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Understanding the neural dynamics of ventromedial hypothalamus in defence and aggression

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Summary

Fear and aggression are evolutionary conserved emotional responses that can be evoked by different stimuli. One of these stimuli is exposure to a threatening conspecific that depending on the context and history of previous encounters can elicit either defence and avoidance or approach and aggression. The ventrolateral division of the ventromedial hypothalamus (VMHvl) has been recently identified as a structure involved in both behaviours. Neural activity in the ventromedial hypothalamus has been shown to be necessary for defensive and aggressive behavioural responses to conspecific threats. In male mice, inhibition of neural activity in VMHvl reduces avoidance behaviour following exposure to an aggressive male, as well as attack behaviour following exposure to a subordinate male. However, whether the same or different neurons in VMHvl are responsible for defence and aggression toward social threat, how experience affects these responses and the identity of defence neurons in VMHvl remains unknown. Here we performed serial cFos labelling experiments and found that defence and aggression recruited partially overlapping populations in VMHvl. Using *in vivo* calcium endoscopy of VMHvl neuron activity during social defence and aggression we found that strong calcium responses were elicited upon exposure to the social stimulus and these were further modulated as the animal exhibited defensive or aggressive behaviours. Notably, specific neuronal calcium responses were identified that were correlated with defensive behaviours, some of these neurons were reacted to more than one behaviour, showing complex patterns of activity during aggression and defence. Moreover, calcium recordings over several days of either defence or aggression revealed a change in the ensemble activity between defence and aggression and this effect was dependent on the previous experience of an animal. At the same time we performed a series of functional manipulation experiments blocking or activating neuronal activity in different cell types of the VMHvl. We found separate populations of VMHvl Esr1+ and Nos1+ neurons that were able to modulate defensive responses to social threat. Together, these results demonstrate that the VMHvl encodes and controls both specific and overlapping features of defensive and aggressive behavioural responses to social threat.

Zusammenfassung

Furcht und Aggression sind evolutionär konservierte Basisemotionen, die von verschiedenen Reizen hervorgerufen werden. Einer dieser Reize ist die Konfrontation mit einem bedrohlichen Artgenossen. Abhängig von der individuellen Vorgeschichte vorangegangener Begegnungen ruft diese Konfrontation agonistisches Verhalten in der Form entweder defensiven, vermeidenden Verhaltens oder einer aggressiver Annäherung hervor. Der ventrolaterale Teil des ventromedialen Hypothalamus (VMHvl) wurde vor kurzem als Struktur identifiziert, die in beide Verhaltensmuster involviert ist. Es wurde gezeigt, dass diese Hirnregion sowohl für sowohl aggressives, als auch defensives Verhalten als Antwort auf einen aggressiven Artgenossen notwendig ist. So führt die Inhibition der neuronalen Aktivität im VMHvl in männlichen Mäusen in einem sozialen Kontext zu einer Abnahme vermeidenden Verhaltens bei Kontakt zu einem aggressiven Artgenossen, aber auch zu einer Abnahme aggressiven Verhaltens bei Kontakt zu einem unterwürfigen Artgenossen. Offen ist dabei bisher geblieben, ob unterschiedliche oder gleiche neuronale Populationen diese gegensätzlichen Verhalten steuern, welche Neuronen für defensives Verhalten zuständig sind und inwiefern Erfahrung diese Verhaltensweisen beeinflusst. Im Rahmen dieser Arbeit konnten wir mittels serieller cFos-Labelling Experimente nachweisen, dass teilweise überlappende neuronale Populationen im VMHvl sowohl während aggressivem als auch defensivem Verhalten aktiv sind. Mittels *in vivo* Calciumendoskopie konnten wir beobachten, dass der soziale Stimulus durch einen Artgenossen eine starke Aktivierung des VMHvl auslöst, die je nach Art der Interaktion (aggressiv bzw. defensiv) weitergehend moduliert wird. Insbesondere konnten wir eine Korrelation spezifischer Akitvitätsmuster mit bestimmten defensiven Verhaltensweisen identifizieren. Hierbei waren einige der beobachteten Neuronen während mehrerer unterschiedlicher defensiver Verhaltensweisen aktiv. Dies unterstreicht die hohe Komplexität der neuronalen Aktivität während Aggression und Verteidigung. Aufnahmen der Calciumaktivität über den Zeitraum mehrerer Tage zeigten außerdem einen Unterschied zwischen der Aktivität neuronaler Ensembles während Aggression auf der einen, beziehungsweise Defensive auf der anderen Seite. Dieser Effekt ist stark von der vorherigen Erfahrung der Tiere abhängig. Um ein genaueres Bild der Rolle einzelner Zelltypen zu erhalten, führten wir zeitgleich eine Reihe direkter funktionaler Manipulationen der Zellpopulationen im VMHvl durch. Esr1+ und Nos1+ Neuronen

spielen beide eine Rolle in der defensiven Antwort auf soziale Bedrohungen. Dies zeigt, dass VMHvl eine zentrale Rolle in Verschlüsselung und Kontrolle spezifischer und überlappender Merkmale agonistischen Verhaltens einnimmt.

Statement of Contribution

Certain data included in this thesis was produced with help of fellow members of the Dr. Cornelius Gross lab.

Ensemble analysis of *in vivo* calcium imaging data was done together with Beatrice Penna.

Agne Dambrauskaite and Tristan Wiessalla contributed to Nos1 pharmacogenetic inhibition studies by performing behavioural paradigm, some of the surgeries and scoring. They also contributed to the quantification of the overlap between Nos1 and Esr1 neurons in VMHvl.

List of abbreviations

AHN – Anterior hypothalamus

BA – Basal amygdala

BDNF – Brain derived neurotropic factor

BLA – Basolateral amygdala

BNST – Bed nucleus of the stria terminalis

CCK – Cholecystokinin

CEA – Central amygdala

ChR2 – Channelrhodopsin-2

CS – Conditioned stimulus

DAT – Dopamine active transporter

DMH – Dorsomedial hypothalamus

fMRI – Functional magnetic resonance imaging

LA – Lateral amygdala

LDA –Linear discriminant analysis

LS – Lateral septum

MEA – Medial amygdala

NPY – Neuropeptide Y

Oxtr – Oxytocin receptor

PACAP – Pituitary adenylate cyclase-activating peptide

PAG – Periaqueductal grey

PB – Parabrachial nucleus

PCA – Principal component analysis

PFC – Prefrontal cortex

Pgr – Progesterone receptor

PLS – Partial least squares regression

PMD – Dorsal premamillary nucleus

PMV – Ventral premamillary nucleus

US – Unconditioned stimulus

vHip – Hippocampus ventral part

VTA – Ventral tegmental area

VMH – Ventromedial hypothalamus

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1. Introduction

1.1 Emotions

1.1.1 What is an emotion?

What is an emotion? People who have been asked this question can answer in many different ways. Some will start to list different names like fear, anger, happiness or disgust following the work of Ekman, who tried to elucidate basal and distinct emotions shared between different human populations. Others can start describing their bodily sensations like faster blood circulation, piloerection and sweating, pointing out arguments first brought to attention by the 19th philosopher William James. Another way people try to explain emotions is by describing what is not an emotion, contrasting it with planned actions and reasoning circling around what is known as the "Two-factor theory of emotions" and its more modern versions (Ralph Adolphs, 2010; Tye, 2018). The answer to this question is not intuitive, yet very important to anyone attempting to study emotions. To better understand this problem, it is worth looking at the history of thought and attempts to develop a consistent definition and theory by affective neuroscientists during last century.

In 1872 Charles Darwin published his "The Expression of the Emotions in Man and Animals" and was the first to approach this question. On the basis of his theory of evolution Darwin proposed a wide homology and similarity between expressions of emotions across the species. He studied facial expressions of humans, chimpanzees, cats and dogs and noticed similarities and concluded that there is a basic set of emotions common to different species that is easily recognizable by common, partially conserved action patterns (Dalgleish, 2004; Darwin, 1965). Later this idea was elaborated by Paul Ekman who during the 1970s published a series of articles studying human facial expressions. By showing pictures of different facial expressions to people from various countries and cultural backgrounds Ekman found that they could easily recognise a set of emotions, which he later called basic emotions – fear, anger, disgust, happiness and sadness – from photographs of faces with certain muscular patterns (Ekman, 1970). He also thought that emotions could

be grouped into "families" that are based on the basic emotions in the way that emotions belonging to the same family would be very similar to each other (Ekman, 1992).

Shortly after Darwin's publication William James proposed his own theory, stating that emotions are simply experiences of bodily states in response to certain stimuli and that different configuration of those states give rise to different emotions. He stated that we start feeling emotion after an action. For example, we feel sad because we cry or we feel angry because we strike another person who insulted us a few seconds ago (James, 1884). At the beginning of the 20th century James' theory was criticized by Phillip Bard and Walter Cannon, who observed emotions ("sham rage") in decorticated cats (Bard, 1928), arguing that if emotions are bodily feelings, they should rely on intact sensory cortices. Based on their findings they proposed a new theory placing the hypothalamus as a cradle of emotions. This sparked a set of neuroanatomical theories of emotion throughout the 20th century starting with the Papez theory, MacLean Limbic system theory and later theories placing the prefrontal cortex (PFC) and the amygdala as an emotion centre (Dalgleish, 2004). In 1962 a pioneering work showed that the same bodily signals could be experienced differently depending on the context, where those signals occur (Schachter & Singer, 1962), showing that cognitive processes play a role in emotions as well.

More recent theories continue along notions of those two main scientific lines of thought. For example Antonio Damasio's somatic marker theory, based on the observation of patients with frontal injuries argues that changes in body states induced by emotional stimuli are processed by the PFC and facilitate logical reasoning, as well as boost processes of attention and working memory (Damasio, 1996). On the other hand, Jaak Panksepp's work on basic emotions points out that all animals including humans show similar emotional responses to pain like crying or shrieking and that all emotions can be placed along intensity and valence dimensions calling attention to the fact that animals learn to turn off unpleasant stimulations and trigger stimulation, if it evokes positive emotional state (Panksepp, 2011). In the last years new theories have started to emphasize than an emotion is a central state of the nervous system (Anderson & Adolphs, 2014) and that there is a separation between survival/emotional behaviour and cognitive states of feeling the emotion (LeDoux, 2012; LeDoux & Pine, 2016).

1.1.2 Neural circuits of emotions

The study of neural circuits of emotions dates back to the early 20th century with previously mentioned cortical lesion experiments by Bard and hypothalamic stimulation experiments done on cats by Walter Hess. These studies led to the identification of the hypothalamus as a centre of emotions gated by cortical structures. In 1937 James Papez laid out his theory about a circuit governing emotions that was composed of the thalamus as a receiver of sensory input, followed by "emotional thoughts" being produced via a "top" route that included the cingulate cortex and hippocampus, and finally passed down to hypothalamus to provide feedback and regulate emotional responses. The second route that produces emotional "feelings" was proposed to go through the hypothalamus, then back to the anterior thalamus and cingulate cortex. The third route that Papez introduced went to the striatum to produce direct actions (Papez, 1937). Central part in this theory was played by the cingulate cortex that was able to produce feelings and regulate emotive actions. This theory was the first one trying to integrate all aspects of emotions and many of the pathways described by Papez were later discovered to be involved in emotions.

In early 1950s Paul MacLean devised his theory of "triune brain" and coined the term "limbic brain". He elaborated on the Papez and Bard theories by integrating data from more recent works by Klüver and Bucy on monkeys with lesions of the temporal lobe (Klüver & Bucy, 1937). Maclean's triune brain consisted of an ancient reptilian brain, executing actions comprising the basal ganglia and striatum, the youngest brain part – the neocortex – providing cognition and exerting top down control on emotions, and the last part of the triune – the limbic brain that included structures described by Papez and Bard with the addition of the amygdala and PFC (Maclean, 1949). In the proposed system the limbic brain acts as a "mediator" between the ancient reptilian brain and the neocortex (Ralph Adolphs, 2010). The limbic brain was thought to integrate sensory information from the outside world with internal signals of the body state where the outcome of this integration process would produce emotion (Dalgleish, 2004). These early circuit theories have proven to be very useful as foundation for affective neuroscience and many of the described structures like amygdala, hypothalamus, PFC and cingulate cortex were identified as key regions in emotional processing and currently are the main subject of studies of affective neuroscience circuitry.

The amygdala is a highly conserved, small region in the brain consisting of many sub-nuclei. Following the work of Klüver and Bucy the amygdala is implicated in processing emotion and motivation (Janak & Tye, 2015). Work of L. Weiskrantz in 1950s revealed that monkeys with bilateral amygdala lesions exhibit less fearful responses and have shorter extinction time to conditioned avoidance (Weiskrantz, 1956). Later studies of amygdala lesions showed similar effects in rats (Blanchard & Blanchard, 1972). Studies on humans are largely based on a case study of the patient SM who suffered bilateral damage to the amygdala due to the illness. SM has impaired recognition of facial emotions (Adolphs, Tranel, Damasio, & Damasio, 1994) and seems not to experience fear in response to various fearful stimuli like snakes, spiders and haunted house tour (Feinstein, Adolphs, Damasio, & Tranel, 2011). The amygdala is also involved in reward associated behaviour. For example, amygdala lesions we shown to impair amphetamine place preference conditioning (Hiroi & White, 1991). The amygdala also have been indicated to control aggressive behaviours (Unger et al., 2015).

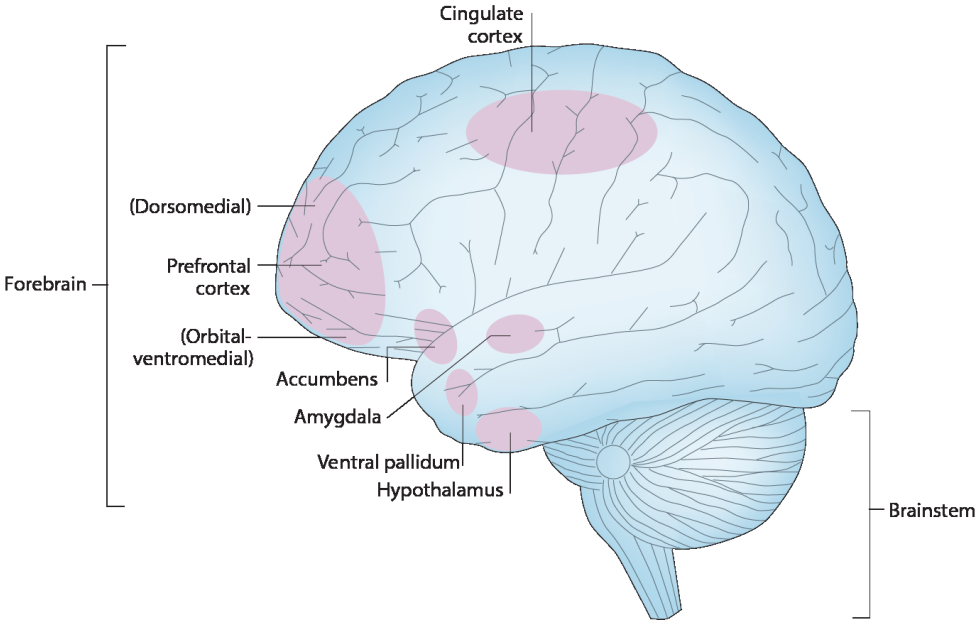

Fig. 1 A graphic depicting regions in the human brain implicated in emotional processing (Addapted from Dalgleish, 2004).

The prefrontal cortex was implicated in emotional processing after studying the case of Phineas Gage who suffered an accident at work which destroyed most of his prefrontal cortices. After the accident he displayed changes in his personality, social behaviours and emotions. Since then the PFC has been implicated in reward processing, primarily in coupling secondary stimuli with unconditioned positive stimuli like food or sex (Dalgleish, 2004). As mentioned above the PFC is also though to integrate body states and facilitate

reasoning according to the research done by Damasio. The prefrontal cortex also participates in a top-down control of emotions particularly aggression, based on the case of Phineas Gage, studies in mice (Takahashi et al., 2014) and humans (Grafman et al., 1996).

Following the studies of Hess in cats, the hypothalamus was implicated in reward processing. Studies by Paul Milner and James Olds showed that rats implanted with electrode in the anterior hypothalamus could learn to self-stimulate (Olds & Milner, 1954). Recently, ventromedial hypothalamic nuclei (VMH) have been implicated in fear responses to social and predatory stimuli, aggression towards conspecifics and sexual behaviours in both male and female mice (Lin et al., 2011; Silva et al., 2013; Yang et al., 2013). Various parts of the hypothalamus were identified to play a role in motivations like hunger (Atasoy, Betley, Su, & Sternson, 2012), reproduction and hunting (Yi Li et al., 2018). In addition, the hypothalamus is thought of as a main regulator of visceral activities.

1.1.3 How to study emotions?

Studying emotions is not easy. Emotions, despite intuitive and introspective obviousness of its nature, have proven hard to define in scientific terms. Emotions as we know and feel them can best be studied in humans via introspective self-reports, functional magnetic resonance imaging (fMRI) and rare cases of patients with selective focal lesions of structures involved in emotions. On the other hand, self-report is hard to quantify and compare and fMRI lacks spatial and temporal resolution. Unfortunately, the latest technologies for circuit interrogation like optogenetics or calcium imaging of neuronal activity cannot be applied in human studies. However, animals like monkeys or rodents that allow usage of much finer and powerful techniques cannot tell us if they feel emotions the way we do. Commonly, we use behaviour to attribute emotions to people as well as animals, but since conscious feelings of affect seem to be an inherent part of emotions, we cannot be sure if animal emotions are the same. Even if we agree that mammals are conscious this can vary in the level and content (Tsuchiya & Adolphs, 2007) making findings in animals hard to translate to humans.

In recent years leading affective neuroscientists have tried to tackle this problem by providing various conceptualizations and frameworks to study emotions. The general view

that emotions like fear, anger or sadness are directly transferable from humans to other animals and that circuits mediating behaviours associated with those emotions can be studied as actual emotion circuit has been challenged by James LeDoux. LeDoux argues that the basic emotion concept is flawed and confusing, especially the usage of common words to name emotions, and that different basic emotion theories have a different number of basic emotions and that studying the relevant circuits should be done with regard to innate survival circuits as opposed to human context driven framework of emotions (LeDoux, 2012). Survival circuits would contribute to emotions indirectly, but their main role is to "negotiate behavioural interactions in situations in which challenges and opportunities exist, not to create feelings" (LeDoux, 2012). In this view emotional states would be a conscious realization of the workings of the survival circuits that produces species-specific responses, such as arousal, increasing the probability of certain actions being performed and inducing a global organismic state in response to emotive stimuli. LeDoux argues that neuroscientist should study only the survival circuit aspect of emotion and not conscious feelings, as these are likely to be variable between species (LeDoux, 2012).

Another prominent emotion researcher, Ralph Adolphs, together with David Anderson proposes the view that emotions are central nervous system states that are causative to behaviour and subjective feelings (Anderson & Adolphs, 2014). They characterize these states by four properties: scalability – referring to different intensities of the state, persistence – saying that the state usually lasts longer than the stimulus, generalization – indicating that these states affect subsequent events, and valence. They argue that these emotion central states can be identified by studying behaviours that show some or all of the above mentioned properties (Anderson & Adolphs, 2014). Later Adolphs expanded this theory by adding additional properties to emotion central states like coordination and automaticity – meaning that emotions are prepotent in their control of behaviours (Adolphs, 2017). In 2016 LeDoux and Pine proposed another framework to study fear related emotions by separating circuits causal to behaviours from cognitive circuits that cause feelings of emotion. In this paper the authors point out that feelings of fear do not always correlate well with behaviour and that infants react emotionally before they are able to report it (LeDoux & Pine, 2016). Another point against the theory of a central basic emotion of fear is that people with the amygdala lesions can still feel fear (Feinstein et al., 2013), as well as that subliminal stimuli can activate the amygdala in

human studies (LeDoux & Pine, 2016). They argue that affective neuroscientists should study cognitive fear circuits separately from fear behaviour circuits and that this approach may improve the translational value of discoveries (LeDoux & Pine, 2016). This view was subsequently criticized by Fanselow who claimed that the separation of behaviour and feeling would hurt progress made in the behavioural neuroscience field and that psychiatry had already abandoned self-reports to study feelings for reasons of being semi-quantitive and unreliable (Fanselow & Pennington, 2017). It is worth noting that other views pose hypothesis that emotions are decentralized, cannot be differentiated to separate circuits and that the whole brain participates in their construction and should be studied in a phenomenal way (Barrett & Satpute, 2017).

As reviewed above there is a significant disagreement on how to study emotions going back to fundamental questions about definition of emotion. In this work I take a middle stance by synthetizing different views. I believe that emotions are states of the central nervous system that increase the probability of certain behaviours occurring and that are causative to feelings that arise upon the conscious awareness of those states. I think that emotions can be studied via behaviour and that circuits for behaviour and feelings cannot be completely separated, but may involve some different or additional structures.

1.2 Fear

Fear is an emotion which is primarily defined in humans as a conscious feeling arising in situations with a direct exposure to subjectively perceived dangerous stimuli. In animals we usually look for behaviours associated with this emotion in order to be able to attribute a fear state to them. As previously exemplified with the definition of emotion, not surprisingly there is ongoing discussion about the true nature of fear. The main questions are whether defensive behaviours are actually governed by fear circuits or are separate circuits distinctive from cognitive circuits involved in fear, and if animals are able to consciously “feel” fear (Ralph Adolphs, 2013; LeDoux, 2014). In this work I apply the same framework as for emotions: I believe fear is a central nervous system state that is causative of behaviours and feelings and both behaviours and feelings are mediated by a common circuitry, where different structures contribute to different parts of the fear emotion. I also argue that fear is a scalable state which illustrates a range of different

responses to fearful stimuli as well as might provide a partial explanation for the fact that we can be in fear state without realizing it (Winkielman & Berridge, 2004).

1.2.1 Fear stimuli and defensive behaviours

Innate fear stimuli, which are defined as stimuli that can elicit defensive behaviour without a prior experience (Blanchard, Griebel, & Blanchard, 2003), can be divided either by class or by the sensory modality via which they are detected (Silva, Gross, & Graff, 2016). Division by class is as follows: predatory threats, which are the presence of a predator itself or other clues such as its smell or shadow, conspecific threats like overcrowding or aggression and internal stimuli like pain or chemical signals. If stimuli are divided on the basis of modality, it could be visual cues like a shadow (looming stimulus), a sudden motion or high place. Olfactory clues are smell of stressed conspecifics and urine or saliva of predators. Auditory clues are loud noises or sounds of predator. The last category are internal stimuli such as pain, organ failure, suffocation, hypoxia and high levels of CO_2 (Ralph Adolphs, 2013; Caroline Blanchard et al., 2003; Schimitel et al., 2012; Silva, Gross, et al., 2016). Most of the mentioned above innate stimuli are common to land mammals with small differences based on the habitat and potential predators.

Another class of fear stimuli are learned clues. One way to acquire new fear stimuli is by fear conditioning. Briefly, the animal is presented with a novel cue, for example, a specific sound that does not elicit any behavioural response, then every time this new cue is presented it co-occurs with an innate fear stimuli eliciting defensive behaviour. The pairing of a chosen sound (conditioned stimulus - CS) and innate cue (unconditioned stimulus – US) is repeated many times. After this procedure the animal will display defensive behaviours in response to CS presentation alone, therefore, acquiring a new fear stimulus (Herry & Johansen, 2014; J. E. LeDoux, 2014; S Maren, 2001; S Maren, 2011). Such paring can be extinguished by multiple presentation of CS without US, but this process is time consuming and can be easily reversed by reacquisition, reinstatement or renewal processes (Goode & Maren, 2014). Interestingly, also the context where pairing of CS and US has occurred can elicit defensive behaviours in a conditioned animal. Another way to obtain “a new fear” is social learning. Social learning is a predominant way how humans learn novel fearful stimuli (Olsson & Phelps, 2007). Animals show this form of learning as well. Young monkeys learn defensive behaviours from their parents, rats show

the amygdala activation when housed with another rat that was conditioned with foot shock (Knapska et al., 2006). Even *Drosophila* can emit CO_2 in response to innate fear stimuli that induce avoidance in other flies (Ralph Adolphs, 2013; Suh et al., 2004). The only uniquely human feature according is that we can trigger fear feeling just by thinking about a freighting stimulus or a previously scary situation.

After describing fear stimuli we can now look at the most common defensive responses triggered by them in animals and humans. In mammals, especially rodents, all measures are behavioural, whereas in humans in addition there are various forms of self-report like fear inventories and questionnaires. Laboratory tests on humans usually involve skin conductance, facial expression, auditory startle, cortisol level, pupil dilation or heartrate measurements (rewied in Ralph Adolphs, 2013). In rodents, in addition to autonomic measurements listed above, we can observe direct behavioural responses. While presented with predator, rodents display array of defensive behaviours: avoidance, freezing, flight, vocalization, defensive attack, upright postures, and risk assessment behaviours like head orientation or stretch (Blanchard & Blanchard, 1989; Blanchard, Flannelly, & Blanchard, 1986; Blanchard et al., 1998). Interestingly, animals display different defensive behaviour dependent on the context and intensity of the stimulus, for example the distance to the predator. As the predator approaches preferred responses switch from flight and avoidance to freezing and in very close inescapable situations to defensive attack, vocalization and upright postures (Blanchard, Parmigiani, Agullana, Weiss, & Caroline Blanchard, 1995). A successful escape is usually followed by a prolonged period of risk assessment behaviour which is thought to serve as information gathering and can be classified as an anxiety measure (Blanchard & Blanchard, 1989). Laboratory tests for this kind of stimuli usually involve a controlled presentation of a predator in various contexts. For social challenge paradigms there are various tests usually involving the introduction of a dominant or aggressive male (Blanchard, McKittrick, & Blanchard, 2001). Common tests are the social defeat test, social interaction test, three chamber social approach test, social preference avoidance test, and social instability test (reviewed in Toth & Neumann, 2013). In response to aggressive conspecifics rodents show similar defensive behaviours as to predators, including avoidance, flight, freezing, defensive aggression and risk assessment with the addition of what is thought be social threat behaviours like tail rattle (Blanchard & Blanchard, 1988; Caroline Blanchard et al., 2003; Blanchard et al., 2001; Silva et al., 2013). A prolonged exposure to a dominant male may lead to social stress, which can

further change the behaviour of an animal to make it less aggressive (Blanchard et al., 1995), less sexually active (D'Amato, 1988) and more anxious (Rodgers & Cole, 1993). Internal stimuli like pain cause avoidance and induces freezing in the context where pain (for example, foot shock) happened (Silva et al., 2013). Other stimuli like organ failure or high CO_2 levels can induce escape and panic (Feinstein et al., 2013).

1.2.2 Neural circuits of fear

During the decades of research many structures have been implicated in fear processing. Widely used fear learning paradigms identified important brain structures participating in this process: the amygdala, the prefrontal cortex (PFC), the ventral hippocampus (vHip) and the periaqueductal grey (PAG). Whereas studies using innate threats pointed to an important role for the medial hypothalamus in addition to structures mentioned above (Gross & Canteras, 2012; Herry & Johansen, 2014; J. LeDoux & Daw, 2018; Silva, Gross, et al., 2016; Tovote, Fadok, & Lüthi, 2015).

The amygdala has been proposed as a key structure involved in fear. It has two main parts, a cortical-like part with two main subnuclei: the lateral amygdala (LA) and the basal amygdala (BA), and a striatal-like part consisting of the central amygdala (CEA) and the medial amygdala (MEA) nuclei (Swanson & Petrovich, 1998; Swanson, 2000). LA is thought to receive visual and auditory input from cortical (McDonald, 1998) and thalamic sites and nociceptive signals from PAG (Herry & Johansen, 2014). LA projects this information to BA and these nuclei are thought to integrate non-olfactory information about predators (Martinez, Carvalho-Netto, Ribeiro-Barbosa, Baldo, & Canteras, 2011), as well as play an important role in fear conditioning showing plasticity during CS and US pairing (Amano, Duvarci, Popa, & Paré, 2011; Quirk, Repa, & LeDoux, 1995). Both of these nuclei project to CEA whose medial part in turn projects to the ventrolateral PAG to promote freezing (S Maren, 2001). A part of BA, the basomedial amygdala, sends projections to the medial hypothalamus particularly to the VMH that provide non-olfactory information about conspecific and predatory threat (Gross & Canteras, 2012). CEA is composed of two parts, the aforementioned medial CEA (CEAm) and the lateral CEA (CEAl). CEAl has been showed to inhibit neurons in CEAm and gate fear expression (Ciocchi et al., 2010) and to project to basal forebrain nuclei promoting risk assessment (Gozzi et al., 2010) and to PAG itself, likely modulating fear expression (Penzo, Robert, & Li, 2014). CEA is also a

recipient of pain sensory information sent via ascending fibres from the parabrachial nuclei (PB) (Bernard & Besson, 1990). The medial amygdala receives olfactory inputs and shows activation to predator and conspecific threats (Martinez et al., 2011). It was shown that the posterior-dorsal MEA projects to the ventrolateral VMH (VMHvl) which is a part of the hypothalamic conspecific fear circuit, while posterior-ventral MEA projects to the dorsomedial VMH (VMHdm) that is a part of the predator fear circuit (Gross & Canteras, 2012; Silva et al., 2013). The amygdala is viewed as a key centre for fear processing, but a recent study showed that humans with bilateral lesions of the amygdala can experience fear or even a panic attack as response to internal fearful stimuli like CO_2 inhalation (Feinstein et al., 2013).

The medial hypothalamus is hypothesized to be the home of two separate fear circuits: for innate threats of conspecific and predator (Gross & Canteras, 2012; J. LeDoux & Daw, 2018; Silva, Gross, et al., 2016). The predator responsive hypothalamic circuit is composed of three heavily interconnected nuclei, VMHdm, the anterior hypothalamic nucleus (AHN), and the ventrolateral part of the dorsal premammillary nucleus (PMDvl) (Cezario, Ribeiro-Barbosa, Baldo, & Canteras, 2008; Gross & Canteras, 2012). Pharmacogenetic inhibition of VMHdm leads to a decrease of fear responses to a predator, but not to conspecific or foot shock (Silva et al., 2013). Optogenetic activation leads to various defensive responses including freezing and flight (Kunwar et al., 2015; Wang, Chen, & Lin, 2015). Cytotoxic lesions to PMD reduce rat defensive responses to cat (Cezario et al., 2008), but not to foot shock (Caroline Blanchard, Canteras, Markham, Pentkowski, & Blanchard, 2005). Nuclei in the predator responsive circuit project to the dorsal PAG structure involved in the expression of defensive responses (Bittencourt, Carobrez, Zamprogno, Tufik, & Schenberg, 2004; Silva et al., 2013). This circuit was also shown to be necessary for fear memory encoding (Kunwar et al., 2015; Silva, Mattucci, et al., 2016). The conspecific fear circuit is comprised of VMHvl, the ventral premammiliary nuclus (PMV), the medial preoptic nucleus (MPN) and the dorsomedial part of PMD (PMDdm). Exposure to an aggressive conspecific activates these nuclei and lesions of PMD (Motta et al., 2009) and vlVMH (Silva et al., 2013) decrease defensive responses to conspecific. Both PMD and VMHvl send projections to PAG (Lo et al., 2018) activating defensive response to conspecific. Not all predator cues activate the medial hypothalamus. For example, defensive responses to looming stimulus are mediated by superior colliculus projections to the dorsal PAG (Evans et al., 2018).

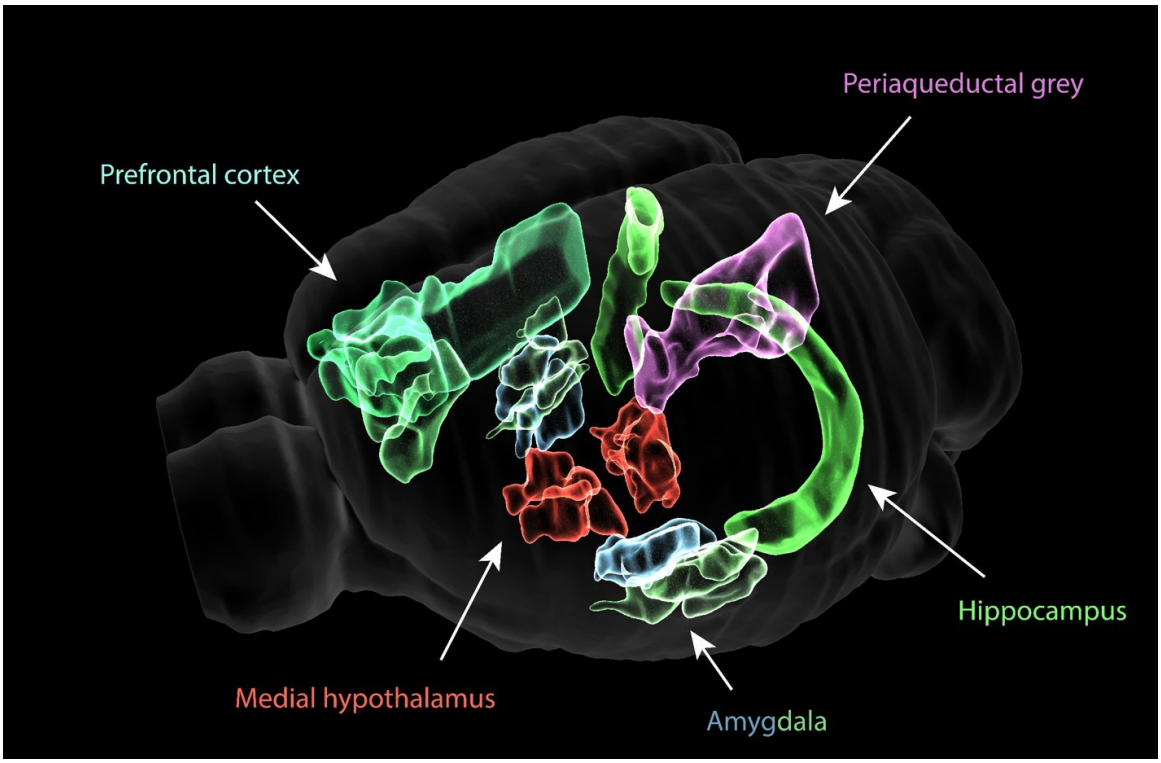

Fig. 2 An image presenting regions implicated in fear in mouse brain. (Image was produced using Blue Brain Brain Atlas application)

The periaqueductal grey is a major output of the amygdala and medial hypothalamic nuclei mediating defensive responses to innate and learned threat (Johansen, Tarpley, LeDoux, & Blair, 2010; Kim, Rison, & Fanselow, 1993; Silva et al., 2013). As mentioned earlier, PAG receives inputs from the amygdala and medial hypothalamus. dPAG mediates flight responses to innate stimuli and its activity correlates with flights, whereas vlPAG mediates freezing responses during fear conditioning (Deng, Xiao, & Wang, 2016; Silva et al., 2013; Tovote et al., 2016; Watson, Cerminara, Lumb, & Apps, 2016). PAG also plays an active role in fear conditioning since an activation of this structure during fear conditioning can support learning and serve as US (Kim et al., 2013).

PFC and the ventral hippocampus (vHip) play important roles in the learning of fear response to novel stimuli and context. The hippocampus is known to encode contextual information and lesions to vHip impair fear expression in a context of a predator and pain stimuli (Caroline Blanchard et al., 2005). PFC modulates fear expression of learned fear by integrating inputs from the amygdala and vHip (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009) and lesions of this structure diminish conditioned fear response (Keene & Bucci, 2008). PFC also controls fear expression by directly synchronizing inputs to BA (Courtin

et al., 2013). It is worth mentioning that PFC also projects directly to PAG and the medial hypothalamus possibly modulating innate fear responses as well (Franklin et al., 2017).

1.3 Aggression

Anger is defined as a negative emotion with an approach/appetitive drive that is rather unique because negative emotions usually create an urge to avoid causative stimulus (Berkowitz & Harmon-Jones, 2004). Anger can be triggered by threat, frustration due to goal obstruction or a personal insult (reviewed in Gilam & Hendler, 2017). The behaviour most commonly associated with anger is aggression. Aggression is an evolutionary conserved innate behaviour with hostile intention to injure another individual (Nelson & Trainor, 2007), which is important for survival and used to compete for and defend resources like territory, food and mating partners. Aggression is classified into two forms: impulsive or reactive aggression, which is usually associated with a perceived threat, frustration and anger, and proactive or instrumental aggression defined as planned and goal-oriented behaviour without high arousal and the presence of the threat that are characteristic to the impulsive one. The latter form of aggression is also called predatory aggression (Siever, 2008). In this work I will review current knowledge about conspecific aggression in rodents associated with the reactive form of aggression. This form recruits the and the medio-basal hypothalamus, as opposed to predatory aggression, that activates the lateral hypothalamus (reviewed in Haller, 2017).

1.3.1 Aggressive behaviours and their triggers

Aggression can be elicited by different circumstances, in their paper Blanchard and Wall described five types of behavioural aggression depending on the situation and agents involved: play fighting behaviour occurring between young mammals of the same species that usually does not involve injurious biting, predatory aggression behaviour directed to hunt and eat prey, maternal aggression behaviour aiming to protect pups from danger and occurring only in the presence of the litter, defensive aggression behaviour elicited in response to an attack where the individual tries to prevent injury to its body and offensive aggression behaviour seen as a response to a challenge to available resources by another conspecific (Blanchard, Wall, & Blanchard, 2003). Interestingly, the occurrence of

offensive aggression can be modulated by several factors, for example, social hierarchy. Dominant males display more aggressive behaviours than subordinate males, moreover, in the presence of a dominant male subordinate males will not fight between each other (Blanchard, Griebel, & Blanchard, 2001). Also the history of fights seems to play an important role in the aggressiveness of rodents in the way that recent wins against another conspecific will increase aggression and defeats will attenuate it (Blanchard et al., 2003). Territoriality is another factor, after establishing their territory males will actively attack other male conspecifics to defend it. Consistent with this impulse, it is common laboratory practice to isolate male mice to induce aggression when an unfamiliar male is introduced in their cage (Miczek, Maxson, Fish, & Faccidomo, 2001). This approach is often used in aggression laboratory studies and is called the resident-intruder paradigm (Koolhaas et al., 2013). Lastly sexual experience or cohabitation with females is another factor that is known to increase conspecific aggression in males probably by enhancing territoriality related aggression and this effect seems to be testosterone dependent (Albert, Dyson, Walsh, & Petrovic, 1988).

Aggression behaviours in rodents occur according to a specific pattern. Usually series of bursts of aggressive activities are separated by periods of relative calm social and non-social behaviours (Miczek et al., 2001). During aggressive bursts animals can display lateral threat which is a sideways rotation of the body in relation to the opponent, chasing of the other animal, tail rattle which serves to threaten an opponent and often precedes direct attack, “put down” behaviour when the animal is on top of its opponent and often actively keeps it pinned down with its limbs, rolling and tumbling of both animals and attacking bite (Blanchard, O’Donnell, & Caroline Blanchard, 1979; Blanchard et al., 2003; Miczek et al., 2001; Scott, 1966). Interestingly, placement of the bites during offensive aggression seems not to be random in rodents, with animals preferring to bite the back of the opponent and avoiding the head and belly. These features were suggested to differentiate offensive and defensive aggression as the latter targets head areas (Blanchard et al., 2003). Between aggression bouts mice usually display grooming, rearing, ano-genital sniffing and walking (Miczek et al., 2001). Typical measures of aggression in rodents are latency to attack, duration and frequency of attacks, tail rattles and chases and number of bites (Blanchard et al., 2003; Koolhaas et al., 2013; Miczek et al., 2001).

1.3.2 Neural circuit of aggression

Throughout the history of aggression research, many brain structures have been associated with processing of aggression, either by cFos and activation studies or direct manipulation of neuronal activity. The main areas identified include the medial hypothalamus, the amygdala, the lateral septum (LS), the bed nucleus of the stria terminalis (BNST), PFC and PAG (Anderson, 2016; Nelson & Trainor, 2007; Takahashi & Miczek, 2014; Yamaguchi & Lin, 2018).

In the amygdala the primary structure involved in aggression is MEA. As mentioned previously the medial amygdala receives olfactory inputs from the accessory olfactory bulb and relays them further to the medial hypothalamus. In fact beyond important connections to VMHvl, MEA also projects to other structures implicated in aggression like the lateral septum and BNST (Canteras, Simerly, & Swanson, 1995). It was shown that optogenetic activation or inhibition of GABAergic neurons in MEA increases or decreases aggression respectively (Hong, Kim, & Anderson, 2014). Another study identified aromatase expressing neurons in MEA that mediate aggression in males and maternal aggression in females via ablation and pharmacogenetic inhibition (Unger et al., 2015). CEA and BLA also show activation during predatory aggression (Tulogdi et al., 2015).

Following the initial experiments of Hess and Bard in the 1920s researchers gathered further evidence of an important involvement of the medial hypothalamus in aggression (Kruk et al., 1983; Lipp & Hunsperger, 1978). This resulted in the definition of a hypothalamic attack area in rats in which electrical stimulation elicited attack (Siegel, Roeling, Gregg, & Kruk, 1999). Also an injection of a vasopressin antagonist into this area in hamsters decreased their aggression, pointing to the possibility of neuromodulation of aggression in this region (Potegal & Ferris, 1989). Recently VMHvl was identified as a central structure governing aggression (Lin et al., 2011). A specific population of neurons co-expressing estrogen receptor alpha (Esr1) and progesterone receptor (Pgr) were identified as mediating aggressive behaviour. Lesion and optogenetic inhibition of those neurons caused a reduction of aggression, while optogenetic activation elicited attack in both males and females (Hashikawa et al., 2017; Lee et al., 2014; Yang et al., 2013). Interestingly, activation of those neurons seems to control behaviour in a scalable way, where low activation promotes mounting and higher activation (increase in light power)

promotes aggression (Lee et al., 2014). An activity of the neurons is synchronized with a social contact and can predict attack behaviour (Falkner, Dollar, Perona, Anderson, & Lin, 2014). VMHvl neurons also seem to be involved in aggression seeking behaviour as showed by their activity during an operant aggression seeking test (Falkner, Grosenick, Davidson, Deisseroth, & Lin, 2016). VMHvl receives direct afferents from other structures involved in aggression like the lateral septum (LS), BNST, PFC and MEA. Another medial hypothalamic nucleus associated with aggression is AHN and an electrical stimulation or an injection of vasopressin into this locus results in increased aggression (Ferris et al., 1997; Kruk et al., 1984). Also PMV was implicated in aggression and this nucleus receives strong inputs from MEA and provides output to VMHvl. An optogenetic stimulation of PMV neurons expressing the dopamine transporter gene (DAT) as well as selective activation of its axonal projections in VMHvl promotes aggression (Stagkourakis et al., 2018).

Structures connected to the medial hypothalamus are often connected amongst themselves. For example BNST receives inputs from MEA and sends inputs to VMHvl and PMV and lesion to this nucleus results in decreased aggression (Nelson & Trainor, 2007). LS sends inputs to VMH and activating these inputs optogenetically can stop the ongoing aggression (Wong et al., 2016). PFC sends inputs to the amygdala and medial hypothalamus and lesions cause increase aggression behaviours (De Bruin, Van Oyen, & Van De Poll, 1983) whereas activation leads to a small decrease in aggression (Takahashi et al., 2014). PAG as previously mentioned is an important output target of the medial hypothalamus and GABAergic signalling in the caudal PAG was shown to regulate maternal aggression (Lee & Gammie, 2010). Also, the ventral tegmental area (VTA), a midbrain structure associated with reward processing was linked to aggression by experiments in which an optogenetic stimulation of its dopaminergic neurons increased aggression (Yu et al., 2014). Curiously, structures involved in aggression like VMHvl and BNST connect directly or indirectly (via the lateral hypothalamus) to VTA, creating a self-regulating feedback loop that could control aggression levels via dopaminergic mechanism, although the source of dopamine fibres in the hypothalamus is not known (Yamaguchi & Lin, 2018).

1.4 Ventromedial hypothalamus (VMH)

1.4.1 Development and anatomy of VMH

VMH is a complex and evolutionary conserved structure with similar organization in reptiles, fish and mammals, including human (Flanagan-Cato, 2011; Kurrasch et al., 2007). It is identified as a cell dense bilateral oval structure surrounded by a cell-poor capsule located in the caudal mediobasal hypothalamus next to the third ventricle. VMH is divided in to three sub-nuclear parts based on neuronal markers, cell density and neuronal projections (Canteras, Simerly, & Swanson, 1994; Millhouse, 1973; McClellan, Parker, & Tobet, 2006). The major subdivisions of the VMH are: 1) the dorsomedial VMH (VMHdm) – a cell-dense oval region close to the third ventricle in the dorsal extent expressing Nr5a1, 2) the ventrolateral VMH – a cell-dense area at the bottom of VMH close to the edge of the brain expressing Esr1 and, 3) the central VMH – a region in between them with much lower cell density, containing scattered Gad2 expressing cells (personal observation). The VMH is surrounded by a capsule or shell, also called fiber plexus or neuropil. The VMH shell is mainly composed of afferent fibres from structures sending projections to VMH, but it also contains sparse neurons (Millhouse, 1973). Neurons in VMH are mainly glutamatergic as shown by expression of Vglut2 (Ziegler, Cullinan, & Herman, 2002) with somas bigger in VMHvl than VMHdm (Madeira, Ferreira-Silva, & Paula-Barbosa, 2001). VMH is highly interconnected with about half of spines remaining in the nucleus after deafferentation (Nishizuka & Pfaff, 1989) Dendrites of VMH neurons often extend to the neuropil where they receive connections from surrounding fibres and their density is influenced by estrogen (Calizo & Flanagan-Cato, 2000). VMH structure is also different between the sexes. It was shown that males possess a significantly bigger VMH than females (Matsumoto & Arai, 1983) and that this difference is mainly attributable to bigger somas and fiber plexus, as well as longer dendrites in male VMH (Madeira et al., 2001; Matsumoto & Arai, 1986). Female VMH on the other hand has been reported to have more dense spines than in males (Griffin & Flanagan-Cato, 2009; Madeira et al., 2001).

Molecular markers of VMH neurons have been widely studied and their localization mapped in respect to aforementioned parts of the VMH (Kurrasch et al., 2007; McClellan et al., 2006; Segal et al., 2005). Nr5a1 (or SF-1) is a transcription factor that was found to

be a unique marker of VMH. It is expressed in the dorsomedial and central VMH in adulthood, and throughout the entire VMH during development (Cheung, Kurrasch, Liang, & Ingraham, 2013). Nr5a1 expression is necessary for the normal development of VMH and its deletion leads to a disruption of VMH formation (Cheung et al., 2013; Shinoda et al., 1995). In adult mice Nr5a1 neurons were shown to mediate fear responses, obesity and glucose responses (Dhillon et al., 2006; Klöckener et al., 2011; Kunwar et al., 2015; Silva et al., 2013). Another transcription factor, Nkx2.1, is crucial to entire medial hypothalamus development and in adult mice is expressed in VMHvl neurons (Krause & Ingraham, 2017). Those neurons were shown to mediate locomotion and energy homeostasis in females (Correa et al., 2015). Also other transcription factors were found to be enriched in VMH like Vgll2, Lhb2, Nkx2.2, Dax1, Sox14, Satb2 and Grlh1, but their functions have not been described (Kurrasch et al., 2007; Segal et al., 2005). VMH is also enriched with neuropeptide expressing neurons. For example pituitary adenylate cyclase-activating polypeptide (PACAP) is expressed in the entire VMH and brain derived neurotrophic factor (BDNF) and neuropeptide Y (NPY) is expressed in VMHdm/c and is suggested to be involved in a glucose response and energy homeostasis (Dube, Kalra, Crowley, & Kalra, 1995; Hawke et al., 2009; Kamitakahara, Xu, & Simerly, 2016; Khodai et al., 2018). Other neuropeptides like cholecystokinin (CCK), somatostatin (SST), encephalin (ENK) or Tac1 (coding the protein precursor for several neuropeptides from the tachykinin family) are enriched in VMHvl, although the function of these was not investigated so far. Neurons expressing Nos1, coding for nitric oxide synthase 1, are located in VMHvl. Nitric oxide (NO) is important for synaptic plasticity and easily diffuses through brain tissue (Tricoire & Vitalis, 2012). Recently, the function of these neurons was probed using a Nos1::Cre mouse line and shown to be involved in a glucose response and immobility behaviour (Faber et al., 2018; Fioramonti et al., 2010). Unfortunately, it is known that this line expresses Cre protein also in VMHdm/c (unpublished lab data) therefore, these results might be not an accurate depiction of the function of Nos1 expressing neurons in VMH. Interesting membrane receptors are also enriched in VMH for example serotonin receptors Htr1b and Htr2a, cannabinoid receptor-1 (Cnr1), GABAa and GABAb receptors, cholecystokinin receptor B (Cckrb) (Kurrasch et al., 2007) and oxytocin receptor (Oxtr) known to be expressed in VMHvl. GABA signalling in VMH is known to be involved in fear related responses (Zagrodzka, Romaniuk, Wieczorek, & Boguszewski, 2000). Although role of other mentioned receptors in VMH was not investigated, it is known for example, that serotonin is involved in aggression regulation (Audero et al., 2013), the whole

brain Oxtr knockout mice exhibit higher aggression levels than littermate controls (Takayanagi et al., 2005), Cckrb neurons in VMHdm might be involved in a hypoglycaemia

Selected neuronal markers	Region of the VMH	Probable Function	Selected References
SF-1	**DM, C**	predator defence, energy expenditure, glucose response, VMH development	(Dhillon et al., 2006; Kunwar et al., 2015; Silva et al., 2013)
BDNF	**DM, C**	hypoglycaemia	(Kamitakahara et al., 2016)
PACAP	**DM, C, VL**	energy balance and glucose response	(Hawke et al., 2009; Khodai et al., 2018)
NPY	**DM, C**	hyperphagia	(Dube et al., 1995)
ERα	**VL**	mating, aggression	(Hashikawa et al., 2017; H. Lee et al., 2014)
PR	**VL**	mating, aggression	(C. F. Yang et al., 2013)
NOS1	**VL**	glucose response*	(Fioramonti et al., 2010)*
Tac1	**VL**	unknown, subset of ERα, enriched in females	(Krause & Ingraham, 2017)
Nkx2.1	**VL**	locomotion, energy expenditure in females	(Correa et al., 2015)
Oxtr	**VL**	locomotion	(Narita, Murata, & Matsuoka, 2016)
CCK	**VL**	unknown	-
SST	**VL**	unknown	-
ENK	**VL**	unknown	-

Table 1. Selected neuronal markers and their putative function in VMH (* Nos1:Cre line was used that is known to express Cre in VMHdm/c).

response as one study proved a link between parabrachial neurons expressing CCK projecting to VMH and a counter-regulatory response to hypoglycaemia (Garfield et al., 2014). Neuronal population expressing estrogen receptor alpha (ERα) and progesterone receptor in VMHvl were shown to be regulating sexual and aggressive behaviours in both sexes (Hashikawa et al., 2017; Lee et al., 2014; Yang et al., 2013).

Development of the ventromedial nucleus of the hypothalamus in mice starts at E9.5 with an onset of neurogenesis in proliferative zone around the third ventricle (Cheung et al., 2013; McClellan et al., 2006). SF-1 marker becomes visible from E9.5, while the first projections at E10.5, suggesting that their development starts at the very beginning of neurogenesis (Cheung et al., 2013; Krause & Ingraham, 2017). SF-1 is expressed in all VMH neurons at the beginning of neurogenesis, but later around E14.5 becomes supressed in VMHvl (Cheung et al., 2013). SF-1 cells occupy VMH area from E10.5, but start to coalesce only around E14.5 – E17.5 forming a proper nucleus structure visible by Nissl staining (Cheung et al., 2013; McClellan et al., 2006). Interestingly SF-1 is not required for initial neurogenesis and migration of VMH neurons, but for terminal differentiation and maintenance of VMH at later stages and development of a proper pattern of neuronal projections (Ikeda, Luo, Abbud, Nilson, & Parker, 1995; Krause & Ingraham, 2017; McClellan et al., 2006; Tran et al., 2003). In order to form VMH, neurons start migrate radially form the third ventricle along helper radial glia (McClellan et al., 2006). Migration progresses in the way that neurons, which started migrating first, end up in the most distal part of VMH from the third ventricle (vlVMH) (Altman & Bayer, 1986). GABA has been proposed as one of neurotrophic factors guiding migration of VMH cells (McClellan et al., 2006). Interestingly, recent study using two different approaches to study SF-1 lineage tracing suggests, as argued previously by some, that region adjacent to VMHvl (the tuberal nucleus) might belong to the ventrolateral part of VMH (Canteras et al., 1994; Cheung et al., 2013). Finally, prenatal projections as early as day E14.5 match the same pattern as VMH projections in the adult mice (Canteras et al., 1994; Cheung et al., 2013).

1.4.2 Inputs and outputs of VMH

The ventromedial nucleus of the hypothalamus receives inputs and sends outputs to over 60 different locations in the brain (Canteras et al., 1994; Fahrbach, Morrell, & Pfaff,

1989; Lo et al., 2018; Toth, Fuzesi, Halasz, Tulogdi, & Haller, 2010). Although inputs and outputs to the whole VMH were described (Canteras et al., 1994; Fahrbach et al., 1989) they received much less attention than VMHvl afferents and efferents and only a few differences were detected between them (Canteras et al., 1994; Fahrbach et al., 1989; Lo et al., 2018; Toth et al., 2010). In this work I will review main inputs and outputs to VMHvl and try to link them with a potential function with relation to social fear and aggression, which are the main focus of this study.

The amygdala, particularly MEA, is one of the main inputs to VMHvl. This structure relays olfactory information to the VMH. Other modalities related to a threat are also sent from the amygdala to VMHvl – small inputs form BLA and CEA were detected in the recent study (Lo et al., 2018). BNST is another big source of inputs, this input structure is implicated in anxiety, reward processing and addiction (Avery, Clauss, & Blackford, 2016). It sends mainly inhibitory input to VMH. The ventral hippocampus and the subiculum send strong projections which most likely can convey spatial information to VMHvl (Stewart, Jeewajee, Wills, Burgess, & Lever, 2014). Inputs from the medial PFC might provide information to help to fine tune responses by VMH since this region of cortex is involved in both aggression and fear processing integrating inputs from the hippocampus and the amygdala (D. Caroline Blanchard et al., 2005; De Bruin et al., 1983). The lateral septum (LS) provide a strong input to VMHvl. This structure was shown to be involved in social and contextual fear related processing (Zoicas, Slattery, & Neumann, 2014). Intra-hypothalamic inputs come mainly from the medial preoptic nucleus and PMV – both were mention before as a part of a conspecific fear circuit. Other hypothalamic structures projecting to VMHvl are the dorsomedial hypothalamic nuclei, the arcuate nuclei implicated in feeding, and AHN described before as a part of a predator fear circuit. VMH also receives inputs form the midbrain structures like VTA involved in reward processing and from PAG and the parabrachial nuclei (PB), most likely conveying information about internal signals in the body (Lo et al., 2018).

Most of the outputs of VMHvl overlap with input structures with the exception of areas far anterior to VMH like the subiculum and the medial PFC (Canteras et al., 1994; Lo et al., 2018; M. Toth et al., 2010). Nonetheless, we can pinpoint structures whose output to input ratio is very low, indicating that they are mainly input structures like MEA, LS or the subfornical organ. On the other hand, structures that are considered to be mainly output

areas with high output to input ratio are PAG, the midbrain reticular nucleus (MRN), the paraventricular thalamic nuclei, and the superchiasmatic nucleus (Lo et al., 2018). VMHvl connections are highly recurrent and mostly within subcortical areas with the exception of PFC and the ventral hippocampus, but using rabies anterograde tracing it was shown that VMHvl is connected with those areas indirectly (Lo et al., 2018). Outputs of VMHvl neurons collateralize very broadly, but two subpopulations were discovered: one preferring structures to the VMH, localized in more caudal part of VMHvl, and another one preferring posterior areas, localized in more rostral part of VMHvl (Lo et al., 2018). Overall, a connectivity pattern of VMH is very complicated, but comparing structures using output to input ratio show a general flow of information from sensory/cognitive structures like MEA, PFC and the subiculum to pre-motor areas in the midbrain like PAG and MRN (Lo et al., 2018). It was proposed that this connectivity pattern together with high interconnection with other hypothalamic and amygdala structures poses the ventrolateral VMH as a regulator, controlling social behavioural decisions and an internal state of animal (Lo et al., 2018).

1.4.3 Functional characterization of VMH

The ventromedial hypothalamic nucleus, particularly dmVMH, has been long implicated in a weight regulation and energy homeostasis (Kim et al., 2011). Initial studies reported obesity in mice with VMH lesions, as well as altered levels of insulin and glucagon attributing it to decreased activity of sympathetic nervous system (Hetherington & Ranson, 1940; Inoue, Campfield, & Bray, 1977). In 2002 the group of Parker studied SF-1 knockout mouse and discovered that mice develop obesity mainly due to the decreased movement (Majdic et al., 2002). After knocking out a leptin receptor in SF-1 neurons in VMH, mice displayed not only obesity, but also a dysregulation in food intake and impaired energy expenditure as measured by the levels of CO_2, heat production and O_2 intake (Dhillon et al., 2006; Kim et al., 2011). Also, knocking out Socs3 and disrupting PI3K signalling in SF-1 neurons resulted in the same phenotype (Xu et al., 2010; Zhang et al., 2008). Another study by deleting Vglut2 from SF-1 neurons showed that this effect is mediated by glutamatergic connections of VMH. Vglut2-deficient mice displayed diet induced obesity and hypoglycaemia (Tong et al., 2007). Interestingly, a deletion of Nkx2.1 in VMHvl induced reduced locomotion activity which resulted in obesity only in females underlying sex differences in VMHvl (Correa et al., 2015). In addition to sensing leptin, VMH neurons

are proven to be glucose sensitive, two populations glucose inhibited and glucose excited neurons were discovered (reviewed in Routh, 2010). These neurons are thought to be crucial in a systemic regulation glucose levels and hypoglycaemic response (Chan & Sherwin, 2013; Shimazu & Minokoshi, 2017).

Sexual behaviour is one of the social tasks that was showed to be heavily influenced by VMHvl especially in females. Studies in late 1970s showed that lesions of this region caused diminished sexual behaviours in female rats (Pfaff & Sakuma, 1979), whereas an injection of estrogen and progesterone into the VMH area increased lordosis behaviour in females. This effect was abolished by the VMH lesion (Pfeifle, Shivers, & Edwards, 1980). Both estrogen and progesterone are known to be crucial in regulating female sexual receptivity and VMHvl expresses receptors for both of them. Indeed, estrogen and progesterone, when administered to VMH, cause structural changes in the dendrites of VMHvl neurons (Griffin & Flanagan-Cato, 2008). Estrogen increases the density of the spines and the length of the dendrites, progesterone, on the other hand, exhibits opposite effects (Calizo & Flanagan-Cato, 2000; Griffin & Flanagan-Cato, 2008). The ventrolateral VMH also expresses oxytocin receptor. Oxytocin is a neuropeptide implicated in reproductive functions in mammals like sexual activity, parturition and lactation (H.-P. Yang, Wang, Han, & Wang, 2013). It has been suggested that estrogen increases levels of the oxytocin receptor in VMHvl (de Kloet, Voorhuis, Boschma, & Elands, 1986), but its function remains unknown. VMHvl is placed within sexual receptivity, lordosis circuits in the brain – it receives inputs from MEA containing pheromone information and from MPO region long implicated in reproductive behaviours (Lo et al., 2018; Toth et al., 2010). VMHvl is viewed as an integrator of the hypothalamic processing of sexual information and an output structure (Micevych & Meisel, 2017). As described earlier, it projects to PAG structure that in turn controls lordosis behaviour by projecting to motor areas in spinal cord (Micevych & Meisel, 2017). In addition, VMHvl was also recently implicated in a control of male sexual behaviours. Studies demonstrated that, on one hand, an ablation of progesterone receptor expressing neurons diminished both sexual receptivity in females and mounting behaviours in males (Yang et al., 2013), on the other hand, the low light intensity optogenetic activation a of Esr1+ neuronal population elicits mounting in males (Lee et al., 2014). This data indicates that indeed VMHvl is important for the expression of reproductive behaviours in both sexes.

As mentioned before, medial hypothalamus was identified as a region important for aggressive behaviours already in 1930s and later studies narrowed the findings to the hypothalamic attack area that comprises of VMHvl and adjacent tuberal nucleus (Anderson, 2016; Kruk et al., 1983). In recent years VMHvl was discovered to play a crucial role in eliciting aggression behaviour in mice. Various studies including pharmacogenetic inhibition of VMHvl region, ablating specific PR+ neurons, or optogenetic inhibition of Esr1+ cells revealed a decreased aggression in male mice (Lee et al., 2014; Lin et al., 2011; Yang et al., 2013). In contrary, an optogenetic activation of Esr1+ population in VMHvl induced attack behaviour in males and females (Hashikawa et al., 2017; Lee et al., 2014). Also, certain inputs to VMHvl were showen to control aggression. For example, activating a projection from the lateral septum inhibits attack (Wong et al., 2016). Other inputs from the subparaventricular zone (SPZ), that in turn receive inputs form suprachiasmatic nucleus (SCN), regulate daily rhythm of aggression (Todd et al., 2018). Electrophysiological recordings revealed that the activity of some (~15%) of VMHvl neurons correlates with attack and can predict future aggression (Falkner et al., 2014). In addition, it was found that those neurons also participate in the appetitive phase of aggression since they show activation during self-initiated aggression seeking task (Falkner et al., 2016). Notably, the same population of VMHvl neurons is also implicated in sexual behaviours as described previously (Lee et al., 2014; Lin et al., 2011). These results place VMHvl as a crucial structure in aggression circuit indispensable for eliciting attack behaviour.

Ventromedial hypothalamus is a part of two defensive circuits: predator fear circuit that involves VMHdm and social fear circuit containing VMHvl (Gross & Canteras, 2012). An exposure to a predator or its odour activates cFos in the region of VMHdm (Caroline Blanchard et al., 2005; Canteras, Chiavegatto, Ribeiro Do Valle, & Swanson, 1997). Furthermore, a pharmacogenetic inhibition of SF-1 neurons in VMHdm reduces defence behaviours selectively to a predator (Silva et al., 2013). Conversely, an optogenetic activation of SF-1 neurons caused freezing and panic-like behaviour in mice in a scalable manner (Kunwar et al., 2015). This response was further dissected to a specific projections, showing that projection to AHN induces panic and projection to PAG – freezing (Wang et al., 2015). Also, a contextual predator fear memory seems to be depended on VMHdm since its inhibition at the time of a memory test impaired defence response to the context in mice and the stimulation of SF-1 induce conditioned place avoidance (Kunwar et al.,

2015; Silva, Mattucci, et al., 2016). Interestingly, also in humans stimulation of VMH induces panic attacks (Wilent et al., 2010). On the other hand, VMHvl is implicated in social fear because an exposure to a dominant conspecific induces cFos expression in this region (Motta et al., 2009). Additionally, a nonspecific inhibition of neurons in VMHvl decreases fear responses to an aggressive conspecific, but not to a predator or foot shock (Silva et al., 2013). Also, an optogenetic reactivation of cFos labelled cells after defeat causes avoidance behaviour towards males, but not females (Sakurai et al., 2016).

All together evidence suggests that VMH is involved in regulating various behaviours and metabolism. VMHdm provides a link between predator defence and a metabolic activity since such responses usually involve a release of cortisol and glucose to the blood stream to prepare the body for a predicted high energy expenditure. Whereas in VMHvl some evidence suggest common mechanism for regulating aggression and mating in males. In contrast, not much is known how this structure contributes to fear responses towards conspecific and how it balances defence versus aggression to akin threatening stimulus of the male intruder.

1.5 Aims of the study

In the previous sections, I reviewed the current scientific knowledge about emotions, particularly fear and aggression, as well as the emerging role of VMHvl in mediating behavioural responses, commonly associated with these two emotions.

The main aim of this study is to understand the mechanism for regulating fear and aggression by VMHvl. This ambitious goal can be divided to a couple of smaller questions. The first is to address whether the same neurons participate in fear and aggression, and if not, to find the identity of neurons mediating fear response. The second is to inquire about the neuronal activity and computation in the VMHvl circuit, as well as the function of particular neuronal populations and the relationship between them. Here, I aim to answer these questions by systematically mapping and monitoring the neuronal activity in VMHvl, which is followed by the functional manipulation of the molecularly identified subsets of neurons in VMHvl.

2. Methods

2.1 Animals

Animals were maintained in temperature and humidity controlled environment with food and water provided *ad libitum*. Light-dark cycle was 12h/12h with lights on at 7:00am. Some experimental mice were kept on reverse dark-light cycle (lights off at 9:00am). All animals used for experimental procedures were 2 to 7 months old. Wild type BALB/c and C57BL/6J mice were obtained from local EMBL colony. CD-1 animals were adult retired breeders obtained from Charles Rivers Lab (Calco, Italy).

Transgenic mice lines used in the study are listed in the table below:

Name	Zygosity	Source
***cFos*::CreER^{T2}**	Heterozygous	JAX No. 021882 (Guenthner et al., 2013)
***arc*::CreER^{T2}**	Heterozygous	JAX No. 021881 (Guenthner et al., 2013)
***ESR1*::Cre**	Heterozygous	JAX No. 017913 (Lee et al., 2014)
***Nr5a1*::Cre**	Heterozygous	JAX No. 012462 (Dhillon et al., 2006)
***nNos*::CreER**	Heterozygous	JAX No. 014541 (Taniguchi et al., 2011)
***Oxtr*::cKo**	Homozygous	JAX No. 008471 (Lee et al., 2008)
***RC*::LSL-tdTomato**	Heterozygous	JAX No. 007914 (Madisen et al., 2010)
***RC*::LSL-sun/GFP**	Heterozygous	JAX No. 021039 Nathans

Table 2. Transgenic lines used in the study.

In addition *cFos*::tdTomato, *Arc*::tdTomato and *Esr1*::tdTomato mice lines were obtained by crossing respective Cre drivers with *RC*::LSL-tdTomato line. *Nr5a1*::OxtrKO line was obtained by crossing *Nr5a1*::Cre line with *Oxtr*::cKO and *cFos*::Sun/GFP line crossing *cFos*::CreER^{T2} line with *RC*::LSL-sun/GFP. All of the above lines were kept in heterozygosity, except *Nr5a1*::OxtrKO where OxtrcKO allele was in homozygosity.

2.2 Behaviour

2.2.1 Behavioural apparatus

Plexiglas custom made behavioural apparatus consists of three detachable parts: home cage with dimensions 25x25x25 cm with Y-shaped slit, 4 cm wide at the bottom serving as an entrance closed by sliding doors, stimulus chamber identical to home cage and a corridor 46x12x30 cm connecting both home cage and "far chamber". Apparatus is a modified version of the one previously described (Silva et al., 2013).

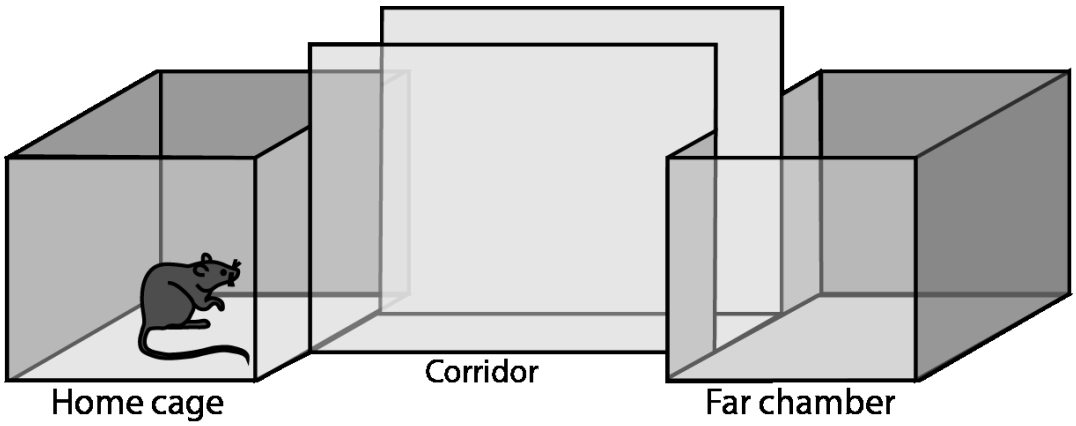

Fig. 3 Schematic representation of behavioural apparatus.

2.2.2 Habituation to the apparatus

Prior to any testing, each mouse was habituated to the apparatus for 3 days right before the first experimental day. During habituation home cage was attached to the rest of the apparatus and mouse was allowed to explore whole apparatus freely for 20 min each day. Apparatus was washed with detergent and 50% alcohol between each mouse to avoid any remaining smell from a previous animal.

2.2.3 Social defence test module

Experimental mice were placed in a home cage part of the behavioural apparatus 7 days before the start of the test, moved into a reverse light-cycle room and single housed there until end of the testing period with water and food *ad libitum*. Test was conducted

during animal's night period. The test had two parts: one day of social defeat and a memory day. On a social defeat day, a mouse was allowed to explore the apparatus freely for 5 minutes, after that time animal was locked in the stimulus chamber where an aggressor (see below) was introduced. Defeat lasted for 10 min, after which mouse was released from the stimulus chamber and aggressor stayed confined within it. After defeat mouse was allowed to explore apparatus freely for period of 5 minutes. Memory test was conducted on the following day after social defeat, during which mouse could explore the apparatus freely. Apparatus was washed with detergent and 50% alcohol between each mouse to avoid any reminiscent of smell that could influence behaviour of the test subject.

CD-1 aggressors were screened for an aggression levels before the test. Screening procedure was 3 days long. Every day an intruder was placed in aggressor's cage for 3 minutes. Only mice that attacked an intruder on every occasion were selected.

2.2.4 Social aggression test module

As in the social defeat test, experimental mice were single housed in a home cage starting seven days before the test in a reverse light-cycle room with water and food *ad libitum* until the end of paradigm. During the test an experimental mouse was confined to a home cage and BALB/c intruder was introduced into it. The test lasted for 10 min after which BALB/c mouse was removed from a home cage. BALB/c intruders used for this test were 7-10 weeks old and co-housed with 3-5 mice per cage.

Aggression test module was modified for Nr5a1::OxtrKO mice where a 7-10 weeks old C57B/6J mouse was used instead of BALB/c mouse, to test aggression versus the same strain.

2.3 Stereotactic surgeries

Mice were anesthetised before surgery using isoflurane 3% (Provet) in oxygen and placed in stereotaxic frame (Kopf Instruments Inc.). Anaesthesia was maintained with continued 1-2% isoflurane administration in breathing air enriched with oxygen. During surgery skull was exposed, aligned and cleaned with hydrogen peroxide solution (0.3%).

For pharmocogenetic inhibition experiments 0.1-0.2 µl of viral vector AAV5-*hSyn*::DIO-hM4DmCherry-WPRE (UNC vector core) was delivered to vlVMH bilaterally using glass capillary. For control mice virus described above was co-injected with AAV1/2-*hSyn*-iRFP670 virus (produced in the lab) in a ratio 2:1 to mark place of injection. Coordinates in relation to Bregma were used, L:+/-0.67, A/P: -0,98 and D/V: -5,75. After infusion, capillary was kept in a place for at least 5 min before a slow retraction.

For optogenetic activation experiments 0.1-0.2 µl of AAV5-*EF1a*::DIO-hChR2(E123T/T159C)-EYFP or AAV5-*EF1a::*DIO-EYFP (UNC Vector Core) virus was infused bilaterally into vlVMH area, using the same coordinates as described above. After 5 min waiting period glass capillary was retracted and custom-made optic fibre connectors were implanted (0.66 NA, 200 µm core fibre and ceramic ferule with 230 µm /1250 µm internal/external diameter). Coordinates in relation to Bregma L: +/-0.67, A/P: -0,98 and D/V: -5,55 and L: +/-1.14, A/P: -0,98 and D/V: -5,6 at 5° angle.

For *in vivo* calcium imaging 0.2-0.3 µl of AAV5-*hsyn*::GCaMP6s or AAV1/2-*CAG*::DIO-GCaMP6s (Penn Vector Core) virus was injected unilaterally into vlVMH. Then endoscope lens (Snap-imaging cannula model L type E, Doric Lenses) was implanted at very slow rate with coordinates L: +/-0.67, A/P: -0,98 and D/V: -5,7.

All implants were secured to the scull using miniature screws (RWD), and dental cement (Duralay). The wound was cleaned and skin was stitched around the implant. After the surgery mice received intra peritoneal injection of 0.4 ml saline and were placed in heated cages and drinking water containing paracetamol for recovery period.

2.4 Histology

All animals were deeply anesthetized and transcardially perfused first with 1xPBS (Invitrogen) followed with 4% PFA (Sigma) in 0.1M PB solution. After that brains were post-fixed in the same 4% PFA solution at 4°C for 24h. Brain tissue was cut using vibratome (Leica VT 1000s) in PBS. 80µm thick slices were sectioned for injection or implant location check, while 50 µm thick slices were cut for any staining procedure. If not used immediately, sections were stored in PBS solution containing 0.1% sodium azide.

For all stainings the same general procedure was used. First sections were washed three times in PBS solution for 10 min, next sections were blocked with 10% Normal Goat/Donkey Serum, and 0,2% Trition-X in PBS for at least 1h. Sections were then incubated with primary antibody solution containing 5% Normal Goat/Donkey Serum and 0.2% Triton-X overnight at 4°C. Next day primary antibody solution was aspirated, sections washed three times in 1x PBS and incubated with appropriate secondary antibody solution containing 10% Normal Goat or Donkey serum for 2 hours at room temperature. After that sections were washed twice with PBS and nuclei were stained with DAPI solution added for 15 min to counter stain the slice. Sections were washed again twice with PBS before mounting them on SuperFrost Plus slides (ThermoScientific) with Moviol.

Primary antibodies used: Nos1 – SC-5302 cat. number DO814 from Santa Cruz Biotechnology, c-Fos – SC-52G Lot FO215 from Santa Cruz Biotechnology and Anti-Estrogen Receptor α (rabbit) Lot number OB-935 from Millipore. Secondary antibodies used: Alexa Fluor 488/546/647 from Invitrogen.

2.5 Mapping activated neurons in *cFos*::CreERT2 mice

To map neurons activated during defence and aggression behaviour, *cFos::*CreERT2 mice crossed with *RC*::LSL-tdTomato were used. Mice were isolated 7 days before the experiment. To map defence neurons, after a standard 3 day habituation, social defeat module test (without memory day) was performed. Right after the defeat (within 1-2min) a single dose (50mg/kg) of 4-hydroxytamoxifen (4-OHT) (Sigma, z-isomer 70%) was injected intra-peritoneally to induce tdTomato expression in neurons activated by defeat. After 7 days mice were again habituated for 3 days to the same apparatus and went through social aggression module. Exactly 1,5h after the second behavioural experiment each mouse was trans-cordially perfused and brain tissue was stained for cFos protein to visualize cells activated by second behaviour. For different experimental mice order of behaviours was swapped. The combinations were as followed: defence-defence, aggression-aggression, defence-aggression, aggression-defence in each combination instance of 4-OHT was injected right after the first behavioural experiment.

Stained sections were imaged under widefield microscope (Widefield Leica DMI 6000 B) with 10x objective and mosaic function allowing to reconstruct whole brain area into one image. Labelled neurons were quantified blindly using ImageJ software. Briefly, a region of interest (ROI) corresponding to VMHvl (according to Paxinos and Franklin Mouse Brain Atlas) was drawn on each image using DAPI channel. Then number of tdTomato cells was manually quantified inside the ROI. Next cells positive for cFos immunofluorescence and tdTomato were quantified, then quantified images were overlayed and neurons positive for both colours were counted.

2.6 Pharmacogenetic inhibition experiments

In order to inhibit specific neuronal populations in vlVMH, *Esr1*::Cre and *nNos*::CreERT2 mice were used. After stereotactic injection of viral vector described above mice were given 2 weeks of recovery. To activate transgene expression in Nos1 line, mice were injected with Tamoxifen (Sigma, 40mg/kg dose) for five consecutive days during the second week of recovery period.

Mice were isolated 2 weeks before the experiment start and moved to a reverse cycle room. Standard 3 days of habituation to apparatus was performed followed by social defeat test module. Exactly 30 min before the start of social defeat each mice was injected with CNO (Sigma, 3mg/kg dose) or saline solution at random ensuring 1:1 ratio between the groups. Week after this experiment mice were trained to become aggressive by carrying out social aggression test module for 4 days. On day 4 mice were again injected with CNO (Sigma, 3mg/kg dose) or saline solution 30 minutes before beginning of the test module.

Behaviour was scored manually using Observer XT11 software (Noldus Information Technology). To score defensive behaviour, 3 min right after the defeat was analysed, while to score aggressive behaviour whole duration of the experiment was used (10min). Behaviour was quantified blindly without prior knowledge of the mouse treatment. Mice with off-target viral injections and the ones that never displayed any aggressive behaviour were removed from the analysis.

2.7 Optogenetic stimulation

To activate Esr1 and Nos1 expressing neuron populations in vlVMH specifically, described previously respective Cre driver mice lines were used. After stereotactic injection of viral vector described above mice were given 2 weeks of recovery. To activate the expression of transgene in Nos1 line mice were injected intraperitoneally with tamoxifen (Sigma, 40mg/kg dose) for five consecutive days during the second week of recovery period.

Animals were housed in Plexiglas home cages separately in a reverse light-cycle room for two weeks before beginning of the experiments. Every animal was handled and habituated to optical wires for at least 2 days prior to any behavioural procedure.

Overt behaviour test was done in animal's home cage. Stimulation protocol was as follows: each animal was stimulated with 30s long trains of light at 20 Hz every 1-2min for 30s to 2min period. First stimulation (after 2 min of free exploration period) was using ~0.5mW light power. Then each consecutive stimulation was done with higher light power: 1mW, 3mW, 6mW and 10mW. After this protocol, stimulation that gave the strongest response was repeated.

For stimulation in a presence of conspecific a version of the social aggression module test was used which lasted for 15 min. During the first 3 min of free exploration was allowed with one 30s stimulation at 20Hz and power determined during overt behaviour test. After this period a BALB/c intruder was introduced into the cage. Then animal was stimulated at least 3-5 times with the same parameters targeting periods of social interaction. At the end of the test BALB/c intruder was removed from the cage.

To test the effects of stimulation after experiencing defeat, Esr1 animals were moved to a novel plexiglas stimulus cage and allowed 1 min free exploration. After that an aggressive CD-1 intruder was placed in the cage. Defeat lasted for 5 min, after which both animals were removed from the cage. This procedure was carried for total of 2 days. On the following day animal was stimulated in presence of non-aggressive intruder in its home cage as described above.

Whole duration of the experiment was scored for defensive and aggressive social behaviours, and overt behaviour (grooming, freezing, darting and cornering). In the analysis the time was divided into following bins: 30sec before stimulation, stimulation and 30sec post stimulation. Animals where viral injection or optic fibre connector were miss-targeted were excluded from the analysis.

Optical stimulation of ChR2 was achieved using LED light (Plexon Plexbright) attached to rotatory joint to which 1m long patch cables (Plexon Plexbright High Performance) were connected. Tips of the patch cables were attached to connectors on mouse head. Power of light at the end of patch cables was measured before each experiment with portable optical power meter (Thor Labs). Stimulation trains were generated using V2.2 Raditant software (Plexon). Rotatory joint was manually operated.

2.8 *In vivo* calcium imaging

For deep brain *in vivo* calcium imaging experiments in vlVMH, miniaturized fluorescence microscopy system was used (Doric Lenses). System consists of a computer connected to fluorescence microscope driver controlling miniaturized microscope and a connectorized LED lamp (Doric Lenses). Driver and connectorized LED (458nm) are connected to pigtailed optic-fibre and electric rotary joint (AHRJ OE_PT_ AH_12_HDMI, Doric Lenses) to which miniaturized microscope body (Model L, Doric Lenses) is connected via pigtailed electric 1.5 meter long cable and 1 meter long mono fiber-optic patch cord (MFP 200/230_900_0.48_0.8_FC_CM3, Doric Lenses). System is operated with DoricStudio software (Doric Lenses).

Behaviour test battery was a combination of social fear and aggression test modules. Animals were allowed 3-4 weeks of recovery period after optical cannula implantation surgery. Mice were isolated for 2 weeks before start of experimentation and moved into reverse light-cycle room. Each mouse was habituated to microscope plugging and unplugging procedure for at least 3 days.

Standard three-day habituation to the behavioural apparatus was performed, microscope body was attached to previously implanted imaging cannula on mouse head

during day 2 and 3 during habituation. The following week mice were subjected to two social defeat test modules one after another for a total of four days. In addition immediately after a social defeat module a non-aggressive CD-1 or BALB/c mouse was introduced to experimental mouse home for 5 min period (Social investigation test). Then after 2-3 days break period, three social aggression test modules were performed spanning over 3 days. All behavioural tests were performed in the dark room with red lights on.

Behaviour was scored during all calcium recordings. Scored behaviours are as follows: locomotion, sniffing, ano-genital sniffing, upright posture, freezing, flight, assessment/immobility, defence action, domination/keep down, face conspecific, cornering, stretch, avoiding conspecific, attack and follow. During a memory test also location of the animal was scored as well: home, home corridor, stimulus corridor, stimulus cage.

All *in vivo* calcium recordings were done with 15-25% LED intensity using 50ms or 100ms exposure. Recording time longer than 5 minutes was avoided to prevent drop in fluorescence due to possible photo bleaching.

Calcium imaging data was collected from 15 animals out of 24 implanted with imaging cannulas. Animals that had wrong placement of endoscope or had very few neurons were excluded from analysis, also animals that did not complete successfully behavioural tests or had very few instances of relevant behaviours were not included in the study (6 out of 15).

2.9 Data analysis

2.9.1 Image pre-processing

Image processing for calcium *in vivo* recordings was done using Fiji software (NIH). Calcium recording was first loaded as a stack of images in .tiff format. Stack was duplicated and for each frame of the recording separate background frame was generated using band pass (lower band - 100µm higher band - 10000µm) filter function. Next each frame of the recording was divided by a corresponding frame from the background stack.

Resulting background filtered stack was aligned using TurboReg plugin in Fiji using translation batch algorithm. Neuronal ROIs were manually selected using max intensity projection image, where detection was aided by inspecting recoding at increased speed to detect dim neurons with slow dynamics. When a complete map of ROIs for each recording was generated, a mean intensity ROI traces were extracted and ΔF/F was calculated where F stands for mean intensity over whole recording period.

To track ROIs over different recordings and different days, ROI mask was projected onto new recording and translated, to account for possible field of view movement between recordings. If field of view moved in the way that some ROIs could not be mapped back to the previously produced ROI mask, they were treated as new ROIs and added to the analysis.

2.9.2 Analysis of in vivo calcium recoding data

For the analysis of neuronal activity during various behaviours, transition of behaviour analysis, and area under the receiver operating characteristic curve (auROC) analysis custom scripts written in Python were used.

Briefly for transition behaviour analysis all starting points of behaviour of interest were picked, then period of 1-2 seconds was selected before and after the start point. All data was z-scored and used to generate heat maps displaying meaned z-scores over all trials/transitions or to perform Wilcoxon signed rank-sum statistical test. For auROC analysis all frames of selected behaviour and all frames of different behaviours in the same recording were used to generate separate distributions of calcium values labelled as true positive and true negative respectively. Then ROC curve graph was generated for each neuron plotting ratio between true positive rate and false positive rate and area under this curve (auROC) was calculated.

For linear discriminant analysis and partial least squares regression data was normalized within days by calculating z-score for each recording separately. Each frame was then expressed as vector containing calcium values. For calculating and generating particular graphs custom written Python scripts were used. The distance between clusters

was quantified by calculating average distance between data points of a given cluster and all other data points from other clusters. This calculation was then repeated for every cluster.

2.9.3 Statistical analysis

Prism Graphpad 5 software or custom scrips in Python were used to generate graphs and perform statistical analysis, t-test was used for optogenetic and pharmacogentics experiments with only two groups, ANOVA with Bonferroni correction was used for experiments with multiple groups or conditions. For calcium imaging statistical analysis sklearn package (Pedregosa et al., 2012) in python was used.

3. Results

3.1 Mapping social fear and aggression cells in VMHvl

VMHvl is involved in many different behaviours that seem to be on the opposite ends of the emotional spectrum. It was discovered that mounting and aggression in males are governed by the same Esr1 expressing population of neurons in a scalable way (Lee et al., 2014). On the other hand, no similar work has been conducted to investigate social fear neurons and their identity is still unknown. To investigate this question and test a hypothesis that different populations of neurons are involved in social fear than in aggression, we decided to map neurons in VMHvl activated by these stimuli. To map VMH neurons activated in social fear and aggression in the same animal, we decided to use an immediate-early gene based approach. Immediate-early genes (IEG) are a set of genes that is rapidly activated upon stimulation of the cell and does not require synthesis of *de novo* proteins (Herrera & Robertson, 1996). In the brain the expression of an IEG is associated with neuronal activity. It was shown that the expression of IEGs like *cFos* and *Arc* is rapid, specific and transient in neurons responding to a certain stimulus or behaviour (Minatohara, Akiyoshi, & Okuno, 2016).

Previously, approaches based on IEGs were used to visualise neuronal activation to two different stimuli. These approaches utilize time difference either between an expression peak of *cFos* RNA and cFos protein or between different localization of the RNA in the cell by employing FISH and cFos staining techniques (Lin et al., 2011; Xiu et al., 2014). These methods are tedious and, in addition, require two behaviours to be performed in a very short stretch of time, which is very hard to achieve in case of defeat and aggression in the same animal. Another method based on the combination of a transgenic mouse and viruses was used recently to investigate this question (Sakurai et al., 2016), but it also has limitations because a virus injection is local, it causes brain damage, which is known to induce *cFos* expression, ultimately resulting in an unspecific labelling.

In this study we decided to use TRAP system mice combined with cFos protein staining. TRAP system has two variants *cFos*::CreER^{T2} and *Arc*::CreER^{T2} mice that can be used to express desired protein permanently in transiently activated neurons (Guenthner et

al., 2013). We crossed both of the lines with RC::LSL-tdTomato mouse line obtaining *cFos*::tdTomato and *Arc*::tdTomato mice lines respectively, and proceeded to validate the system. First, we created a timed expression profile of *cFos* RNA and cFos protein in VMH by using *in situ* hybridization and immunohistochemistry, respectively, on the brain tissue from defeated mice. Quantification showed that the RNA expression peaks between 30 minutes and 1 hour and it is gone 3 hours after the stimulus, whereas protein peaks between 1 hour and 3 hour mark and starts decreasing 5 hours after the stimulus (Fig. 4D). Next, we checked if we can capture activated neurons using TRAP lines by a single injection of 4-hydroxytamoxifen (4-OHT). We could successfully capture cells in both mouse lines by injecting 4-OHT 30 min before or right after the stimulus with comparable numbers, while the background tomato levels induced by injection but without stimulus were low (Fig. 4A-C and Supplementary Fig. 1).

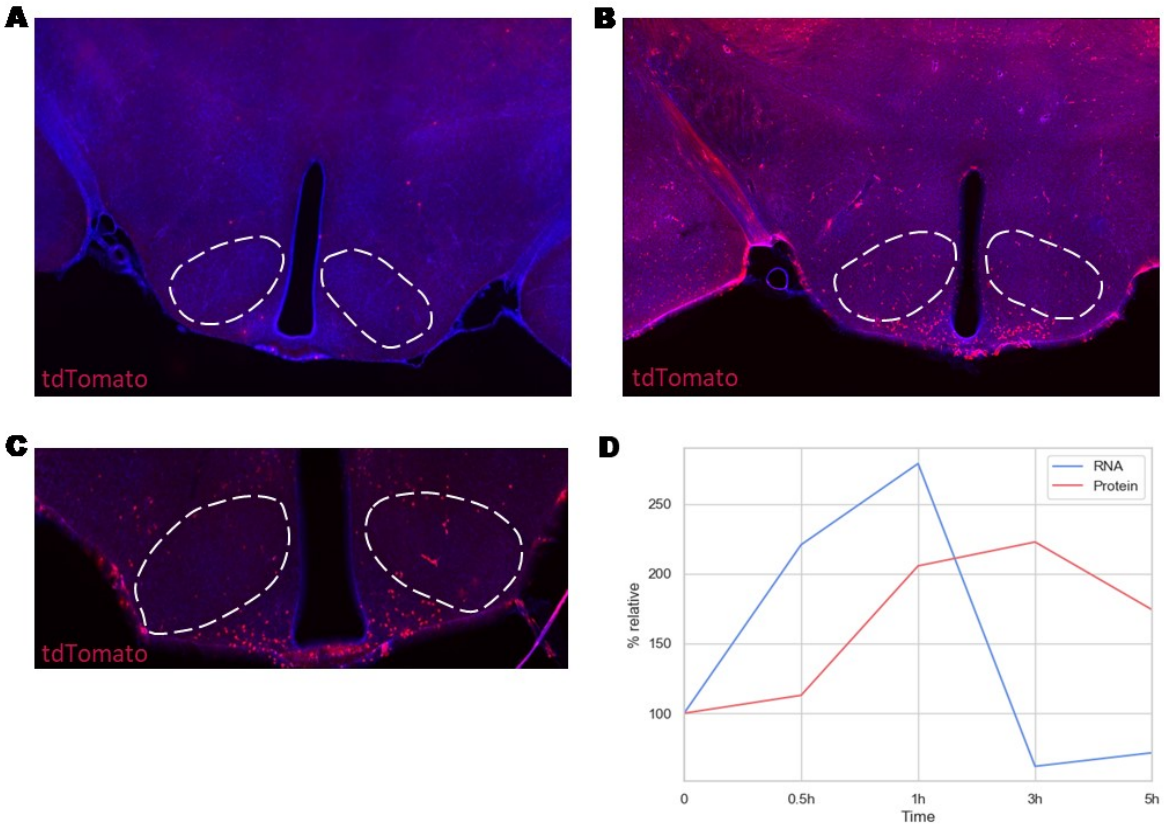

Fig. 4 Validation of cFos::tdTomato line and a time profile of cFos expression in VMH. (**a**) Activation of VMH area (white outline) in control condition - no stimulus given and after the 4-OHT injection before the test. (**b**) Activation of VMH neurons after the rat exposure and the 4-OHT injection right after the test. (**c** VMH activated after the rat exposure and after the 4-OHT injection 30 min before the test. (**d**) Levels of *cFos* RNA and protein after the rat stimulus in relation to time (values expressed as a percentage change relative to time 0 right after the rat exposure).

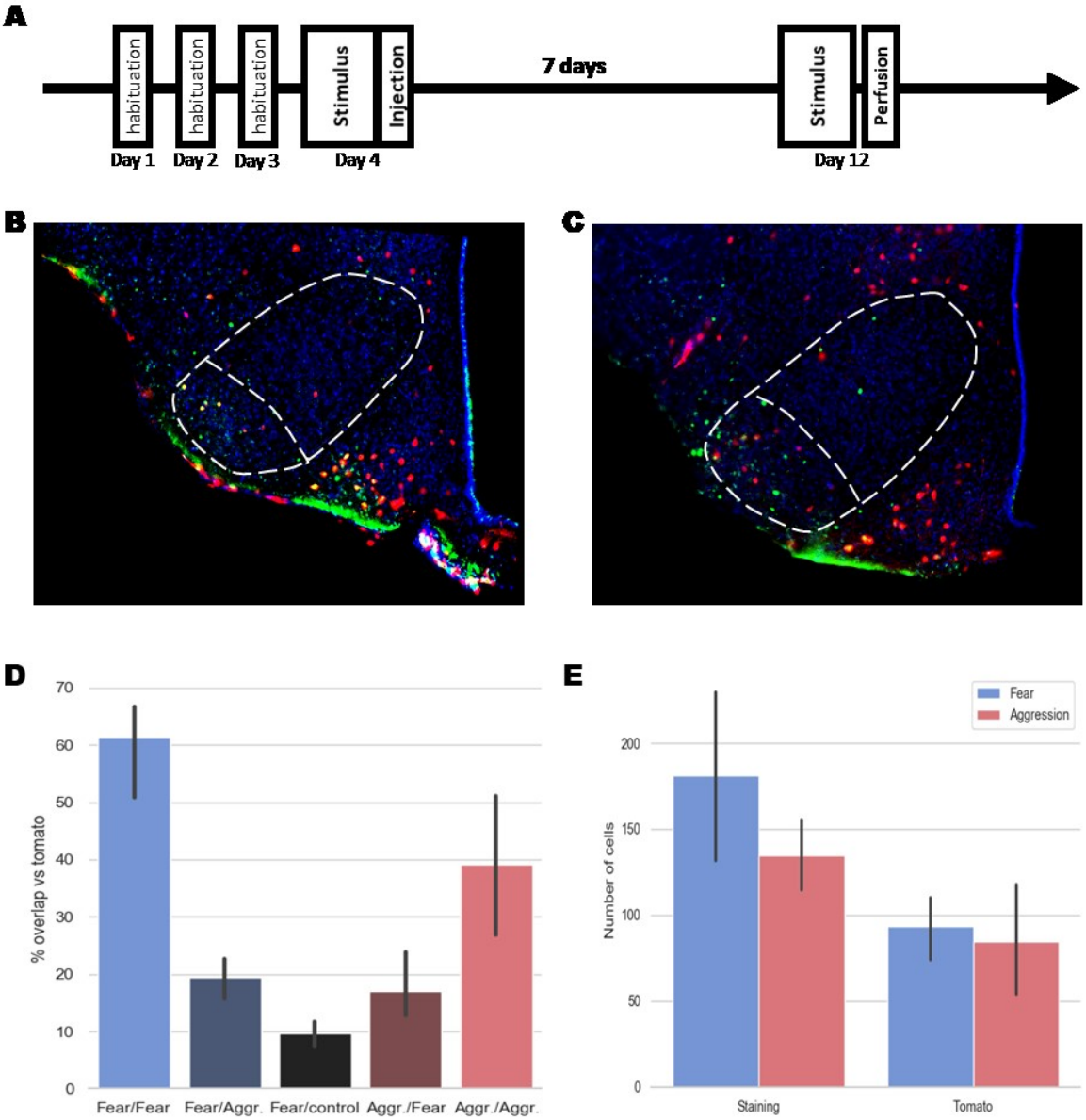

Fig. 5 Fear and aggression activated neurons only partially overlap in VMHvl. (**a**) The time line of the behaviour paradigm. (**b**) A representative brain section from the animal presented twice with an aggressive conspecific (defeat). Neurons in VMHvl show a considerable overlap. (**c**) VMHvl section from the animal presented with an aggressive conspecific and a submissive conspecific (aggression) showing little overlap. (**d**) A percentage of overlap between neurons labelled by TRAP and neurons stained for cFos in different stimulus configurations (from left fear + fear n=3, fear vs aggr. n=3, fear vs control n=3, aggr vs fear n=3, aggr vs aggr n=3, error bars represent SD). (**e**) Social fear activates more neurons than aggression and cFos staining labels more neurons than TRAP n = 12).

After the successful establishment of the cFos mapping system, we proceeded to test the hypothesis by conducting a series of double labelling experiments for defence and aggression in different combinations (Fig. 5A). We have found that an overlap of social fear activated neurons and aggression activated neurons is around 20%, indicating that

those two behaviours are mediated by largely non-overlapping populations in VMHvl. Overlap between the same behaviours was 60% for social fear and 40% for aggression (Fig. 5B-D). This may indicate a degeneracy in the circuit performing this action or that consecutive similar experiences are inherently different. Social fear seems to activate more neurons than aggression as measured by cFos staining but this difference was not seen using TRAP system. Additionally TRAP was less efficient in capturing cFos activation than immunohistochemistry (Fig. 5E). Altogether this result shows that TRAP can be used for a double cFos labelling in a single animal and that there is a difference between social defence and aggression populations that cannot be accounted to different trial and degeneracy of processing in VMHvl. Indeed, this result is in agreement with the results recently obtained using viral based system (Sakurai et al., 2016).

3.2 Monitoring activity in VMHvl neurons.

Mapping neurons activated with cFos suggested that there are largely separate populations involved in social fear and aggression. However it is known that cFos is expressed only in approximately 50% of the activated neurons (Lin et al., 2011) and as shown before TRAP method is not as efficient as staining, therefore the observed results are true only for this relatively small subset of cFos expressing neurons. Taken this into account, we decided to record VMHvl neurons *in vivo.* To do so, we have chosen recently developed technique of *in vivo* calcium imaging with miniaturized fluorescence microscope. This technique has several advantages: it allows mice to move freely while imaging many neurons at the same time and keeping track of them for many days, which was crucial in our experiments. We expected that this approach would allow us to confirm our findings from cFos mapping experiment and what was particularly exciting, to record neuronal activity in VMHvl during social fear (something that has never been done before) and compare it to recent findings from calcium and electrophysiological recordings, which indicated that neuronal activity in VMHvl is correlated with aggression (Falkner et al., 2014; Lin et al., 2011; Remedios et al., 2017).

To begin with we established *in vivo* calcium fluorescence microscopy in our laboratory using system designed by Doric Lenses (Fig. 6A-D). Endoscope *in vivo* calcium imaging in the deep and relatively small hypothalamic structure is not an easy task. Therefore, a total of 24 mice were used out of which only 9 met the criteria for further data

processing and at the end 7 mice were selected for this study. Remaining 15 mice were excluded due to various reasons, for example: bad quality of an image, wrong placement of the endoscope or failure to successfully complete behavioural paradigm. Experimental mice were divided into two groups: one that first underwent social fear followed by aggression trials, and the second begun the paradigm with aggression trials (Fig. 6E).

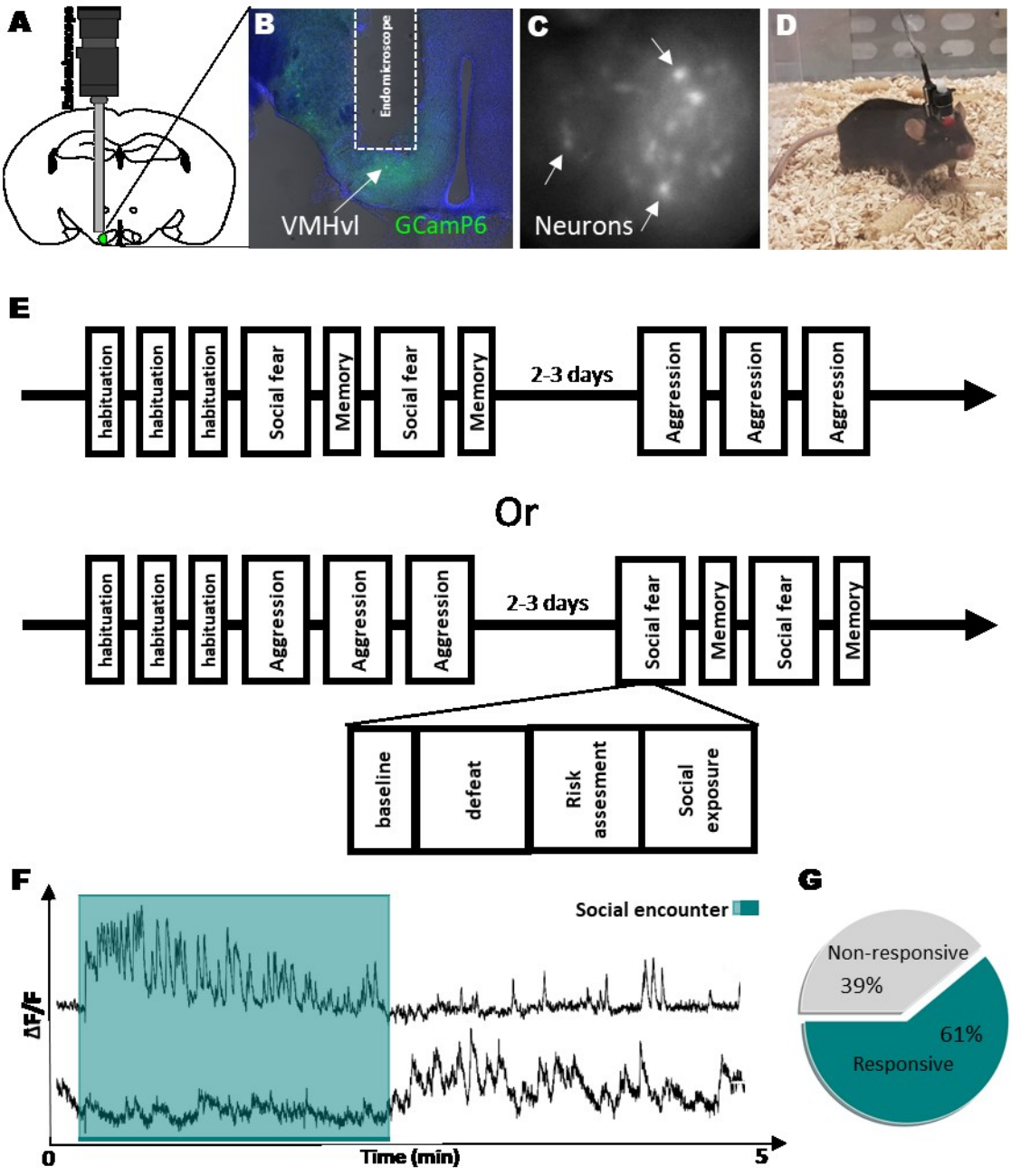

Fig. 6 Implantation and behaviour scheme for calcium in vivo imaging. (**a**) Scheme of endomicroscope implantation modified from Paxinos brain atlas and (Remedios et al., 2017). (**b**) An exemplary image of a brain section with a marked grin lens trace. (**c**) An example frame showing active neurons. (**d**) An experimental mouse with implanted and connected endomicroscope. (**e**) Schematics of two variations of behavioural paradigm used. (**f**) An example of ΔF/F traces of neurons during social encounter (green – close presence of conspecific). (**g**) Percentage of neurons responsive to social behaviours.

Previous electrophysiological recordings in VMHvl revealed that the spike timing of these neurons is predictive to attack behaviour and distance to conspecific and that neurons are activated by social clues (Falkner et al., 2014). On the other hand, recent calcium imaging study during the resident intruder paradigm showed only a general activation of these neurons during social encounter with males and females (Remedios et al., 2017). Surprisingly, our experiment not only confirmed that most of the neurons in VMHvl are activated during direct encounter with male conspecific but also identified a small subset of inhibited neurons. In total ~61% of the neurons in VMHvl responded to one or more of the behaviours during testing (Fig. 6F-H). Moreover, we also identified subsets of neurons highly correlated with fear behaviours and a small population of neurons responding during aggression trails. We found that stretching behaviour during risk assessment phase of social fear activates around 40% VMHvl neurons in a time-locked manner (Fig. 7A-B). Another fear behaviour correlated with neuronal responses was flight, during which 14% of neurons showed increased activity and 30% decreased activity (Fig. 7C-D). Later analysis revealed that the flight inhibition was mainly due to the activation during stretching behaviour right before flight (Fig. 7C and Fig. 10B). Third fear related behaviour was a direct defending (being attacked and bitten and actively trying to prevent it) during defeat phase, during which approximately 25% of recorded neurons raised their activity and ~10% were inhibited (Fig. 7E-F). Since the activity evoked during social trials was very high and persisted during different behaviours, to determine a significance of activation for a particular behaviour we used "choice preference" which is a receiver operator curve (ROC) based statistic (Remedios et al., 2017; Shadlen, Britten, Newsome, & Movshon, 1996). This method compares two distributions of activity values during two different conditions – in our case we always compared behaviour of choice with all the remaining behaviours during the trial. Figure 7G, 7H and 7I show histograms of neurons with their area under ROC score for stretching, flight and defending respectively. For some of the presented behaviours that were repeated at least 8 times we validated neuron *vs*. behaviour correlation with Wilcoxon signed-rank test comparing period before and right after the behaviour onset (Supplementary Fig. 2). These results confirmed involvement of VMHvl in social fear behaviours, especially in the risk assessment, and are in agreement with earlier functional studies (Sakurai et al., 2016; Silva et al., 2013).

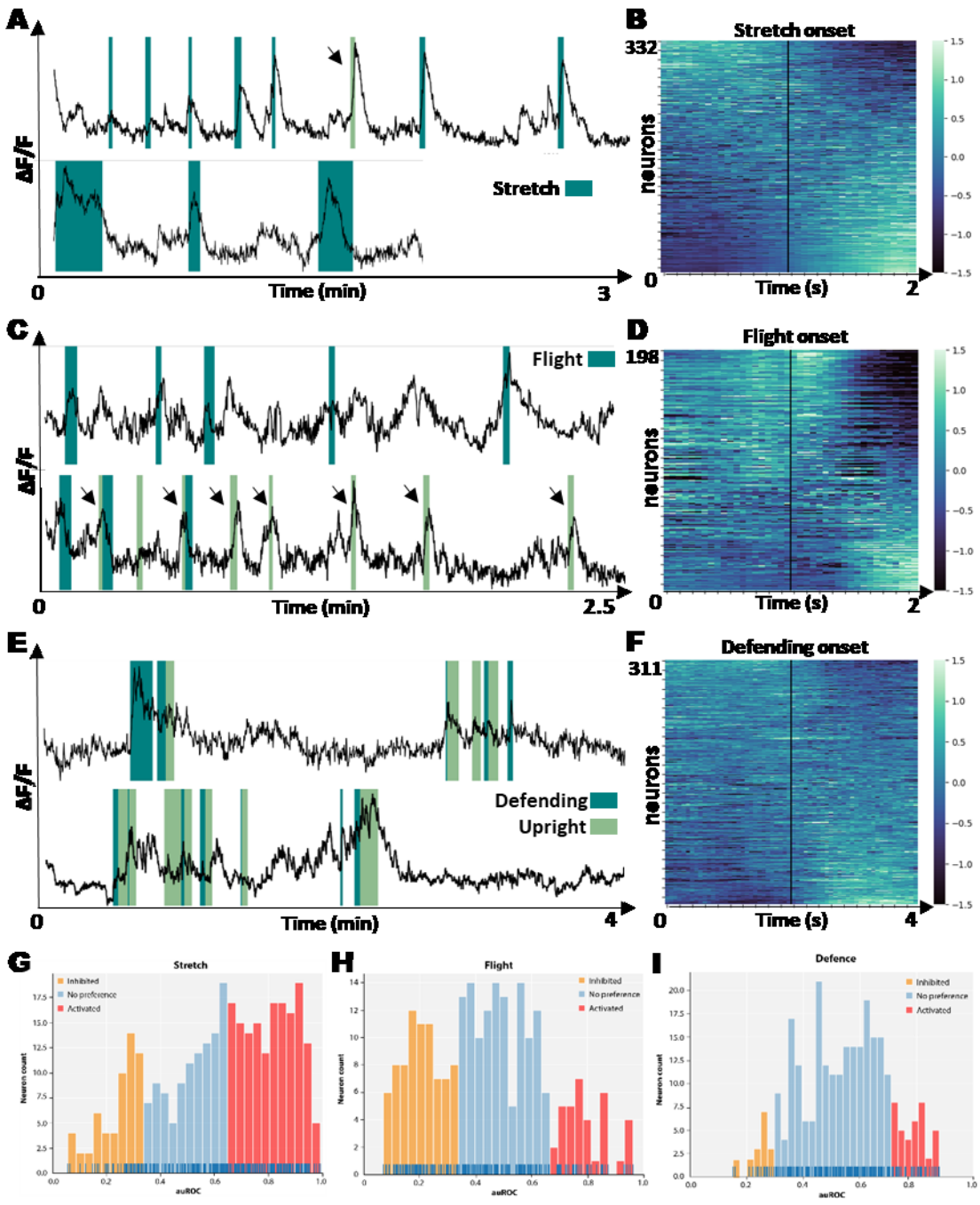

Fig. 7 Neurons in VMHvl are tuned to defensive responses. (**a**) An example of ΔF/F traces of neurons correlated with stretching behaviour (dark green – stretching behaviour periods, arrow indicates risk assessment without stretch). (**b**) All neuronal traces (each row represents one neuron) maened across trials and aligned to onset of stretch behaviour. (**c**) An example of ΔF/F traces of neurons correlated with flights (dark green – flights periods, arrow indicates stretching). (**d**) Neuronal traces of all neurons maenad across trials and aligned to onset of flight period. (**e**) An example of ΔF/F traces of neurons correlated with defending behaviour (dark green – defending periods, light green – upright postures). (**f**) All neuronal traces maened across trials and aligned to onset of defending behaviour. (**g,h,i**) Histogram of area under ROC curve scores (choice preference) for each neuron comparing stretch (g), flight (h), defending (i) with other behaviours during recording session (red - activated neurons auROC > 0.65, yellow - inhibited neurons auROC < 0.35, blue – non responding neurons).

During the aggression paradigm we identified two behaviours that were correlated with an activity of small groups of neurons. The first population was neurons tuned to sniffing where ~15% amongst all of the recorded neurons were activated and less than 4% inhibited during sniffing according to auROC statistic (Fig. 8A-B and E). The other group showed response during attack with approximately 10% of activated and inhibited neurons (Fig. 8C-D and 7F). These numbers are slightly lower than reported by Remedios *et.al* probably because they recorded only Esr1 expressing neurons that were shown to control aggression, whereas we captured all the neurons in VMHvl. Also, it is worth noting that auROC scores were the lowest for these two populations when compared with other social

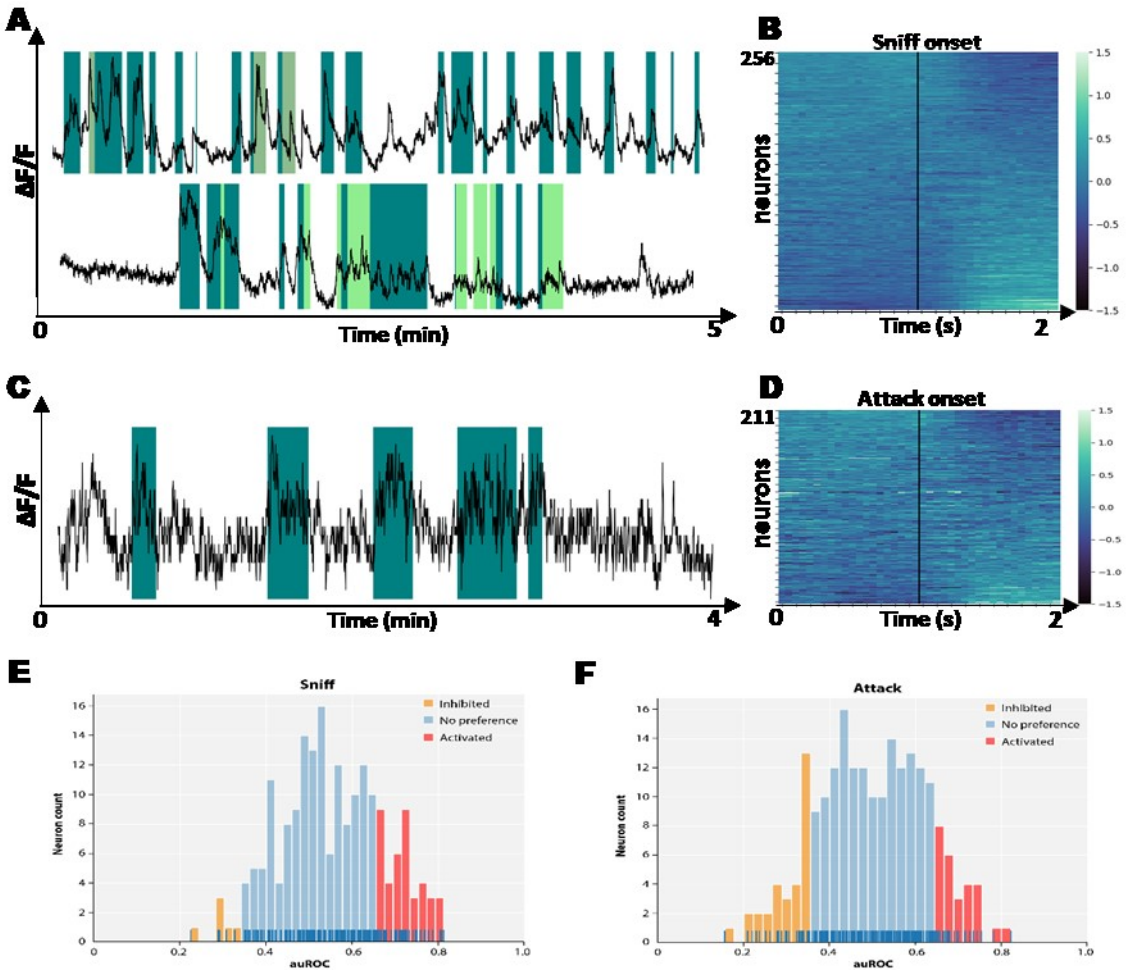

Fig. 8 VMHvl neurons are tuned to attack and sniffing during aggression trial. (**a**) An example of ΔF/F traces of neurons correlated with sniffing behaviour (dark green – sniffing behaviour periods, light green – ano-genital sniffing). (**b**) All neuronal traces meaned across trials and aligned to onset of sniff behaviour. (**c**) An example of ΔF/F traces of neuron correlated with attack (dark green – attack periods). (**d**) Neuronal traces of all neurons meaned across trials and aligned to onset of attack bout. (**e-f**) Histogram of area under ROC curve scores (choice preference) for each neuron comparing sniffing (e) and attack (f) with all other behaviours during recording session (red - activated neurons auROC > 0.65, yellow - inhibited neurons auROC < 0.35, blue – non responding neurons).

behaviours (Fig. 7-9). Another explanation could be different behavioural paradigms used in these two studies. In our experiments, half of the mice experienced defeat before aggression trial. In this case numbers of neurons tuned to aggression and sniffing were lower than in mice with reversed behavioural order, which might suggest some form of plasticity within VMHvl circuit.

One of the most surprising findings was that neurons in VMHvl showed highly time-locked responses during the memory test. During this point of the test, neural activity did not correlate with the behaviour (except in some instances of stretching) but with location of the mouse. Two main populations of neurons were found: one that shows high activation in a home chamber with 22% activated neurons and 30% of inhibited neurons (Fig. 9C-D and 9F), and the second population that was responsive in a far chamber, where the defeat took place. 27% of neurons displayed activation to the stimulus chamber, whereas 17% of neurons were inhibited (Fig. 9A-B and 9E). This result shows that activity in the hypothalamus encodes spatial information during the fear memory retrieval. Interestingly, there is one study showing a necessity of VMHdm for a fear retrieval in the context, 24 hours after rat exposure (Silva, Mattucci, et al., 2016). An activation of neurons in the far chamber of the apparatus suggests that spatial information independent from olfactory clues is processed in VMHvl, although the activation in the home cage might be mediated by a familiar smell. In support of this finding another recent study discovered a direct connection between the ventral subiculum, which is a part of the hippocampal formation and VMHvl, providing a putative link between context encoding and fear (Lo et al., 2018).

Next step in the analysis was to compare neurons in different populations and check for their co-regulation, independence or counter-regulation. First, we looked at defence behaviours and found that defending and stretch populations are highly co-regulated with almost 45% of stretch neurons overlapping with 62% of defending responsive neurons (Fig. 10A). Interestingly, when compared with flight neurons, both subsets revealed counter-regulation with more than 70% of flight inhibited neurons overlapping with either stretch or defending activated neurons (Fig. 10B-C and J). This makes sense, because defending and stretching are directed towards a threat, whereas flight is an avoidance behaviour. All together the high overlap between these neurons indicate that they represent coherent encoding of threat or fear related behaviours. When compared with aggressive behaviour,

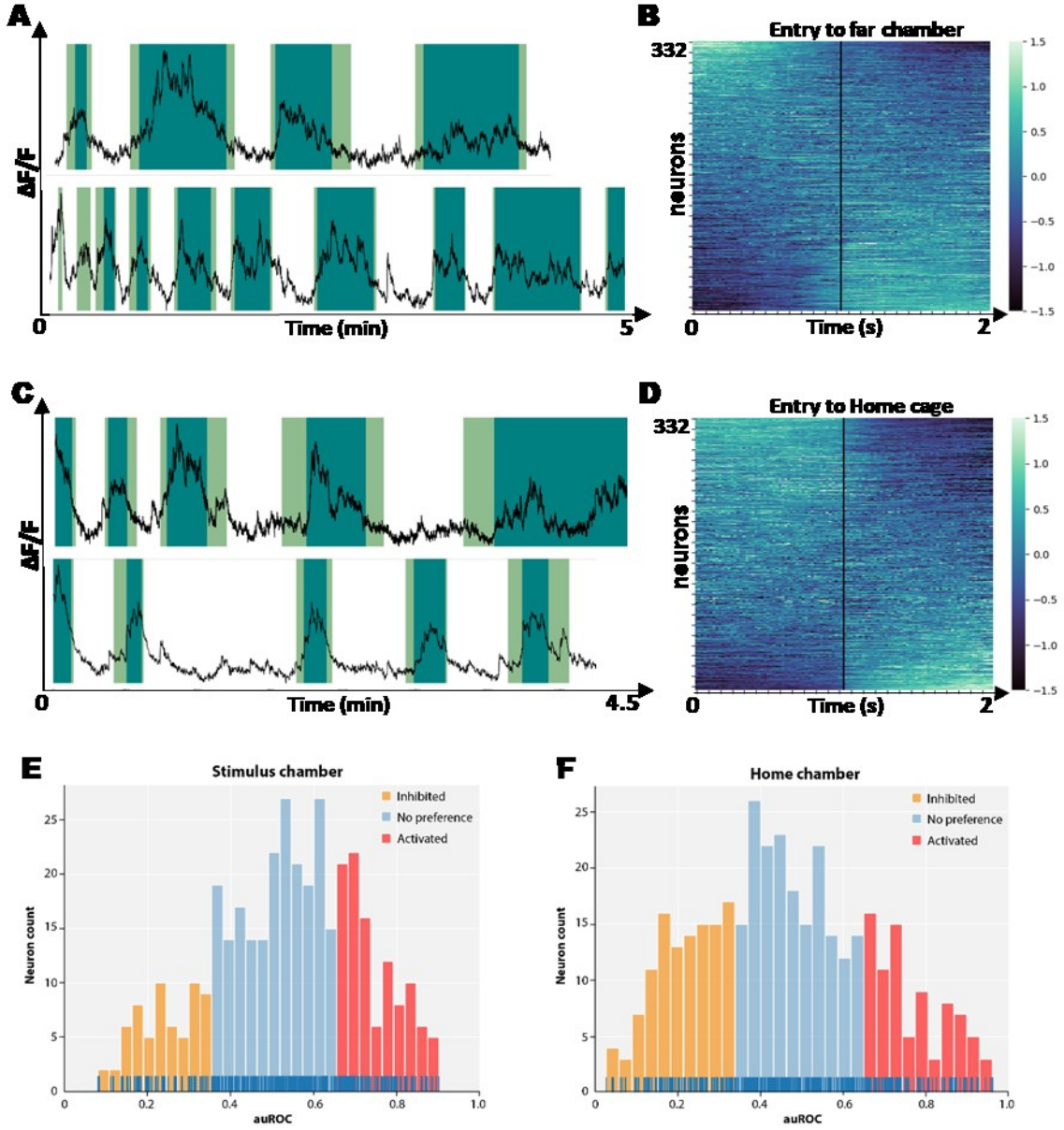

Fig. 9 Neurons in VMHvl encode location during fear memory retrieval. (**a**) An example of ΔF/F traces of neurons correlated with the far chamber location (dark green – stimulus chamber, light green – corridor part close to the far chamber). (**b**) All neuronal traces meaned across trials and aligned to the onset of the far chamber entry. (**c**) An example of ΔF/F traces of neuron correlated with the home cage location (dark green – home cage, light green – corridor part next to the home cage). (**d**) Neuronal traces of all neurons meaned across trials and aligned to the moment of the home cage entry. (**e-f**) Histogram of area under ROC curve scores (choice preference) for each neuron comparing the far chamber (e) and the home cage (f) with all other locations during recording session (red - activated neurons auROC > 0.65, yellow - inhibited neurons auROC < 0.35, blue – non responding neurons).

defence behaviours exhibit much less co-regulation and shift towards independence. Nonetheless, around 34% of attack neurons overlap with ~15% of defending population, while there is almost no overlap between attack and flight responsive neurons (Fig. 10D-F and K). This result fits with cFos mapping data, where an overlap was slightly below 20%.

Curiously, the most of attack-defending overlapping neurons (9/11) came from mice undergoing aggression first. In the same mice an attack-defending overlap is almost doubled, again, suggesting that defeat might induce some plasticity in VMHvl. If we

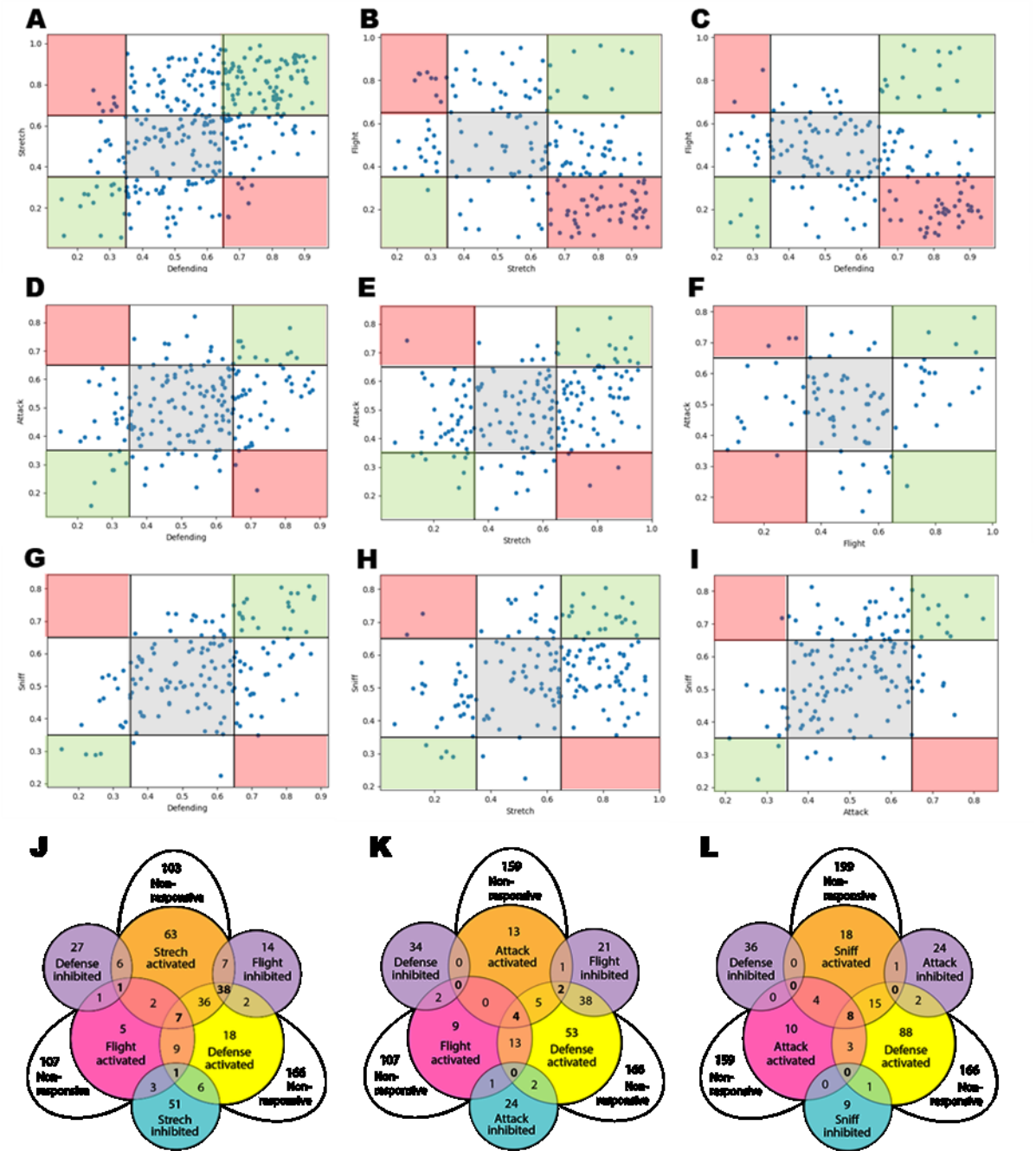

Fig. 10 Relationship between different neuronal populations responding to social behaviours. (**a-i**) Scatterplots of neuron auROC scores plotted for different pairs of behaviours: (a) defending – stretch, (b) flight – stretch, (c) flight - defending, (d) attack – defending, (e) attack – stretch, (f) attack – flight, (g) sniff – defending, (h) sniff – stretch, (i) sniff – attack (red quadrants – counter-regulation, green quadrants – co-regulation, white quadrants – independent activity, grey quadrant – non-responsive neurons). (**j-l**) Venn diagram showing overlap between: (j) defending, stretch and flight (**k**) attack, defending and flight. (**l**) Sniff, defending, attack responsive neurons.

compare sniff with attack and defending populations, we see that around 50% of sniff neurons overlap with defending population (18%) and 30% of sniff neurons overlap with 34% of attack population (Fig. 10G-I). Such high overlap confirms that smell is important factor in social contact in mice. It is interesting to notice that half of the overlapping neurons are actually shared between the three behaviours suggesting an existence of a population simply indicating a presence of conspecific or its smell (Fig. 10L).

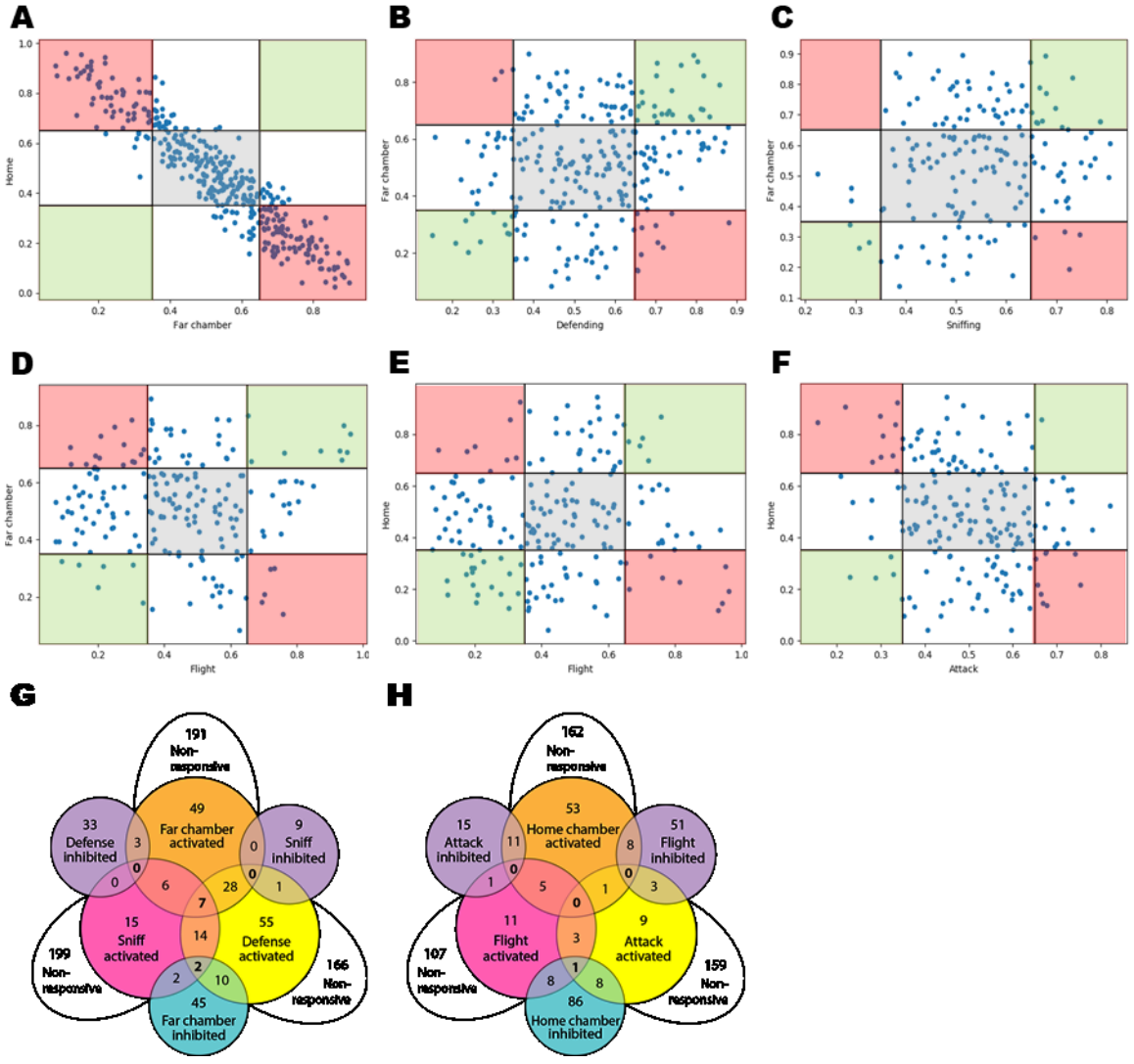

Fig. 11 Relationship between spatial and social neuronal populations. (**a-f**) Scatterplots of neuron auROC scores plotted for different pairs of spatial and behavioural populations: (a) home cage – far chamber, (b) far chamber – defending, (c) far chamber - sniffing, (d) far chamber – flight, (e) home cage – flight and (f) home cage – attack (red quadrants – counter-regulation, green quadrants – co-regulation, white quadrants – independent activity, grey quadrant – non-responsive neurons). (**g**) Venn diagram showing overlap between far chamber, defending and sniff populations. (**h**) Venn diagram displaying overlaps between home cage, flight and attack responsive neurons.

After finding interesting relationships between neuronal populations correlated with behaviours, indicating a separation between defence behaviours and aggression, as well as some degree of plasticity, we sought to link them with spatial coding populations. At the beginning we compared home cage and far chamber populations themselves. Surprisingly, they formed a perfect negative correlation, which means that these two populations are strictly counter-regulated. There was not a single neuron that would be activated or inhibited in both locations (Fig. 11A). Then to test a hypothesis that spatial encoding might be driven by different experiences that is defeat in far chamber and aggression in home chamber, we compared stimulus chamber population with defence populations and sniffing (Fig. 11B-D). Over 30% of the far chamber neurons are co-regulated with 30% of defending neurons, whereas only 14% of them are co-regulated with 30% of sniff population neurons. In turn at least half of sniffing neurons overlapped with defending population (Fig. 11G) supporting view that at least in part behaviour might be driving the spatial coding in VMHvl.

Further we compared home cage neurons with attack and flight neurons. Unexpectedly, both of the comparisons showed high independence but also counter-regulation (Fig. 11E-F). For flight and home cage this is expected since hypothetical flight neuron would be active outside home cage and *vice versa*. For aggression, on the other hand, if we assume that behaviour would drive spatial differentiation as defence-far chamber correlation suggested, we could expect a co-regulation of these populations because aggression test took place in the home cage. Additionally, there was strong a co-inhibition of flight and home cage neurons (30%) and half of those overlapped with far chamber activated neurons, suggesting possible relationship of this population with a threat level (Supplemental Fig. 3).

Having carefully described single neurons and relations between them we sought to examine activity patterns of the whole ensemble of recorded neurons. In particular we wanted to test a hypothesis that VMH might be a structure involved in the generation of a central emotional state as suggested in recent publications (Ralph Adolphs, 2017; Kunwar et al., 2015; Lo et al., 2018). One of the possible features of such structure could be different activity during different central states. Both aggression and defence are thought to be behavioural manifestations of anger and fear respectively, therefore, we decided to compare VMHvl neuronal activity during consecutive days of our behavioural paradigm including

all social behaviours into the analysis. Dimensionality reduction methods are very useful in finding patterns in the big data like RNA sequencing and were recently applied to the *in vivo* calcium imaging data (Ying Li et al., 2017; Remedios et al., 2017). First, we decided to use a principal component analysis (PCA) using only calcium imaging frames as data points without any additional information. This method detected very little to none differential activity in VMHvl during different days (Supplementary Fig. 4). Encouraged by PCA results we decided to use partial least square (PLS) method that allows to optimize principal components to additional information, which in our case is the existence of different days in the data structure. This approach yielded an interesting discovery that the

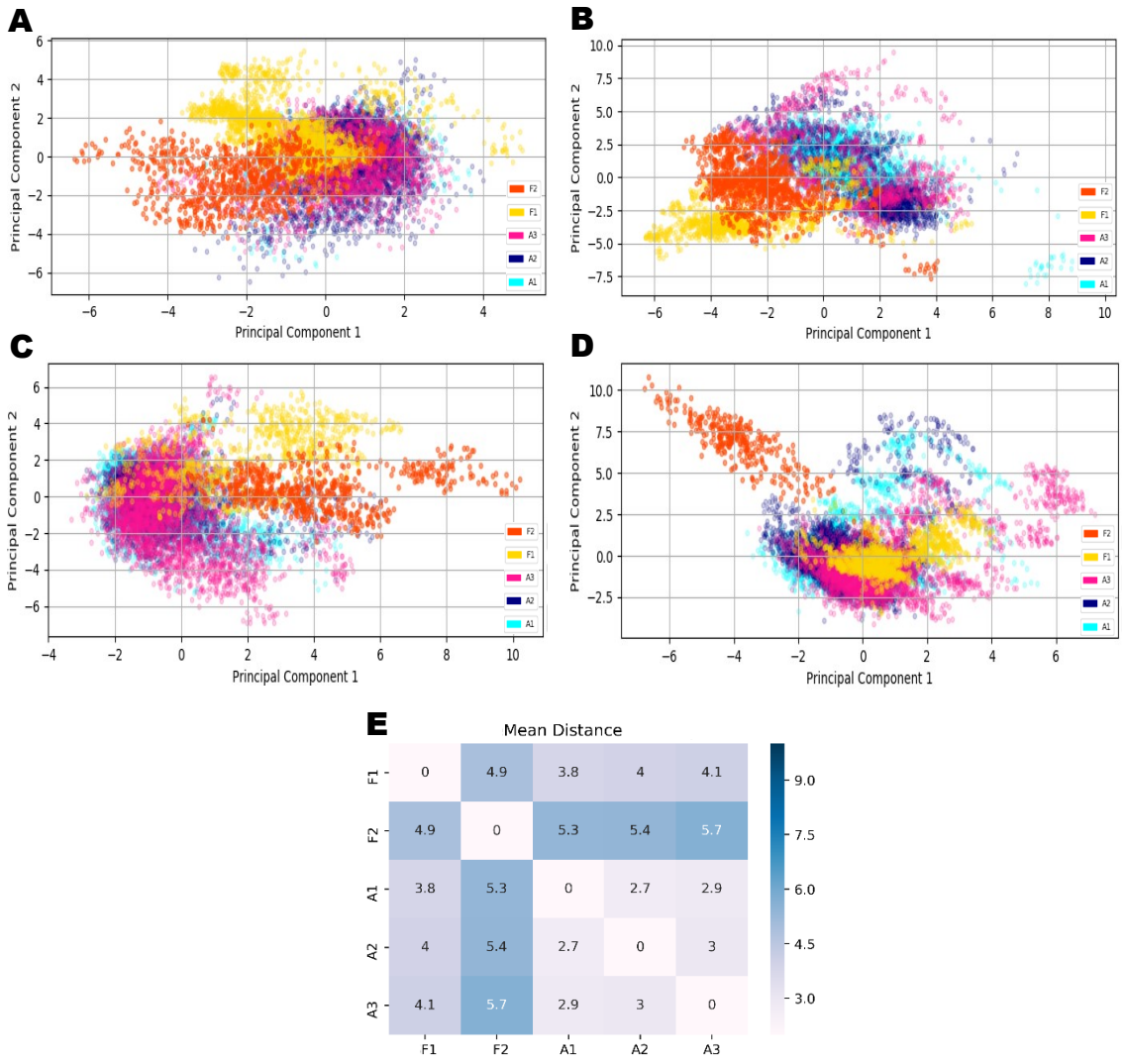

Fig. 12 Differential ensemble activity for defence and aggression. (**a-d**) Partial least square regression analysis results plotted in principal component space comparing the neuronal ensemble activity during social fear and aggression days for 4 different mice (orange F2 – social fear day 2, yellow F1 social day 1, pink A3 – aggression day 3, dark blue A2 – aggression day 2, light blue A1 – aggression day 1, each dot corresponds to one frame of recording). (**e**) Mean distance between clusters of the frames from different days from all mice (n=4). Only the periods of social interaction were used in this analysis.

ensemble activity during social fear days differs from the activity on aggression days in each of the tested mouse (Fig 12). Interestingly, there was also a difference between social fear day 1 and 2. On average aggression days were most similar to each other and then progressively more difference was discovered between aggression days and social fear day 1 and 2. Such structure suggests that there might be some learning induced by social fear paradigm in the VMHvl neuronal ensemble. Next, we applied the same method to mice that went through the reversed paradigm with aggression days before the social fear paradigm. Surprisingly, this time PLS failed to detect any difference between the days in all of the tested mice (Fig 13). We interpret this result as evidence that neuronal activity in VMHvl during social behaviours is shaped by previous social experience – claim that is supported by Remedios *et.al*. study (Remedios et al., 2017), which revealed that VMHvl neurons learn to differentiate between male and female conspecifics. To validate these results we used linear discriminant analysis (LDA) – another dimensionality reduction method that uses exactly the same data as PLS but slightly different algorithm to find differences in the data

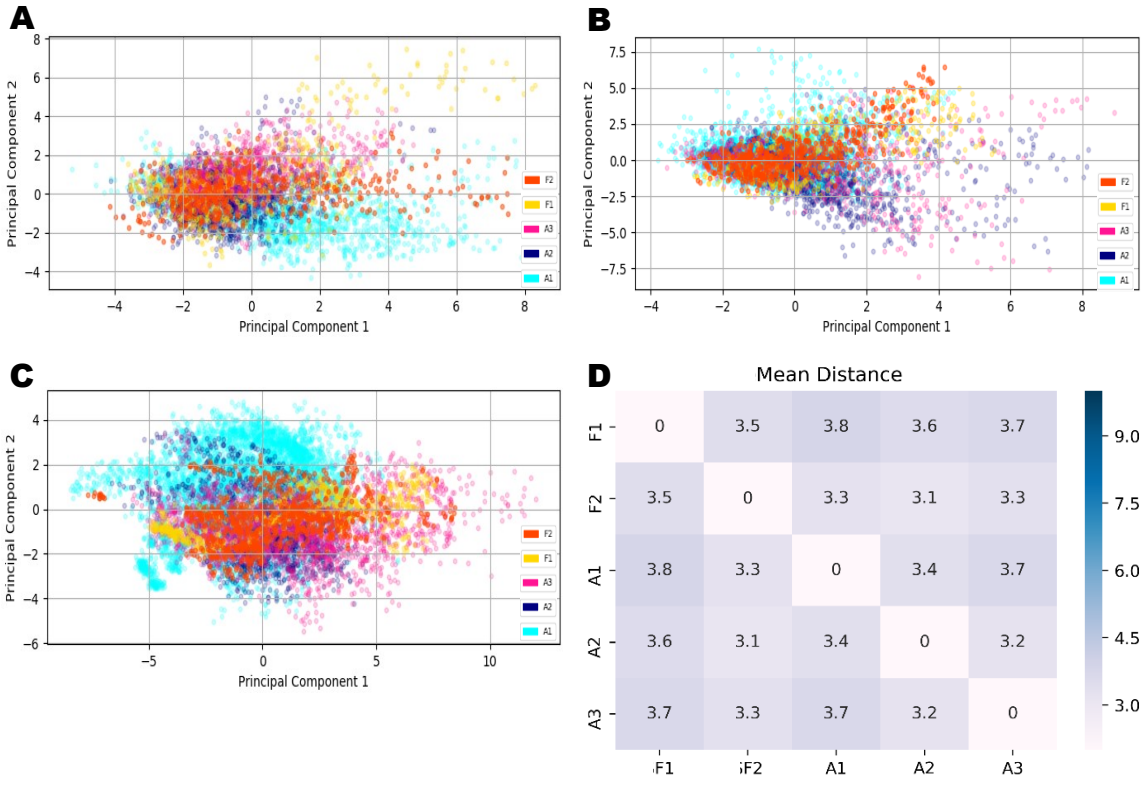

Fig. 13 Experience influences the ensemble activity in VMHvl. (**a-c**) Partial least square regression analysis results plotted in principal component space comparing the neuronal ensemble activity during aggression and social fear days for 3 different mice that underwent reverse order paradigm (orange F2 – social fear day 2, yellow F1 social day 1, pink A3 – aggression day 3, dark blue A2 – aggression day 2, light blue A1 – aggression day 1, each dot corresponds to one frame of recording). (**d**) Mean distance between clusters of the frames from different days from all mice (n=3).

structure. This analysis similarly to previously described PLS detected the difference only between social fear and aggression days in mice that completed the “normal” paradigm (Fig. 14A-D and Fig. 15A-D). Moreover, differences were even more profound than in PLS when mean distance between day clusters was compared (Fig. 14E). LDA also failed to detect changes in the neuronal ensemble activity amongst days in the reverse paradigm, thereby confirming results obtained earlier.

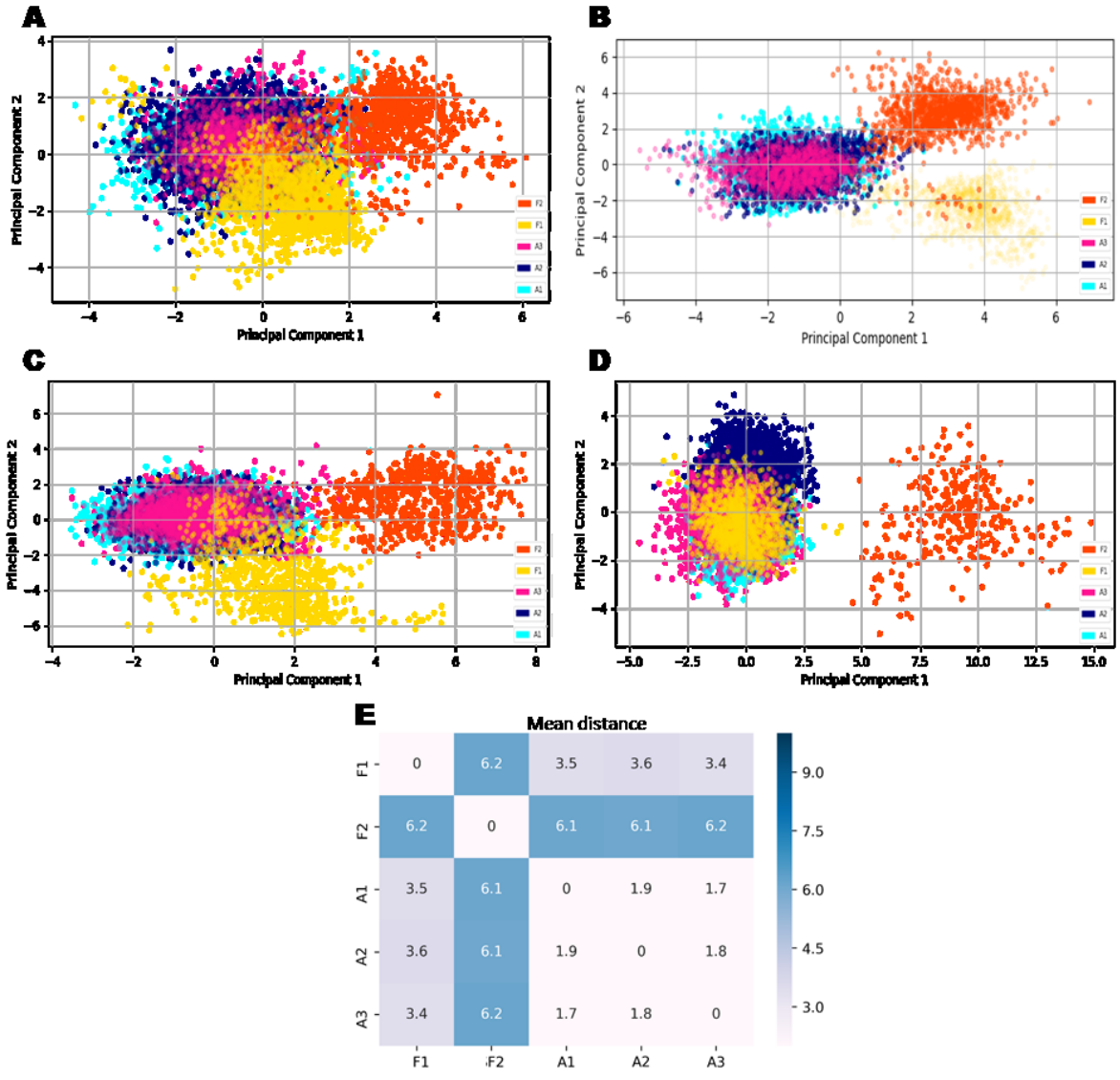

Fig. 14 LDA detects similar differences between social fear and aggression. (**a-d**) Linear discriminant analysis results plotted in principal component space comparing the neuronal ensemble activity during social fear and aggression days for 4 different mice (orange F2 – social fear day 2, yellow F1 - social day 1, pink A3 – aggression day 3, dark blue A2 – aggression day 2, light blue A1 – aggression day 1, each dot corresponds to one frame of recording). (**e**) Mean distance between clusters of the frames from different days from all mice (n=4)

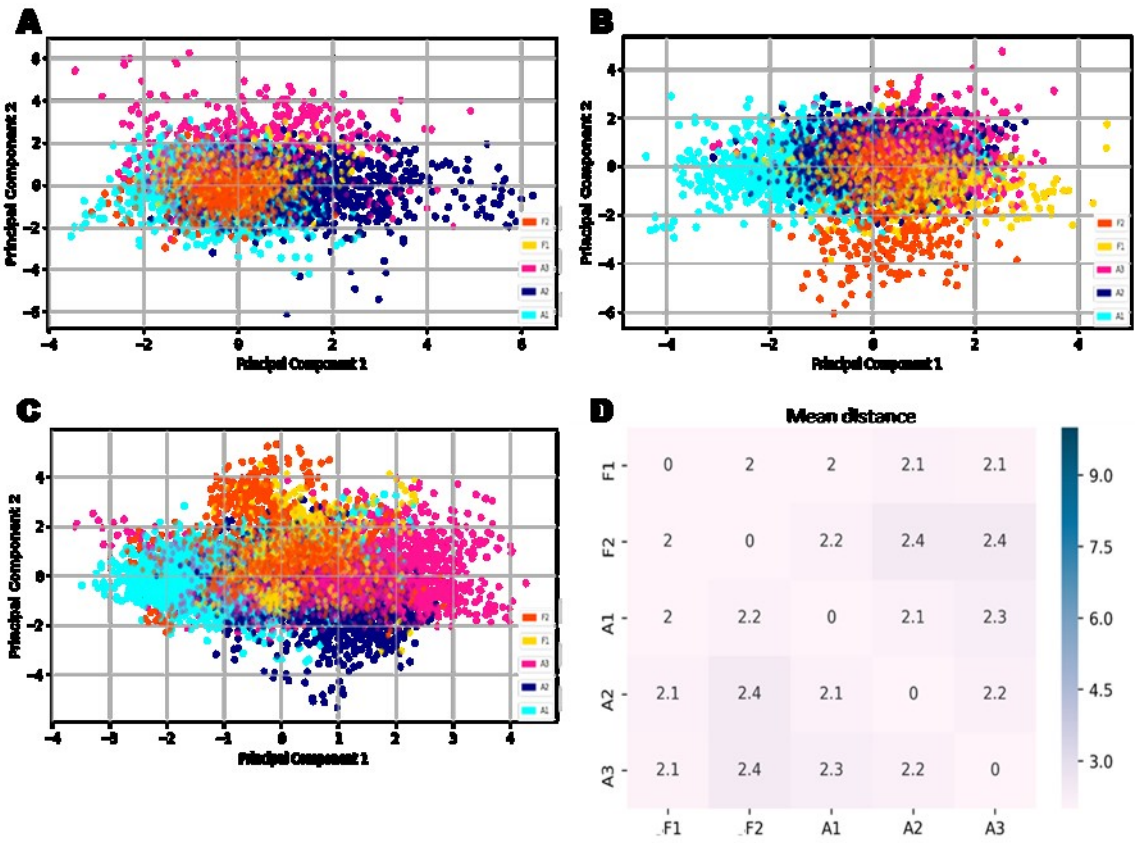

Fig. 15 LDA detects no differences between days in the reverse order mice. (**a-c**) Linear discriminant analysis results plotted in principal component space comparing the neuronal ensemble activity during social fear and aggression days for 3 mice that completed the reverse behavioural paradigm (orange F2 – social fear day 2, yellow F1 - social day 1, pink A3 – aggression day 3, dark blue A2 – aggression day 2, light blue A1 – aggression day 1, each dot corresponds to one frame of recording). (**e**) Mean distance between clusters of the frames from different days from all reverse order mice (n=3)

Next we asked if it is possible to predict a day of the paradigm from the neuronal activity of the whole ensemble. To test this hypothesis we employed a machine learning paradigm based on an LDA algorithm and used K-folds cross-validation to estimate accuracy of the prediction. Indeed, using this approach of comparing each possible pair of days we were able to distinguish between social fear day 2 and other days with 95% accuracy on average. For social fear day 1 accuracy was around 80%. On the other hand, the algorithm could not effectively distinguish between aggression days (Fig. 16A). These results were achieved only for mice that underwent paradigm where social fear part was first followed by aggression, whereas in mice with the reverse paradigm algorithm failed to distinguish between social fear and aggression pairs (Fig. 16B). Nonetheless, accuracy around 70% was achieved for some day pairs revealing small differences in neuronal activity between different days that remained undetected using global dimensional reduction approaches (Fig. 16B).

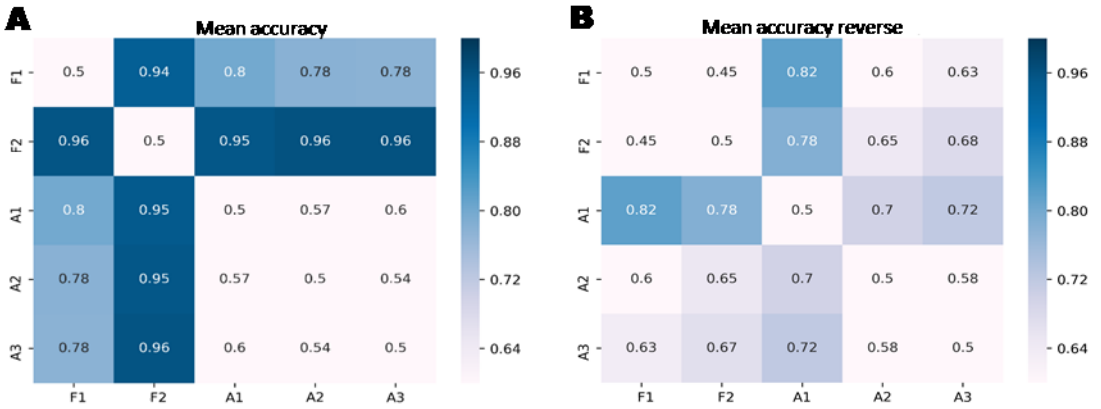

Fig. 16 Machine learning algorithm learns to distinguish social fear and aggression days (a) Mean accuracy of discrimination between all possible day combinations using LDA based machine learning approach in normal order mice (n=4, K folds = 10). (**b**) Mean accuracy of discrimination between all possible day combinations using LDA based machine learning approach in the reversed order mice (n=4, K folds=10).

3.3 Functional manipulation of VMHvl neurons.

3.3.1 Manipulation of Esr1+ neurons in VMHvl.

One of the goals for this work was to identify a neuronal population governing social fear in the ventromedial hypothalamus. Monitoring VMHvl neuronal activity identified neurons correlated with a set of defensive behaviours. Furthermore, neurons activated during sniffing and attack have a significant level of overlap with defensive neurons (30-40%). This points to the possibility that these overlapping neurons process common information for both aggression and defence against conspecifics, which in turn led us to hypothesize that a population of neurons known to modulate aggressive behaviours in VMHvl may also contribute to the regulation of defensive behaviours. To test this, we performed a pharmacogenetic silencing of Esr1 expressing neurons during our defeat and aggression paradigm (Fig. 17A-C). As expected from previous studies, an inhibition of ERα expressing neurons during aggression module greatly reduced attack behaviour in resident mice after CNO injection (Fig. 17D), whereas control groups showed increased aggression behaviour compared to the baseline taken a day earlier (Supplemental Fig. 5).

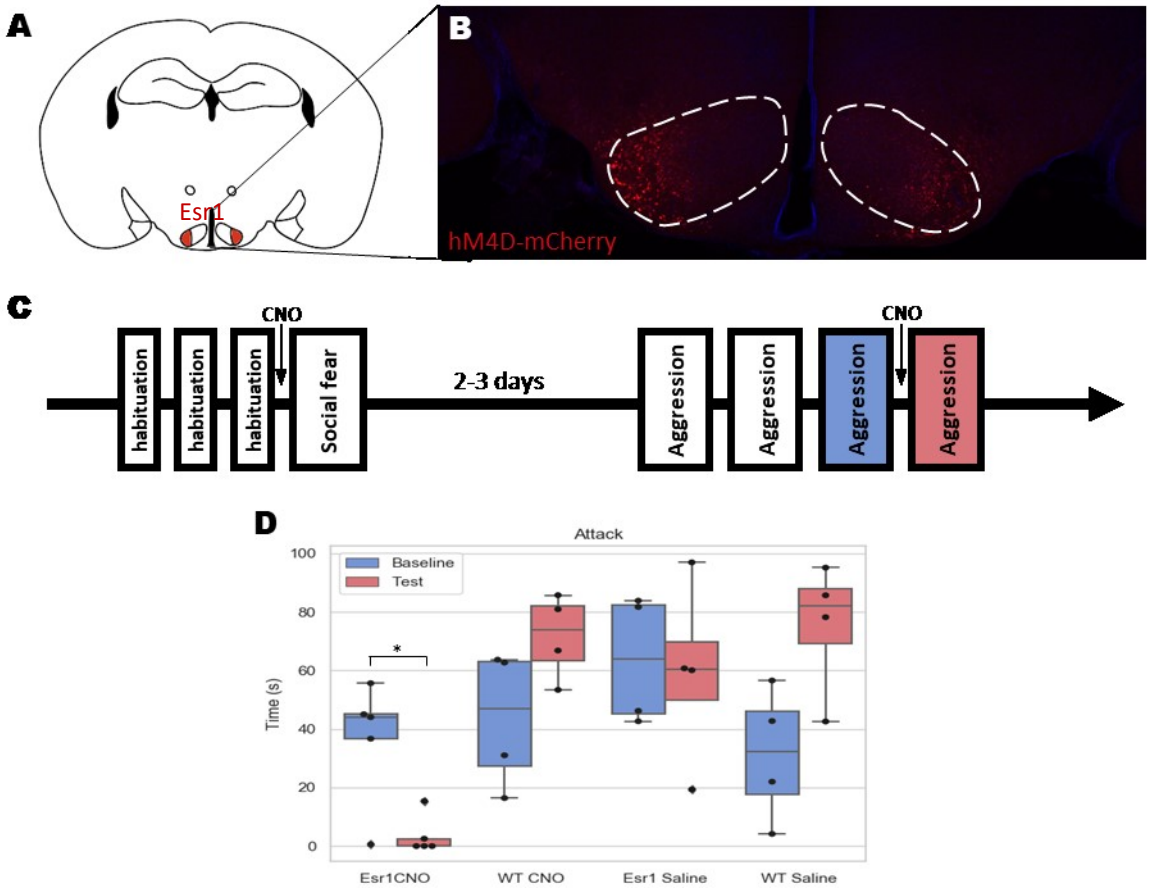

Fig. 17 Pharmacogenetic inhibition of Esr1 neurons reduces aggression. (**a**) Scheme depicting expression of hM4D in VMHvl. (**b**) An image of the brain section with marked VMHvl and hM4d expression in Esr1 neurons. (**c**) Schematics of behavioural paradigm (**d**) Aggression times during baseline (blue) and test day after CNO or saline injection (red) in 4 groups from left: Esr1::hM4D + CNO, WT + CNO, Esr1::hM4D + saline and WT + saline. (n=4/5 for each experimental group, multiple t test, p-value= 0.0107).

In contrary, an inhibition of Esr1 expressing neurons during social fear paradigm was not sufficient to drive any clear change in mice behaviour. Typical fear measures like flight, freezing and stretching did not reveal any significant changes between the groups (Fig. 18A-B). Other more general behavioural measures showed small trends. For example, immobility seemed to be decreased in experimental group and exploration, which is a measure of time spend outside home cage, showed an increase in at least half of the experimental mice (Fig. 18C-D). In general results show high variability within most groups probably indicating dependence of these behaviours on differences that may occur during the defeat. Overall, these results show that inhibition of Esr1 is not sufficient to modulate fear behaviour. This can be due to the fact that social fear tends to recruit more cells in VMHvl than aggression, based on cFos studies and calcium monitoring observations, and silencing only a fraction of them might be not enough to modulate fear behaviours effectively.

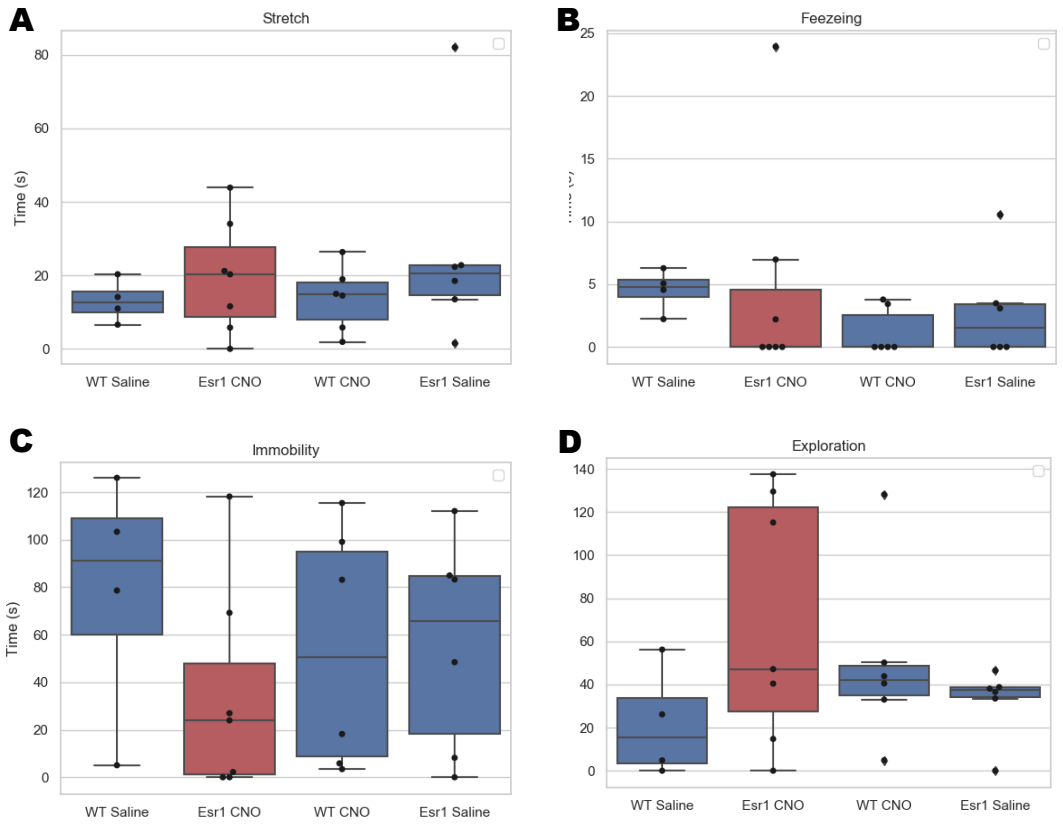

Fig. 18 Inhibition of ERα neurons in VMHvl does not modulate social fear. (**a-d**) A comparison of duration of particular behaviours (a) stretch, (b) freezing, (c) Immobility, (d) exploration during social fear paradigm between experimental group Esr1::hM4D + CNO (red, n=6) and different control groups (blue n=16).

Next, we decided to perform an optogenetic activation experiment. Despite the fact that many studies show that the stimulation of Esr1 neurons in VMHvl during social encounter induces aggression, we focused on the fact that stimulation of the same neurons also leads to an increased mounting showing scalability and possibility that the same neurons can drive different behaviours (Lee et al., 2014; Lin et al., 2011). Moreover, our *in vivo* calcium experiments suggested plasticity in VMHvl after defeat, which might be crucial for inducing social fear behaviours. To check this, we defeated mice expressing channelrodopsin 2 (ChR2) in VMHvl Esr1 neurons in a novel cage with an aggressive conspecific. Then we introduced subordinate male in their home cage with recently changed bedding and stimulated using 20Hz and 5-10mW light protocol. Surprisingly, this stimulation caused a strong fear response in mice. Light induced prolonged periods of freezing and induced flights from the submissive conspecific when he approached (Fig. 19C-D). Stimulation also decreased social interaction time and increasing avoidance in experimental mouse that persisted for a short time after the stimulation (Fig. 19E).

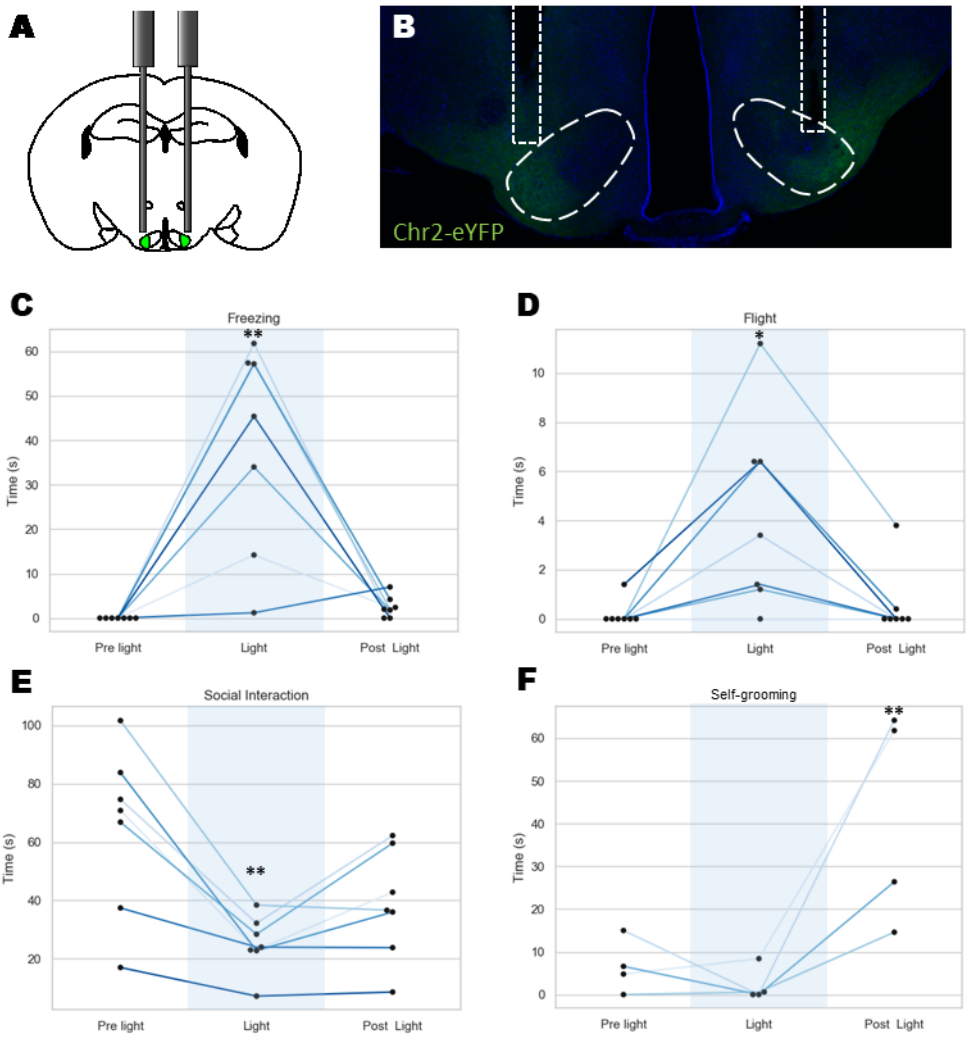

Fig 19 Activation of Esr1 neurons in defeated mice induce fear behaviours. (**a**) A scheme of optic fibres implantation. (**b**) An image of the brain section with marked optic fibre traces and expression of ChR2-eYFP. (**c-f**) Graphs presenting mean duration of (**c**) freezing (n=7, p-value = 0.0055), (d) flight (n=7, p-value 0.0189), (**e**) social interaction (n = 7, p-value = 0.0068) and (f) self-grooming (n=4, p-value = 0.0092) during period previous to light (30s), light stimulation (30s) and after light (30s).

Interestingly, immediately after the light stimulation ended animals started to self-groom excessively. This self-grooming period lasted around 10 to 20s after the light offset. This shows that a stimulation of Esr1+ neurons in VMHvl leads to a display of fear behaviours towards an intruder conspecific in animals that were earlier defeated. All together these results suggest that the function of Esr1 expressing neurons is dependent on the previous experience and possibly current stress levels.

3.3.2 Manipulation of Nos1+ neurons in VMHvl

Silencing of Esr1 neurons failed to modulate social fear behaviours in mice. One possible explanation is that there are other cells mediating this response or that Esr1 expressing neurons are only a subset of those cells and the changes in behaviour escaped resolution of our tests. To investigate this possibility we selected another marker – gene Nos1, whose expression appears to be broader than Esr1 in VMHvl (Allen Mouse Brain Atlas, ISH data). First, we wanted to compare Nos1 expression with Esr1 in VMHvl. To this end, we crossed *Esr1*::Cre mice with *RC::*LSL-tdTomato, stained sections of their brains with Nos1 antibody and quantified number of Nos1 and Esr1 cells and their overlap. Indeed, there was around 20% more Nos1 expressing cells than Esr1 cells in VMHvl but, unexpectedly, those populations did not overlap (< 1%). This suggest that Nos1+ neurons might perform a different role in social behaviour than Esr1+ neurons (Fig. 20A-B).

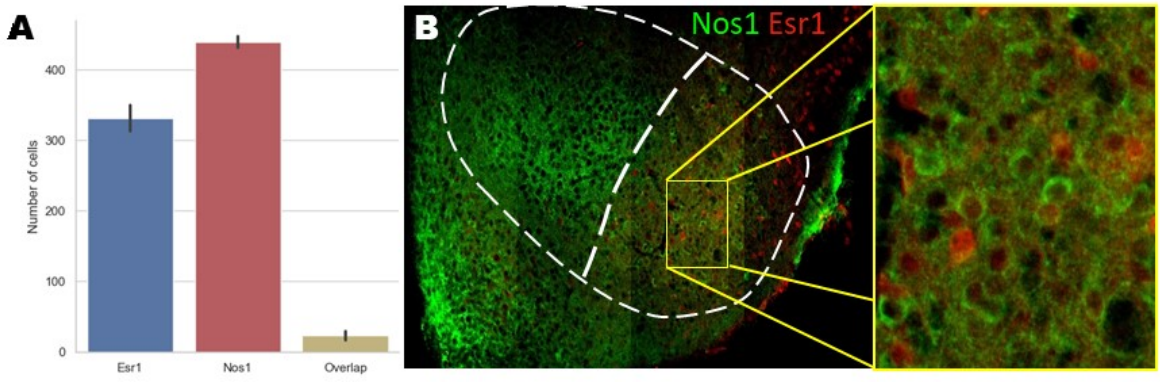

Fig. 20 Esr1+ and Nos1+ neurons are segregated in VMHvl. (**a**) Quantification of Esr1 (blue) and Nos1 (red) cells in VMHvl and an overlap (dark yellow) between them (n=2) (**b**) A representative image of a section of *Esr1*::tdTomato (red) brain stained with Nos1 antibody (green).

Surprised by nearly perfect segregation of those neurons we decided to perform a pharmacogenetic inhibition experiment using our social fear and aggression paradigm (Fig. 21A-C). In contrast to Esr1+ neurons, blockade of Nos1+ neuronal activity in VMHvl during the aggression test had no effect on duration of attacking compared to the baseline, which was measured one day before (Fig. 21D). This result is in line with previous findings that optogenetic inhibition of non-Esr1 neurons in VMHvl failed to reduce aggression (H. Lee et al., 2014). On the other hand, inhibition of Nos1+ cells during social fear revealed a significant differences between groups that seem to be opposite to Esr1+ (Fig. 22). Mice expressing hM4D in Nos1 neurons that received CNO injection showed an increased

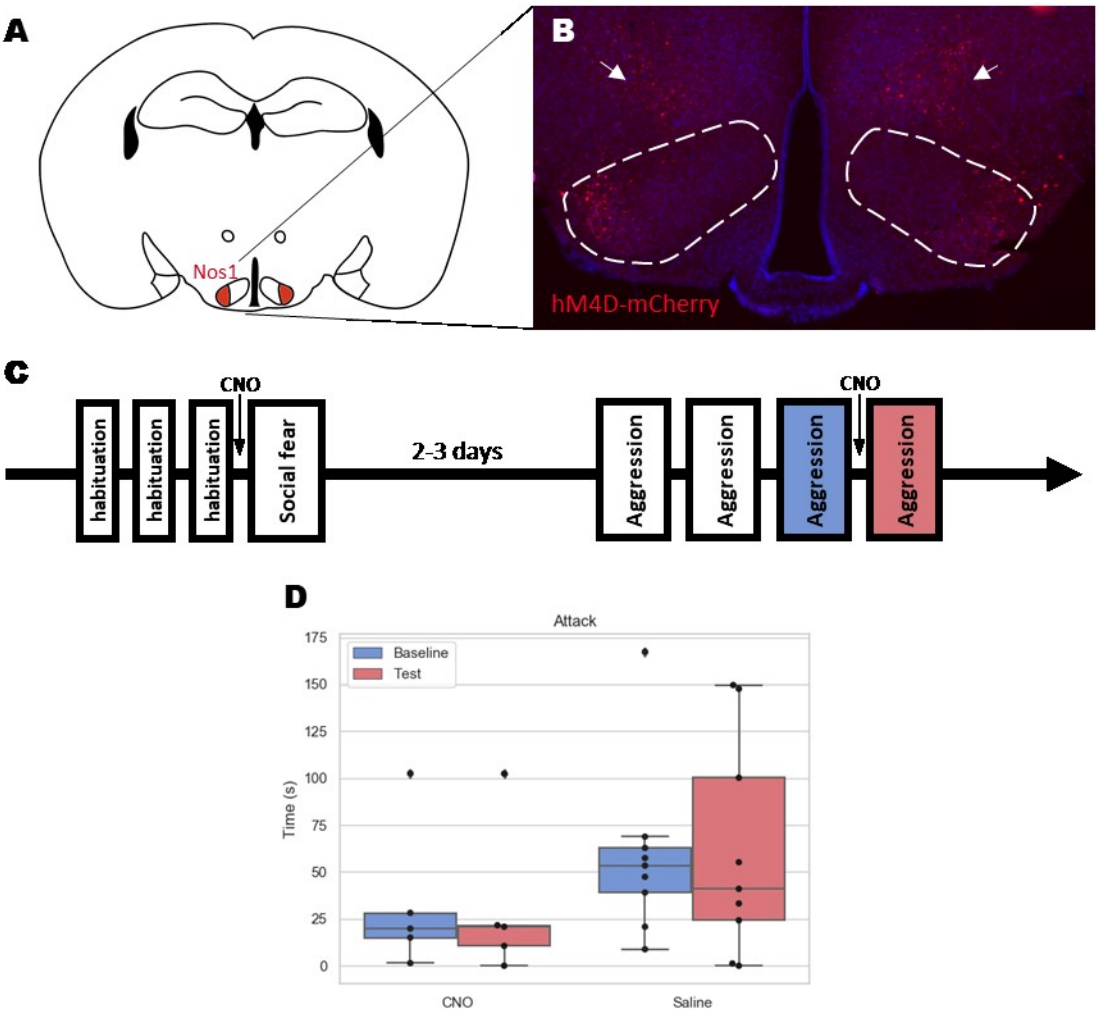

Fig. 21 Inhibition of Nos1+ neurons in VMHvl does not affect aggression. (a) Scheme of expression of hM4D in Nos1+ neurons in VMHvl. (**b**) An image of the brain section with marked VMHvl and hM4D expression in Nos1+ neurons (arrows depict expression in dorsomedial hypothalamic nucleus). (**c**) Schematics of behavioural paradigm (**d**) Aggression times during baseline (blue) and test day after CNO or saline injection (red) in 2 groups from left: Nos1::hM4D + CNO (n=5) and combined Nos1::hM4D/WT + saline (n=9).

immobility, lower levels of exploration and free locomotion in general (Fig. 22A-C). Stretching showed a trend towards decrease as well (Fig. 22D). These results suggest that Nos1+ and Esr1+ neurons in VMHvl are regulating behaviours differently. When inhibited Nos1+ neurons do not affect aggression and seem to have a strong opposite effect on defence in comparison to Esr1 expressing neurons. This data would suggest a counter-regulation between these two populations of neurons. Noteworthy, changes in defence responses induced by Nos1 inhibition are not easy to understand because decrease in stretching would suggest lower fear levels, whereas an increase in immobility and a decrease in exploration advocate for a contrary interpretation. This could be due to the

spread of the virus infection to the dorsomedial hypothalamus (DMH) which occurred in most of the mice (Fig. 21B).

To further investigate the function of Nos1+ population, we decided to optogenetically activate these neurons during exposure to conspecific (Fig. 23A-B). After the introduction of a subordinate conspecific into the cage and activation of the light (20Hz, 5-10mW) a primary response in a group of mice expressing ChR2 but not eYFP was highly stereotyped self-grooming with occasional scratching that could last even for 70% of one minute stimulation (Fig. 23C). Notably, self-grooming was also inducible without conspecific presence and usually persisted for few seconds after the end of the stimulation. Another visible effect of stimulation was reduction in social interaction (Fig. 23D). This could be explained by the fact that mice usually moved to the corner of the cage to self-groom, but also in many cases active avoidance was observed. Moreover, in some mice stimulation of Nos1+ neurons also stopped ongoing social behaviour like sniffing and

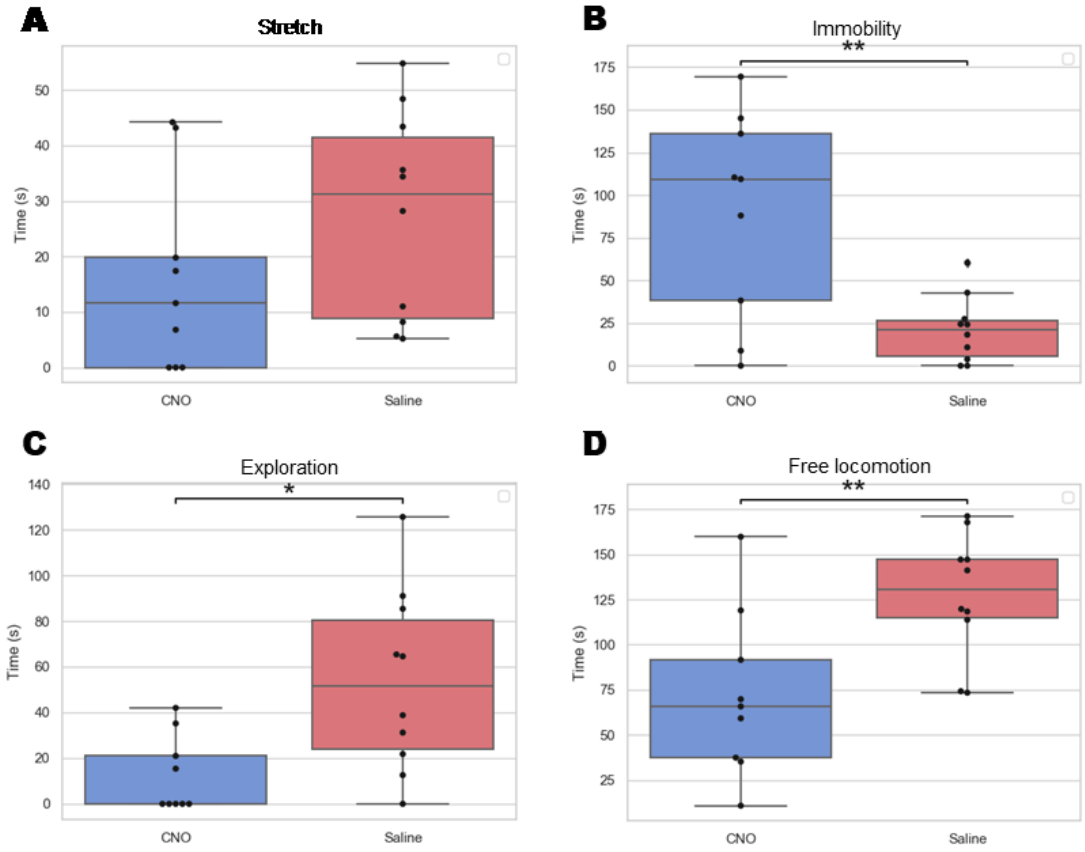

Fig. 22 Defence responses to an aggressive conspecific are altered by inhibition of Nos1 neurons. (**a-d**) A comparison of Nos1::hM4D + CNO (blue, n=9) experimental group and combined Nos1::hM4D/WT + saline (red, n=9) control group and duration of behaviour (**a**) stretch, (**b**) immobility (t test, p-value=0.0036), (**c**) exploration (t test, p-value=0.0105) and (**d**) free locomotion (t test, p-value=0.008) during social fear paradigm.

attack. These results argue in favour of defence and stress inducing role of Nos1 neurons in VMHvl, where, in addition to induced avoidance, self-grooming, especially prolonged one, is viewed as a stress related response (Kalueff et al., 2016). It also provides an interesting link between Nos1 and Esr1 because after stimulation of the latter one, we observed increased grooming, but not during the light period. This again suggests a counter-regulation or some sort of cross-talk between these two neuronal populations.

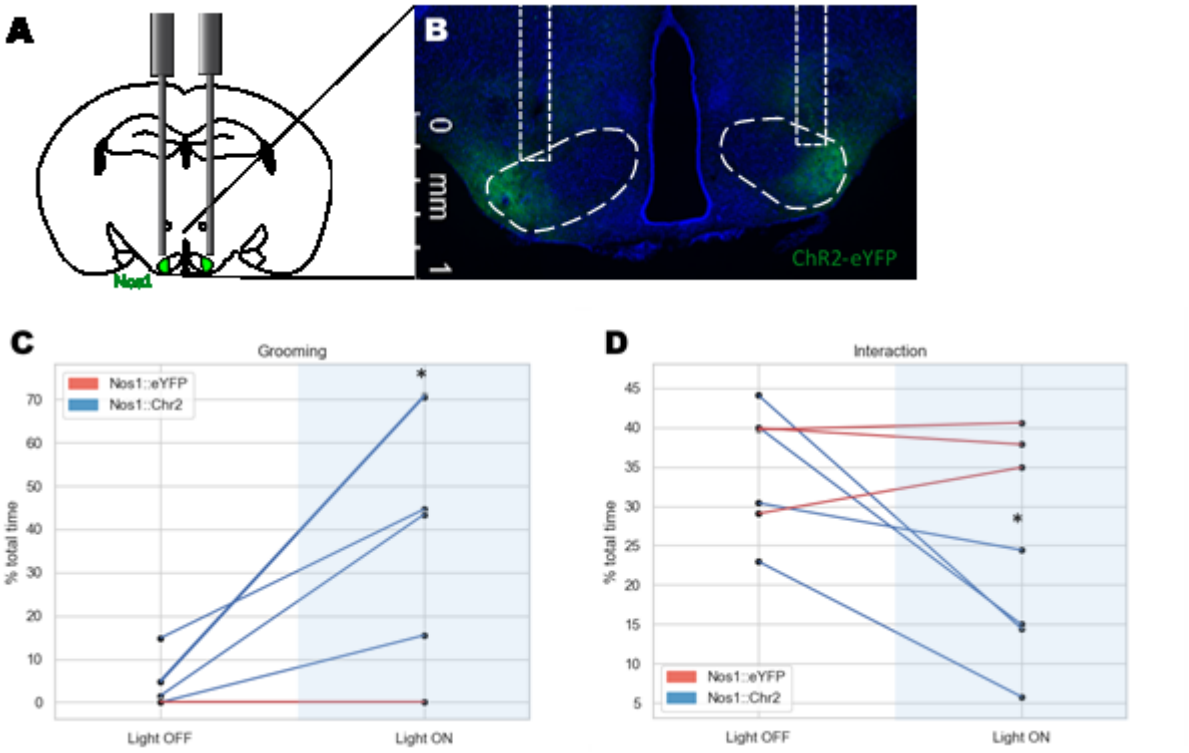

Fig. 23 Optogenetic activation of Nos1+ neurons in VMHvl promotes self-grooming and avoidance. (**a**) Scheme of optic fibres implantation. (**b**) An image of the brain section with marked optic fibre traces and expression of ChR2-eYFP in Nos1+ neurons. (**c-d**) Graphs presenting percent of time spent (**c**) self-grooming and (**d**) interacting with conspecific in experimental mice group (blue) expressing ChR2 and control group (red) expressing eYFP (grooming n=5 vs n=3, t test, p-value=0.0345 and social interaction n=4 vs n=3, t test, p-value=0.048).

4. Discussion

The ventrolateral subdivision of the ventromedial hypothalamus is thought to be a central part of aggression and reproduction circuits in the brain but so far only a few studies suggested its possible role in defence. The main questions about the identity of putative fear cells, how VMHvl controls and processes different behaviours, and a role of experience in mediating these behaviours remain unanswered. In this study we used different approaches to better understand inner workings of VMHvl. Our initial cFos studies revealed partially overlapping neuronal populations for social fear and aggression in VMHvl. Further with an aid of *in vivo* calcium imaging we found neuronal populations mediating particular defence responses, as well as sniffing and aggression towards conspecific. Moreover, we were able to characterize functional cell types by carefully examining their responses during different tasks. Additionally, analysis of the whole neuronal ensemble activity discovered experience dependent plasticity in the VMHvl induced by defeat. Finally, pharmacogenetic and optogenetic studies uncovered two separate populations of neurons namely Esr1 and Nos1 that regulate aggression and defence behaviours in a differential manner.

4.1. Processing of fear and aggression in VMHvl

A recent study examined an overlap between defeat and aggression cells in VMHvl using virus based method and found only a small overlap between them (Sakurai et al., 2016). Authors also activated defence cells captured by this method, which resulted in avoidance in mice only with a high number of infected cells (Sakurai et al., 2016). These results are interesting, but hard to interpret as the viral infection is known to cause unspecific cFos expression in the brain cells. Moreover, authors did not performed control experiments for example, activating neurons labelled by aggressive behaviour, leaving concerns that an optogenetic activation might have stimulated randomly labelled cells in VMHdm, that are known to induce defensive responses (Kunwar et al., 2015; Wang et al., 2015). In this study by using cFos based TRAP method we largely confirmed that mainly non-overlapping populations of neurons are activated during social fear and aggression in VMHvl. We and previous study found similar numbers of overlapping cells: approximately 50% between the same behaviours and around 20% between attack and defence. Such low

numbers can be explained in many ways. First, it is important to stress technical limitations of both methods used: a viral approach is highly dependent on placement and size of the infection, while TRAP method seem to label less cells than staining indicating some level of stochasticity most likely due to the high sensitivity to hardly controllable tamoxifen levels in the tissue. Secondly, cFos is not expressed in all active neurons but only in a fraction approximately 50% (Lin et al., 2011), when compared to electrophysiological recordings. Specific function of cFos is not known but as a transcription factor it is suspected to take part in some form of plasticity in the neurons receiving strong synaptic input (Kawashima, Okuno, & Bito, 2014). Taking this into account, upon certain stimulus cFos expression would reveal neuronal populations undergoing plasticity rather than the whole ensembles of active neurons, making an activity comparison far from accurate and most likely dependent on the previous experiences. Third, 50% overlap between cells labelled by the same behaviour could reflect subtle differences in the interaction with conspecific or phenomena like circuit degeneracy and sparse coding (Spanne & Jörntell, 2015). Despite all mentioned limitations cFos studies can be successfully used as indication of recruitment and activity in the brain regions specific to given stimulus (D. Caroline Blanchard et al., 2005; Herrera & Robertson, 1996; Kawashima et al., 2014; Minatohara et al., 2016). In our study we can use an overlap ratio between different conditions as a clue, that indicates at least partial separation of populations involved in aggression and fear. This conclusion is strengthen by the very similar result obtained using the viral approach (Sakurai et al., 2016).

Calcium imaging of neuronal activity *in vivo* was a second method employed in this study to approach the question on how VMHvl computes aggression versus social fear. Compared to cFos mapping, it allows a real time monitoring of the activity of all neurons in the field of view and direct correlation with ongoing behaviour. Moreover, it makes possible to follow the same neurons along many days allowing for a direct comparison of their activity during different tasks. First, we confirmed previous findings produced during electrophysiological recordings, which show that VMHvl neurons are highly active during social encounter (Falkner et al., 2014; Remedios et al., 2017). In addition to activated cells, we also found a population of neurons that was specifically inhibited during a direct exposure to conspecific, suggesting a possibility of two antagonistic sets of neurons being present in VMHvl. Secondly, our study found for the first time a direct evidence of neurons being activated during specific defensive behaviours. One of the most entrained and

strongest responses during the risk assessment occurred to stretching behaviour. Interestingly, this response was independent from the location of the mouse. Contrary to previous findings (Falkner et al., 2014) this implies that activity of neurons in VMHvl can be autonomous from the distance to the conspecific and presumably olfactory clues. Other behaviour that correlated with the neuronal firing during the risk assessment was flight (rapid return to a home cage). Neurons activated during flight highly overlapped with stretch neurons with the majority of units being activated for stretch and inhibited during flight. Remarkably, a recent publication uncovered a similar populations of neurons in VMHdm during exposure to a predator (Masferrer, Silva, Nomoto, Lima, & Gross, 2018). In contrast to this study, in their work authors were able to show a strong correlation between the firing rate of the assessment cells and a proximity to the rat (Masferrer et al., 2018). This difference could be attributed to the different behavioural apparatus that did not include a home cage, place where a substantial amount of stretching was observed in this study. Another defensive behaviour linked with the activity of VMHvl neurons found here was an active defending. This neuronal population also overlapped (~60%) with stretch population and these responses were mainly co-regulated, which means that particular neurons are activated or inhibited to both stimuli. Together this data, with the fact of a high overlap between neurons responding to all three defensive behaviours, argues for existence of one coherent population of social fear neurons in VMHvl.

A couple of other responsive populations exposed by our recordings were sniffing and attack neurons. This finding is in line with previous studies done with calcium imaging and electrophysiology, which found cells tuned to sniffing and aggression, and in case of electrical recordings that firing of these neurons could be used to predict the timing of the next attack (Falkner et al., 2014; Lin et al., 2011; Remedios et al., 2017). We found approximately five times less neurons tuned to these responses compared to defensive population. Interestingly, aggression activated neurons highly overlapped with stretching neurons, perhaps reflecting approaching to a threat in both behaviours. The overlap between aggression and defending activated neuronal populations was much smaller and was found mainly in mice that underwent aggression testing first. These findings point to some form of learning occurring after the defeat. Sniffing was overlapped at similar level with stretching and defending and considerably less with aggression, which might be due to the nature of the method used to determine this type of tuning. Overall, these findings confirm cFos mapping results, where more cells were shown to be recruited during the

defence than during aggression and to certain extent confirm partial overlap between those two populations.

Generally, the high tonic levels of calcium activity seen throughout the encounter with the conspecific made demixing of the signal and identifying specific behaviourally-locked neuronal responses difficult. Responsive neurons show some level of activity during most of the encounter and their tuning to particular behaviours was determined mostly by the strength of the response. However, this fact does not exclude a possibility that a certain level of activation is shared between different social behaviours. This observation is very interesting as could be viewed as processing of certain qualities or characteristics that are shared between these social behaviours. Moreover, it would be fair to speculate that the modulation of the neuronal signal would roughly correspond to the level of such characteristic. In my opinion, one of such characteristics could be a threat level, encounter of previously unknown conspecific is associated with a certain level of danger, and all responses correlated with neuronal activity in VMHvl are either assessment of this level or a direct response to it. As described before such defensive responses may vary based on the perceived threat and circumstances connected with it (Blanchard et al., 1995). VMHvl could then integrate information from different modalities to compute threat level and promote an appropriate action.

During a memory test we found a surprising activity of VMHvl neurons that was perfectly correlated with the location of the mouse in the apparatus. We discovered two populations of neurons: one highly correlated with a home cage and the second – with a stimulus chamber. These two populations were perfectly counter-regulated, which means that a cell active in a home cage was inhibited in a far chamber and *vice versa.* In fact auROC scores of these cells plotted against each other showed almost perfect anti-correlation. Encoding of spatial information by a hypothalamic structure has never been described before, and only a few previous studies connected VMH with memory. In one of them VMHdm activity was shown to be necessary for fear memory acquisition and expression 24 hours after the rat exposure (Silva, Mattucci, et al., 2016). Another study discovered cFos activation in VMH during auditory fear conditioning and exposure to a paired tone (Butler, Wilson, Gunnersen, & Murphy, 2015). These studies provide evidence of a link between VMH and memory, but at the same time are bound to an expression of particular fear behaviours. In our experiment we see a specific activity that is independent

from a performed behaviour since animals displayed very little to none defence behaviours during this memory test. Furthermore, this activity seems to be dependent solely on non-olfactory clues as a smell of an aggressive conspecific had been carefully removed by washing the cage on a previous day. These results could be explained by linking the location to a threat level learned during defeat. As a threat integrator VMHvl would receive contextual information from recently discovered afferents of the ventral hippocampus (Lo et al., 2018) and compute a certain threat level even in the absence of inputs from different modalities, which would be reflected in the activity of discovered neurons. Actually, this explanation fits with low noise and almost perfect correlation between activity and location since the interpretation of a single modality input would be easier and less noisy. Alternatively, revealed neuronal activity could reflect territoriality, and provide a mechanism for recognition of the "own" territory (home cage) and a territory belonging to other conspecific (stimulus chamber). This thought could be supported by the fact that an elevated aggression in isolated mice is believed to be due to an increased territoriality (Miczek et al., 2001).

4.2. Experience and plasticity in VMHvl

Analysis of the whole neuronal ensemble by dimensionality reduction methods PLS and LDA revealed a specific structure in the data suggesting a separation between aggression and social fear. Recently another group examined neuronal responses to males and females in VMHvl using *in vivo* calcium imaging. In this study authors discovered that initially overlapping cell populations, responding to both male and female stimulus separated from each other over a period of 5 days (Remedios et al., 2017). Later the same group found that this learning effect is dependent on mating experience with females (Remedios et al., 2017). Here we discovered that the difference between aggression and social fear occurred only in animals that underwent social defeat first. In animals that experienced 3 days of consecutive aggression at the beginning of the testing, global neuronal activity remained similar and largely indistinguishable. There are two possible explanations of this observation. One possibility is that aggression behaviour drives the separation and learning in the VMHvl, and the second and more probable one is that the defeat experience is driving plasticity in the global neuronal activity. There are a few pieces of evidence supporting the latter claim: first, our Esr1 activation studies showed that defeat

can shift function of these neurons towards avoidance and second, aggression days in the analysis always overlapped, indicating no further learning after the first aggression trial. On the contrary, two defeat days are separated in the way that defeat on the day 1 is closer to clustered aggression days. This would suggest that aggression-like activity is a default state and consecutive defeats shift network activity more and more causing defeat-like phenotype. Aggression-like activity at the beginning of the recording might be caused by the isolation that these mice experience for the period of at least 3 weeks before the beginning of the paradigm. Moreover, aggression that serves the purpose of defending own territory is an evolutionary preferred strategy resulting in greater reproduction success in mice (Miczek et al., 2001).

Another phenomenon revealed by this data is an apparent resilience to changes in VMHvl activity pattern exhibited by the mice that went through the aggression paradigm first. One of the possibilities is that consecutive victories produce a dominant phenotype. A recent study showed that dominant males employ more successful strategies during fight resulting in less severe defeat (unpublished data, SFN 2018). It is possible that this contributes to a lower threat associated with an aggressive conspecific that could in turn be reflected in VMHvl activity and a single defeat is not enough to change this pattern. On the other hand, an altered neuronal ensemble activity during defence and aggression might be simply driven by the expression of different behaviours during these periods. In this case one could suggest that more active strategies used by dominant males cause similar, aggression-like activity VMHvl.

In general, plasticity dependent on the previous experiences, may represent a correction of a prediction error associated with the computation of the threat level by VMHvl. This could explain changes observed in Remedios *et al.* study by a way of a gradual change in the interpretation of female clues that lowered predicted threat level after subsequent mating events. On the other hand, experiencing defeat would adjust prediction in an opposite direction. To determine whether the whole ensemble activity differences are caused by adjustments in prediction about potential danger represented by a particular conspecific further studies involving all three behaviours are needed.

4.3 VMHvl control of defence and aggression

In this study, we discovered that the activation of VMHvl Esr1 expressing neurons after defeat causes a strong expression of fear behaviour. This is the first molecularly identified neuronal population mediating defence responses in VMHvl. A combination of our findings with data from other studies, demonstrating that the manipulation of Esr1 can induce mounting and aggression (Lee et al., 2014; Lin et al., 2011; Yang et al., 2013) suggests that Esr1 neurons can mediate all behavioural responses attributed to VMHvl. One explanation is connected with aforementioned plasticity in the VMHvl circuitry. In this model previous experiences would shape a global activity pattern and a local circuit in VMHvl causing a shift in the preferred response to the same stimulus. Therefore, Esr1 neurons would cause defence or aggression depending on the state of the VMHvl network. An alternative, a simpler model is the scalable control of behaviour hypothesis. Previous studies revealed that behaviour induced by the activation of Esr1 neurons depends on the power of light delivered during optogenetic stimulation, which in turn correlates with the amount of activated cells (Lee et al., 2014). Low level stimulation promotes mounting, whereas a stronger one causes attack behaviour (Lee et al., 2014). Furthermore, cFos mapping done in our study indicated that the number of activated cells during social fear are higher than that during aggression. Taken together, this suggests a hierarchical organization for VMHvl-mediated behaviour, whereby an increase in the level of VMHvl activation causes animals to shift from mounting, to aggression, and finally to defence. In the experiment where Esr1 stimulation induced social fear, it is possible that the defeat and subsequent exposure to conspecific brought up the VMHvl activity level. Then, the Esr1 stimulation would simply recruit more VMHvl cells, leading to the strong defensive behaviour. This mode of action would go hand in hand with the representation of threat intensity in VMHvl. The increase in VMHvl neurons activity would reflect a rising threat level promoting the most appropriate behaviour to a current threat. The inhibition of Esr1 neurons suppressed aggression behaviour and failed to effectively suppress defensive behaviours, although strong trends in the data were observed, like higher exploration rates and less immobility suggesting that perhaps a higher number of experimental animals would prove significance of this effect. Conversely the lack of a strong modulatory effect on social fear behaviour might be due to the fact that other cells in VMHvl that also mediate this function, and therefore, the inhibition of Esr1 is not sufficient to completely abolish

these behaviours. This view would be consistent with the higher VMHvl activation during social fear.

Strikingly, the activation of Nos1 neurons in VMHvl promoted non-social behaviours such as self-grooming and avoidance. Self-grooming is a naturally occurring behaviour that can express different positive and negative states depending on the context and was previously associated with a stimulation of the paraventricular nucleus of hypothalamus and adjacent areas (Kruk et al., 1998). Generally, prolonged self-grooming is associated with stress (Kalueff et al., 2016). In this study, the motivation for self-grooming exhibited by the animals is not entirely clear, and could be associated with anxiolytic as well as anxiogenic internal state. As mentioned before, the avoidance observed in this study is also ambiguous, as it is hard to separate it from the performance of self-grooming act. Interestingly, self-grooming was also observed right after Esr1 stimulation but not during it, and Nos1 stimulation in some cases was able to supress aggression behaviour. This could be interpreted as a counter-regulation or antagonistic control of behaviour between these two completely separated neuronal populations in VMHvl. This negative feedback mechanism could help to explain the observed bout organization of aggressive behaviour separated by relatively neutral periods (Miczek et al., 2001). Interestingly, a very similar organisation was found in the medial amygdala where stimulation of GABAergic neurons promotes aggression and an activation of a separate glutamatergic population evokes self-grooming and inhibits social interactions (Hong et al., 2014). On the other hand, inhibition of Nos1 neurons during aggression did not show any effect and supressed exploration and increased immobility during social fear. In comparison to activation studies, these results at first seem confusing, but could be explained if observed self-grooming had anxiolytic state properties, then activation and inhibition of Nos1 neurons would decrease and increase anxiety levels respectively. Alternatively, we cannot exclude that the observed co-infection of DMH and in some cases PMV could be the cause of the seemingly opposite effects of the inhibition and activation of Nos1 neurons.

4.4 VMHvl as a central emotional state generator

In order to sum up different concepts and findings in this work, it is worth to turn to a broad perspective of emotion regulation. To start with, I would like to stress that in my view emotions or emotional states are the results of an activity and computations of many brain structures. Therefore, attributing a particular emotion to a single structure would be illogical. Instead, we should turn to concepts that analyse properties of emotional states, infer precise components, processes and computations that are needed to achieve the final goal of producing certain emotion. Equipped with such tools and knowledge we can then begin to search for structures that contribute defined components, qualities and computations to produce a specific emotion or a range of emotions. Recently, a useful framework that attempted to identify certain properties of emotional states that could be later applied in neurobiological studies was proposed (Adolphs, 2017; Anderson & Adolphs, 2014). As mentioned earlier, in this work authors, distinguished four main properties, namely: scalability, persistence, generalization and valence. Below I will try to relate the findings about VMHvl activity and functions to these properties.

Different emotions are usually mapped to a two-dimensional space describing their valence and intensity (Barrett & Russell, 1999), but mapping or categorization can also be done using additional dimensions like persistence (Anderson & Adolphs, 2014). This would imply that these properties are universal amongst emotions and structures involved in their production should exhibit all or at least some of them (Anderson & Adolphs, 2014). Scalability is a property connected to the intensity of an emotion. Studies in VMHvl, examining the relationship between sexual and aggressive behaviours, determined that they are controlled in a scalable way (Lee et al., 2014). In this study, the evidence provided by cFos mapping, calcium imaging and optogenetic activation of Esr1 neurons supports the point of view that social fear is also regulated using this mechanism. Therefore, indicating that VMHvl is encoding the intensity of the stimulus which can be necessary for producing a correct emotional response to the conspecific. In terms of valence, there is not much evidence that VMHvl neurons encode such property. Nevertheless, studies showed that aggression behaviour can be appetitive and that mice can learn to perform an operant task (nose poke) to get access to subordinate conspecific. Moreover, neuronal activity in VMHvl is correlated with the aforementioned process and stimulation of VMHvl neurons shortens

intervals between nose pokes (Falkner et al., 2016). In addition, initial studies in my lab suggest that activation of Nos1 neurons might be used for a real-time place preference test. On the other hand, valence in its simplest form can be seen as an approach and avoidance, and as demonstrated above VMHvl can produce both of the behaviours and an activity of single neurons can be correlated with those behaviours (Falkner et al., 2014). Persistence is a property that highlights the fact that emotional behaviours often outlast the stimuli that triggered them (Anderson & Adolphs, 2014). Evidence that VMHvl activity has this property comes from the activation studies of both Esr1 and Nos1 neurons. After an Esr1 stimulation attack behaviour persisted for around 10 seconds after the light period (Lee et al., 2014) and here we show that stimulation evoked self-grooming also persisted after the optostimulation. One additional component of persistence could be an endocrine signalling for example, an increase in stress hormones. Indeed, studies by M. Kruk showed that electrical activation of hypothalamic attack area influences levels of corticosterone and prolactin in blood (Kruk et al., 1998). The last property mentioned by Anderson and Adolphs is generalization. According to them, generalization can be divided into context generalization, stimulus degeneracy and pleiotropy. To my knowledge, the first property has not yet been demonstrated yet as no one has tested if an activation of neurons in VMHvl can serve as an US in conditioning. However, studies done in VMHdm have confirmed this property (Kunwar et al., 2015) Stimulus degeneracy was shown in our *in vivo* calcium recording experiments, which revealed that activity in VMHvl was induced in the absence of a conspecific during the memory test. Pleiotropy is an ability to cause multiple parallel effects (Anderson & Adolphs, 2014). As mentioned earlier, besides evoking very different behavioural responses, VMHvl also influences the levels of hormones in the blood (Kruk et al., 1998; Lee et al., 2014). Another feature of VMHvl that supports its role in generation of emotional states is architecture of its connections. VMHvl receives multiple inputs from different brain regions implicated in emotional processing like, PFC, ventral hippocampus, amygdala, lateral septum and other hypothalamic nuclei, and in turn projects to multiple structures involving many recurrent connections and high collateralization between them, providing evidence for a “fan-in” organization of its inputs and a “fan-out” organization of its outputs needed for generalization property.

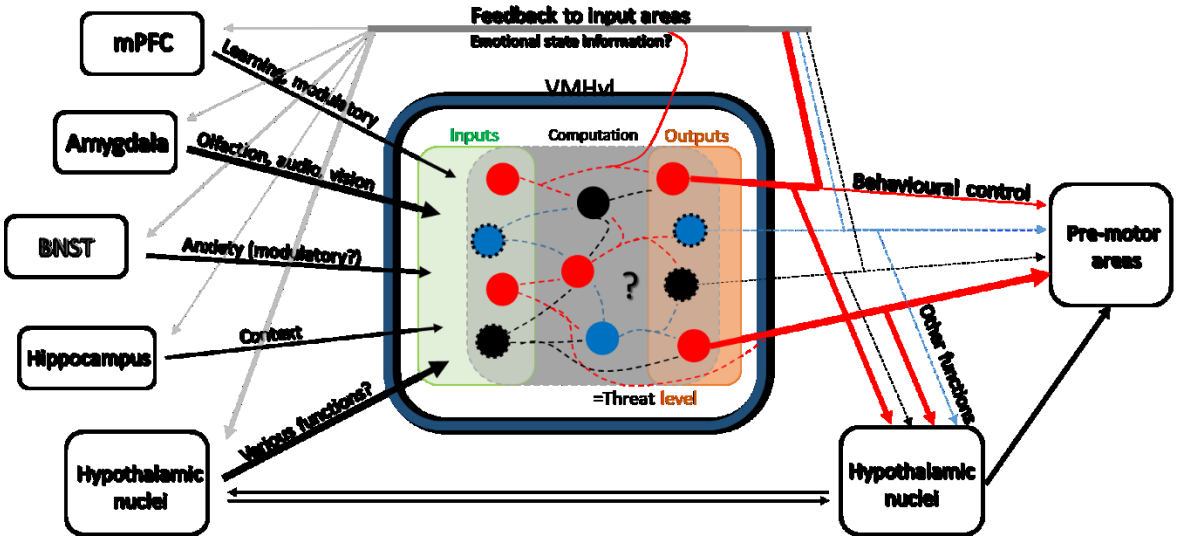

Fig. 24 Schematics representing putative circuitry and functions of VMHvl. (blue – Nos1, red –Esr1)

To conclude, VMHvl can be viewed as a structure that controls behavioural responses or emotional states towards conspecifics. One of the proposed mechanisms underlying its behavioural function, involves computing a prediction of the threat level associated with the encounter and relaying this information downstream to effector centres as well as providing feedback to upstream structures to correct errors in the prediction. Further studies are needed to determine particular functions of distinct projections to and from VMHvl, and the exact nature of information encoded in this structure.

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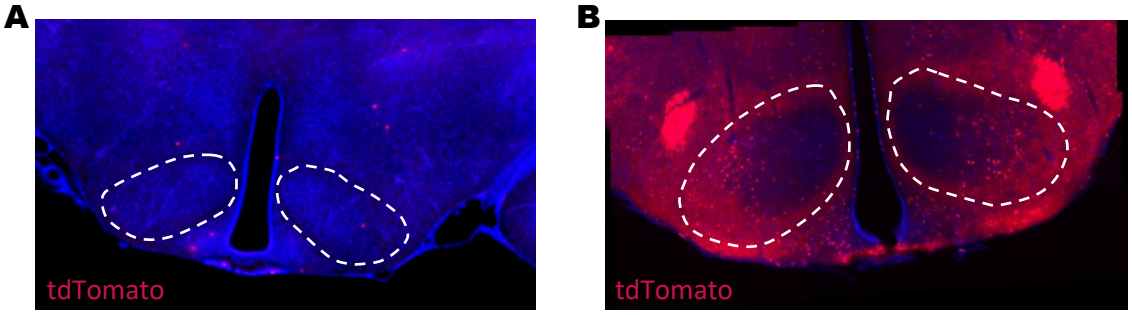
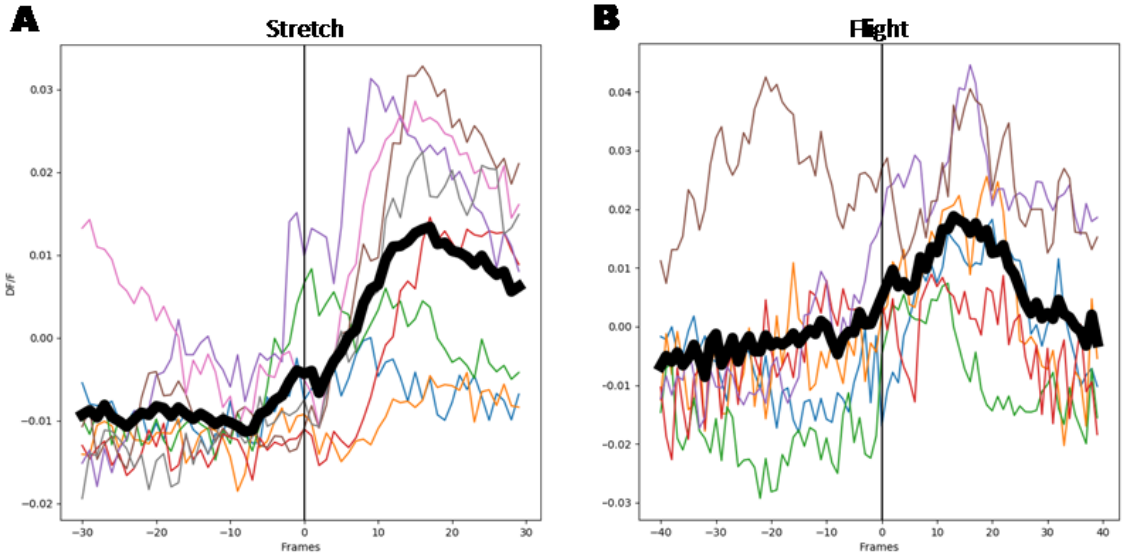
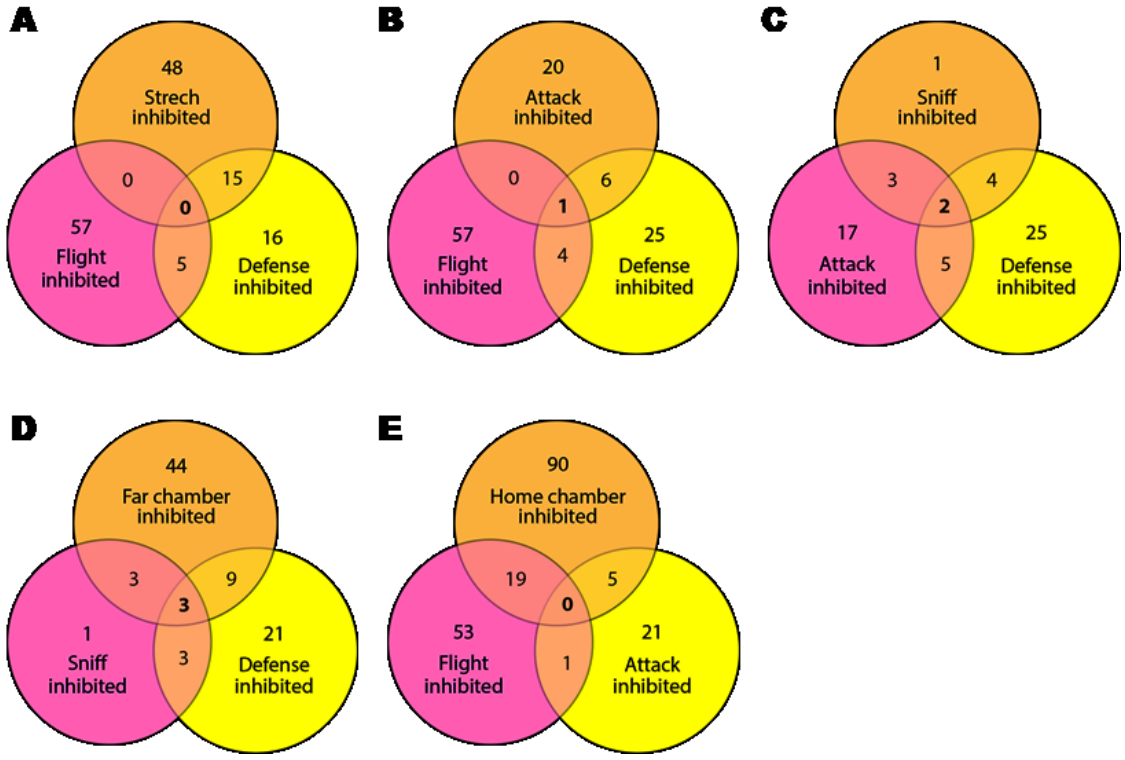
Supplementary Figures



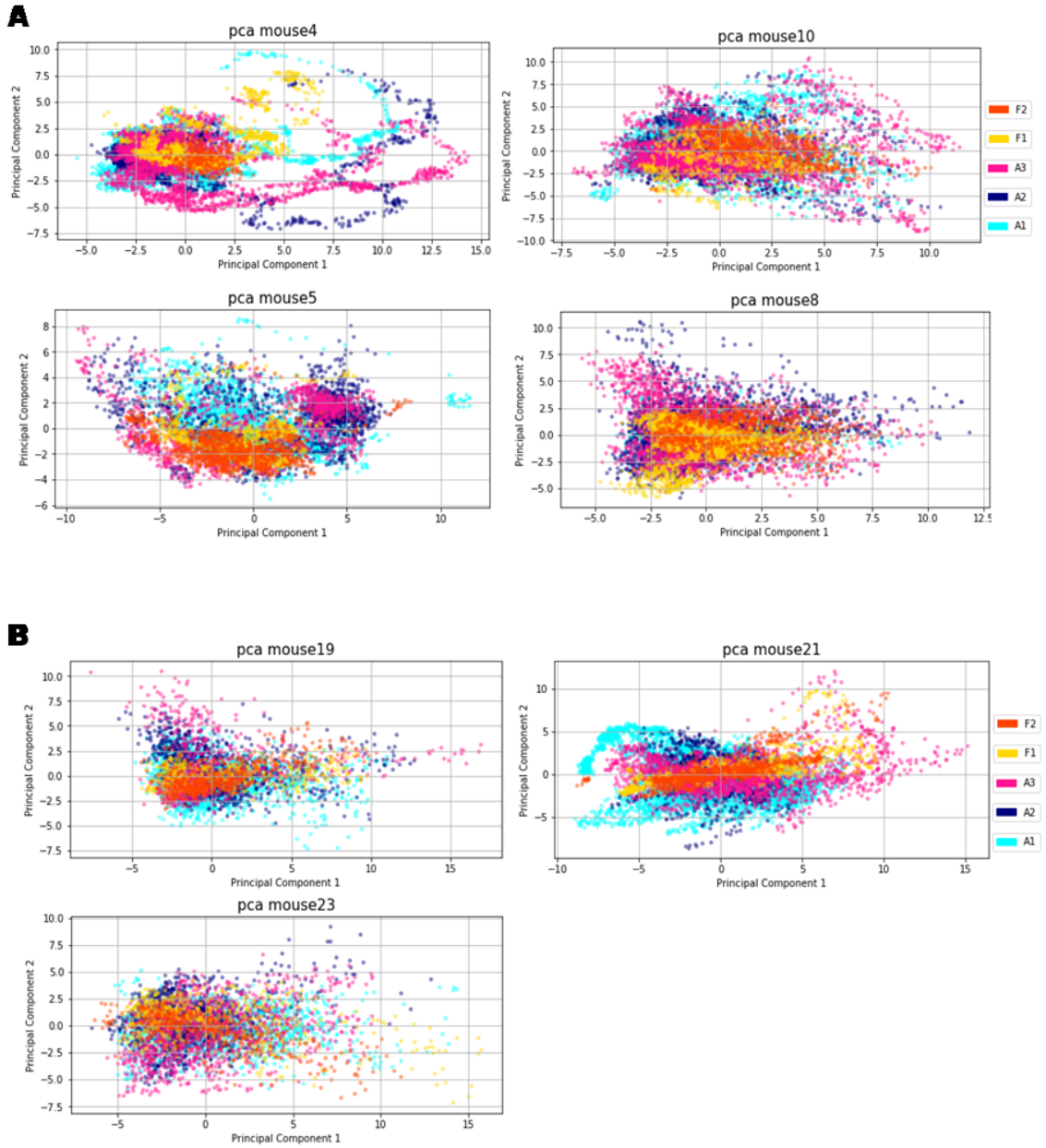
Supplementary Fig. 1 Validation of Arc based TRAP system. (**A**) Activation of VMH area (white outline) in control condition - no stimulus given and 4-OHT injection before the test. (**b**) Activation of VMH neurons after conspecific exposure and 4-OHT right after the test.



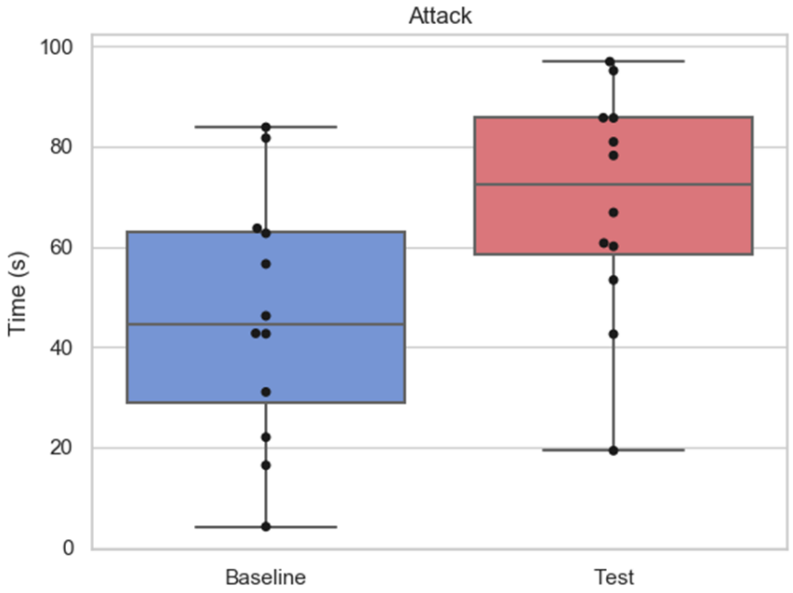
Supplementary Fig. 2 Validation of auROC scores with rank sum test. (**A-B**) Mean activity of an example neurons aligned to (**A)** stretch onset (rank sum – p_value=0.0019, auROC=0,78) (**B**) flight onset (rank sum – p_value=0.0011, auROC=0,76) (Black – Mean, Colours – different trials).



Supplementary Fig. 3 Relations between inhibited neurons in VMHvl. *(A-E)* Venn diagram showing overlap between: (**A**) defending, stretch and flight (**B**) attack, defending and flight (**C**) sniff, defending, attack responsive neurons (**D**) far chamber, defending and sniff populations (**E**) home chamber, flight and attack.



Supplementary Fig. 4 PCA analysis do not reveal changes between aggression and social fear. (**A**) PCA analysis result for Social fear – Aggression order mice plotted in principal component space (**B**) PCA analysis result for reverse order mice plotted in principal component space.



Supplementary Fig. 5 Aggression in control mice rises over days. (**A**) A graph comparing aggression during baseline day (blue) and test day (red) t test, p_value=0.03.