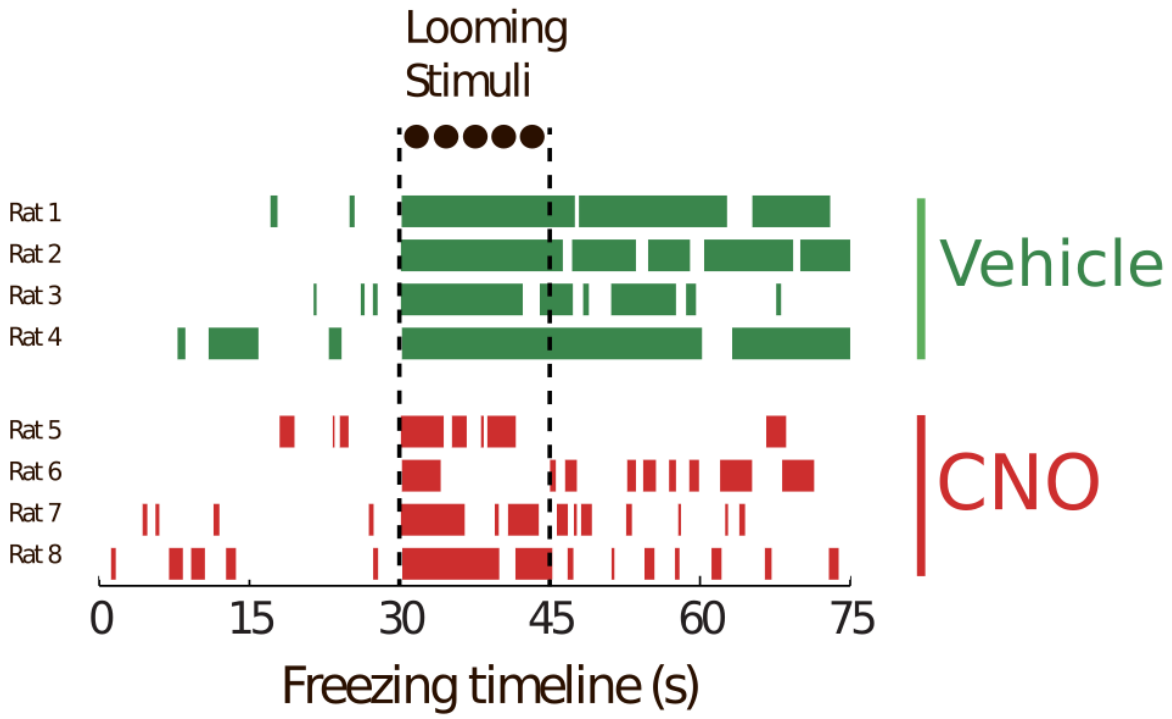

Figure 20: The effect of silencing LHA with CNO in a looming stimulation task. Total time spent freezing (x-axis) time during and after looming stimulus (black dots). Each raster (red or green) indicates manual scoring of a freezing event, defined as cessation of all movement except respiratory movement for any given time. One group of animals (CNO; red; rat 5-8) had DREADD expressed in neurons on the LHA, and received an intraperitoneal injection of CNO before the experiment. The other group of animals (Vehicle; green; rat 1-4) received an intraperitoneal injection of vehicle before the experiment.

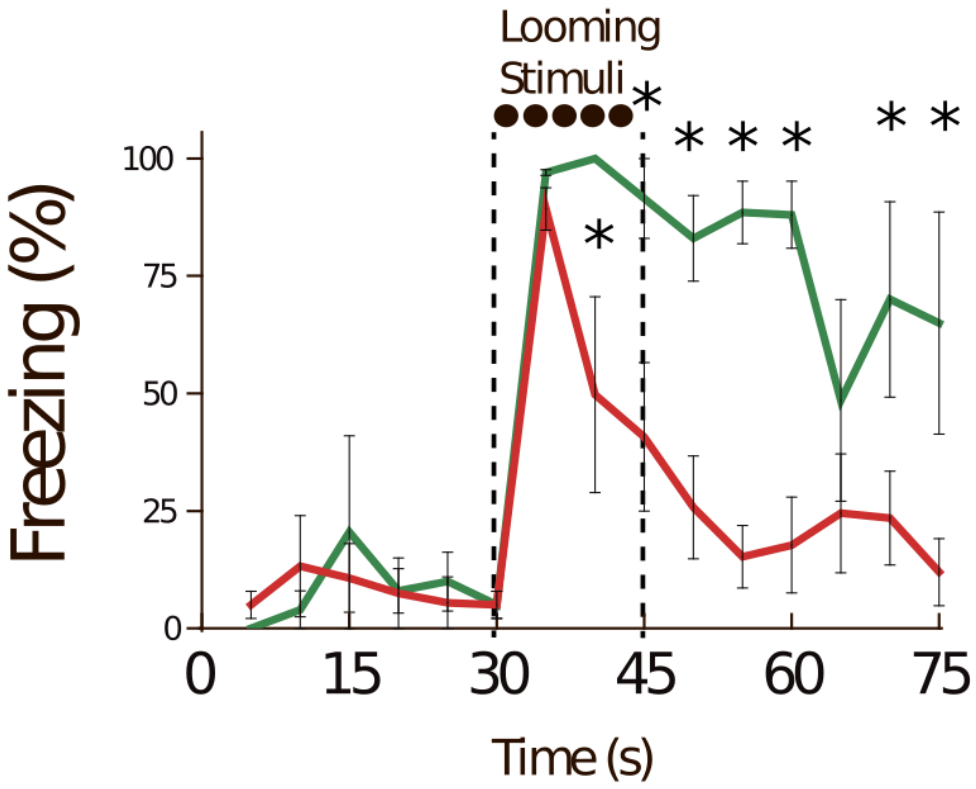

Figure 21: Timeline showing time spent freezing as a percentage at the y-axis, as a function of five second bins on the x-axis. The red graph shows the CNO injected group, while the green graph shows the vehicle injected group. This data was analysed with a two-way ANOVA. Stars above each time point showing significance ($p < 0.05$).

Importantly, the baseline movement (Figure 22) was not significantly different between the CNO (6.8 cm/s) and vehicle group (6.0 cm/s).

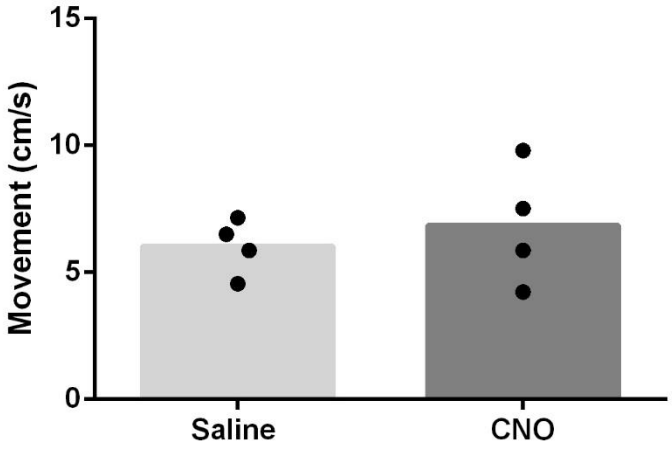

Figure 22: Baseline locomotor activity for vehicle and CNO group. The data was analysed with the Mann-Whitney test.

In summary, this experiment showed that LHA activity is necessary for sustained defensive freezing to a looming shadow.

3.3.2 The effects of DREADD in the contextual fear conditioning group

Previous studies indicate that expression of innate fear is mediated by a different pathway than conditioned fear (Blanchard et al., 1972; Corcoran et al., 2007).

In order to test whether LHA controls innate fear specifically, we next asked whether disruption of LHA activity would also influence learned fear.

We transduced LHA neurons using AAV8-HM4Di-mCitrine followed by fear conditioning to examine whether LHA silencing would disrupt freezing to a learned threat. A total of 15 animals were used. Two animals failed to express mCitrine and were omitted from the analysis.
The animals received foot shocks in a conditioning chamber, and the following day we injected with CNO or vehicle prior to a second exposure the following day to the same context but without foot shocks, allowing for memory retrieval of the conditioned context.

Unlike the prior experiment using the innate looming threat, no significant difference in conditioned freezing was observed in the CNO group compared to the vehicle group (Figure 23).

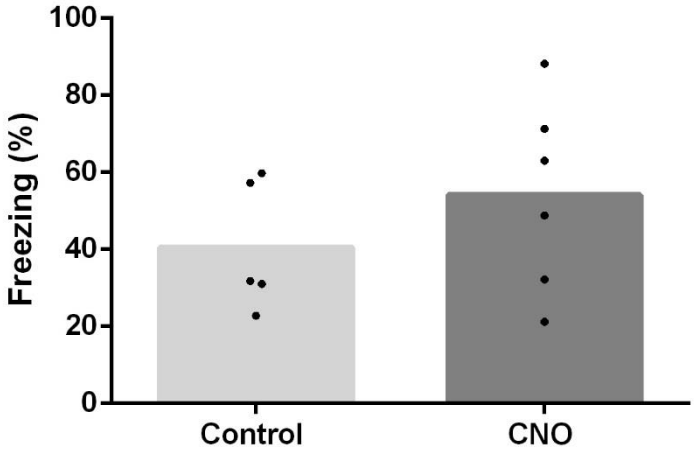

Figure 23: Freezing as a percentage of time in a fear conditioning chamber the day after a contextual fear conditioning training session for the vehicle/control and CNO group. Each dot represents one animal. The data was analysed with a Mann-Whitney test.

To verify that the lack of freezing behaviour was not due to a lack of viral expression in the LHA, mCitrine expression was verified. However, one animal failed to express any mCitrine at all, while another expressed inadequate mCitrine, and consequently these two subjects were omitted from the results.

4 Discussion

This study demonstrates that optogenetic activation of the LHA is sufficient to elicit defensive and ingesting behaviours. The ventral regions of the LHA were strongly associated with freezing. In a classic loss of function experiment using chemogenetics to silence the LHA, we also found that LHA is necessary for sustained freezing following innate fear towards a looming shadow. In contrast, chemogenetic silencing of LHA failed to influence freezing during contextual fear memory retrieval.

4.1 Methodical considerations

Optogenetics using ChR2 is a reliable method for inducing neuron activation using light. However, due to the fact that it forcibly activates cells and circuits, it is difficult in a behavioural context to assess how these cells and circuits govern natural behaviour. As such, we attempted a dual approach using chemogenetic silencing and looming shadows to induce innate fear behaviour and observe loss of function. The looming shadow is a reliable method to induce freezing in rodents (Yilmaz et al., 2013). In addition to the looming shadow experimental setup, we also used the Pavlovian fear conditioning paradigm using mild electric foot shocks, a robust method for inducing fear memories in animals (Maren, 2001).

There are several potential advantages to using chemogenetics compared to optogenetics for silencing. In addition to being less surgically invasive, it allows for us to inhibit larger areas in the brain using CNO. Although new optogenetic probes are continuously being developed, CNO currently allows for a long lasting deactivation compared to optogenetics which until now has been limited to repeated light pulses which may generate heat which may cause tissue damage. Although CNO is an inactive metabolite, and theoretically should not interfere with normal biological function, measures should be taken to ensure that there are no behavioural interferences after injecting CNO.

The control group in our DREADD experiments are virus-treated animals with vehicle injected in place of CNO. Baseline movement was measured for both groups of animals, and no measurable differences in CNO injected animals versus vehicle injected animals were detected. Therefore, it is unlikely that CNO had any significant effects on motility in our

experiments and should not have an effect on freezing scores. A more suitable control group, however, would be naïve animals injected solely with CNO.

4.2 The role of the hypothalamus in defensive behaviour

4.2.1 Optogenetic activation of the lateral hypothalamic area

Our optogenetic experiments indicated distinct LHA regions associated with specific behaviours, and demonstrated that selective activation of the LHA is sufficient to induce defensive and eating behaviours.

The region found to be strongly associated with freezing has been found to contain distinct neurons that strongly interconnect with PAG (Alvarez-Bolado et al., 2015), an area known to be important for freezing behaviour (Brandão et al., 2008; Vianna et al., 2003).

This region was dubbed the parvafox nucleus, due to its expression of FoxB1 and parvalbumin (Figure 24).

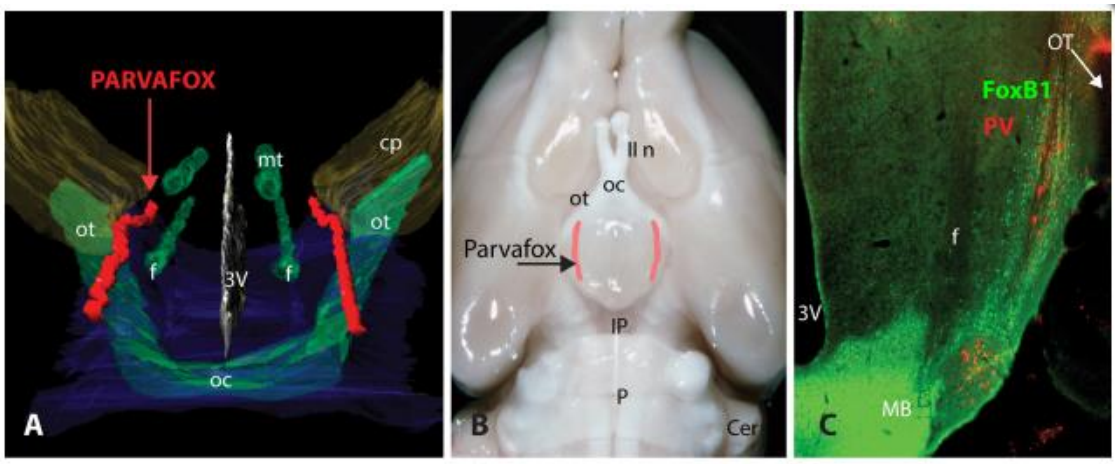

Figure 24: The parvafox nucleus A) A 3D reconstruction of the chain of parvalbumin neurons in the ventral LHA. Shown in relation to the fornix (f), the optic tract (ot), third ventricle (3V), mammillothalamic tract (mt), optic chiasm (OC), and cerebral peduncle (cp). B) The parvafox nucleus seen from the inferior surface of the rat brain. C) A section showing immunostaining for FoxB1 (green) and parvalbumin (red) (Alvarez-Bolado et al., 2015).

Unpublished work by Wigestrand and co-workers has also found, by the use of retrograde tracing, that the LHA is strongly interconnected to PAG and lateral habenula, both of which are important areas for defensive behaviours (Pobbe et al., 2010) (Figure 25).

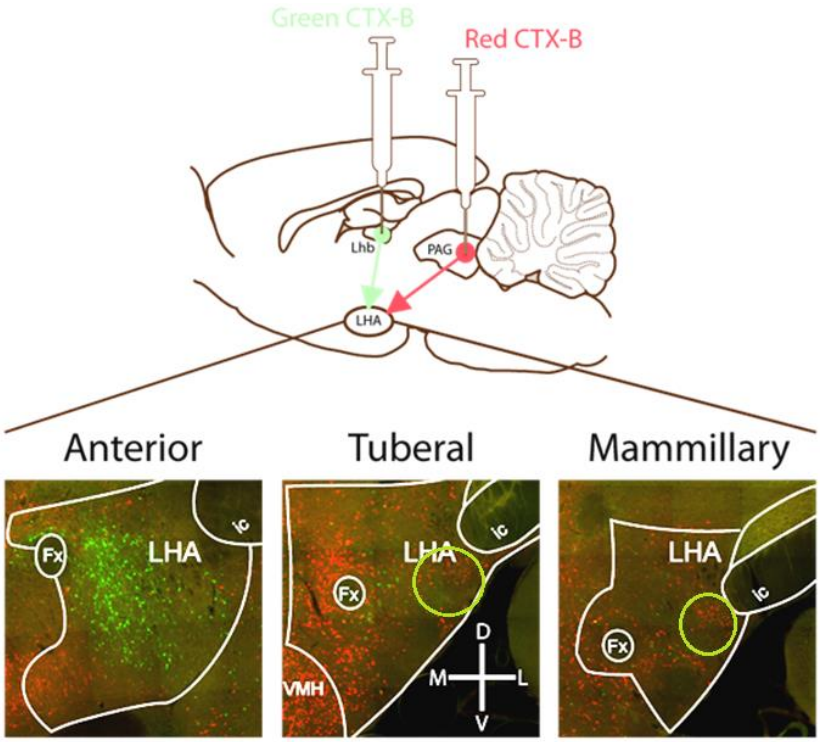

Figure 25: Retrograde tracing connecting the lateral habenula (Lhb) and PAG to specific areas of the LHA The anterior LHA contains cell groups with projections to the lateral habenula, while the tuberal and mammillary part of the LHA strongly interconnect with the PAG. Parvafox nucleus outlined green (Wigestrand and co-workers unpublished).

These findings notably overlap with the parvafox nucleus findings, as there appears to be distinct clusters of cells in the parvafox that propagate to PAG in a similar pattern. There is a strong interconnectivity between LHA and PAG, and the parvafox nucleus suggests complex circuitry between molecularly distinct cells in the LHA and PAG.

4.2.2 Innate versus learned defensive behaviour

Previous studies have found that destruction of neurons in the LHA results in disruption of autonomic function, such as blood pressure, in a classical fear conditioning experiment using tones paired with footshocks. However, no effects on freezing behaviour were observed during fear memory retrieval (Iwata et al., 1986; LeDoux et al., 1988).

In contrast, one previous study found that lesions centred on the perifornical hypothalamus abolished behavioural responses of conditioned fear retrieval (Furlong et al., 2007). Our chemogenetic experiments showed strong expression of mCitrine in the perifornical area of the LHA, and consequently resulted in inhibition of this area during context dependent fear

memory retrieval. However, in our contextual fear conditioning setting, chemogenetic silencing of the lateral hypothalamus also proved to be ineffective in reducing behavioural effects during fear retrieval.

Fear conditioning has been a widely used method to investigate defensive behaviours. However, fear conditioning may not necessarily induce innate defensive behavioural responses. Thus, there might be separate circuits governing innate defensive behaviour and conditioned defensive behaviour, and as such dismissing the role of the LHA in governing defensive behaviours may have been premature.

Ventromedial hypothalamic neurons have been found to elicit defensive states in mice during optogenetic stimulation (Kunwar et al., 2015). Our optogenetic experiments yield similar results upon stimulation of neurons in the LHA. Kunwar and co-workers also attempted to destroy these ventromedial hypothalamic neurons, and found a significant reduction in fear responses after tone-conditioned fear conditioning. In addition, they found a significant reduction in ability to acquire conditional freezing during training.

Furthermore, in their data, mice showed no significant reduction of defensive responses during looming shadow stimuli. Our data are in accordance with these, suggesting that the LHA is involved in innate threat responses to visual threat, such as looming stimuli, while the medial hypothalamic area may be important in eliciting defensive behaviours in response to tone-conditioned fear conditioning.
Studies have also found a crucial link between orexin neurons in the hypothalamus and locus coeruleus in relation to fear learning, supporting the idea that the hypothalamus has a role in fear memory acquisition (Sears et al., 2013).

4.3 Parallel streams and proposed circuitry

There are likely several parallel streams governing defensive behaviours depending on the nature of the stimuli; whether visual, auditory or olfactory, as well as innate and acquired fear. Fear retrieval of conditioned fear to predatory stimuli and painful stimuli such as footshocks, likely depend on different circuits. There are strong interconnections between the central amygdala to the the ventral LHA and to the ventrolateral PAG an area known to induce freezing and likely functions as a part of a circuit for memory retrieval of painful stimuli.

Lesions in central amygdala appear to prevent the expression of conditioned fear to painful stimuli, but do not prevent the expression of fear towards a predator (Martinez et al., 2011; Wilensky et al., 2006). Indeed, central amygdala is an important region for processing pain information (Hasanein et al., 2008). Conversely, lesions in the medial hypothalamic area appear to impair defensive responses predators, but not conditioned fear to pain (Blanchard et al., 2005).

There are strong interconnections between medial hypothalamic area, the dorsolateral PAG, and the medial amygdala. Experiments have confirmed that there is an increased expression of the c-Fos activity marker in the medial hypothalamus during olfactory induced threat (Dielenberg et al., 2001). Overall, the evidence indeed suggests parallel streams governing defensive behaviours towards predatory odours and conditioned fear to pain.
During our looming shadow experiment, we observed a rapid onset of freezing behaviour as a response to a looming visual threat. Its rapid onset is likely the result of a direct pathway from the retina to ventrolateral PAG via the superior colliculus to initiate an immediate defensive response (Dean et al., 1989; Shang et al., 2015; Wei et al., 2015) (Figure 26).

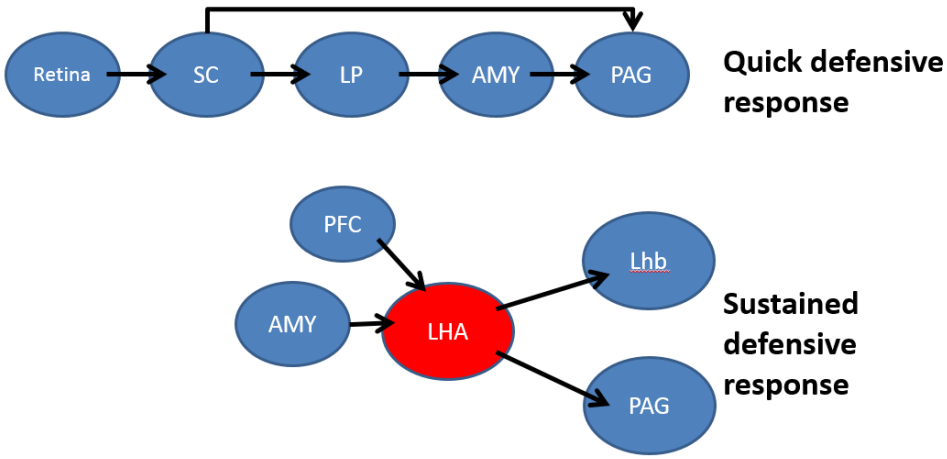

Figure 26: Proposed circuitry, divided in to the quick onset defensive response circuitry and the sustained defensive response circuitry. The quick defensive response consists of a pathway from the retina to the superior colliculus (SC) and subsequently to the lateral posterior nucleus (LP), amygdala (AMY) and periaqueductal gray (PAG). In addition, a direct pathway from SC to PAG is depicted, thought to elicit the rapid onset freezing response seen during looming stimulus. The sustained defensive response is thought to have several pathways, both originating from the amygdala and the prefrontal cortex (PFC) to the lateral hypothalamic area (LHA) further propagating to PAG and lateral habenula (Lhb) to initiate freeze and flight.

The decrease in sustained defensive behaviour in the chemogenetically silenced group suggests post processing and evaluation following innate visual threat which appears to be dependent on activity in the LHA. As such, the LHA might be necessary for evaluating and acting upon threat beyond the initial defensive response, initiating sustained freezing after threat evaluation.

Silencing the LHA resulted in no change in freezing behaviour during memory retrieval of context dependent conditioned pain, and is therefore unlikely to be involved in circuitry governing defensive behaviour for that particular setting. However, we did not investigate all aspects of fear conditioning, and it may be that silencing of LHA may have an effect on retrieval and processing of other kinds of fear memories.

Our data suggests that the LHA might potentially function as a behaviour control centre in the sense that it appears to be involved in post-encounter processing when exposed to visual threat. This conflicts with previously held beliefs that the amygdala serves as a processing region, whereas the hypothalamus only acts as a relay station acting upon processed signals from the amygdala, an idea also challenged by the optogenetic experiments by Kunwar and co-workers (2015) who found similar tendencies in the medial hypothalamic area.

Due to their interconnectivity, there is a possibility that the central amygdala uses the LHA as relay during sustained freezing. However, the LHA also receives input from the prefrontal cortex as well as the ventral hippocampus, which indeed places the LHA in a position to potentially function as an evaluation and processing centre.

4.4 Regulation of appetitive and defensive behaviours

Feeding and defensive behaviours are both essential for the survival of an organism, and failure to evaluate when to feed or when to avoid a predator would be detrimental. When investigating pre-encounter defensive behaviour, it is apparent that threat detection and evaluation influences foraging and feeding behaviour. Our data shows that stimulation of the LHA is sufficient for inducing both feeding and defensive behaviours.

There are known regulatory mechanisms for feeding behaviour within the hypothalamus and between different areas of the brain. Within the LHA, disruption of GABAergic neurons and GABA antagonists causes reduced food intake (Turenius et al., 2009; Wu et al., 2015).

It has been suggested that a part of the extended amygdala, the nucleus accumbens shell (NAcSh), has projections to the LHA that might be involved in controlling feeding behaviour in a context dependent manner, such as predatory threat. Dopamine D1R-expressing NAcSh neurons inhibit GABAergic neurons in the lateral hypothalamus, resulting in cessation of feeding behaviour, even during prolonged hunger (O'Connor et al., 2015).

BNST is also strongly interconnected with the LHA (Oh et al. (2014); http://connectivity.brain-map.org/), and BNST neurons show increased activity during food consumption (Angeles-Castellanos et al., 2007). Optogenetic experiments have found important inhibitory circuits between BNST and LHA to orchestrate feeding through vesicular GABA transporter-expressing (Vgat) GABAergic neurons (Jennings et al., 2013). Intriguingly, these Vgat-expressing GABAergic neurons appear to be distinct and separate from melanin-concentrating hormone and orexin expressing neurons, both of which are known to be important for controlling food intake and regulating energy balance in the hypothalamus (Marsh et al., 2002; Sakurai et al., 1998), suggesting a separate complex network of GABAergic cells in the LHA to control appetitive and ingesting behaviours. Additionally, these cells appear to be concentrated in the dorsal parts of LHA, the very same area shown to induce eating behaviour during our own optogenetic experiments (Jennings et al., 2015).
This may suggest separation within the LHA where the dorsal regions mediate feeding, and ventral regions coordinate defensive behaviours. Interactions between these two regions may be important to balance feeding and defensive behaviours.

There are likely complex networks wherein LHA may serve as a node for orchestrating a delicate balance between defensive and feeding behaviours in a context dependent manner. Thus LHA would be rendered as an important region for governing behaviours in relation to the predatory imminence continuum.

4.5 Future perspectives

An interesting finding in this study was that the defensive circuitry of the LHA appeared to function independently from defensive circuitry related to memories of a fear inducing context involving pain. What we did not further investigate, however, was the role of the LHA during memory acquisition, and it would be interesting to chemogenetically silence the area during fear memory formation to examine whether this circuitry needs to be activated in order to acquire memories of a fear inducing context involving pain. As chemogenetically silencing the LHA appeared to reduce defensive post-stimuli responses to a looming shadow, it may be that LHA circuits contribute to memory formation of the threatening event. In addition, not involved in acquisition of context dependent fear conditioning using pain, it may take part in acquisition of other kinds of conditioned fear.

Another approach would be examining the direct connections that exist between the retina and the LHA (Canteras et al., 2011). While there is evidence that rapid onset defensive behaviour following visual responses follows a pathway from the retina, to the SC to the amygdala and PAG, it has yet to be disclosed whether or not the direct pathways from the optic tract to the hypothalamus may play a role.

During optogenetic stimulation we found clear clusters of behaviour. Animals engaging in flight or feeding did not simultaneously freeze. In addition, defensive behaviours clearly have an effect on foraging and feeding behaviours, altering both frequency and intake amount for each feeding session. As such, there are clearly regulatory mechanisms between regions eliciting feeding and defensive behaviours to maximize caloric intake despite changes in levels of threat. It is not known where these regulatory circuits reside, and as such a potential research prospect would be to map if there are specific circuits in the amygdala, BNST, hypothalamus, prefrontal cortex, PAG or maybe even premotor neurons that regulate defensive and appetitive behaviours.

In addition, the parvafox nucleus should be further investigated. Selective optogenetic activation of these molecularly distinct cells using cre-recombinase expressing mice or rats, as well as electrophysiological recordings in the parvafox nucleus region during looming shadow stimulation might ameliorate its function in relation to defensive behaviours.

4.6 Conclusions

Based on our findings, we conclude that the LHA indeed plays an important role in governing defensive behaviours. Optical stimulation throughout the LHA results not only in eating behaviours, but also flight and freezing behaviour. As such, stimulation of the LHA is sufficient to induce defensive and ingesting behaviours. In addition, our chemogenetic silencing also shows that the LHA is necessary to process innate visual threat.

There was a significant reduction in sustained, but not immediate, defensive behaviour, suggesting the LHA may serve as a post-encounter processing behaviour control centre, further underlining its importance in governing defensive behaviour.

This is the first study to highlight the importance of the LHA when processing innate visually induced threat. Chemogenetic silencing following fear conditioning shows that LHA is not necessary for retrieval of context dependent fear memories, and supports the idea that there are separate circuits involving different types of fear (Gross et al., 2012).

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6 Appendix

6.1 Index of abbreviations

AAV	Adeno-associated virus
A(C)H	Anterior hypothalamus
BSA	Bovine serum albumin
BNST	Bed nucleus of *stria terminalis*
ChR	Channelrhodopsin
CNO	Clozapine-N-oxide
DM(H/N)	Dorsomedial hypothalamic nucleus
DREADD	Designer receptors exclusively activated by designer drugs
DMSO	Dimethyl Sulphoxide
FX	Fornix
GABA	Gamma-Aminobutyric acid
GFP	Green fluorescent protein
HR	Halorhodopsin
IC	Internal capsule
LHA	Lateral hypothalamic area
LHAa	Anterior lateral hypothalamic area
LHApf	Perifornical lateral hypothalamic area

Lhb	Lateral habenula
LP	Lateral posterior nucleus
NAcSh	Nucleus accumbens shell
NSAID	Non-steroidal anti-inflammatory drug
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PMd	Dorsal premammillary nucleus
PMV	Ventral premammillary nucleus
SC	Superior colliculus
ST	Stria terminalis
VM(H/N)	Ventromedial hypothalamic nucleus
ZI	Zona incerta

6.2 Chemicals and solutions

6.2.1 10X PBS

80 g NaCl

2.0 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

Dissolve in 800 mL dH_2O, adjust to pH 7.4, and adjust volume to 1 L.

Dilute 1:10 with dH_2O for a 0.01 M solution.

6.2.2 Block solution

10 mL 0.01 M PBS

0.1 g BSA

20 µL Triton-X

Gently dissolve to avoid foaming.

6.2.3 Paraformaldehyde

40 g PFA

1 L 0.01 M PBS

Heat to 50-60 °C. Stir until dissolved. Filter before use.

6.3 Immunohistochemistry protocols

6.3.1 Staining of the c-Fos activity marker and endogenous viral GFP

Day 1

Rinse sections three times in 0.01 M PBS for five minutes each at room temperature during gentle shaking.

Block for one hour in 3 mL 1 % BSA (Sigma-Aldrich, USA), 0.2 % Triton 100-X (Sigma-Aldrich, USA) in 0.01 M PBS at room temperature during gentle shaking.

Incubate overnight in 2.5 mL 1:2000 0.01 M PBS/primary antibody solution (anti c-Fos goat + anti-GFP chicken).

Day 2

Rinse sections three times in 0.01 M PBS for five minutes each at room temperature during gentle shaking.

Incubate for one hour in 2.5 mL 1:200 0.01 M PBS/secondary antibody solution (Alexa Fluor® 488 goat anti-chicken and Alexa Fluor® 594 goat anti-rabbit).

Rinse sections three times in 0.01 M PBS for five minutes each at room temperature during gentle shaking.

Mount on glass slides and allow to dry for at least 30 minutes.

Rinse slides in dH_2O three times.

Add ProLong Gold Antifade solution with DAPI to slides and add coverslip.

Allow to dry overnight.

6.3.2 Staining GFP

Day 1

Rinse sections three times in 0.01 M PBS for five minutes each at room temperature during gentle shaking.

Block for 1 hour in 3 mL 1 % BSA (Sigma-Aldrich, USA), 0.2 % Triton 100-X (Sigma-Aldrich, USA) in 0.01 M PBS at room temperature during gentle shaking.

Incubate overnight in 2.5 mL 1:2000 0.01 M PBS/primary antibody solution (anti-GFP chicken).

Day 2

Rinse sections three times in 0.01 M PBS for five minutes each at room temperature during gentle shaking.

Incubate for one hour in 2.5 mL 1:200 0.01 M PBS/secondary antibody solution (Alexa Fluor® 488 goat anti-chicken).

Rinse sections three times in 0.01 M PBS for 5 minutes each at room temperature during gentle shaking.

Mount on glass slides and allow to dry for at least 30 minutes.

Rinse slides in dH_2O three times.

Add ProLong Gold Antifade solution with DAPI to slides and add coverslip.

Allow to dry overnight.

6.3.3 Nissl staining

Incubate sections on 0.1 % Triton X-100 / 0.01 M PBS for one hour.

Wash sections twice in 0.01 M PBS for five minutes each at room temperature.

Incubate in 1:100 Neurotrace Fluorescent Nissl Stain diluted in 0.01 M PBS for 30 minutes at room temperature.

Incubate in 0.1 % Triton X-100 / 0.01 M PBS for 10 minutes in room temperature.

Wash twice in 0.01 M PBS for five minutes each at room temperature.

Mount sections onto glass.