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Independent hypothalamic circuits for social and predator fear

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ABSTRACT

Independent hypothalamic circuits for social and predator fear

Fear is a distressing negative sensation induced by a perceived threat. This emotion is necessary for the survival of the individual, since it guarantees appropriate responses to life challenging threats. In the last decades research on the neural mechanisms underling such emotion both in humans and in animal models have been mostly focused on the amygdala. In particular fear models in rodents typically rely on foot shock based paradigms. However, innate and learned fear elicited by other stimuli such as predators or aggressive members of the same species has been shown to be regulated by other circuits where the triggering, coordination and the expression of fear seem to be centered in the hypothalamus and periaqueductal grey. Nevertheless very little is known about the function and physiology of these structures in fear processing.

To study the function of the medial hypothalamic fear circuit, we developed a novel behavioral paradigm to measure innate and conditioned fear responses to social and predator threats in mice. We subsequently created tools to selectively inhibit specific hypothalamic nuclei during the fear and we observed the inhibition of the ventromedial hypothalamus, a nucleus previously studied for its function in feeding, sex and aggression, specifically impaired social and predator fear but not foot shock fear. Moreover we demonstrated that different portions of this nucleus account for fear to different threats with the dorsomedial portion, previously implicated in feeding function, processing predator fear, and the ventrolateral portion, previously implicated in sex and aggression, processing social fear.

Our results demonstrate that the hypothalamus plays a crucial role in fear processing even if it is not recruited during foot shock exposure, suggesting that it might be a good target for the treatment of fear related disorders like panic or phobias and we are now trying to identify possible drugs specifically acting in this area. On the other hand, we showed that specific hypothalamic subnuclei are recruited selectively during social or predator fear, corroborating the hypothesis that different types of fear are processed by separate brain circuits. Such evidence opens the possibility of targeted therapy of pathological fear in humans. Interestingly these same hypothalamic structures are fundamental regulators of non-fear motivated behaviors that are essential for survival such as feeding behavior, aggression and sex and we are now investigating how the same nuclei can orchestrate multiple functions.

INTRODUCTION

1 Fear

Fear is an unpleasant emotional state caused by the awareness or anticipation of a danger. Fear is a very powerful emotion that affects our behavior, choices and attitudes in our daily life. This emotion plays the fundamental function of inducing an organized pattern of responses that serves to anticipate, avoid and cope with dangerous situations. However, in some cases, fear can degenerate into pathological states like general anxiety, panic disorder, or phobias, where this emotion is experienced in the absence of a real threat. Although pathological fear states are extremely common in the population, their neural correlates remain unclear.

Defense against harm is a fundamental requirement of life and fear responses can be observed in virtually all animal species suggesting that this is an evolutionary conserved neural response. In complex organisms (invertebrates and vertebrates), fear processing consist of three main functional elements: the detection of the threat via transmission of threat-derived stimuli through sensory systems, the generation of a mental state through the integration of the various sensory information, and the generation of an organized fear response. All these functions are orchestrated by specialized defense brain circuits, which are likely to be conserved across animal species.

Fear can be divided into two main classes, namely innate (or unconditioned) and learned (or conditioned) fear. Innate fear responses do not require pain or any previous encounter with the threat in order to strongly and systematically induce defensive behaviors and are relatively resistant to habituation. Stimuli that induce innate fear are species-specific and in most mammalian species they include predators, aggressive members of the same species and dangerous features of the environment such as heights or fire. Moreover innate fear can be triggered by internal stimuli like oxygen deprivation or myocardial infarction (Ziemann, Allen et al. 2009; Feinstein, Adolphs et al. 2011). Learned fear responses are defensive responses to an innocuous stimulus previously associated to a threatening stimulus. Importantly, what is learned in this type of fear are not the defensive responses per se, but the association that makes a normally innocuous stimulus a warning of danger that elicits them in anticipation of a actual threat.

As discussed above a wide variety of stimuli can induce fear, spanning from physically harmful stimuli to predators or social threats. However, the research

attempting to unravel the neural basis of fear has mainly focused on fear induced by painful stimuli and how fear of other stimuli is processed in the brain remains unclear. In particular, it is not known if a single brain circuit processes fear induced by any threat or if distinct dedicated circuits process fear of different threats. The aim of this study is to address this question with a particular focus on the neural circuits underlying predator and social fear that are largely unknown despite their immense relevance in both healthy and pathological fear states in humans.

1.1   Modeling fear in rodents

Mice and rats have been widely used as model organisms for the study of the neural basis of fear. Behavioral paradigms to assess fear responses both in mice and in rats are mostly based an electrical foot shock, used as an unconditioned stimulus, paired with a tone or a specific context as a conditioned stimulus. The use of these tests is mainly focused on the understanding of the mechanism at the basis of fear learning. On the other hand, innate fear has been studied less intensively. The investigation of innate fear circuits is typically based on exposure to predators or predator odors or more rarely to aggressive members of the same species.

1.1.1   Defense in rodents

In rodents fear is elicited in response to various actual or potential threats to the animal's life or body. Threats can be divided into three categories: predators, dominant conspecifics and threatening features of the environment (light, fire, high places and water). Rodents display immediate defensive behaviors in the presence of actual threats that vary depending on the nature of the threat but also on the environment where the threat is presented. On the other hand, rodents also show anticipatory defensive behaviors when facing potential threats. Both in mice and in rats defensive responses include (Dielenberg and McGregor 2001):

Flight: rapid movement away from the threat source

Hiding or sheltering: entering and remaining in a place where the animal is less visible.

Freezing: immobility, associated with high alertness and muscle tone.

Defensive threat: defensive upright posture facing the threat.

Defensive attacks: biting the oncoming threat.

Risk assessment: a pattern of investigation of the threat source, including scanning it from a distance and stretch posture approach or attend in which the animal adopts a

stretched low-back posture while oriented towards the threat source and shows approaches interspersed with periods of immobility. Closer approaches and even contacts may occur.

Defensive burying: discrete objects may be covered with bedding or other materials.

Hypoalgesia: also called "fear dependent analgesia" prevents injuries from interfering with the defensive system.

Autonomic arousal: diffuse activation of the sympathetic system. The consequent increase of the energetic metabolism is required for coping with dangerous situations (Cannon 1929).

HPA axis activation: the release of corticotropic releasing hormone by the hypothalamus induces the secretion of corticotropin (ACTH), which, in turn, promotes the synthesis of corticosteroids by the adrenal gland (Korte 2001).

The factors that influence the choice of a specific pattern of defensive behaviors include the type of threat, intensity of the threat and the environment where the threat is encountered (Blanchard 1997). Indeed some threats are salient, immediate and clearly recognizable by specific cues, whereas some others are ambiguous. A localized threat can be efficiently avoided by flights and an embodied threat makes defensive attacks effective. On the other hand ambiguous threats mainly induce risk assessment behaviors characterized by cautious scanning of the environment adopting stretched-out posture. Such posture minimizes the chances of the animal to be detected and allows for the clarification of the nature of the threat (Pinel and Mana 1989). The situation where the threat is encountered is another very important feature that determines the nature of defensive behaviors. Escapable threats facilitate flights, the presence of shelters promotes hiding and tunnel guarding, manipulable substrates facilitate defensive burying, whereas a confined space with no escape possibility facilitates freezing (Blanchard, Shepherd et al. 1991; Dielenberg and McGregor 1999). This is very important to consider when studying fear in laboratory paradigms. Indeed often the only defensive behavior that is observed in laboratory animals is freezing because they are tested in small boxes with no shelters, exit, or conspecifics. In our study we aimed to compare fear induced by pain, aggressive conspecifics and predators in mice. In order to be able to analyze defensive responses to different threats we constructed a behavioral setup where the three stimuli were presented in the same manner (Silva, Mattucci et al.). Moreover, we put the mice in the condition to be able to express a broader panel of defensive behaviors besides immobility. We

introduced a space between the threat box and the home cage through a corridor where mice showed numerous risk assessment and flights events that would not have been possible if the stimulus was presented in a classical fear-conditioning box.

1.1.2 Foot shock based behavioral paradigms

Electrical foot shock has been by far the most used stimulus to induce fear in the laboratory. Nevertheless it is rarely used to investigate innate fear responses in rodents. Typically, the experimental setup consists of a small box with a metal grid covering the floor, through which the electrical shock is delivered. The length of the electrical shocks can vary in time and the intensity normally spans from 0.1 to 1 mA for 0.5 to 5 seconds and can be presented at different frequencies. Typically fear behavior is assessed comparing the freezing time before and after the stimulus, and in such an environment mice show mainly freezing to the shock due to the inescapability of the danger. It is important to keep in mind that the post shock response may not be an entirely innate fear response, but instead may be a conditioned response to the context where the shock was received (de Oca and Fanselow 2004). Innate responses to the shock itself are very limited and normally restricted to jumps in the fraction of time when the shock is perceived. These immediate behaviors are normally considered as a pain avoidance response rather than an ethological defensive response (Fanselow 1994). For these reasons foot shock-based test are not used as a model for the study of innate fear behaviors. Electrical foot shock, instead, is used as an unconditioned stimulus in fear conditioning paradigms. In these behavioral paradigms the animals are trained to associate the foot shock (US) with an otherwise innocuous stimulus (CS) like a tone, a light, an odor or a tactile stimulus. Rodents also have fear responses conditioned to the setting in which the discrete CS and shock US was presented. Such stimuli, are made up of many separate features, are referred to as contextual stimuli.

1.1.3 Predator based behavioral paradigms

In contrast to foot shock based behavioral fear tests, which are almost exclusively used to study fear conditioning, predators based ones are the most used to investigate unconditioned fear. Predators are frequent threats for rodents and they have evolved a very robust and complex innate defensive system against them. Behavioral paradigms based on predator fear provide therefore very ethologically meaningful information. Studies of fear induced by the presence of a predator were mainly performed in rats

exposed to cats or to cat odor (Canteras, Ribeiro-Barbosa et al. 2001; Dielenberg and McGregor 2001). Defensive behaviors displayed by rats exposed to actual predators differ significantly from those displayed when they are exposed to cat odor. Predator odor tends to elicit risk assessment behavior without defense that rely on the presence of a corporal threat while live predator virtually elicit all defensive behaviors including flight if an escape route is available and subsequently freezing (Endler 1986). Predators and predator odor are also used as unconditioned stimuli in contextual fear conditioning paradigms. Nevertheless, not all predator- derived odors can induce conditioning with the same efficacy. For example, conditioning can be obtained exposing rats to cat fur- derived odor but not to cat feces-derived odor even if both of them elicit very similar acute defensive responses upon direct exposure (Fendt and Endres 2008). This difference may be the result of the fact that fur-derived odors dissipate faster and therefore have a greater value in the prediction of the presence of a predator. On the other hand, feces, and its odor dissipate very slowly. This is probably the reason why predator fear tests based on TMT, a molecule derived from fox feces, may have limited value to understand predator fear.

Predator fear tests in mice are based on exposure to rats or their odor, as they are natural predators for mice. Mice exposed to rats or rat odor display innate defensive behaviors without any need to be injured or previously exposed to them. Two main paradigms have been reported, the rat exposure test and the mouse defense test battery (Yang, Augustsson et al. 2004). The mouse exposure test apparatus consists of a large chamber divided in the middle by a wire mesh. One side of the chamber is connected by a corridor to a small shelter where the experimental mouse can hide, while a rat is placed on the other side of the chamber. The presence of a safe area allows the experimental mouse to control its own proximity to the threat and therefore to display risk assessment behaviors (Yang, Augustsson et al. 2004).

In the mouse defense test battery the experimental mouse is placed in a large oval runway while the experimenter holds an anaesthetized rat and varies the distance to the predator. In such a setting animals show a very diverse set of defensive behaviors including flight, avoidance, risk assessment, vocalization, defensive attack and escape attempts with each behavior preferentially elicited by a specific feature of the threat stimulus and situation (Blanchard and Blanchard 1989; Blanchard, Hebert et al. 1998).

A disadvantage of using natural predators to induce innate fear is that they consist by definition in a very variable, complex and difficult to standardize stimulus. To address this issue investigators have showed that exposure to ultrasonic tones at the frequency of 20 Hz induces a wide array of defensive behaviors like flights, jumping and freezing, indicating that it can be used as an innately aversive stimulus. Moreover, animals exposed to these innately aversive ultrasounds showed a brain activation map similar to subjects exposed to real predators. This evidence provides a further indication that in mice ultrasounds can be used as a more discrete and standardizable stimulus as an alternative to live predators (Mongeau, Miller et al. 2003).

1.1.4 Aggressive conspecifics based behavioral paradigms

Aggressive conspecifics have been rarely used as a stimulus in fear behavioral paradigms; they are more often used in studies investigating social defeat as a model a chronic social stress. Nonetheless, encounters with an aggressive conspecific induce a clear pattern of defensive behaviors and can be used as a conditioning stimulus for contextual fear conditioning (Motta, Goto et al. 2009; Faturi, Rangel et al. 2013).

The classical test to study social defeat is the resident-intruder test. This test is very well established test where acute defensive responses to an aggressive conspecific can be investigated. In such paradigm an intruder male mouse is introduced into the cage of the resident mouse that displays aggression towards the intruder. The attacked mouse displays the typical array of active and passive defensive behaviors including freezing, upright postures, defensive attacks and escape (Koolhaas, Coppens et al. 2013).

An aggressive member of the same species is more complex as a stimulus than a predator or a painful stimulus like an electrical foot shock as it does not elicit innate fear codified by very clearly identifiable cues. For example odors, sounds and images associated with a predator are all able to induce fear and avoidance independently when presented to a naïve mouse. This is not the case for conspecifics as mice are social animals and they have an instinctual drive to investigate a social stimulus. The presence of another male mouse induces a certain level of arousal and initiates a mechanism that will eventually lead to the establishment of dominance. During this period, the submissive subject displays fear responses that are likely to derive from the association of detection of a member of the same species (visual auditory and pheromonal) and the outcome of the encounter (nociceptive). Therefore the brain

circuits of social fear are likely to involve structures associated with both fear and with social functions in the mouse.

1.2 Circuits supporting innate fear

Threats must be detected through sensory systems and information must reach a specialized defense circuit that orchestrates the defensive response. Unconditioned threat stimuli are species specific as they normally involve other animals such as predators or aggressive members of the same species. In rodents, the most obvious set of innately wired stimuli include predator cues, such as predator odor (Blanchard, Blanchard 1990), moving shadows in the upper visual field (Morris 1979), and high frequency predator warning calls emitted by conspecifics (Litvin, Blanchard et al. 2007). All these stimuli are able to activate innate defensive responses independently and without the need to be associated with an actual predator, suggesting that they activate an innately wired defensive circuit. In rodents, cues associated with environmental danger like a bright open space, or heights also trigger innate fear responses even if the behavioral outcome differs from the one actuated towards predators (Thompson, LeDoux 1974). Bolles identified a specific set of innately determined species specific defensive reaction like flight or freezing. He theorized that when an animal faces a threat its behavioral repertoire becomes restricted to this limited set of defensive behaviors (Bolles 1970). Later Fanselow proposed the existence of a unique fear circuit underling such behaviors in response to all types of threat (Fanselow 1994). However, growing evidence from many studies over the last two decades suggests the existence of distinct parallel circuits processing fear to different types of threat. In particular three circuits responsible for responses to predators, pain and aggressive conspecifics have been proposed (Gross and Canteras 2012), (Silva, Mattucci et al.). All these circuits have a common structure composed of three main functional parts: a sensory center where primary inputs from different sensory modalities are gathered together based in the amygdala, an integration center, based in the hypothalamus and an output center, based in the midbrain periaqueductal grey (Figure 1).
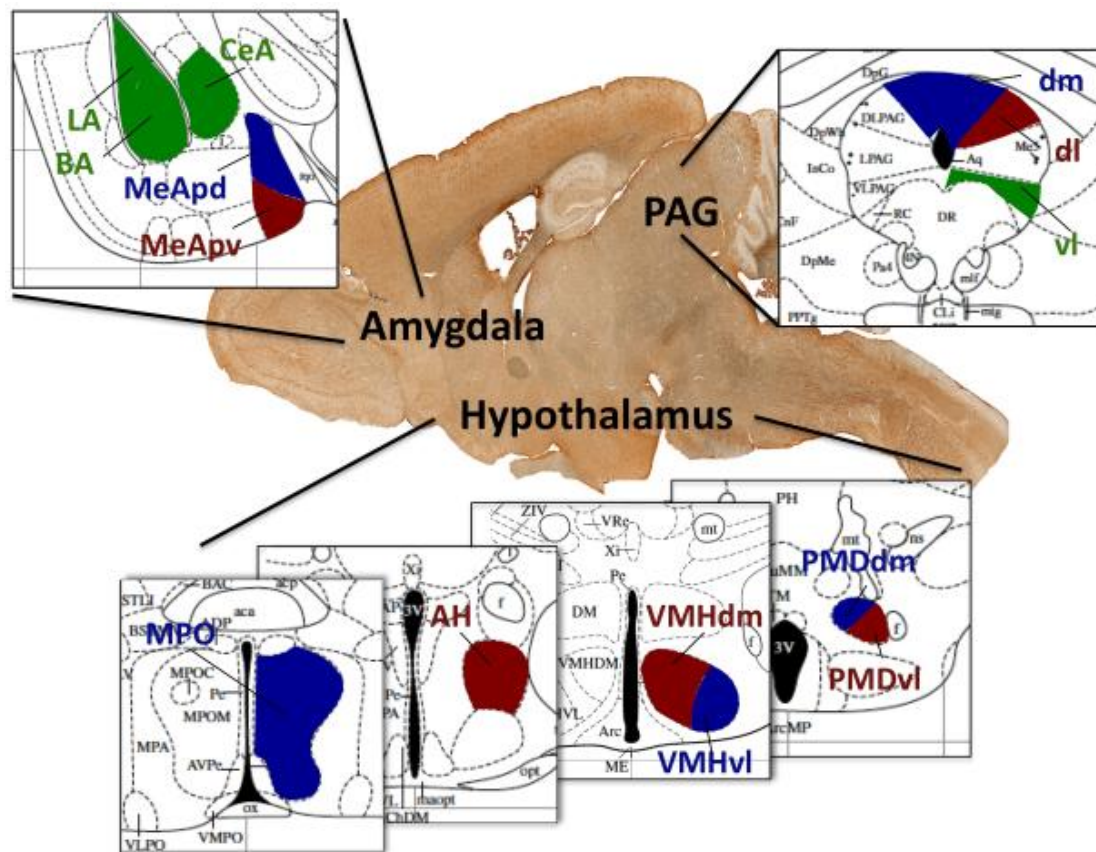
Figure 1. Different brain areas are C-Fos activated upon exposure to social (blue), predator (red) and foot shock (green) fear in the amygdala, medial hypothalamus and periaqueductal grey. Central amygdala (CeA), lateral amygdala (LA), basolateral amygdala (BA) and ventrolateral periaqueductal grey are activated by foot shock exposure. Medial amygdala posterior dorsal (MeApd), medial preoptic nucleus (MPO), ventromedial hypothalamus ventrolateral (VMHvl), dorsomedial portion of the dorsal premammillary nucleus (PMDdm) and dorsal periaqueductal grey (dPAG) are activated by exposure to aggressive conspecifics. Medial amygdala posterior ventral (MeApv), anterior hypothalamic nucleus (AH), ventromedial hypothalamus dorsomedial (VMHdm), ventrolateral portion of the dorsal premammillary nucleus (PMDvl) and dorsal periaqueductal grey (dPAG) are activated by exposure to predators (Canteras 2002).

1.2.1 The amygdalar sensory information center

The mammalian amygdala is a heterogeneous structure located medially in the temporal lobe. It is composed of more than six different nuclei that show either a cortical or striatal cyto-architecture, while functionally, they belong to the olfactory, autonomic and frontotemporal cortical systems (Swanson and Petrovich 1998). The amygdalar olfactory component includes the cortical amygdalar nucleus (COA) and the nucleus of the lateral olfactory tract (NLOT) that are part of the olfactory cortex as well as the postpiriform transition area (TR) and the piriform amygdalar area (PAA).

All these structures receive major projections from the main olfactory bulb and project to other amygdalar regions of the olfactory system, the posterior amygdala (PA), and the basomedial amygdala (BMA) (Scalia and Winans 1975). These areas receive projections also from structures processing other sensory information like the parabrachial nucleus (Bernard, Alden et al. 1993), which carries visceral and nociceptive information and from thalamic regions possibly targeting auditory and somatosensory information (LeDoux, Farb et al. 1990). Moreover these amygdalar structures are highly connected to other cortical areas like the medial prefrontal, the agranular insular and perirhinal cortical areas (Romanski and LeDoux 1993; McDonald, Mascagni et al. 1996).

A different set of nuclei processes olfactory information deriving from the accessory olfactory bulb, that, in turn, receives pheromonal information from the vomeronasal organ. Indeed the medial amygdala (MEA) and the posteromedial cortical amygdalar nucleus (COApm) represent the only major field of projections of the accessory olfactory bulb (Scalia and Winans 1975). The major outputs of the accessory olfactory components of the medial amygdala are the cerebral cortex, the nucleus accumbens and the CEA, the medial hypothalamus and the mediodorsal thalamus (Canteras, Simerly et al. 1995). Notably, the hypothalamic projections are restricted to the systems that control innate reproductive, defensive and ingestive behaviors (Risold, Thompson et al. 1997).

The autonomic division includes the striatal like structure denominated as central amygdala (CEA). It receives a wide range of sensory information from various descending cortical inputs including massive projections from the other amygdalar systems (Pitkanen, Savander et al. 1997). Moreover it receives ascending projections from the midbrain and brainstem including projections from the parabrachial (Bernard, Alden et al. 1993) and nucleus of the solitary tract (Ricardo and Koh 1978) and from the prigeniculate thalamus (LeDoux, Farb et al. 1990). In addition the CEA receives projections from the paraventricular thalamus, which is highly innervated by the hypothalamus. The major outputs of the CEA include projections to autonomic related centers like the dorsal motor nucleus of the vagal nerve, the nucleus of the solitary tract, the parabrachial nucleus, periaqueductal grey and lateral hypothalamus (Hopkins and Holstege 1978; Bandler and Shipley 1994). These nuclei are also involved in the somatomotor aspects of defensive behaviors.

The frontotemporal component includes the lateral (LA) and basolateral amygdala (BLA). Both nuclei show bidirectional projection to the olfactory system and prefrontal and insular regions whereas the LA has unique connections with the temporal and hippocampal regions and the BLA with somatosensory motor areas in the frontal and parietal lobes (McDonald and Mascagni 1996). Both parts innervate the caudatoputamen and nucleus accumbens, whereas the main output of the LA is the CEA (Pitkanen, Savander et al. 1997).

The amygdala has been widely implicated in fear. In particular a number of studies have indicated that the lateral and central amygdala play a major role in acquisition and expression of foot shock induced fear conditioning (Maren 2001). As regards fear induced by predators or dominant conspecifics the available data are not as comprehensive. Nevertheless there is evidence indicating that specific distinct amygdalar portion may act as a gate structure for sensory information in fear to predators, aggressive conspecifics or pain. In particular, functional studies based on c-Fos mapping in rodents indicate that the exposure to different threats recruits different amygdalar nuclei. Fear of painful stimuli such as an electrical foot shock activates the central nucleus (Ciocchi, Herry et al. 2010) while fear of predators and of aggressive conspecifics activates two different portions of the posterior medial amygdala, respectively, the ventral and the dorsal ones (Canteras, Ribeiro-Barbosa et al. 2001; McGregor, Hargreaves et al. 2004). Similarly, lesions at the level of the central nucleus block freezing to a tone that has been associated with a foot shock, but do not impair freezing responses to the exposure of a predator or to a context associated with the predator (Martinez, Carvalho-Netto et al. 2011). On the other hand, lesions to the medial amygdala impair predator fear but not conditioned responses to a foot shock (Blanchard, Canteras et al. 2005; Martinez, Carvalho-Netto et al. 2011). The pattern of neuronal connections of the different amygdalar nuclei reflects their differential function. Indeed, the central nucleus receives a wide range of sensory information for pain and contextual cues. As mentioned above, nociceptive and visceroceptive information derive from the brainstem parabrachial nucleus and nucleus of the solitary tract, while somatosensory and auditory information derive from the pregeniculate thalamus (LeDoux, Farb et al. 1990). On the other hand, the medial amygdalar nuclei get their main source of inputs from the accessory olfactory bulbs, which, in turn, process inputs from the vomeronasal organ, therefore gathering pheromonal information that are the main cues in the detection of other animals such

as predators or aggressive conspecifics (Dulac and Torello 2003). The lateral and posterior basomedial amygdalar nuclei also serve as relay for the predator detection receiving inputs from the medial amygdala and from visual and auditory association areas. Also the outputs of the different amygdalar nuclei are different and reflect their function. The central nucleus projects to the midbrain ventrolateral periaqueductal grey and to other brainstem autonomic centers like the dorsal motor nucleus of the vagus nerve and the parabrachial nucleus (Hopkins and Holstege 1978; Bandler and Shipley 1994). Accordingly, these regions are c-Fos activated by pain. In contrast, the medial amygdala mainly projects to the hypothalamic medial zone and in particular, the ventral portion, which is activated by predator odor, projects to those areas involved in predator fear, while the dorsal portion, which is activated by conspecifics odor, projects to the hypothalamic nuclei involved in social fear behavior and reproduction (Swanson and Petrovich 1998). Taken together, these suggest that fear circuits are dissociated at the level of the amygdala.

1.2.2 The hypothalamic defensive circuits

The hypothalamic medial zone is a structure involved in the integration of sensory information for the organization of coordinated behavioral, autonomic and endocrine responses to specific stimuli. It plays a role in a number of fundamental functions necessary for the survival of the animal including feeding, drinking, sex, aggression and defense. The schematic analysis of anterograde and retrograde tract-tracing, c-fos mapping and lesions studies has led to the identification of two non-overlapping circuits underlying reproductive and defensive behaviors (Canteras 2002). The defense circuit shows c-Fos expression upon predator exposure and is composed of the anterior nucleus, the dorsomedial portion of the ventromedial hypothalamus and the ventrolateral portion of the dorsal premammillary nucleus. On the other hand, the reproductive circuit includes the medial preoptic nucleus, the ventrolateral portion of the ventromedial nucleus, the dorsomedial portion of the dorsal premammillary nucleus and the ventral premammillary nucleus (Canteras 2002). Interestingly the nuclei of the defensive circuit are selectively activated by exposure to predators but not to an electrical foot shock or to aggressive conspecifics. On the other hand the nuclei belonging to the reproductive circuit are c-Fos activated upon exposure to dominant conspecifics (Motta, Goto et al. 2009). Studies based on c-Fos expression indicate that social and predator fear are processed by different circuits in the

hypothalamus. However, the limitation of these studies is that they were performed in very different behavioral setups where rodents performed very different behavioral responses, which could, in turn, lead to differences in brain activation. In our study we performed c-Fos mapping after exposure to different threats in our novel behavioral paradigm where mice exposed to a predator, a foot shock or an aggressive conspecifics and perform very similar defensive responses. C-Fos mapping confermed previous studies indicating that, at the level of the VMH, non overlapping sets of neurons are activated by the three stimuli, with predators recruiting the dorsomedial portion and aggressive conspecifics recruiting the ventrolateral one. Interestingly, the predator and reproductive circuits are very highly connected within themselves but almost completely segregated one to the other (Canteras 2002).

The **predator responsive circuit** receives inputs from the two amygdalar paths that integrate predator related cues (Figure 2). The first one is related to predator pheromonal cues sensed in the vomeronasal organ and conveyed to the posteroventral part of the medial amygdala via the accessory olfactory bulb and the second consists of the lateral and posterior basomedial amygdalar nuclei, known to receive inputs from visual and auditory association areas (LeDoux, Farb et al. 1990). Importantly, both amygdalar areas show cFos activation upon predator exposure (Dielenberg and McGregor 2001) and target the predator-responsive medial hypothalamic circuit mostly by projecting to the dorsomedial part of the ventromedial nucleus (Sesack, Deutch et al. 1989). The predator-responsive medial hypothalamic circuit also receives inputs from the hippocampal septal path presumably conveying contextual cues (Figure 2). The densest projections from this path come from the ventrolateral zone of the rostral part of the lateral septum, which innervates predominantly the anterior nucleus with only minor projections to the VMH and PMd (Risold and Swanson 1997). This septal structure contains a large population of GABAergic neurons that are likely to provide inhibitory inputs to the predator defensive circuit (Risold and Swanson 1997). The predator-responsive medial hypothalamic circuit also receives projections from other hypothalamic structures such as specific regions of the lateral hypothalamus including the retinoceptive and perifornical regions (Comoli, Ribeiro-Barbosa et al. 2000). The retinoceptive hypothalamic region is located dorsally to the supraoptic nucleus and is likely to provide information about the environmental light and darkness. These modulatory inputs are likely to be important, as different behavioral responses are more efficiently elicited in specific

light conditions, e.g. freezing is a more effective defensive response in the night. The perifornical region receives inputs from the parabrachial nucleus and, thus it is likely to convey nociceptive information (Bester, Besson et al. 1997).

A number of studies have also shown that the hypothalamic medial zone receives direct control by cortical structures. In particular the infralimbic and prelimbic areas of the prefrontal cortex provide a moderate projection to the AH and PMd (Comoli, Ribeiro-Barbosa et al. 2000). However, it is important to keep in mind that the most prominent regulation of defensive behaviors by the prefrontal cortex is mediated by projections to the periaqueductal grey (Sesack, Deutch et al. 1989). Moreover, a few brainstem sites provide inputs to this hypothalamic system, namely the precommissural nucleus, the dorsolateral part of the periaqueductal grey, the parabrachial area and the ventral tegmental area (Canteras 2002).
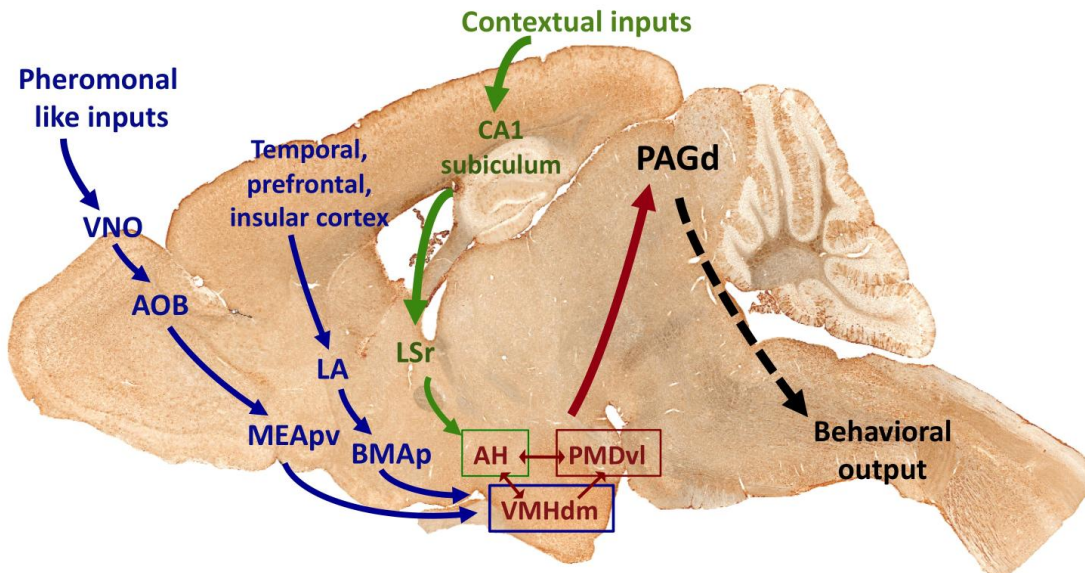
Figure 2. The predator responsive circuit. The medial hypothalamic defensive circuit has a central position in the predator responsive circuit. It is composed of the anterior hypothalamic nucleus (AH), the dorsomedial portion of the ventromedial hypothalamus (VMHdm) and the ventrolateral portion of the dorsal premammillary nucleus (PMDvl). The AH mainly integrates contextual inputs from the subiculum and CA1 via the rostral lateral septum (LSr) The VMHdm receives the strongest inputs from the posterior ventral medial amygdala (MeApv) processing inputs from the vomeronasal organ (VNO) and basomedial amygdala (BMA) that integrates inputs from the temporal, insular and prefrontal cortex via the lateral amygdala (LA). The main source of projections to the PMDvl derives from the other nuclei of the medial hypothalamic defensive system. The AH, VMHdm and PMD project to the dorsal periaqueductal grey (PAGd), which ultimately regulates the behavioral and autonomic outcome via projections to the medulla (Canteras 2002).

The **reproductive medial hypothalamic circuit** is composed of the median preoptic nucleus (MPO), the ventrolateral portion of the ventromedial hypothalamic nucleus (VMHvl) and ventral premammillary nucleus (PMV) (Figure 3). These structures are sexually dimorphic, express steroid hormones receptors and have been implicated in a wide range of social behaviors like copulatory, parenting and aggressive behaviors in females and males (Kollack-Walker and Newman 1995; Coolen, Peters et al. 1996). Surprisingly, the same set of nuclei is activated in animals exposed to aggressive conspecifics, suggesting that they may be the main regulators of social fear (Motta, Goto et al. 2009). The only exception to this surprising overlap between the reproductive and social fear activated nuclei is the dorsomedial portion of the PMd, whose C-fos activation was reported upon the encounter with dominant conspecifics, but nor after aggression or mating (Motta, Goto et al. 2009). Like the predator responsive system, also the reproductive system is dominated by amygdalar inputs conveying pheromonal information. Although, a different part of the medial amygdala shows c-Fos activation during social fear, the anterodorsal and posterodorsal portions, which in turn receive conspecifics related pheromonal information from the vomeronasal organ (Choi, Dong et al. 2005; Isogai, Si et al. 2011). Contextual information may derive from the septo-hippocampal system via projections to the lateral hypothalamus. Moreover, the VMHvl receives inputs from the parabrachial nucleus, which convey nociceptive information thus providing information about the level of threat represented by a conspecifics (Saper and Loewy 1980).

What needs yet to be unraveled is how these different responses to a member of the same species such as aggression, defense or reproduction can be prioritized and organized in the same circuit. Specific pheromonal cues are certainly critical to drive different social and reproductive behaviors. A recent study showed the presence at the level of the VMHvl of two distinct neuronal populations involved in aggression and mating, where neurons activated during attack were inhibited during mating suggesting a potential substrate for competition between these opposite social behaviors (Lin, Boyle et al. 2011). Less obvious is the switch between aggressive and defensive responses to a conspecific. In such function we hypothesize the presence of two components, one a priori driven by the internal state of the subject and by cues coming from the conspecifics such as testosterone levels and the second driven by the initial outcome of the encounter mainly driven by noxious stimuli.
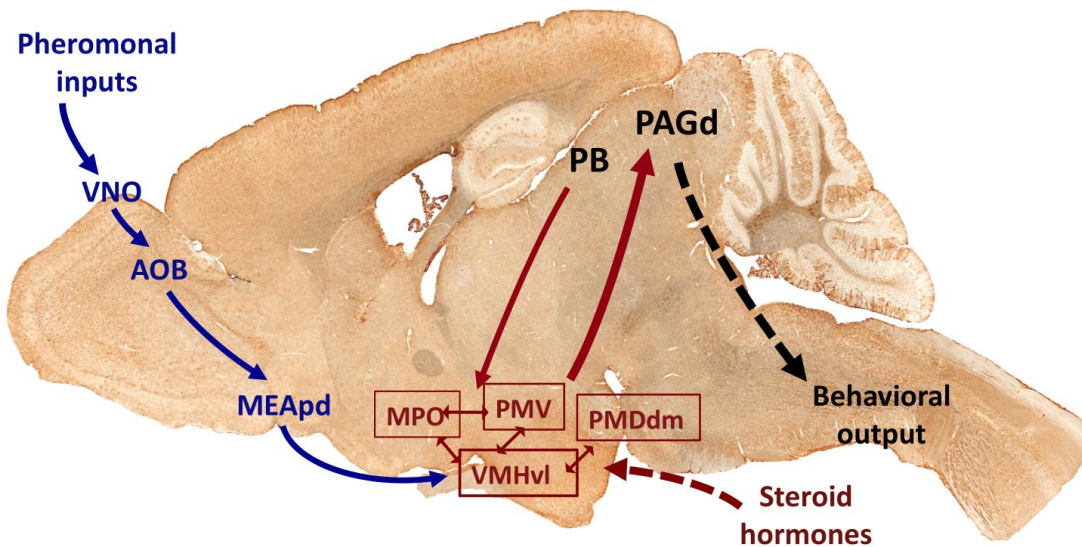
Figure 3. The social fear responsive circuit. The medial hypothalamic reproductive circuit is composed of the medial preoptic nucleus (MPO), the ventrolateral portion of the ventromedial hypothalamus (VMHvl) and the ventral premammillary nucleus (PMV). The same circuit is recruited by the encounter with aggressive conspecifics with the addition of the dorsomedial portion of the dorsal premammillary nucleus (PMDdm). The VMHvl receives the strongest inputs from the posterior ventral medial amygdala (MeApv) processing inputs from the vomeronasal organ (VNO). The nuclei belonging to the reproductive circuit are sexually dimorphic and express steroid hormones receptors. The VMHvl also receives projections from the parabrachial nucleus (PB). The social fear responsive hypothalamic nuclei target the dorsal periaqueductal grey (PAGd) (Canteras 2002).

The **foot shock responsive circuit** does not seem to recruit the hypothalamic medial zone. However, it is important to know that foot shock-induced fear is associated with c-Fos activation of the lateral hypothalamus may be mediating physiological arousal (Maren 2011).

1.2.3 The periaqueductal grey behavioral output system

All the three defensive circuits described above have as a common target the periaqueductal grey (PAG), a brainstem structure critical for the production of organized fear responses. However, circuits responsive to predators, foot shock or aggressive conspecifics seem to have at least a partial segregation also at the level of the PAG. For example the vlPAG receives direct projections from the medial portion of the central amygdala and has been shown to be critical for the expression of conditioned fear responses including freezing, vocalization and conditioned analgesia (Maren 2001).

On the other hand, the predator responsive hypothalamic circuits mainly targets the dorsolateral part of the PAG (dlPAG) via projections from the VMHdm and PMD. The PAGdl shows C-Fos activation in rats upon exposure to predators or to cues associated with predators like ultrasounds vocalizations (Mongeau, Miller et al. 2003) or predator odor (Cezario, Ribeiro-Barbosa et al. 2008). Accordingly lesions at the level of this structure block a wide range of defensive responses including flight/freezing responses, which are displayed when the predator threat is imminent, and risk assessment, displayed in response to more ambiguous predator threats like predator odor (Sukikara, Mota-Ortiz et al. 2010). The c-Fos activation in response to dominant conspecifics is more prominent in the dorsomedial and lateral portions of the PAG, reflecting the projections pattern of the dorsomedial PMD, where lesions impair passive but not active defensive responses (Motta, Goto et al. 2009).

Even though the dorsomedial and dorsolateral portions of the PAG seem to account for social and predator fear respectively, such segregation is not as strong as for other brain structures like the hypothalamus. The whole dorsal PAG shows c-fos activation to both stimuli with only an enrichment in the different portions for the two stimuli. Further investigation needs to be done in order to assess the extent of overlap between predator and conspecifics fear at the level of the PAG. Importantly, the PAG receives massive projections from the medial prefrontal cortex. The function of such projections has not been investigated in detail but it may act as a top down control of defensive responses.

1.2.4 Circuits supporting innate fear, outstanding questions

Most of the knowledge on the neural circuits supporting innate fear comes from the combination of c-Fos studies, which permit to identify which brain areas are recruited during fear, with tract-tracing studies, which permit to unravel how they are connected to each other (Canteras 2002). These studies suggest that different brain circuits are recruited during fear to different threats. However, these techniques have some intrinsic limitations. The first problem is that c-Fos mapping studies in animals exposed to predators or aggressive conspecifics were performed in very different behavioral paradigms where animals showed very different behavioral responses which could be the reason why different brain regions were activated (Motta, Goto et al. 2009). For example, foot shock based paradigms are typically performed in a confined environment with no possibility to escape and rodents mainly show freezing.

On the other hand, behavioral setups where animals are exposed to predators normally provide a safe shelter and rodents tend to escape to that. In order to run a systematic mapping of brain c-Fos activation in animals exposed to predators, foot shock and conspecifics we designed a new behavioral paradigm where animals could be exposed to different threats in the same environment and express comparable defensive responses.

The second major limitation of c-Fos studies is that they only provide correlative information but they do not give any information about causality. The main question that needs to be addressed is if the nuclei recruited during fear to different threats are actually necessary for the processing of fear. To address this question it is necessary to specifically inhibit them and examine if fear responses are impaired. This issue has been addressed for foot shock–based fear conditioning in the lateral and central amygdala (Maren 2001), but very little is known about the medial hypothalamic circuits processing predator and social fear. The only evidence indicating that these nuclei may be required for fear comes from lesions or muscimol inhibition studies that where performed only on the PMD within the hypothalamus and dPAG within the PAG. The limitations of these studies, beside the fact that they were performed on a very limited set of nuclei, is that they lack in temporal precision and cell types specificity. To address this question we took advantage of a designer receptor specifically activated by designer drug (DREADD), a newly developed pharmacogenetic inhibitory tool (Armbruster, Li et al. 2007) that we targeted specifically to two hypothalamic structures activated upon social and predator fear, the VMHvl and VMHdm respectively. This inhibitory tool consists of a modified version of the human muscarinic receptor M4 that has virtually no affinity for its endogenous ligand acetylcholine, but high affinity for an otherwise biologically inert drug named clozapine-N-oxide (CNO). In the presence of CNO this receptor gets activated and inhibits the neuronal activity. This allowed us to specifically inhibit neurons belonging to the VMHdm and VMHvl and address two main questions: if they are necessary for fear and if their function is specific for fear responses to a specific threat.

The third fundamental question that needs to be addressed is how the same hypothalamic circuits modulate different functions like reproduction and social fear or feeding and defense. For example the same set of nuclei are c-Fos activated during sex, aggression and social fear suggesting that they process information about the

encounter with conspecifics. However how the proper organized behavioral response gets prioritized remains unknown.

1.3   Circuits supporting memory of fear

In contrast to innate fear, fear memory related to different types of threats appears to share common brain circuits involving the hippocampus and the lateral amygdala as well as cortical areas such as the anterior cingulated area, the retrosplenial, and postrhinal area (LeDoux 2000; Maren 2001; Maren 2011). Fear learning has been mainly investigated with classical Pavlovian fear conditioning paradigms and the lateral amygdala has been shown to be the primary site where associations between the conditioned and the unconditioned stimulus (usually a foot shock) are formed and stored (Maren 2001). On the other hand, lesions of the posterior basomedial and lateral amygdalar nuclei seem to impair both acquisition and recall of fear memory associated with predator threats (Takahashi, Hubbard et al. 2007; Martinez, Carvalho-Netto et al. 2011). Projections of the lateral amygdala to the central amygdala mediate fear conditioning to painful stimuli, while its projections to the VMH through the basomedial nucleus are likely to mediate conditioning to predator cues (LeDoux 2000; Gross and Canteras 2012). The hippocampus is recruited in fear conditioning to contextual cues associated with both predators and painful stimuli. In particular, lesions at the level of the ventral hippocampus including intermediate and ventral regions of field CA1 and subiculum reduced conditioned defensive responses to the exposure to a context where a predator or its odor had been encountered (Pentkowski, Blanchard et al. 2006).

As concerns higher order association cortical areas, indirect paths seem to involve the anterior cingulate and retrosplenial area that influence contextual fear processing through their projections to the postrhinal area, which in turn projects to the hippocampal formation and lateral amygdala. Notably, lesions at the level of the retrosplenial area before or immediately after training impair the expression of contextual fear but not of tone specific fear. Fear-related information reaches these cortical areas via projections from the midline and intra-laminar thalamic nuclei (McNally, Johansen et al. 2011; Pavesi, Canteras et al. 2011), which collect inputs from the medial hypothalamus and the periaqueductal grey from structures responsive to both foot shock and predator cues. In particular the PMD, a structure highly activated by predator fear, provides dense projections to the anteromedial thalamic

nucleus, where lesions completely block predator related conditioned fear responses leaving intact defense to a live cat (Carvalho-Netto, Martinez et al. 2010). On the other hand, vlPAG seems to be responsible for conveying foot shock-related fear information to the cortex via its projections to the intra-laminar thalamic nuclei.

Much less is known about the circuits underlying memory of social fear. A recent study has shown the inhibition of the premammillary nucleus and the dorsal PAG impair conditioned responses to a context previously associate with the encounter with an aggressive conspecific (Faturi, Rangel et al. 2013). This suggests that it may share the same circuit as predator fear memory with the PMD projections to the anteromedial thalamus.

1.4   Fear circuits in humans

Fear circuits in humans have been mainly investigated by inducing fear in healthy individuals during positron emission tomography (PET) or functional magnetic resonance (fMRI) scannings. In human studies fear is typically induced by Pavlovian fear conditioning where an aversive unconditioned stimulus is repeatedly coupled with a normally neutral stimulus (CS), which after several pairings starts to elicit a conditioned fear response. Similarly to what was found in rodents, in humans functional neuroimaging studies have reported amygdalar activation during fear conditioning (Buchel, Morris et al. 1998; LaBar, Gatenby et al. 1998; Phelps, O'Connor et al. 2001; Morris and Dolan 2004). During fear conditioning most studies also reported activation of the anterior cingulated cortex (ACC), insular cortex and hippocampus (Buchel, Dolan et al. 1999; Alvarez, Biggs et al. 2008). Alternatively, specific pharmacological agents such as cholecystokinin-4 or procain can induce fear in humans. These studies also report activation in the amygdala, ACC and insular cortex (Benkelfat, Bradwejn et al. 1995). However it is important to keep in mind that it is impossible to discriminate the brain areas recruited by fear to the areas directly activated by the pharmacological agents. Another important piece of evidence on human fear circuits comes from neuroimaging studies in healthy humans exposed to emotional stimuli. These studies report amygdalar activation by a wide range of emotional stimuli suggesting a broader role of the amygdala in response to emotionally arousing situations. Collectively studies have highlighted the amygdala, the mPFC, the hippocampus and the brainstem as main centers of the fear circuits (Shin and Liberzon 2010). However it is important to remember that functional

neuroimaging typically only provides correlative non-causal linking neural structures and behavioral states.

Evidence that the amygdala is involved in human fear processing comes from patients with damages to this structure. Patients affected by Urbach-Wiete disease have amygdalar degeneration and, when the extent of the damage is sufficiently large, lack any sense of fear or perception of danger (Feinstein, Adolphs et al. 2011). However, in a recent study three patients with Urbach-Wiete disease were exposed to a CO_2 inhalation test and all of them developed panic attacks, demonstrating that the amygdala is not necessary for the expression of fear and panic but rather appears to be required for the gating of environmental threat stimuli (Feinstein, Buzza et al. 2013).

In our study we showed that the ventromedial hypothalamic nucleus and the periaqueductal grey are necessary for predator fear in mice. Unfortunately, data on hypothalamic activation in humans are challenging to obtain by functional neuroimaging studies due to the artifacts deriving from the close proximity of the third ventricle. However, evidence for an implication of the ventromedial hypothalamus in human fear comes from a recent deep brain stimulations study that reported anguish, autonomical arousal and panic attack in a patient receiving stimulations in the VMH (Wilent, Oh et al. 2010). Such evidence, together with the enhanced CO_2-induced panic seen in amygdala-damaged patients suggests the possibility that the VH, most likely the VMHdm is part of a circuit supporting panic attacks.

On the other hand, the PAG, which plays a crucial role in the production of fear responses to all kinds of threats in rodents, rarely shows changes in its activity in fMRI studies during fear in humans. Nevertheless, a recent study showed priaqueductal gray activation by imminent threats. In particular they used a tarantula approaching to the subject's foot and they showed correlation between the distance of the threat and the activation of PAG (Mobbs, Yu et al. 2010; Hermans, Henckens et al. 2012). This study suggests that also in humans the PAG is recruited in fear responses and that probably its activation was not detected in previous fMRI studies due to the fact that the stimulus used in the experimental fear paradigm did not represent a threat imminent enough.

1.5　Anxiety disorders in humans

1.5.1　Neural correlates of anxiety disorders, lessions from fMRI studies

Anxiety disorders are marked by excessive fear (and avoidance), often in response to specific stimuli and in the absence of true danger, and they are extremely common in the general population. According to a recent epidemiological study, the lifetime prevalence of any anxiety disorder is 28.8% (Kessler, Berglund et al. 2005). Since excessive fear is a key component of anxiety disorders, the investigation of the neural circuits underling fear in animal models has been crucial for the identification of human brain mechanisms of anxiety. According to the DSM IV the following disorders are classified as anxiety disorders: panic disorders, posttraumatic stress disorder, social phobia, specific phobia, generalized anxiety disorder and obsessive-compulsive disorder.

Panic disorder is characterized by the experience of recurrent, unexpected panic attacks, followed by persistent concern about having future attacks, or worry about the implications or consequences of the attacks, or a significant change in behavior related to the attacks (American Psychiatric Association. 2000). A panic attack is a discrete episode of intense fear, discomfort, and sympathetic nervous system arousal that occurs in the absence of true danger (DSM-IV, 2000). Symptoms of a panic attack include palpitations, sweating, trembling, shortness of breath, chest pain, dizziness, fear of dying, paresthesias, Panic attacks can be classified as unexpected, when the individual does not associate the onset with an internal or external situation trigger, situationally bound, when they almost invariably occur upon exposure to the situational cue or trigger, or situationally predisposed, when they are facilitated by a specific situation but they do not invariably happen around it.

Functional neuroimaging studies during panic normally require the artificial induction of a panic attack. CCK-4 injection is assumed to be an ideal and valid agent for the experimental induction of panic attacks, since CCK-4-induced panic attacks closely resemble spontaneously occurring panic attacks experienced by panic disorder patients, In studies investigating the functional neuroanatomy of CCK-4-induced panic, CCK-4 and placebo injections are delivered during PET or *f*MRI scanning and brain activity is recorded meanwhile contrasting brain activity during CCK-4, placebo, and periods of anticipatory anxiety with baseline activity then reveals what brain regions might be involved in the generation of panic attacks. Eser et al found

large responses to CCK-4 injection in the ventral anterior cingulate cortex (ACC), middle and superior frontal gyrus, precuneus, middle and superior temporal gyrus, occipital lobe, sublobar areas, cerebellum, and brain stem.

Moreover neuroimaging studies on patients affected by panic disorder indicated differential function in the amygdala, hippocampus, mPFC, insular cortex and brainstem. As pointed out above, patients with bilateral amygdalar lesions underwent panic attacks when exposed to CO_2 challenge indicating that the amygdala is nor required for such responses.

Posttraumatic stress disorder (PTSD)

PTSD can develop in individuals who were exposed to or witnessed an event that involved the threat of death or serious injury and reacted with intense fear, helplessness or horror (American Psychiatric Association. 2000). Individuals with PTSD reexperience the traumatic event in the form of nightmares, intrusive recollections, flashbacks, and physiological arousal and distress in response to reminders of trauma. These patients may attempt to avoid reminders of the trauma and may experience a restricted range of effect, especially positive effect. Finally, patients with PTSD report hyperarousal symptoms, such as hypervigilance, exaggerated startle, and difficulty sleeping or concentrating (American Psychiatric Association. 2000).

Several neuroimaging studies have shown hyperresponsivness of the amygdala in PTSD patients and some studies have reported that amygdala activation is positively correlated with PTSD symptom severity (Shin, Orr et al. 2004; Armony, Corbo et al. 2005; Dickie, Brunet et al. 2008). Other studies have also reported altered mu-opioid and GABA binding in PTSD patients, which may be causing the hyperresponsiveness. On the other hand, portions of the vmPFC (including the rACC) are hypo-responsive in PTSD and thus fail to inhibit the amygdala (Bremner 1999; Lanius, Williamson et al. 2001; Lindauer, Vlieger et al. 2004; Shin, Orr et al. 2004). It is not clear which of the two structures drives this pathological state, but a hyperresponsive amygdala and hyporesponsive mPFC may potentially lead to deficits in extinction, emotion regulation, attention, and contextual processing (Liberzon and Sripada 2008).

Moreover some neuroimaging studies have reported decreased hippocampal activity during symptomatic states and memory tasks in PTSD patients. The hippocampal dysfunction may account for the deficits in contextual processing and impairments in memory and neuroendocrine function.

Social phobia

Also called (or social anxiety disorder) is characterized by a marked and persistent fear of social or performance situations involving possible scrutiny by others (American Psychiatric Association. 2000). The fear of embarrassment and distress can lead to avoidance of social situations and impairment in social, occupational, and academic functioning. The amygdala and medial prefrontal cortex have been considered important regions of interest in this disorder (Stein 1998; Amaral 2002; Stein, Goldin et al. 2002).

Also in social phobia the amygdala seem to be the key structure responsible of the disease. Several studies have reported amygdalar hyperresponsiveness during public speaking (Tillfors, Furmark et al. 2001), the anticipation of public speaking (Tillfors, Furmark et al. 2002; Lorberbaum, Kose et al. 2004), negative comments (Blair, Geraci et al. 2008), and in response to neutral, angry, contemptuous, happy, and schematic angry facial expressions (Shin and Liberzon 2010). The role of the rACC and insular cortex in social phobia remains contradictory with some studies reporting exaggerated rACC and insular cortex activation and some others finding no significative changes in the activity of these structures.

Specific phobias

Specific phobias are marked by excessive, unreasonable and persistent fear of specific objects or situations such as small animals, flying, enclosed places, heights, and blood/injury (American Psychiatric Association. 2000). The fear and avoidance causes significant distress and/or impairment in occupational, academic, or social functioning. Specific phobia is a relatively common disorder, with a lifetime prevalence of 7–11% (American Psychiatric Association. 2000).

The amygdala, dACC and insular cortex all appear to be hyperresponsive to phobia-related stimuli in specific phobia. These abnormalities tend to normalize with successful treatment. The findings are few and mixed with regard to the rACC.

Obsessive–compulsive disorder (OCD)

Patients with obsessive–compulsive disorder (OCD) experience recurrent, unwanted thoughts or images (obsessions) that cause distress, and engage in excessive ritualistic behaviors or mental acts (compulsions) that are typically carried out in response to the obsessions (American Psychiatric Association. 2000).

The fear/anxiety-related brain regions pointed out so far such as the amygdala, mPFC, insula, and hippocampus do not appear to mediate the core OCD symptomatology. In

contrast, abnormalities in thalamo-cortico–striatal loops have been posited to account for the repetitive quality and the cognitive and motor content of the obsessions and compulsions in OCD. The current model of OCD pathology hypothesizes that the striatum (caudate nucleus) functions abnormally, leading to inefficient gating in the thalamus (Graybiel and Rauch 2000). This may lead to hyperactivity in the orbitofrontal cortex and the anterior cingulate cortex, which may mediate intrusive thoughts and anxiety, respectively.

Generalized anxiety disorder (GAD)

Generalized anxiety disorder (GAD) is characterized by excessive diffuse anxiety and worry that is difficult to control. Patients with GAD may experience restlessness, fatigue, irritability, muscle tension, and sleep and concentration difficulties (APA, 2000). The neural correlates of generalized anxiety are poorly understood. Some studies indicated amygdalar hyper- function in GAD patients exposed to aversive photographs or angry faces, as well as hyper-activation of the prefrontal cortex. However relatively few neuroimaging studies exist on generalized anxiety and evidences for brain function alterations in humans remain elusive.

1.5.2   Studying the hypothalamic fear system, implications for understanding anxiety disorders

Can hypothalamic fear circuits help us better understand the neural correlates of human anxiety disorders? While fear consists in the physiological emotion induced by the presence or the anticipation of a danger, anxiety disorders are characterized by excessive fear, in the absence of a true threat. As discussed above, anxiety disorders consist of a very heterogeneous set of disorders all characterized by a somehow over-reactivity of the fear system including: panic disorder, generalized anxiety, phobias, post traumatic stress disorder and obsessive compulsive disorder. These disorders are very diverse and what is malfunctioning in the brain circuits remains unclear.

In our study we showed that independent hypothalamic circuits process fear induced by different threats, suggesting that also anxiety disorders may be characterized by non-overlapping neural correlates. Understanding which fear circuits are preferentially affected could help finding targeted pharmacological interventions. For example, we identified a specific hypothalamic circuit processing social fear, which could be a potential target for the treatment of social phobias. On the other hand,

stimulations of the VMH in a human patient induced a panic attack suggesting a potential implication of the medial hypothalamic defensive circuit in panic disorder. Moreover, our study showed that hypothalamic and periaqueductal grey circuits play a fundamental role in fear, suggesting that altered physiology at this level could potentially contribute to the etiology of anxiety disorders. However, hypothalamic and brainstem networks were rarely considered in human studies on anxiety disorders, probably due to the fact that they are mainly based techniques like functional magnetic resonance (fMRI) where imaging structures situated close to the ventricles, like the hypothalamus or PAG, is particularly challenging.

2 Functional architecture of the hypothalamus

The hypothalamus is a core brain structure fundamental for the regulation of a number of basic functions necessary for the survival of the individual and of the species such as feeding, drinking, sleep, reproduction and defense. In particular it plays a crucial role in the generation of integrated hormonal, autonomic and behavioral responses in all these basic functions. Indeed, fear responses are necessary for the survival, as they allow the individual to avoid and react to life threatening situations and they recruit the hormonal, autonomic and behavioral systems. Despite the fact that the hypothalamus has been shown to be a central regulator of integrated defensive responses, most of the research that has studied the neural basis of fear has focused on a different brain area, the amygdala, and little is known of the hypothalamic circuits regulating fear. For this reason we have centered our research trying to understand the role of a specific hypothalamic nucleus, the ventromedial hypothalamus (VMH) in the regulation of fear responses.

2.1 Morphological organization of the hypothalamus

Both in rodents and in humans the hypothalamus occupies the ventral half of the diencephalon on both sides of the third ventricle and lies immediately above the pituitary grand. Dorsally, the hypothalamus is bounded by the zona incerta and the medial edge of the cerebral peduncle corresponds to its lateral border. Caudally, the hypothalamus merges with the periaqueductal grey and the ventral tegmental area of the midbrain, while rostrally it is bordered by the anterior commissure and nucleus of the diagonal band of Broca.

On the basis of neurochemical and hodological studies the hypothalamus can be divided into three longitudinal zones: periventricular, medial and lateral. A further subdivision of the hypothalamic structure comprises four rostrocaudal levels designated as the preoptic, anterior, tuberal and mammillary.
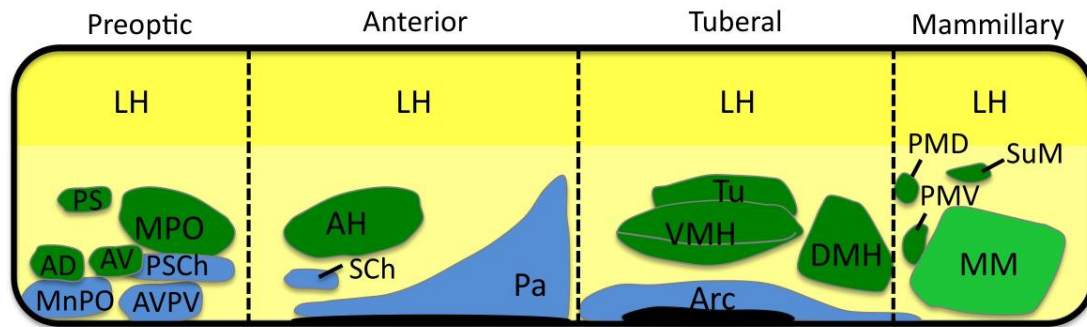
Figure 4. Morphological organization of the hypothalamus. Hypothalamic nuclei seen in a flatmap representation of the hypothalamus (Top = lateral, bottom = medial, left = rostral, right = caudal). The periventricular nuclei are shown in blue. The black area represents the ventricle. The nuclei of the medial zone are represented in dark green, the mammillary bodies in light green. The lateral zone is represented in yellow. Preoptic region: preoptic periventricular nucleus (PePO), Median preoptic nucleus (MnPO), anteroventral periventricular nucleus (AVPV), suprachiasmatic preoptic nucleus (PSCH), medial preoptic nucleus (MPO), anterodorsal preoptic nucleus (AD), anteroventral preoptic nucleus (AV), parastrial (PS). Anterior region: suprachiasmatic nucleus (SCh), paraventricular (Pa), anterior hypothalamic nucleus (AH). Tuberal region: arcuate nucleus (Arc), ventromedial nucleus (VMH), dorsomedial nucleus (DMH). Mammillary area: dorsal premammillary nucleus (PMD), ventral premammillary nucleus (PMV), mammillary complex (MM), suprammammillary nucleus (SuM).

2.1.1 The periventricular zone

Functionally, the periventricular zone contains most of the neurons that express hormone-releasing hormones and represents the final pathway for the neural control of the pituitary gland. Hormones produced in periventricular neurons are secreted into the portal brain-pituitary blood system through axons to the medial eminence and have their targets in the anterior pituitary gland that, in turn, controls the endocrine function of the whole body (Harris 1948). The only two exceptions are the hypothalamic supraoptic nucleus and gonadotropin releasing hormone neurons that reside outside of the periventricular zone.

Cytoarchitectonically, the periventricular zone is characterized by small, vertically oriented fusiform neurons and is traversed by ascending and descending fibers connecting it with midline thalamus and midbrain periaqueductal grey.

The preoptic region of the periventricular hypothalamic zone contains four identifiable cell groups, the periventricular preoptic nucleus (PePO), the median

preoptic nucleus (MnPO), the anteroventral periventricular nucleus (AVPV) and the suprachiasmatic preoptic nucleus (PSCh).

The MnPO is a dense cluster of cells located in the lamina terminalis playing a critical role in neural circuits controlling cardiovascular responses and fluids homeostasis. Consistent with this role it receives inputs from the subfornical organ and the parabrachial nucleus and sends projections to the paraventricular nucleus and dorsomedial hypothalamic nucleus.

The AVPV and the MnPO are located immediately caudal to the vascular organ of the lamina terminalis. The AVPV is involved in the regulation of gonadotropin secretion sending outputs to the gonadotropin- releasing hormone expressing neurons in the region adjacent to the vascular organ of the lamina terminalis as well as to the tuberoinfundibular dopaminergic neurons in the arcuate nucleus. It receives strong inputs from the posterior and medial amygdala and from the principal nucleus of the stria terminalis that convey olfactory information. Moreover it receives inputs from the lateral septum and from all the parts of the periventricular hypothalamic zone, from the dorsomedial hypothalamus, medial preoptic nucleus and ventral premammillary nucleus.

The anterior region of the periventricular hypothalamic zone contains three distinguishable nuclei: the suprachiasmatic nucleus (SCh), and the paraventricular, and anterior periventricular nucleus (Pa).

The SCh receives direct inputs from the retina and plays a critical role in the control of circadian and diurnal rhythms. The SCh also receives serotoninergic projections from the raphe and inputs from NPY expressing neurons in the lateral geniculate nucleus (Moore 1983).

The paraventricular nucleus is responsible of the release of most of the hypothalamic hormone-releasing hormones playing a major role in mediating endocrine responses to stress, feeding and drinking behavior and takes part in various autonomic responses. The Pa contains two major populations of neurons, one composed of small-sized cells that project to other parts of the brain or secrete hypothalamic-releasing hormones to the median eminence and a second magnocellular neurosecretory one that produces oxitocin and vasopressin released in the blood stream through axons in the posterior pituitary gland. The small-sized neuronal population secretes a number of hormone-releasing hormones including corticotropin releasing hormone (CRH) (Antoni, Palkovits et al. 1983), thyrotopin releasing

hormone (TRH) (Kawano, Tsuruo et al. 1991), somatostatin (Kawano and Daikoku 1988), growth hormone releasing hormone and dopamine that are released through axons in the median eminence. Moreover they contain several other neuropeptides including angiotensin II, atrial natriuretic peptide, bombesin, AVP, CART, CCK, PACAP, neurotensin, peptide histidine leucine, enkephalins, galanin, and vasoactive intestinal peptide (Swanson, Sawchenko et al. 1986).

The **tubera**l region contains the intermediate periventricular nucleus and the arcuate nucleus (Arc). The arcuate nucleus also contributes to the release of hypophisiotropic hormones from the terminals located in the median eminence into the hypophysial portal system with neuroendocrine neurons secreting dopamine and GHRH. On the other hand, centrally projecting neurons in the arcuate play a major role in the regulation of food intake and energy balance. This neuronal population contains both orexigenic and anorexigenic cells producing neuropeptide Y (NPY), agouti related peptide (AgrP) and GABA or proopiomelanocortin (POMC) and CART respectively.

The mammillary part of the periventricular zone is occupied solely by the caudal part of the posterior periventricular nucleus which surrounds the posterior end of the third ventricle. These cells are normally considered part of the arcuate nucleus.

2.1.2   The medial zone

The medial zone consists of an undifferentiated hypothalamic grey matter in which several cellular condensations, or nuclei, are embedded. These nuclei, collectively, play key roles in the initiation of motivated behaviors such as aggressive, sexual, defensive and appetitive behaviors. Accordingly, they are connected with widely distributed parts of the telencephalon, diencephalon and brain stem, that are fundamental for the somatomotor integration necessary for the elaboration of appropriate adaptive responses to specific external cues (Canteras 2002). Indeed they are in a good position to receive information from all sensory modalities. This sensory information is relayed by nuclei in the limbic region of the telencephalon and from brain stem nuclei that relay visceral inputs (Risold and Swanson 1996). Interestingly nuclei in the medial zone share very strong bidirectional projections with the structures that provide their inputs; moreover they share strong intra-hypothalamic connections with each other, with the lateral zone and with the periventricular zone, therefore providing a mean for the limbic system to modulate the neuroendocrine function. As the periventricular zone, the medial zone can also be divided into four

rostrocaudal levels, preoptic, anterior, tuberal and mammillary. The mammillary region primarily processes cortical information, while other nuclei are more directly involved in the regulation of essential behavioral responses to visceral, gustatory and olfactory stimuli.

In **the preoptic region** five distinct cell groups are embedded in the undifferentiated grey, the medial preoptic nucleus (MPO), the anterodorsal preoptic nucleus (ADPO), the anteroventral preoptic nucleus (AVPO), the parastrial nucleus (PS) and the posterodorsal preoptic nucleus (PD).

The MPO occupies the largest part of the medial preoptic area. It is highly sexually dimorphic and mainly involved in the regulation of sexual and maternal behavior. In addition to its extensive intrahypothalamic inputs it receives projections from the posterior and medial nuclei of the amygdala, the BST, the caudal and ventral lateral septum, the ventral tegmental area, the nucleus of the solitary tract and parabrachial nucleus (Berk and Finkelstein 1981; Canteras, Simerly et al. 1992). Its outputs include regions important for the regulation of the neuroendocrine, autonomic and somatomotor components of the reproductive and maternal function. Accordingly, they include a number of equally sexually dimorphic brain regions such as, the ventral lateral septal nucleus, the ventrolateral part of the ventromedial hypothalamus, the ventral premammillary nucleus, the principal nucleus of the BST, and the medial amygdala. Moreover it sends projections to some hypothalamic periventricular zone nuclei involved in the regulation of hormone secretion from the pituitary.

The AVPO contains GABAergic and galanin neurons that project directly to the tuberomammillary nucleus and play an important role in the regulation of sleep and body temperature.

The ADPO is often merged with the ventral part of the lateral septum nucleus.

The parastrial nucleus receives strong inputs from the AVPV and projects to the paraventricular nucleus therefore it s thought to be involved in the regulation of fluid homeostasis.

The PDPO has been implicated in the regulation of male sexual behavior.

The anterior region is almost completely occupied by the anterior hypothalamic nucleus (AH) with only an additional cluster of cells in the dorsal portion denominated stigmoid nucleus. The AH has been sown to play a crucial role in defensive responses and in particular in the processing of contextual information. It receives inputs from the hippocampal formation through lateral septum and

subiculum, BST and strong intrahypothalamic connections from the medial and lateral zones. It sends efferents to all hypothalamic zones with particular dense projections to the ventromedial, premammillary nucleus and perifornical region. In addition it targets the paraventricular nucleus of the thalamus and periaqueductal grey.

The tuberal region contains two large well-defined cell groups, the dorsomedial hypothalamic nucleus (DMH) and the ventromedial hypothalamic nucleus (VMH). In addition a smaller cell aggregation denominated tuberal nucleus lies laterally to the VMH. The VMH occupies the largest part of the nucleus and will be discussed in detail later. Most neurons in the tuberal nucleus express estrogen receptors and have projection patterns very similar to the ventrolateral portion of the VMH. Nevertheless they seem to provide stronger inputs to regions regulating neurosecretion such as the arcuate, anterior periventricular and paraventricular nucleus of the hypothalamus (Simerly 1990).

The DMH is located dorsally to the VMH and receives inputs from the BST, lateral septum and all parts of the brainstem that project to the MPO. In addition it receives inputs from most nuclei of the hypothalamus. Its projections are mostly intrahypothalamic, however it also connects to the periaqueductal grey, Barrington's nucleus, parabrachial nucleus and nucleus of the solitary tract. It has been implicated in the regulation of ingestive behavior, stress, reproduction, circadian rhythms and thermogenesis.

The mammillary region is occupied by the mammillary body, the supramammillary and premammillary nuclei, the posterior hypothalamic area and the tuberomammillary nucleus.

The mammillary body is composed by a medial mammillary nucleus (MM) that occupies the majority of the mammillary region and a lateral mammillary nucleus (LM). The most important set of inputs to the mammillary body is represented by the postcommissural fornix that conducts projections from the hippocampal formation. Projection axons form two main fiber tracts, a descending mammillotegmental tract that terminates in the tegmental nuclei of Gudden and the ascending mammillothalamic tract that terminates in the anterior thalamus. Anterior thalamic nuclei project, in turn, to the limbic cortex including the anterior limbic area, retrosplenial ara, presubiculum and parasubiculum. In contrast to the rest of the medial zone the mammillary body is mostly influenced by visual and auditory indormation (Simerly 1995).

The ventral premammillary nucleus is part of the sexually dimorphic hypothalamic circuit regulating maternal and reproductive functions. It expresses steroid hormones receptors and is highly connected to other intra- and extra-hypothalamic nuclei of this circuit including the posterior nucleus of the amygdala, the MPO, the BST and VMHvl. The PMV provides strong inputs to the periventricular zone.

The dorsal premammillary nucleus as been designated as the final hypothalamic structure of the defensive circuit, receiving inputs from the anterior hypothalamic nucleus and dorsomedial portion of the ventromedial hypothalamic nucleus. The PMD sends projections to the anterior thalamus, periaqueductal grey, superior colliculus and adjacent parts of the reticular formation.

The supramammillary nucleus (SuM) receives inputs from the ventral parts of the lateral septum, the BST, the medial preoptic nucleus and the lateral zone of the hypothalamus. It projects to most major telencephalic structures including the entire cortex, dentate girus, central nucleus of the amygdala and entorhinal cortex.

The function of this nucleus is not well understood; however, it has been shown that it contains cells that regulate the slow rhythmical activity of the hippocampus and based on its anatomical connectivity people have hypothesized transforms information to achieve integration of cognitive and emotional aspects of goal-directed behavior (Pan and McNaughton 2004).

The posterior hypothalamic area is the most caudal and dorsal portion of the hypothalamus and shares a lot of connections with the periaqueductal grey. It receives inputs from the amygdala, the septum, the hippocampal formation and much of the hypothalamus. Many of these connections are bidirectional (Cavdar, Onat et al. 2001). In addition it provides significant inputs to cortical regions related to the limbic system including the perirhinal, insular, limbic and prelimbic cortex and therefore it has been suggested that it may be implicated in the processing of various aspects of the emotional behavior (Vertes, Crane et al. 1995).

In the tuberomammillary nucleus most neurons express histidine decarboxvlase indicating that the use histamine as a neurotransmitter. They receive catecholaminergic inputs from C-C3 and A1-A2 cell groups in the brain stem, as well as serotoninergic inputs from the B5-B9 cell groups. Its projections pattern has much in common with the one of the locus coeruleus and dorsal raphe, being distributed to widespread parts of the brain. Its connections have led to the idea that it may play a role in the modulation of arousal and behavioral state. However its strongest

projections are to the periventricular hypothalamus suggesting a role also in the regulation of the endocrine function.

2.1.3   The lateral zone

The lateral hypothalamic zone is one of the most complex structures of the brain since it consists of an undifferentiated grey where very few if any nuclei can be identified based on cytoarchitectural or neurochemical grounds and for this reason in some cases it is considered as an extension of the reticular formation (Simerly 1995). Moreover it is traversed by the medial forebrain bundle that is a very complicated fiber system containing ascending and descending projections that involve a very large variety of brain areas extending from the cortex to the spinal cord (Veening, Swanson et al. 1982). All these projections send collaterals that contact the hypothalamic lateral zone. Specific cell populations in the lateral zone has been implicated in the processing of sensory information and the expression of behaviors associated with hunger and thirst (Berthoud and Munzberg 2011). Moreover functional evidences suggest it is involved in mediating general arousal and sensory sensitization associated with motivated behavior and may modulate spinal pathways and therefore regulate the likelihood that a specific behavioral response will take place (Simerly 1995). However, based on its strong connections with telencephalic regions such as the cerebral cortex, amygdala and septum, and its connections with the periventricular zone it is in a good position to coordinate motivated aspects of behavior with visceromotor responses (Swanson 1987). In classical anatomical studies it is divided into two portions, the lateral preoptic area and the lateral hypothalamic area.

2.2   The ventromedial hypothalamic nucleus (VMH), a key structure of behavior integration

2.2.2   Anatomy and cell identities

The VMH is identified as a cell dense area surrounded by a cell-poor fiber-rich zone located close to the base of the diencephalon, adjacent to the third ventricle above the median eminence and pituitary complex. It is a bilateral cell group that has an elliptical shape, stretching more laterally as it extends rostral to caudal. Based on cellular density, neuronal cytology, neuronal ultrastructure, fiber projections and cell identity it can be subdivided into three different subnuclear regions, namely,

dorsomedial, central and ventrolateral VMH (Millhouse 1973). Another cell-dense zone located at the ventrolateral side of the VMH, designated as tuberal nucleus, is also annotated by some as part of the VMH complex based on neuronal birthdates and cell phenotype (Simerly, Chang et al. 1990; Canteras, Simerly et al. 1994).

The synaptic organization of the VMH is not well understood, but VMH neurons have long primary dendrites that may be uniquely positioned to contact afferents terminating in the fiber plexus surrounding the VMH and fewer short primary and secondary dendrites that may receive inputs from local interneurons.

Structural sex differences have been observed in the adult VMH. Based on Nissl stain the VMH results to be 25% larger in males than in females (Dorner and Staudt 1969; Madeira, Ferreira-Silva et al. 2001; Dugger, Morris et al. 2007; Martini, Di Sante et al. 2008). This difference seems to be caused by soma size and amount of neuropil rather than by neuronal number. Moreover the dendritic arbor is more prominent in males than in females (Griffin and Flanagan-Cato 2009). Interestingly the sexual dimorphism does not seem to be confined to the ventrolateral portion, region where it was originally hypothesized based on its function in the regulation of sexual behavior and expression of steroid hormones (Griffin and Flanagan-Cato 2009).

Molecular markers of the VMH have been extensively investigated. Gene expression profiling in the adult and neonatal hypothalamus identified a number of VMH-enriched genes (Kurrasch, Cheung et al. 2007). Their studies identified the Nr5A1 (also denominated nuclear receptor SF-1) as the highest expressed VMH transcript. This gene appears to be expressed in all three VMH subregions during development, but after birth its expression is restricted to the dorsomedial and central portions. For this reason, Nr5a1 has been widely used as a tool for the selective manipulation of VMHdm neurons (Dhillon, Zigman et al. 2006).

Interestingly VMH neurons seem to be mainly glutamatergic as indicated by strong V-Glut2 expression (Allen Brain Atlas, http://www.brain-map.org/) unlike all surrounding areas that show prominent expression of GABAergic markers. Several other markers identify the cell types in the different VMH subregions. They can be classified into four families: transcription factors, neuropeptides, membrane receptors and GABA and its receptors as summarized in McClellan et al. 2006 (McClellan, Parker et al. 2006).

Transcription factors: in addition to Nr5a1 microarray experiments revealed the enrichment of the mRNA of other transcription factors in the VMH including Vgll2,

Sox14, Satb2, Fezf1, Dax1, COUPTFII, Nkx2-2, Ldb2, Fbxw7, Lcorl, Nkx2.1, Grhl1, Neud4, and Isl1 (Kurrasch, Cheung et al. 2007). Of these transcription factors, known roles in hypothalamic development have been described only for Nr5a1 and Nkx2.1, where loss of NR5a1 impairs the maintenance of normal VMH cytoarchitecture (Shinoda, Lei et al. 1995), the VMH terminal differentiation (Tran, Lee et al. 2003), and proper condensation of the VMH nucleus (Davis, Seney et al. 2004) and loss of Nkx2.1 results in the disruption of the entire basal hypothalamus. Steroid hormone receptors are expressed in stereotypical locations within the VMH in many species. Estrogen and progesterone receptors are localized strongly to the ventrolateral regions of the nucleus, while androgen receptors are expressed throughout the nucleus. A number of studies have shown that the neurons within the VMH containing steroid hormone receptors, particularly those of the ventrolateral region, are involved in regulating female sexual behavior in adult animals (Simerly, Chang et al. 1990).

Neuropeptides: brain derived neurotrophic factor (BDNF), pituitary adenylate cyclase-activating polypeptide (PACAP), and Slit3 (Nguyen-Ba-Charvet and Chedotal 2002) are expressed throughout the entire nucleus. Neuronal nitric oxide synthase (nNos), somatostatin (SST), enkephalin (Penk), and cholecystokinin (CCK) are expressed in the ventrolateral region with nNos reaching more dorsolaterally through the nucleus. Of these, functions in the VMH are best described for BDNF, in which it acts as a satiety factor downstream of melanocortin signaling (Xu, Goulding et al. 2003). PACAP signaling has been proposed to regulate the sympathoadrenal axis affecting the release of adrenal steroids (Hashimoto, Shintani et al. 2006), and substance P release from ER-positive VMH neurons is proposed to affect sexual behavior (Daniels, Miselis et al. 2003).

Membrane proteins: Cannabinoid receptor-1 (CB-1) is expressed throughout the entire nucleus while the leptin receptor delineates the dorsomedial portion. Both factors, together with insulin receptors and the neonatally enriched the ATP-dependent potassium channels, the potassium inwardly rectifying channel Kcnj11 (Kir6.2, 1) are part of the signaling pathway involved in the regulation of energy bakance of the VMH. The oxytocin receptor (OTR) and the growth hormone secretagogue receptor (GHSR) are expressed in the ventrolateral region. Interestingly these genes are not strongly expressed in the developing VMH suggesting a prevalent role in the adult signal transduction rather than in the nuclear development (McClellan, Parker et al. 2006). Other membrane proteins were found enriched in the

neonatal VMH: Gpr149, Nmbr, Cckbr, Htr1b, Cnr1, Htr2a, and Gpr176, and ion channels, Gabra5, Kcnj5, Kcnab1, Abcc4, Abcc8, Cacna2d1, Slc17a6, Clca1, and Fxyd7 (Kurrasch, Cheung et al. 2007).

GABA receptors: although GABA is not synthesized by VMH neurons, GABA is made and released in fibers that surround the VMH. GABA$_A$ and GABA$_B$ receptors are found within the nucleus. GABA$_B$ receptor subunits are expressed throughout the entire nucleus. There are GABA$_A$ receptor subunits expressed throughout the entire nucleus as well, however, each subunit has distinct expression patterns. GABA$_A$ receptor subunit α3 is expressed throughout most of the dorsomedial region, GABA$_A$ receptor subunit α5 is localized to the central region, and the GABA$_A$ receptor subunits $\beta\alpha$2, β3, γ3 are expressed in the most ventrolateral portion (McClellan, Parker et al. 2006).

2.2.3   Development of the VMH

The neuronal population that locates in the VMH is born between E10 and E15 in mice, E13 to E17 in rats, and around E30 in primates (Shimada and Nakamura 1973; van Eerdenburg and Rakic 1994; Tran, Lee et al. 2003). Studies based on [$_3$H]thymidine incorporation have shown that cells in the VMH derive primarily from precursors in the proliferative zone surrounding the lower portion of the third ventricle dorsal to the arcuate nucleus (Altman and Bayer 1986).

Following neuronal divisions in the proliferative zone neurons migrate radially away from ventricular zones guided by processes of radial glial cells that extend ventrolaterally from the cell bodies located adjacent to the third ventricle to the pial surface of the brain. In contrast to the inside-out pattern of the cortex, the earliest born cells in the hypothalamus migrate the farthest from the ventricle. The migration process finishes in the mouse around E17, in the rat around E19 (Hyyppa 1969) and in human around gestational week 15 when an oval shaped cell mass starts to be visible (Koutcherov, Mai et al. 2003).

The VMH specific transcription factor Nr5a1 plays a crucial role in the migration process as shown by NR5a1 knock out studies (Davis, Seney et al. 2004). Nr5a1 knock out mice show misplacement of VMH neurons that were phenotipically identified by GFP expression under the control of the Nr5a1 promoter. Interestingly also Isl-1, estrogen receptor α, Nkx2.1, NPY, and galanin positive cells are misplaced in NR5a1 null mice (Dellovade, Young et al. 2000). Among Nr5a1 target gens in the

VMH many cell adhesion and cell guidance proteins were described. These molecules are important for movement of the neurons along radial glial. Also GABA signaling plays a role in the migration of VMH cells as shown by artificial activation of GABA receptors where the movements and the distribution of VMH cells are impaired.

VMH projections start to be visible early at embryonic E10.5 when few postmitotic Nr5a1 expressing neurons have been born (Cheung, Kurrasch et al. 2013) suggesting that formation of VMH circuitry begins at the onset of neurogenesis and not after the neuronal migration and nuclear organization. The most prominent embryonic fibers at this stage extend in the medial forebrain bundle and ventral supraoptic commisure (vSOC), which travels through the dorsal thalamus and targets the PAG. Also the rest of ascending and descending projection from the VMH start to appear early at E17.5 and at P0 the pattern of projections resembles very much the adult one. Very little is known about the molecular mechanisms driving the projection patterning and axon guidance of VMH neurons.

2.2.4   VMH connectivity and functional implications

The efferents of the VMH have been first investigated by Phaseolus vulgaris-leucoagglutinin tract tracing in the rat where this anterograde tracer was injected in the different subregions of the VMH (Canteras, Simerly et al. 1994). Later a very similar pattern of projections was found in a transgenic mouse line expressing GFP under the control of the VMH genetic marker NR5a1 promoter (Cheung, Kurrasch et al. 2013). VMH axons can be divided into ascending fibers projecting to targets rostral to the VMH, and descending fibers projecting caudal to the VMH. The nucleus also shows extensive intrinsic projections.

Ascending fibers course mainly through the medial zone of the hypothalamus and medial portions of the medial forebrain bundle where they contact their hypothalamic targets with the VMHdm and VMHc targeting mainly the anterior nucleus and the VMHvl and tuberal nucleus targeting mainly the medial preoptic nucleus. Some of the fibers ascending through this pathway take a dorsal route and enter the thalamus contacting the paraventricular and parataenial nuclei, as well as the nucleus reuniens. At preoptic levels, a significant number of fibers extend through the septa1region and contacts the main thelencephalic sites. Very dense projections from all VMH subregions are found at he level of the BST and lateral septum at this level. Notably, projections from different VMH subregions seem to segregate to different subnuclei

of these structures. From this ascending pathway some axons continue as far rostral as the infralimbic and prelimbic areas of the medial prefrontal cortex. In addition, a group of ascending fibers takes a lateral course, mainly following the ansa peduncularis through the substantia innominata and the ventral supraoptic commissure system, thus gaining access to several parts of the amygdala, including the capsular and medial parts of the central nucleus, the lateral nucleus, the anterodorsal part of the medial nucleus, and the piriformamygdaloid area. Interestingly each part of the VMH provides a somewhat different pattern of projections to the amygdala. The central and lateral nuclei appear to receive the most abundant inputs from the VMH. A small group of fibers coursing through the ansa peduncularis ends in limited regions adjacent to the amygdala, including the piriform area and endopiriform nucleus as well as the perirhinal, entorhinal, and postpiriform transition areas. In addition a smaller group of axons extends rostrally from the VMH through the rostral parts of the zona incerta just dorsal to the hypothalamus.

Descending fibers from the VMH follow three main routes:

1) the medial hypothalamus and medial forebrain bundle where they contact the premammilary and supramammilary nuclei. Interestingly, the VMHvl provides a sparse input to the ventral premammillary nucleus while the VMHdm projects to the dorsal premamillary nucleus.

2**)** the midbrain periventricular system, where they contact first the posterior hypothalamus and more caudally the subparafascicular nucleus and the peripeduncular nucleus reaching subsequently the periaqueductal grey. This structure represents the most prominent VMH target with fibers from all VMH subregions projecting throughout all the rostro-caudal length of the PAG with specific patterns. Axons from the midbrain periventricular system also project to the deeper layers of the superior colliculus and cuneiform nucleus.

3) the ventral supraoptic commissure system providing projections to the mesencephalic reticular nucleus, where some fibers descending through these pathways merge and appear to extend caudally into pontine and medullary levels of the reticular core of the brainstem.

One of the main points to be drawn is that the anterior, dorsomedial, central, and ventrolateral parts of the VMH present significant differences with regard to their projection patterns, with the VMHvl projecting largely to other sexual dimorphic steroid sensitive areas. Another interesting feature of this nucleus is that it provides

strong outputs to a large number of structures that in turn project to the VMH itself suggesting the existence of important feedback mechanisms.

VMH inputs are also partially segregated to the different subregions.

Lateral septum: the VMH receives projections from the intermediate and ventral subdivisions of the lateral septum and from the ventral divisions of the vertical limb of the diagonal bands (Fahrbach, Morrell et al. 1989). All these projections target only the VMHvl. Interestingly, the lateral septum, like the VMHvl expresses steroid hormones receptors and is part of the sexual dimorphic circuits suggesting an implication of this connection in the reproductive function. The only innervation, although very weak, to the VMHdm comes from the dorsal region of the ventrolateral zone of the rostral part of the lateral septum (Risold and Swanson 1997).

Bed nucleus of the stria terminalis: projection from this structure come mainly from the interfascicular nucleus that innervates all three subregions of the VMH (Fahrbach, Morrell et al. 1989; Comoli, Ribeiro-Barbosa et al. 2000).

Hypothalamus: at the preoptic level, projections from the medial preoptic nucleus target mainly the ventrolateral VMH while projections from the medial preoptic area target the whole VMH (Fahrbach, Morrell et al. 1989).

The strongest VMH hypothalamic input comes from the anterior nucleus that projects mainly to the dorsomedial portion of the VMH (Risold and Swanson 1997). Other very sparse hypothalamic inputs come from the paraventricular nucleus, perifornical portions of the lateral hypothalamus and dorsomedial hypothalamus.

Amygdala and hippocampus: the amygdala, amygdala hippocampal area and ventral subiculum represent the main source of afferents in the VMH. In particular the medial amygdala posterior ventral projects mainly to the VMHdm, providing only sparse innervation to the VMHvl while the MeApd projects exclusively to the VMHvl. Also the basmedial amygdala projects to the VMHdm and vl. In the hippocampal region the main projections come from the ventral subiculum and CA1.

Brain stem: The main source of projections in the brain stem comes from the parabrachial and peripeduncular nucleus (Bester, Besson et al. 1997).

2.2.5   Nr5a1, a specific marker for the VMH

The nuclear receptor 5a1(Nr5a1) or steroidogenic factor 1 (SF1) is an orphan nuclear receptor whose expression within the brain localizes selectively at the level of the dorsomedial and central portions of the VMH. Therefore it has been extensively used

as a marker to identify neurons belonging to this nucleus. In our study we have used Nr5a1 promoter to specifically target the VMHvl neurons. Several anatomical studies have shown an overlapping projections pattern from Nr5a1 expressing neurons and from the VMHdm and VMHc as a whole indicating that the vast majority of VMH projecting cells express Nr5a1 (Cheung, Kurrasch et al. 2013) .

Protein structure and putative ligands

Nr5a1 belongs to the nuclear hormone receptor family and in particular, together with its homologue Nr5a2 (also called LRH1), the Nr5a subfamily. These receptors are characterized by a modular domain structure comprised of an N-terminal zinc finger DNA-binding domain, a ligand binding domain, a C-terminal AF-2 activation domain and an intervening proline rich hinge region containing an AF-1 like activation domain. Nr5a1 also contains a fushi tarazu factor 1 box that mediates specific binding to sequences 5' to the consensus examer (Schimmer and White 2010). As determined by X-ray crystallography, Nr5a1 contacts the DNA as a monomer (Little, Zhang et al. 2006) by binding the major and minor grooves through the core DNA binding domain and the N-terminal segment of the A-box. The crystal structure of Nr5a1 indicates that the ligand binding domain is very large and very hydrophobic suggesting that the lipid environment regulates Nr5a1. In particular, Nr5a1 has been shown to bind sphingosine, which may serve as endogenous Nr5a1 ligands.

Pharmacological ligands compounds with a rigid cis-bicyclo(3.3.p)oct-2ene core structure selectively increase Nr5a1 activity (Whitby, Dixon et al. 2006; Whitby, Stec et al. 2011), while 4-alkyloxy-phenols derivatives act as inverse agonists. These compounds are particularly interesting since they could be used as modulators of the activity of NR5a1 expressing neurons in the VMH and tested for the treatment of fear related disorders like phobias or panic. Moreover they could act on peripheral Nr5a1 and be tested for steroid disregulation diseases like steroid hormone excess, steroid hormone-dependent tumors, obesity and related metabolic disorders.

Nr5a1 peripheral expression patterns and function

Consistent with its role in steroidogenesis Nr5a1 is expressed in steroidogenic tissues including the three zones of the adrenal gland cortex, testicular Leydig and Sertoli cells and ovarian interstitium, theca cells, granulose cells and corpus luteum. Nr5a1 KO mice do not form the adrenal gland and gonads suggesting a key role of Nr5a1 in the development of these structures. Interestingly, even heterozygous mice for the Nr5a1 KO allele show hypoplastic adrenal gland indicating that it may act in a gene

dosage dependent manner. In the adrenocortical cells Nr5a1 increases the expression of corticotropin receptor (McR2), STAR, scavenger receptor B2 (required for the cellular importation of high density lipoprotein cholesterol) and all the enzymes required for cortisol and corticosterone biosynthesis. As regards aldosterone biosynthesis, in the adrenal zona glomerulosa Nr5a1 downregulates aldosterone synthase thereby restricting its biosynthesis.

Analogous to its effects in the adrenal gland, Nr5a1 regulates a number of steroidogenesis factors also in Leydig cells, like the LH receptor, STAR, CYP11a1 and CYP17 required for testosterone biosynthesis. Moreover it increases the expression of various genes required for the development of the male reproductive tract such as insulin-like polypeptide 3 and AMHR2. In Sertoli cells it is required for the expression of testes determining gene products, sex determining region Y, SOX9, FSH receptor and INHA. Nr5a1 role in the ovary is less clear. In theca and granulose cells it regulates the expression of cytochome P450 steroid hydroxylases such as CYP11a1, CYP17 and CYP 19, the inhibin α subunit and steroidogenic acute regulatory protein, thus suggesting a role in steroid hormones biosynthesis.

NR5a1 central expression patterns and function

In the mouse brain Nr5a1 starts to be expressed at E9.5 at the level of secondary prosencephalon (Stallings, Hanley et al. 2002) and at E14 in the pituitary primordium. At later stages it is found in all three regions of the VMH and in gonadotropic cells in the anterior pituitary. Interestingly only at later stages Nr5a1 expression is confined in the VMHdm and central. This is particularly important to consider when NR5a1 promoter is used to drive Cre expression specifically in the dorsomedial portions (Bingham, Verma-Kurvari et al. 2006; Dhillon, Zigman et al. 2006; Tong, Ye et al. 2007).

In GnRH receptor-expressing cells in the anterior pituitary Nr5a1 regulates the expression of the α subunit of the glycoprotein hormones, the GnRh receptor and FSH β, suggesting a role of NR5a1 in gonadotropins production. NR5a1 pituitary specific knockouts have low gonadotropins levels and show severe hypoplasia of the gonads and external genitalia.

The main body of information about the role of the Nr5a1 in the brain comes from knockout studies in the mouse. Full Nr5a1 knockouts are not viable due to adrenal insufficiency; if rescued with adrenal transplant or corticosterone suppletion therapy

incomplete development of the VMH is observed. The same was reported for brain-specific knockouts. In particular Nr5a1 neurons are properly generated but they fail to migrate and form the VMH. These neurons do not develop the pattern of projections observed in wild-type mice, as shown by the lack of axons to the bed nucleus of the stria terminalis, and amygdala in Nr5a1 knockout mice (Tran, Lee et al. 2003). Interestingly, also neurons surrounding the VMHdm showed altered distribution of cell bodies. For example estrogen receptor α expressing cells are normally densely packed in the ventrolateral VMH whereas in Nr5a1 knockouts they are mainly located near the ventricle. This effect is specific for the VMH because ERα expressing cells in other nuclei like the arcuate are distributed normally. This suggests that the embryonic expression of Nr5a1 in the VMHvl may have a role in the migration of these cells as well.

Mice lacking Nr5a1 specifically in the brain have impaired regulation of energy homeostasis and female reproductive function (Kim, Zhao et al. 2009; Kim, Li et al. 2010) and increased anxiety like behavior. Nevertheless, due to the developmental role of Nr5a1 and the consequent misformation of the VMH it is difficult to infer the function of Nr5a1 in the adult brain. A recent study examined the role of VMH expressed-Nr5a1 in the regulation of energy metabolism using a postnatal brain-selective Nr5a1 knockout line. These mice have structurally intact VMH, but showed increased body weight, and impaired thermogenesis upon high fat diet (Kim, Zhao et al. 2011). In this study the authors did not assess if fear responses were impaired in these animals. In our study we showed that inhibition of the neuronal activity of Nr5a1 expressing neurons in the VMH impairs fear responses to predators, therefore postnatal Nr5a1 knock out animals would be a good tool to test whether or not this depends on the activity of the Nr5a1 gene product.

Only a few genes have been shown to be direct Nr5a1 targets in the VMH by functional assays or electrophoretic mobility shift assays. Among these the CRHr2, the cannabinoid receptor 1, BDNF and urocortin 3 genes have been identified, whose regulation might explain the energy metabolism deregulation in knockout mice. Moreover some other hypothalamic Nr5a1 target genes were indirectly identified by in situ hybridization in wild-type and NR5a1 knockouts at P0 (Kurrasch, Cheung et al. 2007). They include cell adhesion molecules like Amigo2, Cdh4, Sema3a, Slit3

and Netrin3 and other hypothalamic enriched genes such as Fezf1, Nptx2, NKx2-2, A2bp1, leptin receptor and BDNF (Kim, Sohn et al. 2011).

Nr5a1 promoter region

Due to its specific expression in the VMH the Nr5a1 promoter has been used as a genetic tool for the selective expression of exogenous constructs in this area. A number of mouse lines have been constructed with different fragments of the Nr5a1 promoter. However, only a few of them recapitulated the endogenous Nr5a1 expression pattern.

The Nr5a1 promoter region in the mouse does not have a TATA box but it contains some other regulatory elements including SRY (sex determining region Y) binding site, an E box, a CCAAT box and an Sp1/Sp3 site. Binding sites for GATA-4, WT1 and Lhx9 are present more upstream in the promoter region. Two additional Sp1/Sp3 sites situated at +10 and +30 also contribute to the activity of the promoter.

An enhancer was identified in intron 6 of the gene; this seems to be the main driver of VMH-specific expression. Structurally, the sequence is conserved among animal species (mouse, human and chicken), thus strongly indicating that the conserved sequence probably function as a VMH- specific enhancer among animal species (Shima, Zubair et al. 2005).

Several transgenic lines have been constructed using the Nr5a1 promoter region. Very short promoter fragments are insufficient to recapitulate all gene expression sites. Promoter fragments from -590 to +85 drive expression in the gonads but not in other areas. Even very large promoter fragments spanning from NR6a1 gene region located 150 Kbs upstream to the exon two failed to achieve full expression, lacking pituitary expression. Genesat has recently made a 201 Kbs BAC transgenic mouse line that seems to match, at least in the brain, the wt expression. In our study we used the same strategy and transgenic mice recapitulated the Nr5a1 endogenous expression in all central and peripheral tissues.

Nr5a1 evolutionary conservation

Nr5a1 is selectively expressed in the VMH throughout the whole brain; this expression pattern makes it the best genetic marker to define the VMH in mammals. The presence of highly similar orthologues of Nr5a1 and of other pan-hypothalamic markers like Nkx2.1 in the majority of vertebrate species would allow to identify VMH like neurons also in non-mammalian species and therefore to test if they have a conserved function over evolution. Since Nr5a1 neurons regulate fundamental

functions for the survival like feeding and defense, we hypothesize that they may have evolved very early and be conserved across a wide range of species.

Nr5a1 is a member of one of the seven nuclear receptor subfamilies, the *NR5A* family. *NR5A* is also called *Ftz-F1*, because it was first identified as the transcription factor that activates *fushi tarazu* (*ftz*) in *Drosophila*. This family has two members in the human and fly genomes, four members in the teleost fish and just one in *C. elegans*. The two orthologues of *NR5A1* in zebrafish, NR5a1a and NR5a1b probably arose by the duplication of the ancestral Nr5a1 gene in a genome duplication event that punctuated ray-fin fish evolution. Interestingly the expression profile for Nr5a1 orthologues in zebrafish displays the same restricted expression pattern in a subpopulation of hypothalamic neurons as in the mouse brain when compared to other hypothalamic markers like Nkx2.1 and fezF1 suggesting the presence of VMH like neurons in the fish as well.

Nr5a1 and human diseases

Several Nr5a1 null mutations in humans were described. In all cases the patients were heterozygous for the mutation and showed some sort of gonadal insufficiency. In many cases patient had complete 46XY sex reversal with strong hypogonadism and adrenal insufficiency. 46 XX patients were often diagnosed adrenal insufficiency and premature ovarian failure, suggesting that homozygous mutations may be lethal in humans. Moreover these mutations did not cause a dominant negative effect suggesting that, in humans, male gonad development and adrenal development in both sexes requires Nr5a1 expression in a dosage sensitive manner. Notably, patients harboring a mutation in Gly-146 exhibited obesity affecting insulin sensitivity and type II diabetes (Liu, Liu et al. 2006). In some cases mood disorders like anxiety and depression were reported in patients with Nr5a1 mutations. It is important to note that Nr5a1 expression pattern in the brain in human hypothalamus has not been reported. Northern blot analysis and RNA microarrays have shown the presence of Nr5a1 transcripts in adult human brain but in situ hybridization studies on human hypothalamus have not been performed (Ramayya, Zhou et al. 1997).

2.2.6   Role of the VMHvl in reproductive behavior and aggression

The VMHvl, together with the medial preoptic nucleus and the ventral premamillary nucleus is part of the hypothalamic sexual dimorphic circuits mediating reproductive behaviors and has been shown to play a crucial role both in females and males sexual

behavior. These nuclei are characterized by the expression of steroid hormones receptors and being highly interconnected within themselves.

Female sexual behavior

Strong evidences suggest that the VMHvl is one of the main regulators of female sexual behavior. Lesions of the VMHvl dramatically reduce lordosis behavior, while electrical stimulations facilitate the expression of lordosis in hormone primed-females (Malsbury, Kow et al. 1977). Sexual behavior in female rodents depends on a sequential exposure to the ovarian hormones estradiol and progesterone, and is triggered by sensory cues originating from a male (Pfaff, Montgomery et al. 1977). Neurons in the VMHvl express estrogen and progesterone receptors and receive pheromonal information from the vomeronasal organ through the medial amygdalar nucleus. Therefore the VMHvl is believed to be the site where sexual hormones exert their function in the regulation of behavior. It has been found that estrogen acts on different genes in the VMHvl to facilitate lordosis behavior. First, estrogen induces the nuclear progesterone receptor in the VMH (Blaustein, King et al. 1988). Administration of progesterone 24 or 48 hours after estrogen priming greatly amplifies the estrogen effect on mating, and this effect disappears after antisense DNA against progesterone receptor mRNA administered onto the VMH (Mani, Blaustein et al. 1994). Estrogen also induces the expression of noradrenergic α-1b and muscarinic receptors in the VMH (Petitti, Karkanias et al. 1992; Kow and Pfaff 1995), which regulate lordosis behavior (Kow, Weesner et al. 1992). These receptors are influenced by ascending noradrenergic or cholinergic paths, likely to signal heightened arousal upon stimulation from the male. In addition, growth in the VMH neurons may also be induced by estrogens, which stimulate the synthesis of ribosomal RNA, leading to dendritic growth and an increased number of synapses (Flanagan-Cato, Calizo et al. 2001). Collectively, estrogen-induced gene expression in the VMH provides the basis for increased synaptic activity and therefore increases the facilitation of sex-behavior output. Interestingly the neuronal excitability of ERα expressing neurons in the VMHvl is regulated by histamine, an important regulator of central nervous system generalized arousal (Zhou, Lee et al. 2007).

On the sensory side, the VMHvl is in a position to integrate pheromonal information relayed through the medial amygdalar nucleus, and sensory inputs arising from the vaginocervical stimulation that is relayed through the parvicellular part of the

subparafascicular thalamic nucleus (Coolen, Veening et al. 2003; Coolen, Veening et al. 2003). On the motor side, the VMHvl influences lordosis behavior through its projection to the periaqueductal gray (Canteras, Simerly et al. 1994; Flanagan-Cato, Lee et al. 2006). Bilateral lesions of the ventrolateral caudal periaqueductal gray inhibit lordosis (Lonstein and Stern 1998). The caudal ventrolateral PAG is a sensorimotor integration site for lordosis; it receives the somatosensory inputs necessary to elicit it, conveyed through the ventrolateral columns of the spinal cord, and descends to premotor sites in the medulla, particularly the nucleus gigantocellularis, which in turn projects to motoneurons that control the trunk musculature involved in postural alterations (Sakuma and Pfaff 1980; Salzberg, Lonstein et al. 2002; Normandin and Murphy 2008). The permissive signal sent by the VMH to the PAG is essential for timing the onset and duration of the period during which lordosis must be activated. However, the PAG is also known to have a role in controlling switches of adaptive behavioral responses (Sukikara, Mota-Ortiz et al. 2006), as in the present case, permitting lordosis while, at the same time, suppressing competing responses that otherwise would interfere with the execution of the lordosis behavior, such as defensive reactions and anxiety. An important conclusion from this information is that there is a large overlap in the hypothalamic neural systems that underlie the different kinds of reproductive and non-reproductive behaviors, and, at the moment, the critical question to be answered is how certain patterns of behavioral responses and not others, at particular points in time are selected in each situation. Hormonal and genetic factors are likely candidates to mediate such behavioral specificity, and to be responsible of the switch between the different hypothalamic mediated responses to different internal and environmental situations, but this question remains quite puzzling, and needs to be thoroughly addressed.

Male sexual behavior

The hypothalamic control of male sexual behavior has been classically centered in the MPN. This area expresses gonadal hormone receptor and integrates input from the medial amygdala carrying pheromonal information. Lesions in the MPN decrease female preference and female pursuit (Paredes, Tzschentke et al. 1998).

Nevertheless a recent study assigned an important role to the VMHvl in the regulation of sexual behavior in males too. Yang et al. ablated specifically progesterone receptor expressing cells in the VMHvl and observed a slight decrease in the number of

mounts, intromissions and ejaculations. Interestingly the ablation of the same neurons completely abolished female sexual behavior indicating that the same neurons in sexual dimorphic regions control the same sexual dimorphic behavior (Yang, Chiang et al. 2013). Moreover electrophysiological recordings in this nucleus showed an increase of neuronal firing in a small fraction of VMHvl neurons during mating in males. However optogenetic stimulation of the VMHvl neurons fails to induce mating like behaviors and only induces aggression (Lin, Boyle et al. 2011).

Aggression

The VMH was first implicated in aggression when electrical stimulations localized to the ventrolateral portion elicited fighting behaviors (Kruk, Van der Poel et al. 1983). In this study the VMHvl was part of the so called "hypothalamic attack area" that included parts of the lateral hypothalamus and tuberal nucleus as well. Recently Lin et al. investigated the VMH function in aggression using more sophisticated neuronal manipulation tools that could be addressed by a more restricted population of neurons. Lin et al. 2011 showed that only the stimulation targeted to the ventrolateral portion of the VMH was sufficient to induce fighting behaviors. Indeed optogenetic stimulation in this structure induced attacks even to female mice and to inanimate objects.

Interestingly, the same hypothalamic portion is implicated in sexual behavior; however, neurons activated during aggression seem to only partially overlap with neurons activated during mating. Interestingly, many aggression-activated VMHvl neurons are actively inhibited by the presence of a female, and a higher intensity of illumination was required to evoke attack towards a female during mating encounters. These data identify a neural correlate of competitive interactions between fighting and mating[i]. Whether this competition originates in VMHvl, or is controlled by descending inputs to this nucleus, is still unclear.

2.2.7   Role of the VMH in defensive responses

Evidence for the involvement of the VMH in the regulation of defensive responses mainly derives from c-Fos and neuroanatomical tracing studies. Indeed c-Fos activation at the level of the VMHdm has been reported in mice and rats upon predator exposure (Canteras, Chiavegatto et al. 1997; Martinez, Carvalho-Netto et al. 2008; Motta, Goto et al. 2009). On the other hand anatomical tracing studies have highlighted the strong connections of this nucleus with the nuclei belonging to the so-

called medial hypothalamic defensive system. This system consists of the anterior nucleus (AH), the VMHdm and the dorsal premammilary nucleus (PMD). These three nuclei are strongly interconnected and involved in the integration of innate defensive responses; however they are almost completely segregated from the rest of the medial hypothalamus. The medial hypothalamic defensive system integrates inputs carrying information of the threat through different sensory modalities. Within this system the VMH is thought to play a major role in the intergration of pheromonal stimuli deriving from predators given its strong inputs from the medial amygdala. Nevertheless, the VMHdm receives afferents also from the basomedial amygdala that conveys auditory and visual information, and may therefore play a broader role in the integration of predator cues.

C-Fos studies have indicated the involvement of the VMH in the processing of predator fear and its specific role can be inferred by its connections, however they do not demonstrate the necessity or sufficiency of this nucleus in fear processing. Only few studies have assessed the effect of selective VMHdm stimulation. Electrical, optogenetic or pharmacological activation of the VMH induced fear behaviors like escape, immobility and stress mediated analgesia (Freitas, Uribe-Marino et al. 2009) (Kruk, Van der Poel et al. 1983; Lin, Boyle et al. 2011). Interestingly, a study where a patient received deep brain stimulation in the VMH underwent a panic attack, suggesting that the VMH may play a similar role in humans (Wilent, Oh et al. 2010). Taken together this evidence indicates that VMHdm activation is sufficient to induce fear responses, however evidence that the VMHdm is necessary for the processing of such responses can be inferred only by inhibition of this nucleus during naturally induced fear, and selective VMHdm lesion studies have not been reported.

On the other hand c-Fos and neuoanatomical tracing studies have linked VMhvl to social fear. These studies have shown activation in this area in defeated animals (Motta, Goto et al. 2009) but functional studies to unravel its role in social fear have not yet be reported.

2.3.7 Role of the VMH in energy balance

Among several hypothalamic nuclei, the VMH was the first site that was recognized as a site for body weight and energy balance regulation (Hetherington, 1941). Since then, the VMH has remained site of interest for body weight regulation and glucose homeostasis (Rothwell and Stock 1979; Minokoshi, Saito et al. 1986; Amir 1990; Dhillon, Zigman et al. 2006; Bingham, Anderson et al. 2008; Klockener, Hess et al.

2011). It has been convincingly shown that VMH lesions directly impact body weight and food intake. The VMH is known to expresses receptors for signals denoting the energy status including leptin, insulin, melanin-concentrating hormone (MVH) and orexin (Storlien, Bellingham et al. 1975; Mercer, Hoggard et al. 1996; Trivedi, Yu et al. 1998; Kokkotou, Tritos et al. 2001); however before the advent of genetic manipulations the molecular and cellular mechanisms remained unclear. At the state of the art most of the data on VMH role in the regulation of energy homeostasis come from the specific manipulation of Nr5a1 expressing neurons. In particular people have taken advantage of Nr5a1::cre lines to delete specific genes in the VMH and unravel the molecular mechanisms at the basis of body weight regulation. These studies have indicated leptin as one of the major regulators of VMH function in energy homeostasis. Deletion of the leptin receptor in Nr5a1 neurons of the VMH (*Nr5a1::Cre, Lepr$^{flox/flox}$* mice) resulted in increase in body weight mainly due to decreased energy expenditure upon high fat diet. Moreover direct application of leptin into the VMH activated Nr5a1 expressing neurons (Dhillon, Zigman et al. 2006) and preferentially increased glucose uptake in skeletal muscle, heart, and brown adipose tissue, and this increased glucose uptake was impaired when the sympathetic nervous system was denervated, suggesting that leptin signaling in the VMH plays crucial roles in mediation of sympathetic tone from the VMH to peripheral tissues (Kamohara, Burcelin et al. 1997; Haque, Minokoshi et al. 1999; Minokoshi, Haque et al. 1999; Toda, Shiuchi et al. 2009). Investigators also used Nr5a1::Cre transgenic mice to examine the metabolic roles of several other genes thought to be associated with metabolic regulation. For example the specific removal of cytokine signaling-3 (SOCS3) a negative regulator of leptin action results in increased insulin sensitivity and improves glucose homeostasis (Zhang, Dhillon et al. 2008). Furthermore, both inhibition and activation studies of SIRT1 in Nr5a1 expressing neurons in the VMH, demonstrated the protective roles of SIRT1 against diet-induced metabolic imbalance (Ramadori, Fujikawa et al. 2011). Glucose also directs transcription of BDNF and TRKB in the VMH and BDNF induces neuronal activity in hypothalamic energy balance centers. BDNF knockouts in the VMH show increase in body weight. These mice have hyperleptinemia, hyoperinsulinemia and hyperglycemia due to increase in food intake (Wang, Bomberg et al. 2010).

Collectively, all the studies that impaired the function of VMH neuronal fraction that expresses Nr5a1 indicate that their main role in the regulation of body weight is the

regulation of energy expenditure and not food intake behavior. Accordingly, neuronal tracing studies showed that Nr5a1 neurons project to several sympathetic autonomic centers including the C1 catecholamine cell group in the ventrolateral medulla and the nucleus of the solitary tract (NTS); moreover it projects to the retrotrapezoid nucleus (RTN), which is important for the regulation of respiration (Cheung, Kurrasch et al. 2013).

On the other hand, non Nr5a1 expressing neurons in the VMH may play a role in the regulation of food intake behavior. Indeed, deletion of long form 3UTR BDNF in the VMH leads to hyperphagia and obesity in mice (Liao, An et al. 2012). Moreover, deletion of ERα in the entire VMH leads to hyperphagia and more profound obesity than that seen when ERα is deleted only in NR5a1neurons. Notably, both BDNF and ERα are abundantly expressed in the ventro-lateral area of the VMH (Musatov, Chen et al. 2007), where Nr5a1 is not expressed in the adult (Cheung, Kurrasch et al. 2013). Thus, it seems that topographically and genetically distinct neurons from Nr5a1 neurons may regulate food intake behavior. VMH neurons also play a role in glucose homeostasis, acting as a central sensor of glucose levels. The first evidence of glucose sensing function by VMH neurons came from directed chemical VMH lesions that exhibited impaired glucagon, epinephrine and norepinephrine responses against hypoglycemia (Borg, During et al. 1994) and local induced glucopenia around the VMH that resulted in increase in plasma glucose in association with elevation of glucagon, epinephrine and norepineohrine. Moreover several electrophysiological studies demonstrated the capability if VMH neurons to sense glucose levels. In addition, the VMH expresses insulin receptors and insulin receptors VMH-specific knockouts (SF-1$^{\Delta IR}$) exhibit improved glucose metabolism and resistance to high-fat diet, and, interestingly, increased cellular activity of POMC neurons (Klockener, Hess et al. 2011).

2.3   Investigating the role of the medial hypothalamus in fear: outstanding questions

The hypothalamus is the brain structure designated for the integrated regulation of behavioral, hormonal and autonomic responses of functions that are necessary for the survival of the individual and of the species like feeding, drinking reproduction and defense. The hypothalamus is divided into three anatomically and functionally separated zones: the periventricular zone that regulates the endocrine function, the

medial zone that regulates the behavioral and autonomic responses and the lateral zone that has a less clear function probably regulating general arousal. Studies on the hypothalamic function have mainly focused on feeding and reproduction and, although a set of specific nuclei located in the medial zone have been shown to play a key role in fear, the hypothalamic role in fear modulation is poorly understood because fear circuits have been classically centered in the amygdala.

For this reason we decided to further investigate the hypothalamic neural circuits of fear. In particular, the vast majority of the studies that have investigated these circuits were based on neurotoxic lesions of anatomically defined brain areas coupled with c-Fos brain activation maps. These techniques do not allow the selective manipulation of the neuronal activity of genetically defined populations of neurons. Therefore we decided to apply novel pharmacogenetic manipulation technologies (Armbruster, Li et al. 2007) to better understand the contribution of different hypothalamic neuronal populations in fear. In particular we have decided to focus on one specific hypothalamic nucleus belonging to the medial zone, the ventromedial hypothalamus.

This nucleus has been implicated in fear by c-Fos studies in mice and rats exposed to predators, but functional evidences of its necessity in fear are lacking. Among all the hypothalamic nuclei we decided to target the VMH for the pharmacogenetic functional manipulations because of its unique features that suggested that it could serve us as a model for the more general medial hypothalamic mechanisms in this and other functions. These unique features include: its peculiar connectivity, the existence of unique genetic markers, its evolutionary conservation, and its crucial role in other functions like feeding, sex and aggression. The connectivity is particularly interesting because it receives and sends projections to areas recruited during social and predator fear; receiving inputs from sensory processing areas like the medial amygdala and sending inputs to both downstream and upstream targets like the amygdala, thalamus and periaqueductal grey. The existence of a genetic marker like Nr5a1, whose expression is restricted to the VMHdm throughout the whole brain, is particularly useful to drive the expression of exogenous constructs for the pharmacogenetic manipulation. In terms or evolutionary conservation this nucleus is present from fish to humans where targeted electrical stimulations have been shown to elicit panic attacks (Wilent, Oh et al. 2010), suggesting possible translational implication to human health. Importantly this is not the case for all hypothalamic nuclei; for example the PMD, which plays a crucial role for predator fear in rodents, is not

present in the human brain. Another reason that made us focus on the VMH is its implication in other fundamental functions like feeding sex and aggression that suggest that it is a multi-modal hub of hypothalamic integration and the understanding of its microcircuitry can be applied to a general model of hypothalamic integration.

The other fundamental question that we wanted to address in our study was whether different brain circuits account for fear to different threats or if there is one single fear circuit that processes the responses to multiple threats. We considered the VMH an ideal structure to address this question since previous studies have reported non-overlapping c-Fos activation within the VMH when mice were experiencing predator or social fear (Motta, Goto et al. 2009). Therefore we took advantage of the newly developed selective pharmacogenetic manipulation tool in order to selectively inhibit the neuronal activity of one or the other neuronal populations during fear to different threats and investigate if they are functionally dissociated.

3 Functional architecture of the periaqueductal grey

In our study we demonstrated that two functionally dissociated populations of neurons in the VMH are required for social and predator fear. We subsequently investigated if this functional dissociation was maintained one step beyond in the fear circuit, at the level of the main VMH downstream target, the periaqueductal grey (PAG). The PAG is believed to be the motor generator structure responsible of the actuation of fear responses. Ours and other's studies suggest that the VMH is the structure responsible of the generation of the mental state of fear; on the other hand, the PAG generates the behavioral outcomes of a given mental state. We showed that this behavioral outcome is very similar when fear is induced by different threats therefore we hypothesized that the PAG could represent the common downstream target of the different fear circuits.

3.1 Morphological organization of the periaqueductal grey

The periaqueductal grey is a cell-dense region located in the area surrounding the cerebral aqueduct at the level of the midbrain. It contains small- to medium-sized, fusiform-, triangular- and stellate-shaped neurons, whose soma and axons are located normally in a rostro-caudal direction. PAG neurons utilize glutamate, GABA, enkephaline, substance P, neurotensin, neurikinin-1 and other neurotransmitters. On the basis of different functional specialization, the PAG can be divided into four

longitudinal columns: dorsomedial, dorsolateral, lateral and ventrolateral. According to this parcellation defensive behaviors and aversion related responses are ascribed to the dmPAG, dlPAG and lPAG, while quiescent behavior and opioid mediated analgesia are attributed to the vlPAG (Swanson 2004; Feinstein, Adolphs et al. 2011). The PAG columnar subdivision can also be defined on neurochemical basis. In particular, the dlPAG stains specifically for NAPH diaphorase, nitric oxide sintase, cholecistokinin, acetilcholinesterase and metenkephalin, but not for other neurochemicals such as glycine-transporter and cytocrome oxidase that are enriched in the dm and lPAG. Among these the dorsolateral columns neurons expressing nNOS are thought to be important for the regulation of antipredator defensive responses, and the ventrolateral column contains a group of dopaminergic neurons, known to be crucial for the adaptive switch between different adaptive behaviors (McGregor, Adamec et al. 2005).

3.2 Anatomical connections

3.2.2 Inputs to the PAG

The PAG receives projections from the forebrain, brainstem and sensory neurons; these projections preferentially target different PAG subregions. The major forebrain input to the PAG comes from the prefrontal cortex, with the caudal prelimbic and anterior cingulated cortex preferentially targeting the dorsolateral column, and the rostral prelimbic cortex terminating predominantly the ventrolateral one (Dielenberg and McGregor 2001). Prefrontal afferents including infralimbic, prelimbic, anterior cingulated and secondary motor areas also represent the most important sources of projections to the rostrolateral and dorsolateral PAG. The function of prefrontalcortical inputs to PAG is poorly understood but people hypothesize that it might play a role in the initiation of behavioral responses.

The inputs from the amygdala mainly originate from the medial part of the central nucleus and target the ventrolateral and rostrolateral columns. These projections have been shown to play a major role in the induction of freezing behavior upon foot shock fear conditioning, where indeed the central nucleus of the amygdala has a pivotal role (LeDoux, Iwata et al. 1988).

The PAG receives strong inputs from the hypothalamus and in particular from the hypothalamic substructure responsible of the modulation of basic behavioral

responses, the hypothalamic medial zone. In particular, the dorsolateral PAG, which is mostly involved in the regulation or antipredator defensive responses, is mostly innervated by hypothalamic sites involved in processing of predatory cues including the dorsomedial part of the ventromedial nucleus and dorsal premammillary nucleus. In contrast, the lateral and ventrolateral columns of the PAG receive inputs from the lateral hypothalamus that may be GABAergic and involve neurotensin (Korte 2001) and these projections are probably associated to the regulation of food intake. Notably, also the superior colliculus has a differential pattern of connection to the PAG, where the lateral part of the intermediate layer, which have been associated to exploration, projects to the lateral PAG and the medial part of the intermediate and deep layers, that are involved in predator escape responses, target the dorsal PAG. These anatomical evidences support the idea of a functional specialization of the different PAG subcolumns, with the dorsal portions regulating active defense responses and the lateral and ventrolateral portions regulating exploration and quiescent behaviors.

The PAG also receives dense noradrenergic and adrenergic projections from the ventrolateral and dorsomedial medulla. In terms of both somatic and visceral sensory inputs, the PAG is innervated by neurons in the spinal cord, medullary dorsal horn and nucleus of the solitary tract. These inputs are directed to the controlateral PAG mainly targeting the lateral and ventrolateral columns and are probably very important for stress-mediated anagesia (Mongeau, Miller et al. 2003).

3.2.3 Outputs from the PAG

The PAG shows a very wide pattern of projections spanning from the forebrain, all the way to the brainstem and spinal cord (Cameron, Khan et al. 1995). Forebrain projections target the thalamus and the hypothalamus with a specific patterning depending on the different columns. In particular the lateral hypothalamic area, region involved in hypotension and bradycardia, is selectively targeted by the ventrolateral column, whereas dorsal and meidal hypothalamic areas, which are involved in hypertension, tachycardia and somatomotor activation, receive inputs from the lateral and dorsolateral columns. The PAG projections to the thalamus likely serve as gateway to the prefrontal cortex, amygdala and basal ganglia, with the ventrolateral column providing the heaviest inputs to the thalamus, specifically to the centromedial, centrolateral, intermediodorsal and paraventricular nuclei. The dorsolateral PAG, in

contrast, projects predominantly to the paraventricular thalamic nucleus. Interestingly the paraventricular thalamus is heavily connected to the VMHdm and all these three regions show strong c-Fos activation upon predator exposure.

The projections to lower brain stem nuclei are believed to be the direct substrate of PAG-mediated somatomotor, cardiovascular and nociceptive adjustments. The ventrolateral, dorsomedial and lateral PAG broadly target the same ventromedial and ventrolateral medullary regions. Nevertheless there is good evidence that dorsal and venrolateral PAG columns elicit opposite physiological effects probably modulating different cellular populations in the medullary regions or using different neurotransmitters. In contrast, the dlPAG has no direct projections to the medulla; instead, it strongly targets the cuneiform nucleus, region from which active defensive behaviors like freezing, flight and hypertension are evoked, and to the superolateral parabrachial nucleus, a region innervating the retrochiasmatic and ventromedial (dorsomedial division) hypothalamic nuclei.

3.3   PAG function

The PAG is believed to mediate the motor output of a number of basic behavioral responses spanning from predator defense to reproductive behaviors, to stress mediated analgesia and maternal behaviors. Recent studies indicate also an integrative role of the PAG in influencing the selection of different adaptive behavioral responses. Moreover the presence of ascending connections from the PAG suggests that it is not simply a final path for behavioral outcomes but may play a role in the coordination and memory formation of events related to these behaviors.

3.3.2   Defensive responses

Since Hunsperger (1963) the PAG has been viewed as the final common path for all defensive responses. Such assumption came from the evidence that fear responses elicited by the stimulation if the amygdala or the hypothalamus can be reversed by PAG lesions but not the other way around (Hunsperger et al 1963). All the PAG columns show increased c-Fos activation after exposure to a predator (Yang, Augustsson et al. 2004), or to its smell (Yang, Farrokhi et al. 2006), after re-exposure to a conditioned aversive context (Blanchard, Griebel et al. 1998) or after electrical stimulation in the medial hypothalamus (Silveira, Sandner et al. 1995) and dorsal PAG (Silveira, Graeff et al. 1994). Later experiments based on electrical stimulations

lead to the conception that the different PAG columns mediate different aspects of the defensive responses. The dorsal PAG is thought to mediate active defense, including escape and freezing associated with muscular tension, tachycardia, hypertention, hypervigilance and hyperreactivity. The sympathoexcitatory responses elicited by lateral and dorsolateral PAG are mediated by neurons of the rostral medulla, which activate sympathetic preganglionic cells. In contrast, vlPAG appears to mediate passive immobility associated with bradycardia, hypotension and hyporeactivity to the environment. The type of freezing associated with the vlPAG has been considered a kind of imposed quiescence characteristic of the recovery component of the defense recuperative process following injuries. The vlPAG, controlled by medial amygdalar projections, is thought to modulate merely the motor aspects of this inhibition of behavior. On the other hand, the dPAG appears to be contolling sensory and affective aspects of active defensive strategies (Antoni, Palkovits et al. 1983; Brandao, Coimbra et al. 1990; Ribeiro-Barbosa, Canteras et al. 2005; Pardo, Alcaraz et al. 2013). Interestingly, upon dPAG stimulation at increasingly intensities alertness and freezing appear before escape, suggesting that a certain level of processing of aversive information is also occurring at this level. The PAG has also been related to panic disorder in human. Functional magnetic and positron emission tomography studies showed that the close proximity of a predator and lactate-induced panic were associated with PAG activation (Mobbs, Yu et al. 2010; Hermans, Henckens et al. 2012). Moreover, patient that received electrical stimulations in the PAG reported fear and the sensation of being chased (Amano, Tanikawa et al. 1982). Interestingly, active defensive responses mediated by dPAG seem to be modulated by serotonin. Both excitatory and inhibitory local interneurons in the dPAG exert a dual control on output neurons. Serotonin seems to have an inhibitory effect on output neurons by activating GABAergic interneurons via 5-HT2 receptors and activating excitatory ones via 5-HT 1a receptors.

3.3.3 Pain modulation

The PAG is part of the descending network modulating pain perception. Such system includes the prefrontal and anterior cingulated cortex, hypothalamus, amygdala, dorsolateral pontine reticular formation, rostral ventromedial medulla and caudal ventrolateral medulla and acts through excitatory and inhibitory projections on nociceptive transmission in the dorsal horn and trigeminal nucleus (Dugger, Morris et

al. 2007; Griffin and Flanagan-Cato 2009). The descending pain modulation system is known to act dynamically inhibiting or facilitating nociception depending on different behavioral, emotional and pathological states (Shimada and Nakamura 1973). Experimental studies have shown that the role of the PAG in the system is inhibition of pain perception. In particular different PAG columns act together and inhibit dorsal horn neurons that relay information carried by C-fibers, with the dorsolateral and lateral PAG mediating sympathetic excitation and non-opioid analgesia upon short lasting skin stimulation, and he ventrolateral PAG eliciting long lasting opioid dependent analgesia associated with vasodepression and immobility upon somatic, visceral or repetitive superficial pain (Altman and Bayer 1986; Cheung, Kurrasch et al. 2013). The PAG exerts it pain modulatory effect mainly through glutamatergic projections to the rostral ventromedial medulla including the raphe and noradrenergic nuclei of pontine tegmentum. Such PAG glutamatergic projection neurons undergo tonic inhibition by local GABAergic interneurons, which are the site of modulation by opioids, endocannabinoids and neurotensin. µ-opioids agonists inhibit local GABAergic interneurons, endocannabinoids such as anandamide tonically control nociception via activation of TRPV1 receptors expressed in the ventrolateral PAG, neurotensin induced the release of endocannabinoids that, in turn, inhibit GABAercic interneurons via CB+ receptors activation (Kim, Zhao et al. 2011).

3.3.4 Other functions in behavioral control

Besides its roles in the modulation of defensive behaviors and nociception the PAG has been implicated in the control of a number of other behavioral responses. In particular it seem to act as a motor generator relay station between the forebrain and brain stem and spinal cord structures often modulating both autonomic and motor outcomes. Recent studies have also suggested an integrative role of the PAG in influencing the selection of adaptive behavioral responses.

The PAG has a critical role in **vocalization** in response to painful stimuli or other stressors. The lateral and ventrolateral columns integrate the expiratory and laryngeal activity required for vocalization through connections with the medullary reticular formation (Storlien, Bellingham et al. 1975). Lesions in the PAG produce mutism both in humans and in experimental animals (Mercer, Hoggard et al. 1996).

The PAG also acts as relay motor output station for **sexual behaviors** integrating inputs from the hypothalamic medial zone reproductive system and projecting to the

medullary reticular formation. Such projections involve the caudal ventrolateral PAG and have been shown to be particularly relevant for the production of lordosis behaviors in females.

The ventrolateral PAG also acts as an interface between bladder afferent input and forebrain modulatory influences controlling **micturition**. It receives Aδ afferents from the bladder and relays this information to the pontine micturition center (Trivedi, Yu et al. 1998).

The ventrolateral PAG also participates in mechanisms of arousal and switch between non-REM and REM **sleep**.

The PAG also shows C-Fos activation upon **aggression, social defeat, maternal behavior and predatory hunting,** however the neuronal mechanisms underling these behaviors are poorly understood. Recent studies indicate that the PAG may play a role in the selection of the most suitable adaptive behavioral responses. For example Miranda-Paiva et al., 2003 have shown that the PAG regulates the opiate mediated inhibition of maternal behavior in the presence of a predator, mediating the switch to defensive behaviors (Kamohara, Burcelin et al. 1997).

3.3.5 Investigating the role of the PAG in fear: outstanding questions

The PAG is the final behavioral generator structure of a number of different functions spanning from reproduction to defense. However, the detailed neural mechanisms underlying such different functions are largely unknown. Very little is known about the contribution of different cell types in this region as well as about the local PAG microcircuitry. In particular, the PAG plays a major role in the generation of fear responses to different threats, but if distinct neuronal populations are involved in responding to the different threats or one unique set of neurons promotes all fear responses regardless of the threat remains unknown. To address this question we took advantage of our novel behavioral paradigm where mice display very similar fear responses to predators, foot shock and aggressive conspecifics, and specifically inhibited the dPAG via local viral delivery of the inhibitory pharmacogenetic receptor hM4D (Armbruster, Li et al. 2007). This approach allows dissecting the role of a specific PAG region in fear of different threats.

AIM OF THE PROJECT

1. Summary of specific aims

The neural circuits at the basis of fear were mainly studied in rodents using paradigms that induce fear with an electrical foot shock. These studies have led to the identification of a fear circuit having as central core the amygdala. Despite their extensive relevance to human fear, the neural basis of fear induced by other more naturalistic threats like predators or aggressive members of the same species remain poorly understood. In our study we aimed to provide a deeper insight into the neural circuits underlying fear to these threats with a particular focus on the role played by the hypothalamus in the processing of such emotional responses. Moreover we were interested in understanding if fear is processed by a unique circuit that can be activated by the presence of different threats or if separate circuits underlie fear to different threats.

1.1. Development of a novel behavioral paradigm for the systematic comparison of defensive responses to different threats in the mouse.

In order to be able to study the neural basis of fear to different threats we needed a reliable behavioral test where defensive responses induced by predators, electrical foot-shock or aggressive conspecifics could be compared side by side in the exact same behavioral set-up. In fact fear to different threats in the past has only been tested by different groups in very different experimental setups where mice display different behaviors. This makes it impossible to tell whether the potential activation in independent brain regions is due to actual dissociated fear circuit or if it simply reflects differences in the behaviors elicited.

1.2. C-Fos mapping of the neural activation pattern in the mouse brain following the exposure to different threats.

C-fos mapping studies have shown differential brain activation upon fear to different threats. In particular the medial hypothalamus resulted to be strongly activated after predator and conspecific fear but not after foot shock-induced fear. Intriguingly predator and conspecific exposure seemed to activate non–overlapping nuclei in this area. Unfortunately, these c-Fos studies were performed by different research groups using very different behavioral setups where mice display different behaviors. Therefore the observed differences in brain activation could simply reflect the

differences in the behavioral responses. For this reason we needed to confirm in our newly developed behavioral test, where mice show comparable defensive behaviors, the previos reported c-Fos data.

1.3. Generation and validation of a BAC transegenic mouse expressing an inhibitory pharmacogenetic tool (hM4D) under the control of a promoter expressed exclusively in the VMHdm.

In order to test the hypothesis that independent brain circuits underlie fear to different threats we needed a tool to selectively and reversibly inhibit a nucleus necessary for one type of fear and not the others. We decided to target the ventromedial nucleus of the hypothalamus because it showed strong C-Fos activation selectively upon predator exposure. Moreover this nucleus was particularly interesting because it is present in the human hypothalamus as well and more importantly it has been related to fear in humans too.

The VMH is particularly interesting also because it regulates other functions related to the survival like feeding and regulation of energy expenditure. Therefore we thought that developing a tool for the selective manipulations of these neurons could be very useful for the more broad understanding of goal oriented behaviors hypothalamic control.

1.4. Development and validation of inhibitory viral vectors for the pharmacogenetic manipulation of other brain areas.

A pharmacogenetic inhibitory tool that could be delivered to the other nuclei involved in fear circuits was needed to prove the double dissociation of predator and social fear circuits. Our strategy is to use the same inhibitory pharmacogenetic tool hM4D (Armbruster et al. 2008) and to deliver by adeno associated viral vectors (AAV).

1.5. Selective pharmacogenetic inhibition of the VMHvl

The ventrolateral portion of the VMH (VMHvl) was shown in our and other's studies to be C-fos activated upon social fear. Therefore we thought it could be a good target to demonstrate the double dissociation of social and predator fear. Indeed if the selective inhibition of this structure impairs social but not foot shock or predator fear

it implies that independent neuronal populations in the same nucleus are responsible of the processing of fear to different threats.

1.6. Selective pharmacogenetic inhibition of the PAG

The medial hypothalamus is considered as an integration center in the processing of fear responses. It is thought to integrate the information from various sensory processing areas and activate downstream structures that are responsible of the production of an organized behavioral outcome. The first crucial motor initiator center in the fear circuit is considered to be the periaqueductal grey (PAG). Our aim was to investigate if neurons involved in fear to different threats were functionally dissociated at the level of the PAG as at the hypothalamic level. In order to specifically target a specific portion of the PAG we could take advatage of our newly developed viral tool for the local targeting of the inhibitory receptor hM4D.

RESULTS

1. Results

1.1 Development of a novel behavioral paradigm for the systematic comparison of defensive responses to different threats in the mouse

We developed a behavioral test in which similar patterns of fear behavior are elicited in mice by exposure to either a predatory rat, an aggressive mouse, or an electric foot shock (**Figure 1**). The experimental apparatus consists of two chambers connected by a narrow corridor. The experimental subjects were habituated to the apparatus for three days. On the fourth day the experimental mice were confined in the stimulus chamber and briefly exposed to a predatory rat, an aggressive conspecific or to a foot shock and then allowed to escape to the other chamber where defensive behaviors (immobility, flight, stretch postures, locomotion) were recorded. This strategy allowed us to score defensive behaviors induced by different threats in the exact same environment. We thought this was crucial because the environment where the threat is encountered has been shown to determine the behavioral outcome (See introduction). On the following day mice were re-exposed to the apparatus in the absence of the threat and defensive behaviors were scored as a measure of contextual fear. Mice showed a significant increase in stretch postures, immobility, and flight and decrease in locomotion following exposure to all threats when compared to their behavior during habituation. As a control mice were exposed to a toy rat. This did not elicit increases in stretch postures, immobility or flight, but did result in a significant decrease in locomotion ($P < 0.0001$) following acute exposure, suggesting that some of the decreased locomotion to threat is a result of the novelty of the stimulus. These data validate our test as a robust method to examine similar fear responses to foot shock, predator, and social threat.
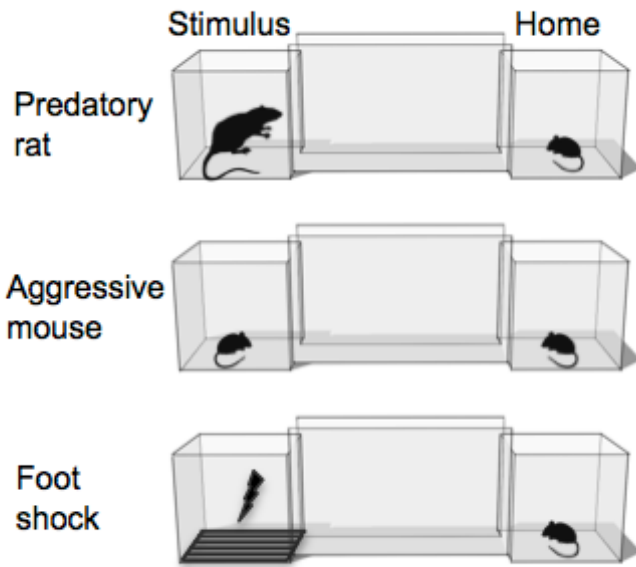
Figure 1. Experimental apparatus. The behavioral testing apparatus consisted of two chambers connected by a narrow corridor. An experimental mouse was continuously housed in one chamber (Home) and allowed to freely explore the corridor and second chamber (Stimulus) once daily for 20 minutes. At the end of the free exploration period on the fourth day, the door to the stimulus chamber was briefly closed to confine the mouse which was then exposed to either a predatory rat (Predator, < 5 s), aggressive mouse (Social, 10 min), electric foot shock (Foot shock, 1 min, 4 x *0.5 s, 0.5 mA), or toy rat (Fake rat, < 5 s) after which free exploration continued for an additional 10 minutes*

1.2 C-Fos mapping of the neural activation pattern in the mouse brain following fear to different threats

In order to investigate whether fear to predators, aggressive conspecifics or foot shock activates non overlapping brain areas under comparable testing conditions where the mice exert similar fear behaviors, we performed c-Fos mapping in our behavioral test. We found C-Fos induced in distinct regions at the level of the hypothalamus as reported in previous studies. In particular the dorsomedial portion of the VMH, an area reported to be important for regulation of energy metabolism, was selectively activated by predator exposure whereas the ventrolateral portion, known to play a key role in sex and aggression, was selectively activated by dominant conspecifics exposure. On the other hand the VMH was not activated by foot shock. Our data demonstrate that the medial hypothalamus is selectively recruited during predator and social fear, and that similar fear behaviors recruit different brain circuits. We also examined c-Fos activation at the level of the main output area of the VMH, the periaqcuiductal gray (PAG). Here we found a partial overlap of activation by different types of threats. This could be explained by the fact that the circuits processing different types of fear are independent at the level of the hypothalamus but they then converge at the level of the PAG where they give rise to similar behavioral patterns.

A second explanation is that the circuits are still functionally dissociated but they are not anatomically separated. Simple C-Fos immunohistochemistry does not allow testing these two hypotheses.

1.3 Generation and validation of a BAC transegenic mouse expressing an inhibitory pharmacogenetic tool (hM4D) under the control of a promoter expressed exclusively in the VMHdm

To determine whether VMH harbors functionally independent circuits for predator and social fear, we used the hM4D–clozapine-*N*-oxide (CNO) pharmacogenetic neural inhibition tool (Armbruster, Li et al. 2007) to rapidly and selectively inhibit neurons in VMHdm. Stable expression of hM4D in VMHdm neurons was achieved by constructing transgenic mice in which *hM4D* was driven by the *Nr5a1* gene promoter (*Nr5a1*::*hM4D-2A-TomatoF)*. We designed a bicistronic construct with the HA tagged hM4 derived DREADD (Armbruster, Li et al. 2007) followed by a membrane-bound fluorescent protein (farnesylated tomato) exploiting the viral P2A sequence. The cassette was inserted in a BAC under the control of the VMHdm specific NR5a1 promoter. Two founders were obtained. Both lines transmitted and showed specific expression of fTomato in VMHdm cells. We subsequently mapped the projections of the fTomato expressing neurons that proved to overlap with what was described in anatomical studies performed in the rats using anterograde tracers injected in the VMHdm (Canteras, Simerly et al. 1994). This indicates that NR5a1 neurons projections pattern recapitulates the one of the whole VMHdm. For all the further studies we picked the line with higher expression levels. The specific expression of the hM4D was checked by co-immunofluorescence against the HA tag and NR5a1. Expression of HA-hM4d was found selectively in the VMHdm NR5a1 expressing neurons.

Finally, to check the efficiency of neuronal activity inhibition by CNO injection we performed slice elecrophysiological studies from transgenic and non-transgenic animals in collaboration with Emanuele Murana and Davide Ragozzino at La Sapienza University of Rome. Infusion of CNO induced a significant decrease in spontaneous firing and membrane potential of *Nr5a1*::*hM4D-2A-TomatoF* neurons but not of wt animals. Taken together, these evidences validate our line as a robust method for the selective inhibition of VMHdm neurons.

1.4 Development and validation of inhibitory viral vectors for the pharmacogenetic manipulation of specific brain areas.

In order to be able to specifically inhibit brain nuclei other than the VMHdm we designed a viral vector carrying the coding sequence for inhibitory pharmacogenetic tool hM4D. Our construct includes also a fluorescent protein for the fast and easy detection of infected cells. Briefly the cassette contains the coding sequences for Venus and hM4d separated by a viral P2A sequence under the control of the synapsin promoter to ensure expression of the cassette only in neurons. The hemagluttinin-tagged hM4D (Armbruster, Li et al. 2007) sequence (HA-hM4D) was excised from pcDNA-5FRT-HA-hM4D (gift of B. Roth, University of North Carolina, Chapel Hill, NC). The viral P2A (Szymczak, Workman et al. 2004) sequence was inserted between Venus and hM4D to produce separate peptides from a single open-reading frame. The Venus-P2A-HA-hM4D cassette was cloned so as to replace the open reading frame of pAAV-Syn-NpHR3.0-EYFP-WPRE (gift of K. Deisseroth, Stanford University, Palo Alto, CA). Production and purification of recombinant AAV (chimeric capsid serotype 1/2) were done in collaboration with Valery Grinevich Schaller Research Group on Neuropeptides, German Cancer Research Center DKFZ) as described (Pilpel, Landeck et al. 2009).

1.5 Pharmacogenetic silencing of different portions of the VMH during fear to different threats.

In order to test whether the VMH is necessary for predator and social fear and to unravel if they are processed by functionally independent circuits we used the hM4D/CNO pharmacogenetic neural inhibition tool to rapidly and selectively inhibit neurons in VMHdm and VMHvl in behaving mice. For VMHdm inhibition we used the *Nr5a1::hM4D-2A-TomatoF* transgenic mouse line. Systemic treatment with clozapine-N-oxide (CNO) induced a significant decrease in defensive responses to predators but not to dominant conspecifics or to foot shock. In order to inhibit the VMHvl we stereotactically delivered an Adeno-Associated Virus (AAV) carrying the same pharmacogenetic inhibitory tool hM4D. Systemic treatment with CNO caused a selective reduction in social fear but not in predator and foot shock fear.

These data demonstrate that the VMH is necessary for predator and social fear responses and that it harbors functionally independent circuits. Interestingly both portions of the VMH were previously implicated in very different functions such as

feeding, aggression and sex. This, together with its role in fear processing, indicates that the VMH is a multi-modal node for motivated behavior.

1.6 Pharmacogenetic silencing of the dorsal peracqueductal grey.

The PAG is the main target structure of the VMH and is commonly considered the output area where organized behavioral patterns are triggered. We expressed the hM4D in the dorsal PAG via AAV stereotactic delivery and systemically injected the CNO in our behavioral paradigm. CNO treated mice showed a decrease in fear responses to predators and preliminary results indicate a decrease in the defensive responses to dominant conspecifics but not to an electrical foot shock. This suggests that predator and conspecific fear circuits are not anatomically separated at the level of the PAG but foot shock fear is. Our findings don't exclude that they are functionally separated but anatomically intermingled. To address this question we will target the hM4D to specific cell types within the PAG injecting a Cre dependent virus in mouse lines that express CRE under the control a of specific genetic markers such as NOSI.

CONCLUSIONS AND FUTURE PROSPECTS

1 Conclusions

1.1 The VMH is necessary for social and predator fear

C-fos studies have shown selective activation of the VMHdm and VMHvl upon predator and social fear respectively. Nevertheless these studies have two major limitations. First, they were conducted in different experimental setups where the animals showed different behavioral responses that could account for the differential activation. Second, c-Fos studies only provide correlative information but they do not tell s anything about causality. In our study we developed a new behavioral paradigm where fear responses to predators, aggressive conspecifics and electrical foot shock were comparable. Using our paradigm, where mice exhibit very similar defensve behaviors, we confirmed previous C-fos studies and showed that the VMHdm and the VMHvl are activated upon predator and social fear respectively. Notably, the VMH was not recruited by foot shock fear indicating that this may be processed by an independent circuit in the brain. In order to address the second point and provide functional evidence for the necessity of the VMH in fear processing, we selectively and reversibly inhibited these neurons and tested fear responses in our behavioral test. Our results provide the first evidence that the VMH, a hypothalamic structure previously implicated in feeding, sex and aggression, is necessary for social and predator fear. C-fos and functional activation studies had indicated the implication of the VMH in the regulation of predator fear responses but its necessity in such process had not been demonstrated. Social fear is extremely relevant in humans and dysfunctions in fear processing may account for several forms of pathological fear. Nevertheless, the neural basis of this type of fear are poorly understood and behavioral tests to model it in rodents are not well established. In our study we established a reliable behavioral paradigm to study social fear and provide the first evidence of VMHvl role in the processing of such emotion.

1.2 Social and predator fear circuits are functionally dissociated at the level of the hypothalamus

Through the selective expression of the pharmacogenetic inhibitory tool hM4D obtained through transgenics targeting or stereotactic viral delivery we managed to selectively inhibit the two different portions of the VMH, namely the dorsomedial (dm) and ventrolateral (vl) parts. The selective inhibition of these two cellular populations during fear to different threats allowed us to demonstrate that the VMH

processes social and predator fear through two distinct non overlapping neuronal populations indicating that these two types of fear are functionally dissociated at the level of this nucleus. Anatomical tracing studies combined with C-fos activation studies suggest that these two circuits are functionally dissociated also in the structures located upstream to the VMH. In particular the main VMH inputs come from two different portions of the medial amygdala, namely the posteroventral portion that is c-Fos activated by predator smell and projects to the VMHdm, and the posterodorsal portion that is activated by conspecific smell and projects to the VMHvl. The idea that social and predator fear are processed by functionally dissociated circuits carries with it important implications for the treatment of pathological fear related diseases in humans. These disorders are extremely heterogeneous, spanning from post traumatic stress disorder to specific phobias or panic disorder (see introduction) and they are characterized by the lack of effective therapies. Our finding suggest that fear in humans may come in different flavors and opens the possibility of targeted therapies for pathological fear.

1.3 Social and predator fear are not dissociated at the level of the PAG

Our and others' findings demonstrate that social and predator fear are functionally dissociated at the level of the hypothalamus and its inputs however, less clear is whether or not social and predator fear circuits are functionally dissociated at the level of structures located downstream to the VMH like the periaqueductal grey. The PAG is thought to be the motor generator structure responsible of the execution of fear behavioral and autonomic responses (see introduction). In order to address if fear circuits are dissociated also at this level we selectively inhibited the dorsal PAG via stereotactic viral injections of a rAAV delivering the inhibitory pharmacogenetic tool hM4d and exposed the animals to different threats in our behavioral paradigm. Upon dorsal PAG inhibition we observed decreased fear responses to predator and aggressive conspecific. Surprisingly the same fear responses where not decrease when they were induced by an electrical foot shock, indicating that they are initiated by a different area like the ventral PAG. Our finding indicate that fear circuits are partially dissociated at the level of the PAG, with social and predator fear overlapping in the dorsal portion. However our results do not allow us to exclude the possibility that these two are regulated by non overlapping neurons located in the same region.

1.4 The VMH is a multimodal hub for different motivated behaviors

The two subregions of the VMH have been previously implicated in the regulation of food intake, sex and aggression. The VMHdm has a function in the reduction of food intake and in the increase of energy expenditure. Neurons located in this area express various molecules implicated in this function like leptin and insulin receptors. Our results demonstrate that this nucleus also plays a central role in predator fear processing. It remains unclear if the same cells exert different functions of if they are processed by non-overlapping neurons intermingled in this structure. Fear and feeding functions are known to be related: on one hand fear inhibits feeding and on the other fear responses need metabolic changes, in particular increased energy expenditure, to be effective. Therefore, we hypothesize that also metabolic challenges like high levels of leptin or insulin may take advantage of a fear nucleus that in turn inhibits feeding and increases energy expenditure.

The VMHvl instead has been classically implicated in reproduction and aggression and now we have shown its role in social fear. All these three functions are strongly related and depend on the interaction with another member of the same species. Indeed the VMHvl receives pheromonal information via inputs from the medial amygdala that allow the conspecifics detection. Once a social stimulus is detected different adaptive behaviors are initiated depending on the nature of the stimulus and on the internal state of the subject. Nevertheless the neural mechanism of the switch between the different adaptive behaviors is poorly understood. We hypothesize a role of the VMHvl that could combine pheromonal inputs, which provide information about the nature of the stimulus, with brain stem inputs, which provide pain information and may account for the initial outcome of the social encounter.

2 Future prospects

2.1 Investigation of the mechanisms of fear modulation in the VMHdm

Our study we showed that the selective inhibition of the VMHdm inhibits fear responses to predators. However this does not allow unraveling the exact contribution of the VMH in the neural process that generates of fear. The VMH could be a simple relay of sensory information or the final generator of fear behavioral responses. Preliminary data suggest that it may play a more complex role, more similar to an

integrator structure responsible of the fear "mental state", also contributing to the formation of fear memory.

When we selectively inhibited the VMHdm during the direct encounter with a predator we observed not only a decrease in acute fear responses but also in learned responses on the day following the exposure, suggesting that the VMHdm plays a role in fear memory acquisition. On the contrary, selective pharmacogenetic inhibition of the PAG during the predator exposure impaired acute fear responses but did not interfere with conditioned responses. These findings suggest that the VMH mediates memory acquisition independently from its projections to the PAG, probably through thalamic outputs via the premammillary nucleus (Carvalho-Netto, Martinez et al.) or through outputs to the amygdala. In order to identify the VMH circuit mediating predator fear memory acquisition we will selectively inhibit the different VMH projections taking advantage of our *Nr5a1::hM4D-2A-TomatoF* mouse line and locally injecting the CNO in the different VMH targets. We hypothesize that the inhibition of VMH projection to the PAG will impair acute fear responses but leave fear memory intact, whereas, the inhibition of other upstream projection like the ones to the amygdala or to the PMD will leave acute fear responses intact but impair fear memory formation. If this were true it would implicate that the VMH contributes to predator fear memory through its upstream projections and promotes fear behaviors through its downstream projections to the PAG.

A second evidence that the VMH does not simply act as a relay station for sensory information, derives from preliminary data where we observed that the inhibition of the VMHdm immediately after the encounter with the predator, reduced learned responses on the day after the encounter with the predator, when the animals where exposed to the context associated with a predator. This result suggests that the VMH undergoes a persistent activation that continues after the fear stimulus is presented independently from the presence of sensory inputs. To have a deeper insight on the neuronal activity in the VMH after acute fear we plan to perform in vivo electrophysiological recordings in this structure. This experiment will allow us understanding how the neural activity in the VMH is changed after the encounter with the predator and how long these changes persist. Moreover we will investigate if the magnitude of the post-stimulus neuronal activation correlates with the intensity of defensive responses to the predatory context. Taken together, these evidence would

indicated that the VMH may act as a integratory structure responsible of the fear "mental state".

Another method to understand the contribution of the VMH in promoting fear is to analyze its neuronal activity during acute fear responses. Therefore we plan to perform in vivo single units recordings in mice exposed to predators and analyze the correlation of the firing activity of the single neurons with the specific fear behavioral responses. In particular, the correlation of the neuronal activity of VMH neurons with the behavioral outcome and not only with the proximity of the threat, will potentially rule out the possibility that the VMHdm acts as a simple relay station of sensory, but instead playing an active role in the generation of fear responses.

2.2 Same nucleus regulating different functions? Feeding and fear in the VMHdm and fear and aggression in the VMHvl

Our findings indicated that the VMH is a poli-functional center for the regulation of multiple goal oriented behaviors, with the VMHdm controlling feeding and predator fear and the VMHvl controlling sex, aggression and social fear. However, the neural mechanism through which the same nucleus, characterized by homogeneous cell types and connections, can regulate different functions is not clear. We want to understand if the same neurons regulate different behaviors or if non-overlapping neuronal populations located in the same anatomical region are specialized for different functions and show differences in the cell identity or connectivity.

To address this question we will take advantage of a double c-Fos detection system that allows the identification of c-Fos activated cells from two different stimuli in the same animal. Such technique is based on a double staining for c-Fos protein and mRNA that are produced in the activated neurons at different time points. Utilizing this technique, we will be able to expose mice to two subsequent fear stimuli, such as a predator ao a dietary challenge or an aggressive or submissive conspecifics, and identify the neuronal populations in the VMH that were activated by each stimulus, figuring out the amount of overlap between these two populations. This approach will allow us to quantify the amount of overlap between neurons activated by the two stimuli.

Subsequently we plan to selectively manipulate the activity of the neurons c-Fos activated by one stimulus and investigate the effect on the other one. This will allow us to unravel if the neuronal populations orchestrating the two functions are

functionally independent or overlapping. To perform these experiments we will take advantage of a knock in mouse line that expresses the inducible Cre under the control of the c-Fos promoter. We will locally infect the VMH of these animals with a hM4D Cre dependent virus and expose these animals to a specific stimulus like predator fear of leptin injection in the presence of tamoxifen. The Cre will only be active in the cells c-fos activated by this stimulus and will recombine the viral DNA and allow the specific expression of the hM4D in this neuronal population.

2.3 Investigation of functional dissociation of predator and social fear at the level of the PAG

Inhibition of the dorsal PAG impaired both predator and social fear, suggesting that they are not functionally dissociated at this level. However, our experimental strategy did not consent to demonstrate such dissociation since we could be inhibiting two completely independent populations of neurons. Similarly, we have performed C-Fos mapping studies in the PAG and we reported activation in the dorsal PAG upon both predator and conspecific fear. We want to understand whether these c-Fos activated cells are the same for the two types of fear or if two distinct populations of dPAG neurons account for social and predator fear. In other words, we are interested in understanding if predator and social fear are functionally dissociated from the sensory all the way down to the motor generator functional elements or if they have a common exit point at the level of the PAG.

To address this question, we will take advantage of a double c-Fos detection system that allows the identification of c-Fos activated cells from two different stimuli in the same animal. Such technique is based on a double staining for c-Fos protein and mRNA that are produced in the activated neurons at different time points. Utilizing this technique, we will be able to expose mice to two subsequent fear stimuli, namely a predator and an aggressive conspecific, and identify the neuronal populations in the PAG that were activated by each stimulus, figuring out the amount of overlap between these two populations.

To further investigate the fear circuits at the level of the PAG we aim to identify specific cell types that may play a specific role in social or predator fear. We will run a set of co-stainings with c-Fos and some markers of the different PAG cell types such as Vglut2, Gad2, Nos1, Tac1 in animals exposed to predators or aggressive conspecifics. Subsequently we will selectively manipulate the neuronal activity of the

different cell types in the PAG and identify their specific contribution in the generation of fear responses. In particular we are now focusing of a neuronal population in the PAG characterized by the expression of nitric oxide syntase 1. We will locally inject in the PAG of a mouse line expressing the Cre under the control of the NOS1 promoter virus that allows the expression of the pharmacogenetic inhibitory receptor hM4D in a Cre dependent manner. The same technique will be applied to other specific Cre driver lines like Vglut2::Cre o Gad2::Cre. These results will potentially be coupled with in vitro electrophysiological slice recordings that will clarify how these different cell types, that may play different roles in fear, are connected to each other.

2.4 Screening for possible drug targets for the selective inhibition of the VMHdm in humans

The pharmacomenetic inhibition of the VMHdm in our *Nr5a1::hM4D-2A-TomatoF* transgenic mouse line lead to a strong decrease of predator fear. Moreover, as mentioned above, there is good evidence to believe that the VMH is a crucial central modulator of the fear "mental state". For these reasons we now want to move to the pathological aspects of fear and investigate if the physiology of this circuit is altered in models of fear related diseases like post traumatic stress disorder, panic disorder or phobias.

Importantly, there is good evidence that this nucleus may be an important regulator of innate fear in humans too, since fMRI studies have reported activation of this structure during exposure to scary videos (Pichon, de Gelder et al.) and deep brain stimulation in the VMH induced panic attacks (Wilent, Oh et al.). As a result the inhibition of the VMH in patients with fear related disorders like panic attacks or phobias may help to moderate the excessive fear states and possibly to reprogram the mal-functioning fear circuits. We are now looking for suitable genes that could be good drug target to effectively inhibit the VMH in humans.

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Independent hypothalamic circuits for social and predator fear

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The neural circuits mediating fear to naturalistic threats are poorly understood. We found that functionally independent populations of neurons in the ventromedial hypothalamus (VMH), a region that has been implicated in feeding, sex and aggression, are essential for predator and social fear in mice. Our results establish a critical role for VMH in fear and have implications for selective intervention in pathological fear in humans.

Studies in laboratory animals have routinely used freezing behavior elicited by exposure to cues associated with electric foot shock to study the neural circuits underlying fear. However, evidence suggests that fear behaviors elicited by other types of threat may not depend on these circuits[1]. In particular, c-Fos mapping studies have shown that exposure to a predator or an aggressive conspecific recruits the medial hypothalamus, a region that has been implicated in motivated behaviors such as feeding, sex and aggression[2]. Notably, exposures to predator and aggressive conspecific activate non-overlapping nuclei in the medial hypothalamus, suggesting that predator and social fear may depend on separate circuits[3]. However, it remains unclear whether the different brain regions recruited by foot shock, predator and aggressive conspecific reflect truly independent fear circuits or arise as a result of differences in the behaviors elicited, differences between innate and learned fear, or differences in testing methodology.

We developed a behavioral test in which similar patterns of fear behavior are elicited in mice by exposure to either a predatory rat, an aggressive mouse or an electric foot shock (**Fig. 1a**). The apparatus consisted of two chambers separated by a narrow corridor. Mice were housed in one chamber and, each day, a door was opened to allow brief access to the corridor and second chamber. On the fourth day, the mouse was confined to the second chamber and briefly exposed to a predatory rat, an aggressive conspecific, a foot shock or a fake toy rat. The door was reopened and defensive behaviors (immobility, flight, stretch postures and locomotion) were recorded. On the following day, mice were again allowed free access to the corridor and second chamber in the absence of threat and defensive behaviors were scored as a measure of contextual fear. Mice showed an increase in stretch postures, immobility and flight and a decrease in locomotion following exposure to all threats when compared with their behavior during habituation (**Fig. 1b–d** and **Supplementary Fig. 1**). Exposure to the conditioned context also elicited an increase in stretch postures, immobility and flight and a decrease in locomotion (**Fig. 1b–d** and **Supplementary Fig. 1**), whereas exposure to a toy rat did not elicit increases in stretch postures, immobility or flight, but did result in a decrease in locomotion following acute exposure, suggesting that some of the decreased locomotion to threat is a result of the novelty of the stimulus. These data validate our test as a robust method to examine similar acute and learned fear responses to foot shock, predator and social threat.

To investigate whether distinct neural activation patterns are induced by foot shock, predator and aggressive conspecific under conditions of similar testing methodology and behavior, we performed c-Fos mapping. c-Fos was induced in different brain regions in accordance with previous reports[2,3]. In particular, predator exposure significantly activated ($P = 0.045$) the dorsomedial division of the VMH (VMHdm), whereas exposure to an aggressive conspecific significantly activated ($P = 0.012$) the ventrolateral VMH (VMHvl; **Fig. 1e–g**). Neither control mice nor mice exposed to foot shock showed activation in VMH, indicating that the medial hypothalamus is selectively recruited during predator and social fear and that similar fear behaviors recruit different brain circuits. Notably, these data suggest that VMHdm, a region that has been extensively implicated in the control of energy homeostasis and metabolism[4], is involved in predator fear, whereas VMHvl, a region that has been implicated in sexual and aggressive behavior[4–6], is involved in social fear.

To determine whether VMH harbors functionally independent circuits for predator and social fear, we used the hM4D–clozapine-*N*-oxide (CNO) pharmacogenetic neural inhibition tool[7] to rapidly and selectively inhibit neurons in VMHdm and VMHvl in behaving mice. Stable expression of hM4D in VMHdm neurons was achieved by constructing transgenic mice in which *hM4D* was driven by the *Nr5a1* gene promoter[8] (*Nr5a1∷hM4D-2A-TomatoF*; **Fig. 2a**). Reporter gene expression in the transgenic mice was found in the dorsomedial and central divisions of VMH, in VMH efferents of the supraoptic commisure and in all known VMH target areas, including dorsal periaqueductal gray (dPAG)[9] (**Fig. 2b–d** and **Supplementary Figs. 2** and **3**). hM4D was selectively expressed in Nr5a1-expressing neurons in the transgenic mice (**Fig. 2e–g** and **Supplementary Fig. 4**). *In vitro* patch-clamp electrophysiology confirmed a significant reduction in spontaneous firing and membrane potential in VMHdm neurons in brain slices from transgenic mice (firing rate = $-32\% \pm 6$, $P = 0.0013$, $N = 8$ recorded neurons from 5 mice; membrane potential = -3.35 mV ± 1.07, $P = 0.0074$, $N = 15$ recorded neurons from 6 mice), but not non-transgenic littermates (firing rate = $7.6\% \pm 25.2$, $P = 0.78$, $N = 8$ recorded neurons from 5 mice; membrane potential = 0.71 mV ± 0.94, $P = 0.47$, $N = 10$ recorded neurons from 5 mice) treated with CNO, a selective agonist of hM4D that is otherwise biologically inert[10] (**Fig. 2h**). Systemic treatment of *Nr5a1∷hM4D-2A-TomatoF* transgenic mice, but not non-transgenic littermate control mice, with CNO before threat exposure resulted in a significant decrease in defensive behaviors ($P < 0.0001$) and an increase

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Figure 1 Similar fear behavior elicited in mice by different classes of threat. (**a**) The behavioral testing apparatus consisted of two chambers connected by a narrow corridor. An experimental mouse was continuously housed in one chamber (home) and allowed to freely explore the corridor and second chamber (stimulus) once daily for 20 min. At the end of the free exploration period on the fourth day, the door to the stimulus chamber was briefly closed to confine the mouse, which was then exposed to either a predatory rat (predator, <5 s), aggressive mouse (Social, 10 min), electric foot shock (foot shock, 1 min, 4 × 0.5 s, 0.5 mA) or toy rat (fake rat, <5 s), after which free exploration continued for an additional 10 min. (**b**–**d**) Time spent performing stretch postures (**b**), immobility (**c**) and locomotion (**d**) was measured during the pre-stimulus (habituation) and post-stimulus (stimulus) free exploration periods, as well as on the day following stimulus exposure (context). Stretch postures and immobility were significantly increased after exposure to predator ($N = 15$, $P < 0.0001$), aggressive conspecific ($N = 9$, $P < 0.0001$) and foot shock ($N = 6$, $P < 0.0001$), but not to toy rat ($N = 6$; stretch postures, $P = 0.005$) when compared with the habituation session. Locomotion was significantly decreased after exposure to all stimuli. Re-exposure to the context associated with social, predator and foot shock, but not fake rat (stretch postures, $P = 0.45$; locomotion = 0.042, ns) threats, elicited a significant increase in stretch postures ($P < 0.0001$) and immobility (predator: $P = 0.195$, social: $P = 0.048$, foot shock: $P = 0.0007$) and a decrease in locomotion ($P < 0.0001$). $**P < 0.01$, $***P < 0.001$). (**e**–**g**) Quantification of c-Fos immunohistochemistry in brain sections from mice exposed to predator and aggressive conspecific in the two-chambered apparatus revealed a significant increase in the number of c-Fos cells labeled in VMHdm (predator, $P = 0.045$; social, $P = 0.87$; **f**) and VMHvl (predator, $P = 0.95$; social, $P = 0.012$; **g**), respectively, when compared with the habituation condition (predator, $N = 5$; social, $N = 4$; foot shock, $N = 3$–4; $*P < 0.05$). Negligible neural activation was seen in VMH following foot shock exposure (VMHdm, $P = 0.49$; VMHvl, $P = 0.48$) or in home cage control mice. Scale bar represents 50 µm. dm = dorsomedial, c = central, vl = ventrolateral. Error bars represent s.e.m.
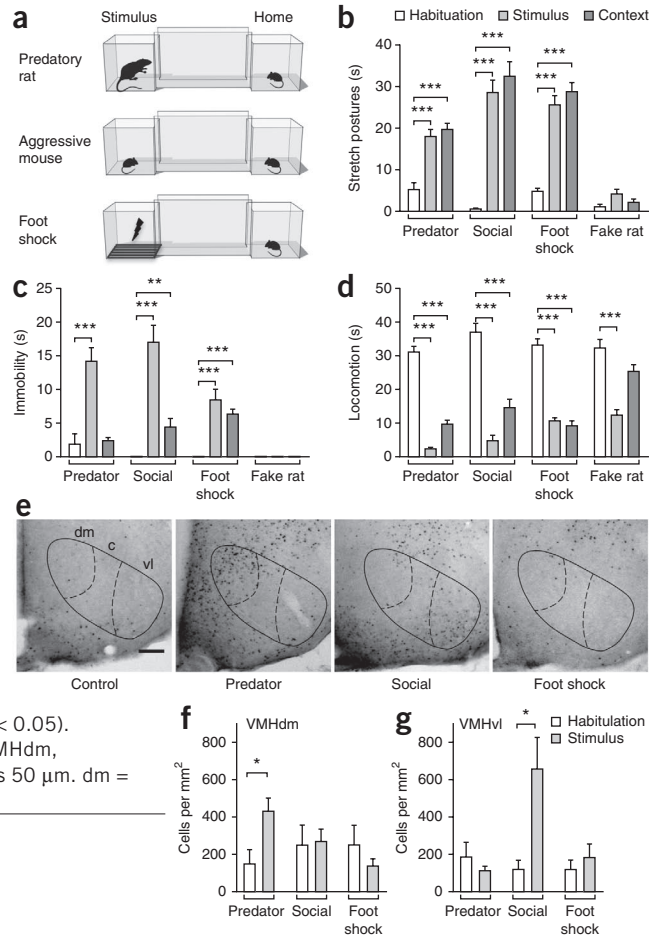
in locomotion ($P = 0.001$) to predator. Similar treatment had no effect on fear behaviors elicited by exposure to aggressive conspecific or foot shock (**Fig. 3a,b**). These data indicate that VMHdm has an essential and selective role in the expression of predator fear behavior.

Expression of hM4D in VMHvl neurons was achieved by local infection with adeno-associated virus (AAV) expressing hM4D (AAV-*Syn*∷*Venus-2A-hM4D*; **Fig. 3c,d** and **Supplementary Figs. 5a**–**f** and **6a**–**f**). CNO treatment of AAV-*Syn*∷*Venus-2A-hM4D*–infected mice before threat exposure

resulted in a significant decrease in defensive behaviors ($P = 0.006$) and an increase in locomotion ($P = 0.02$) to an aggressive conspecific when compared with vehicle-treated controls, but no change in fear behavior was elicited by predator (**Fig. 3e,f**). In some cases, expression of hM4D in virally infected mice extended to the VMHdm (**Supplementary Fig. 6b**) and tuberal nucleus (**Supplementary Fig. 6d**) and we cannot completely rule out that inhibition of cells in these nuclei contributed to the behavioral effects seen. The observation that expression in these structures was significantly lower ($P = 0.035$) than in VMHvl (**Supplementary Fig. 6e**) and that this infection was not associated with a reduction in predator fear, suggests that this ectopic expression was not sufficient to modulate fear behavior. Expression outside the VMH was sparse (**Supplementary Fig. 6c**). Notably, CNO treatment did not affect the number of attacks received nor the submissive behavior during the direct encounter with the aggressor
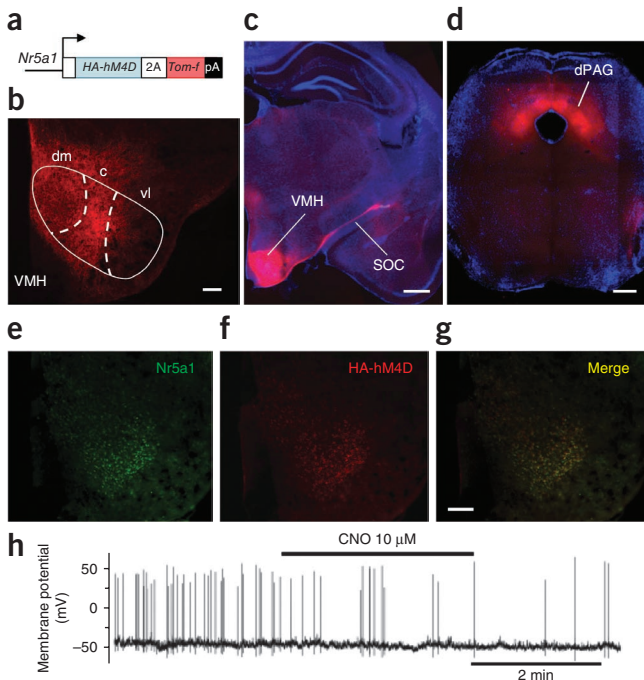

Figure 2 Generation and validation of *Nr5a1*∷*hM4D-2A-tomatoF* transgenic mice. (**a**–**d**) Mice carrying a transgene in which the HA-tagged hM4D pharmacogenetic neural inhibition tool and a farnesylated Tomato fluorescent protein (Tom-f) were expressed under the control of the *Nr5a1* gene promoter (**a**) showed expression of Tom-f in the dorsomedial (dm) and central (c), but not ventrolateral (vl) divisions of the VMH (**b**; scale bar represents 100 µm), in the supraoptic commissure (soc; **c**), and in the dPAG (**d**). Scale bars in **c** and **d** represent 500 µm. (**e**–**g**) Double immunofluorescence staining with (**e**) antibody to Nr5a1 (**e**) and HA (**f**) confirmed selective and robust expression of the transgene in Nr5a1-positive cells (**g**). Scale bar represents 100 µm. (**h**) Sample trace from *in vitro* patch-clamp electrophysiological recordings in VMHdm neurons confirmed a reduction of firing rate and membrane potential following CNO treatment in brain slices from *Nr5a1*∷*hM4D-2A-tomatoF* mice, but not control mice.

Figure 3 Functional dissociation of fear in VMH and PAG. (**a,b**) *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice, but not non-transgenic littermates, showed a significant inhibition of cumulative defensive responses (**a**) and an increase of locomotion elicited by exposure to a predatory rat (predator), but not an aggressive conspecific (social) or electric foot shock (foot shock, 4×0.5 s, 0.5 mA) (**b**), following systemic administration of CNO (3 mg per kg, intraperitoneal; predator: $N = 7$–8, total defense, $P = 0.0001$; locomotion, $P = 0.001$; social: $N = 7$–8, total defense, $P = 0.72$; locomotion, $P = 0.04$; foot shock: $N = 6$–8, total defense, $P = 0.42$; locomotion, $P = 0.68$). (**c,d**) Mice locally infected with an AAV expressing the Venus fluorescent protein and HA-tagged hM4D pharmacogenetic neural inhibition tool (HA-hM4D) under the control of the *synapsin-1* (*Syn1*) gene promoter (**c**) showed expression in the VMHv1 (**d**). Scale bar represents 100 μm. (**e,f**) AAV-*Syn*::*Venus*-2A-*hM4D* infected mice showed a significant inhibition of cumulative defensive responses (**e**) and an increase of locomotion elicited by exposure to an aggressive conspecific (social), but not predatory rat (predator) (**f**), following systemic administration of CNO when compared with vehicle-treated mice (predator: $N = 17$–18, defensive responses, $P = 0.58$; locomotion, $P = 0.54$; social: $N = 17$–19, defensive responses, $P = 0.006$; locomotion, $P = 0.02$). (**g,h**) Mice locally infected with AAV-*Syn*::*Venus*-2A-*hM4D* in the dPAG (**g**) displayed a significant decrease of cumulative defensive responses elicited by exposure to an aggressive conspecific (social) or a predatory rat (predator), but not to an electrical foot shock (foot shock, 4×0.5 s, 0.5 mA) (**h**) following systemic administration of CNO when compared with similarly infected vehicle-treated mice (predator: $N = 5$–13, $P = 0.003$; social, $N = 9$–10, $P = 0.031$; foot shock, $N = 13$–14, $P = 0.67$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bar represents 100 μm. Error bars represent s.e.m.
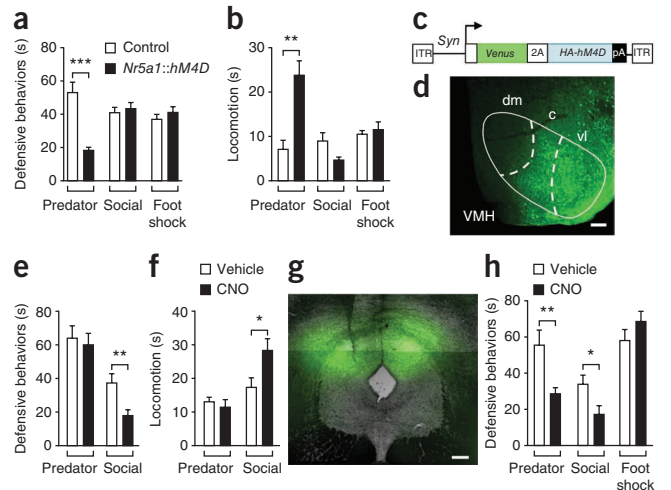
(**Supplementary Fig. 7a,b**). These data suggest a double dissociation of VMH circuits supporting fear behavior to predator and social threats.

Finally, we examined whether fear of predator, aggressive conspecific and foot shock were also functionally dissociable at the level of the PAG, a downstream structure that is involved in motor pattern initiation and has been shown to be critical for the expression of fear responses[11]. Both VMHdm and VMHvl projected prominently to the dPAG[9], and CNO-treated mice with local infection of AAV-*Syn*::*Venus-2A-hM4D* (**Fig. 3g**) in dPAG showed significantly reduced predator ($P = 0.003$) and social ($P = 0.031$), but not foot shock, fear when compared with vehicle-treated, similarly infected control mice (**Fig. 3h**). Although infection often included the overlying superior colliculus (**Supplementary Fig. 8**), treatment of mice explicitly infected in superior colliculus with CNO did not result in a change in fear behavior (**Supplementary Fig. 9**). Although CNO treatment did not affect the number of attacks received, a decrease in submissive behavior was observed during the direct encounter with the aggressor (**Supplementary Fig. 10a,b**), suggesting that dPAG is involved in supporting passive defensive behaviors during conspecific encounters. These data indicate that the neural circuits supporting defensive behaviors to distinct threats are also dissociable at the level of downstream motor initiation centers.

Our findings demonstrate that VMH is a multi-modal hub for the control of motivated behaviors and physiological homeostasis. Nr5a1-expressing cells in VMHdm are leptin responsive and essential for supporting metabolic responses to dietary challenge[4], and our data suggest that a link between metabolic regulation and predator fear may occur at the level of the VMHdm. Consistent with an evolutionarily conserved role for VMHdm in fear, electrical stimulation of VMHdm in humans elicits panic attacks[12]. On the other hand, the dual role of VMHvl in aggression[5,6] and social fear suggests that it functions as a key threat processing circuit during social encounters. Our observation that dPAG is critical for predator and social, but not foot shock, fear further supports the existence of independent fear circuits at both the level of fear processing and expression. These data suggest that fear of different classes of threat are processed in distinct circuits and open the possibility for the selective pharmacological blockade of fear. Finally, our data provide, to the best of our knowledge, the first functional dissection of the neural circuits supporting social fear, an important risk factor for mental illness.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS
B.A.S. designed, carried out and analyzed all of the experiments, except for some of the behavioral experiments, which were carried out and analyzed by C.M. and P.K., and the electrophysiology experiments, which were designed, carried out and analyzed by E.M. and D.R. Viruses were produced and tested by A.I. and V.G. The project was conceived by B.A.S. and C.T.G. with critical input from N.S.C. The manuscript was written by B.A.S. and C.T.G. with input from D.R. and N.S.C.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All mice were derived from local European Molecular Biology Laboratory breeding colonies. Non-transgenic experimental subjects were adult C57BL/6N mice. Predators were adult male SHR/NHsd rats (Harlan). Aggressive conspecifics were adult male CD1 mice selected for elevated aggression as previously described[13]. All animals were housed at 22–25 °C on a 12-h light-dark cycle with water and food *ad libitum*. Males were used for all experiments except for data in **Figure 3a,b**, where both males and females were tested. No sex difference in behavioral responses was observed. All animals were handled according to protocols approved by the Italian Ministry of Health (#231/2011-B, #121/2011-A).

Behavioral testing. The experimental apparatus (adapted from ref. 14) was made of clear Plexiglas and composed of similar detachable home and stimulus chambers (25 × 25 × 25 cm) that were connected by an opening (2.0 cm wide, 2.0 cm high) to a narrow corridor (12.5 cm wide, 60 cm long, 30 cm high). Both openings could be closed by a manual sliding door. The experimental subject was continuously housed in the home chamber with access to food and water for the entire test. Each day the home cage was carried from the housing room to the testing room and attached to the apparatus, and the sliding door opened to give the mouse access to the entire apparatus for 20 min (habituation period). In case of foot shock, a metal grid connected to a scrambled electric shock generator (Med Associates) was placed into the stimulus compartment. On day 4, following 10 min of exploration, the experimental mouse was confined to the stimulus compartment by closing the door and either a rat or an aggressive mouse was placed into the stimulus compartment and allowed to interact (rat, <5 s; mouse, 10 min) before the door was re-opened to allow the experimental mouse to escape. The door was immediately re-closed in the case of the stimulus mouse to prevent escape. In case of foot shock, a scrambled electric current was delivered to the grid over a period of 1 min (0.5 mA every 15 s) before the door was re-opened. To prevent injury to the experimental mouse, the experimenter held the rat during the direct encounter. Defensive behaviors were scored during the first 3 min of free exploration each day and during the first 3 min of the post-stimulus period. CNO (3 mg per kg of body weight, intraperitoneal, in 0.9% saline (wt/vol); Enzo Life Sciences) or vehicle was injected 30 min before the beginning of the test. On day 5, the experimental mouse was given access to the entire apparatus as on the habituation days. Between each subject the apparatus was cleaned first with 50% ethanol (vol/vol) and then detergent and the bedding was changed. The apparatus was washed in an automatic cage washer between testing days to eliminate odors. All the testing was performed during the dark phase under red light illumination (40 W).

Mice were naive to the testing apparatus except in the case of the data shown in **Figure 3e,f,h**, where predator- and foot shock–exposed mice had been previously tested. In these cases, pseudo-randomization of drug treatment groups was performed and no influence of multiple drug treatment was observed. Behavior was scored from videotape using Observer software (Noldus) by an experimenter blind to genotype and treatment. Behaviors were scored as follows: immobility, subject motionless; stretch postures, body stretched forward without movement or mouse moving slowly toward stimulus compartment in an elongated posture; flight, subject quickly running toward home cage; locomotion, ambulatory movement not characterized by stretch posture. Defensive behavior was the sum of stretch postures and immobility. In the case of mice tested for social fear, the number of biting attacks received and the time spent in an upright/submissive posture during the direct encounter was also scored. In two cases, the experimental mouse performed a pronounced number of attacks toward the intruder (>70% of all attacks) and was excluded from the analysis.

Statistical analysis. Data analysis was performed with PRISM software (GraphPad). All data are reported as mean ± s.e. measurement. Statistical significance was determined by repeated-measures ANOVA with behavior during habituation, stimulus and context considered as repeated measures coupled to Bonferroni *post hoc* analysis in case of significance (**Fig. 1b–d** and **Supplementary Fig. 1**), two-way ANOVA (**Fig. 1f,g**), *t*-test (**Figs. 2 and 3**, and **Supplementary Figs. 5e, 6, 8 and 9**) or MANCOVA[15] followed by pairwise correlation analysis (**Supplementary Fig. 5**). In the *in vitro* electrophysiology experiments, the changes from baseline in firing rate and membrane potential were calculated by *t* test. No statistical methods were used to predetermine group sizes. The sample sizes that we chose are similar to those used in previous publications.

c-Fos immunohistochemistry. 90 min after exposure to the stimulus (predator, conspecific or foot shock), the experimental mouse was deeply anesthetized with Avertin (Sigma-Aldrich), perfused trans-cardially (4.0% paraformaldehyde (wt/vol), 0.1 M phosphate buffer, pH 7.4), and the brain was removed, postfixed (4% paraformaldehyde overnight) and cryoprotected (20% sucrose (wt/vol), PBS, 4 C, overnight). The brains were frozen and 40-µm coronal sections were cut with a sliding cryostat (Leica Microsystems) and processed for immunohistochemistry with rabbit antibody to Fos (1:20,000, Ab-5, Calbiochem). The primary antiserum was localized using a variation of the avidin-biotin complex system (Vector Laboratories)[16]. In brief, sections were incubated for 90 min at 22–25 °C in a solution of biotinylated goat antibody to rabbit IgG (PK-6101, Vector Laboratories) and then placed in the mixed avidin-biotin horseradish peroxidase complex solution (ABC Elite Kit, Vector Laboratories) for the same period of time. The peroxidase complex was visualized by a 5-min exposure to chromogen solution (0.05% 3,30-diaminobenzidine tetrahydrochloride (wt/vol, Sigma-Aldrich), 0.4 mg ml^{-1} nickel ammonium sulfate, 6 µg ml^{-1} glucose oxidase (Sigma-Aldrich), 0.4 mg ml^{-1} ammonium chloride in PBS) followed by incubation in the same solution with 2 mg ml^{-1} glucose to produce a blue-black product. The reaction was stopped by extensive washing in PBS. Sections were dehydrated and coverslipped with quick mounting medium (Eukitt, Fluka Analytical).

Fluorescent protein detection. Mice were trans-cardially perfused (4.0% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4) and brains were removed and left overnight in fixative. Coronal sections (70 µm) were cut on a vibratome (Leica Microsystems). All sections were imaged for Venus, TomatoF and DAPI fluorescence with a motorized wide-field microscope (Leica Microsystems).

Double immunostaining. Mice were perfused trans-cardially (4.0% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4) and brains were removed, postfixed (4% paraformaldehyde overnight) and cryoprotected (20% sucrose, PBS, 4 C, overnight). The brains were frozen and 40-µm coronal sections were cut with a sliding cryostat (Leica Microsystems) and processed for immunohistochemistry with antibody to HA raised in rat (1:200, 11867423001, Roche) and antibody to Nr5a1 in rabbit (1:200, K0611, Trans Genic). Before incubation with primary antibodies, the sections were boiled for 10 min in citrate buffer (10 mM) and incubated with blocking solution (1% BSA, 5% NGS in PBS and 0.4% Triton X-100; vol/vol) for 1 h. Primary antibodies were detected with fluorescent-labeled secondary antibodies (1:800, Alexa Fluor 488 goat antibody to rabbit, A-11034; 1:800, Alexa Fluor 647 goat antibody to rat, A-21248; Invitrogen).

Generation of transgenic mice. Recombineering was used to insert a HA-hM4D-2A-TomF-FRT-kan/neo-FRT cassette replacing the translational start of the *Nr5a1* gene in a bacterial artificial chromosome (BAC) clone (RP23-225F7, CHORI-BACPAC). The hemagluttinin-tagged hM4D sequence (HA-hM4D) was excised from pcDNA-5FRT-HA-hM4D (a gift from B. Roth, University of North Carolina). A farnesylation domain (KLNPPDESGPGCMSCKCVLS[17]) was added to the C terminus of the Tomato open reading frame and the viral P2A[18] sequence was inserted between hM4D and TomatoF to produce separate peptides from a single open-reading frame. Modified BAC DNA was prepared (Large-Construct kit, Qiagen), diluted in injection buffer (30 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 100 mM NaCl), and microinjected into the pronucleus of fertilized one-cell stage B6x(B6xD2) embryos. One of two founders showed stronger reporter gene expression was used in all studies and backcrossed to C57BL/6N. Transgenic mice were genotyped by PCR (forward: 5′-CAATCCAGCTGTGTGCCCTACTTCGCC-3′, reverse: 5′-GGCCATAGCGTAATCTGGAACATCGTATGGGTA-3′).

***In vitro* electrophysiology.** Coronal slices (250 µm) containing the VMH were cut at 4 °C using a vibratome (DSK, Dosaka EM) from brains incubated for 5–10 min in ice-cold oxygenated modified artificial cerebrospinal fluid (ACSF; 3 mM KCl, 2 mM MgCl$_2$, 1.6 mM CaCl$_2$, 1.25 mM NaH$_2$PO$_4$, 2 mM MgSO$_4$, 26 mM NaHCO$_3$, 10 mM glucose, 200 mM sucrose) extracted from transgenic and control littermates that were anesthetized with halothane and decapitated. Slices were maintained for at least 1 h at 22–25 °C in oxygenated (95%/5% CO$_2$) ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 1.25 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, 10 mM glucose, pH 7.35). Recordings were performed at 22–25 °C in ACSF perfused at a rate of ~1.5 ml min^{-1}. CNO (10 µM) was applied to the slice by bath perfusion for 3 min. Whole-cell patch-clamp

recordings in current clamp configuration were performed using borosilicate glass pipettes (3–5 MΩ) filled with 140 mM potassium gluconate, 2 mM MgCl$_2$, 5 mM BAPTA, 10 mM HEPES, 2 mM MgATP, 0.4 mM NaGTP, and pH corrected with KOH to pH 7.32. Recordings were performed using an Axopatch 200A amplifier (Molecular Devices); signal was low-pass filtered at 2 kHz, collected at 10 kHz using Clampex10 (Molecular Devices), and analyzed off-line with Clampfit10 software (Molecular Devices). In some experiments 10–50 pA of current were injected to induce firing. Recordings were discarded if membrane potential and/or firing rate were unstable. To determine changes in membrane potential, signals were digitized at 1 Hz and firing frequency was monitored using 30-s duration bins. In both cases, CNO response was assessed 4 min following the start of drug application.

Viral production. The Venus-P2A-HA-hM4D cassette was cloned so as to replace the open reading frame of pAAV-Syn-NpHR3.0-EYFP-WPRE (a gift from K. Deisseroth, Stanford University). Production and purification of recombinant AAV (chimeric capsid serotype 1/2) were as described[19]. Viral titers (>10[10] genomic copies per μl) were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs) and RT-PCR as previously described[20].

Stereotaxic viral injections. Bilateral injection of AAV aimed at the VMHvl (posterior, −0.95 mm; depth, −5.75 mm; lateral, ±0.65 mm to bregma; coordinates empirically adapted from ref. 21) or dPAG (posterior, −3.8 mm; depth, −2.3 mm lateral ±1.0; angle, 26 degrees) was performed using a glass pipette (intraMARK, 10-20 μm tip diameter, Blaubrand) connected to a syringe and a stereotaxic micromanipulator (Kopf Instruments) in deeply anesthetized mice

(Ketavet, ketamine 100 mg per kg, xylazine 10 mg per kg, Intervet). We injected 0.3 μl of AAV-containing solution per side in VMHvl and 0.1 μl per side in dPAG. Behavioral experiments were performed 3–4 weeks after surgery.

Quantification of viral infection. The location of viral infection was determined in all mice injected with AAV. The mice were trans-cardially perfused with 4% paraformaldehyde within 3 d of behavioral testing. The exact position of the brain nucleus of interest was determined by overlaying a reference atlas grid[21] using white matter landmarks on the bright field fluorescent image. Venus signal was thresholded and quantified (ImageJ) and infection efficiency (either % area or total area) was calculated over the total area of the nucleus as determined from the atlas overlay. 15 dPAG-infected mice that showed less than 10% infection of the target area were excluded from the behavior analysis. No VMHvl-infected mice were excluded from the behavior analysis.

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Supplementary information for:

Independent hypothalamic circuits for social and predator fear

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Illarionova, Valery Grinevich, Newton S. Canteras, Davide Ragozzino, Cornelius T.
Gross
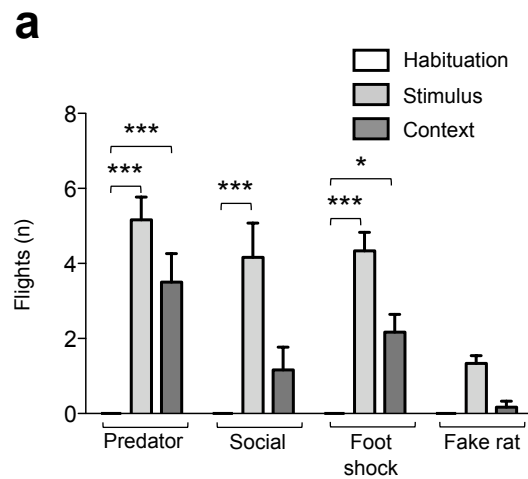
Supplementary Figure 1 (related to Figure 1a-d). Flight behavior elicited in mice by different classes of threat. (**a**) The number of flight behaviors performed during the post-stimulus (Stimulus) free exploration period were significantly increased after predator ($P<$, 0.0001), aggressive conspecific ($P<$,0.0001), and foot shock ($P<$,0.0001), but not toy rat ($P = 0.005$) exposure when compared to the pre-stimulus habituation (Habituation) session. Re-exposure to the context (Context) elicited an increase in flights to predator ($P<$,0.0001) and foot shock ($P = 0.021$), but not aggressive conspecific ($P = 0.21$) or toy rat ($P = 0.77$) (Predator: N = 15, Social: N = 9, Foot shock: N = 6, Fake rat: N = 6, * $P < 0.05$, *** $P < 0.001$).
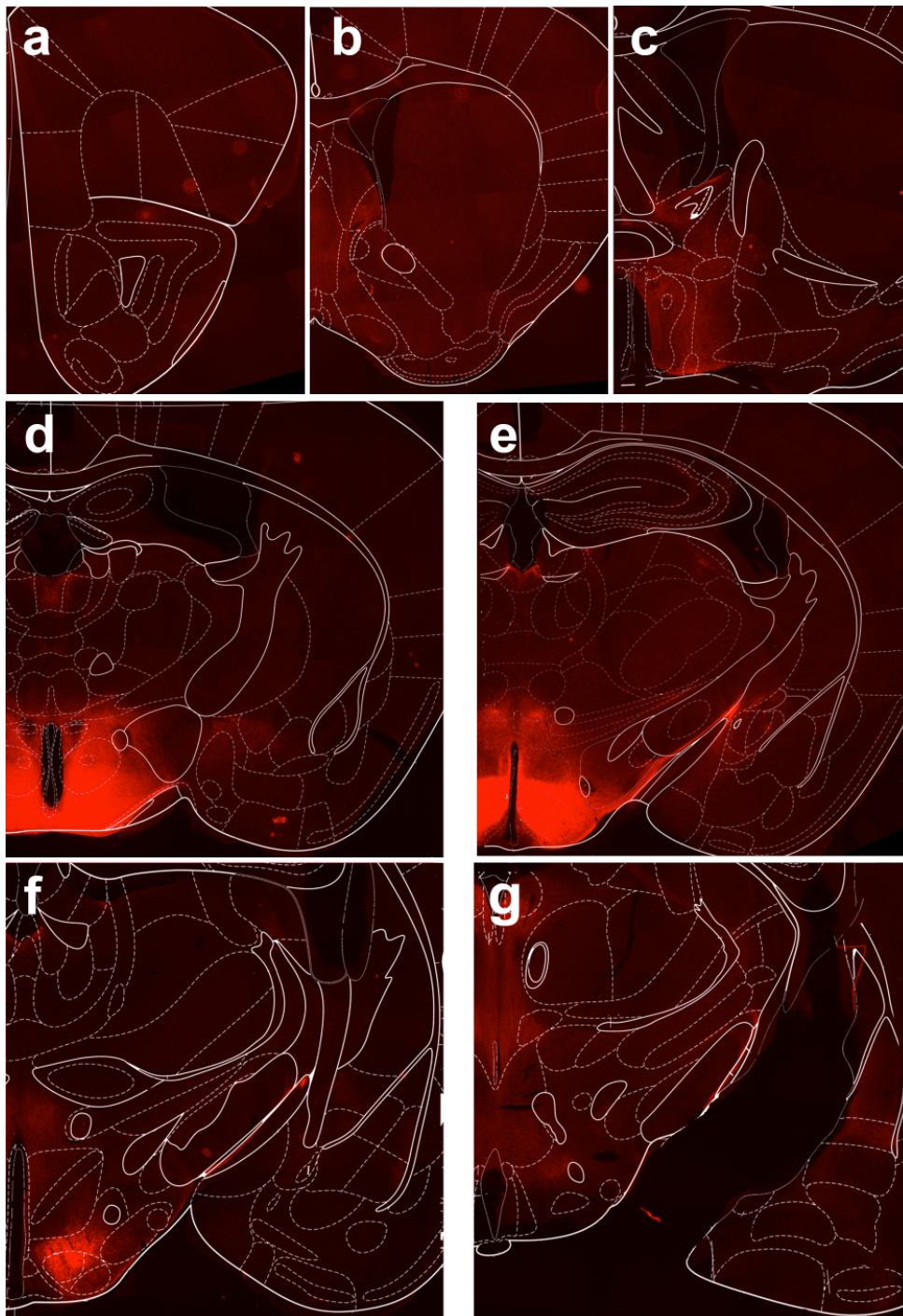
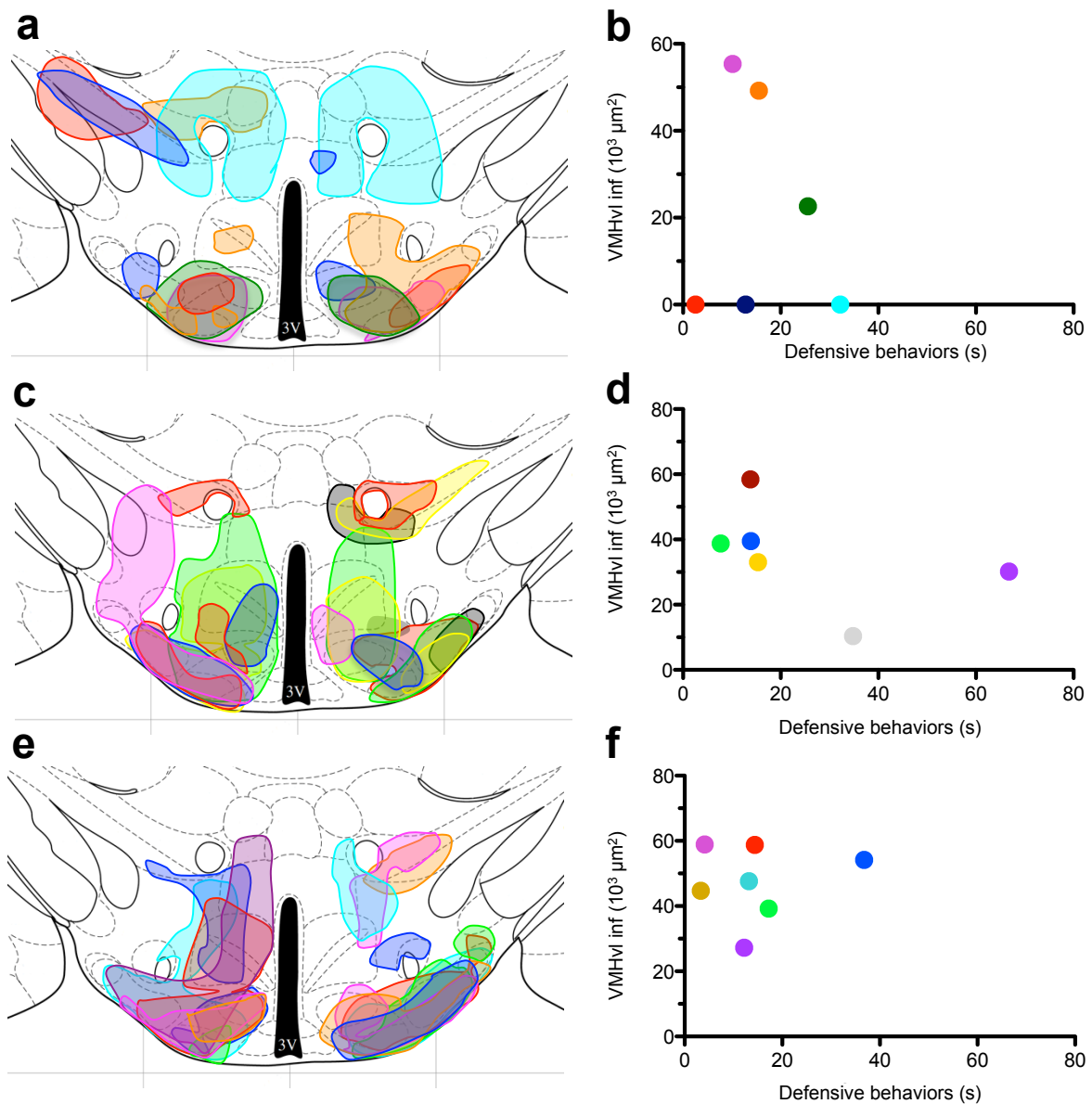**Supplementary Figure 2.** (related to Fig. 2a-d). TomatoF expression in *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice (rostral part). Farnesylated tomato (tom-f, see Fig. 2a) expression was found in cell bodies in the VMHdm and in projections in a number of previously reported target brain regions [10]. Fluorescent images of rostral to caudal coronal brain sections from *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice are shown overlaid with the outlines of mouse brain structures deriving from a standard anatomical atlas [22]. Atlas outlines were morphed in some cases to better match the sections.
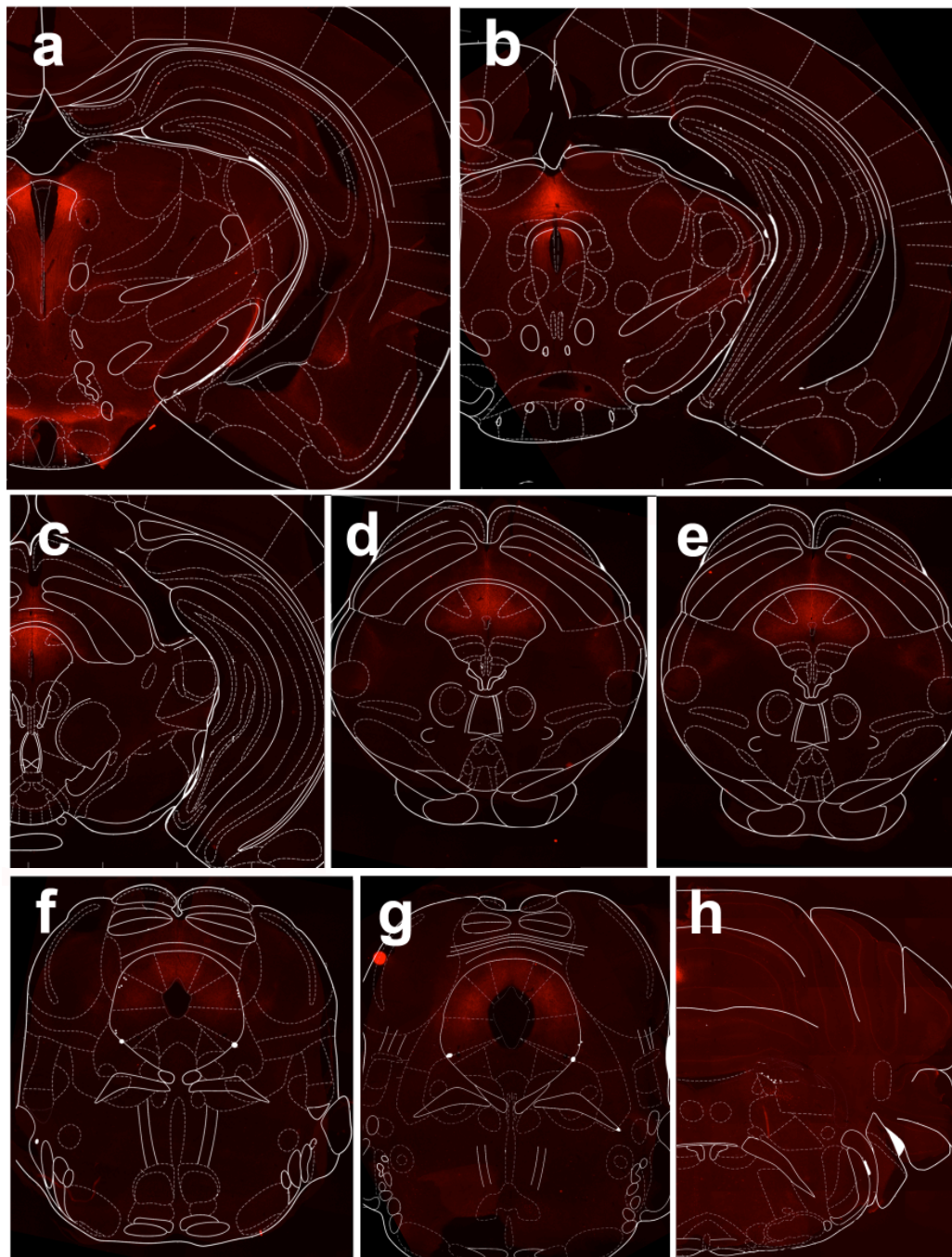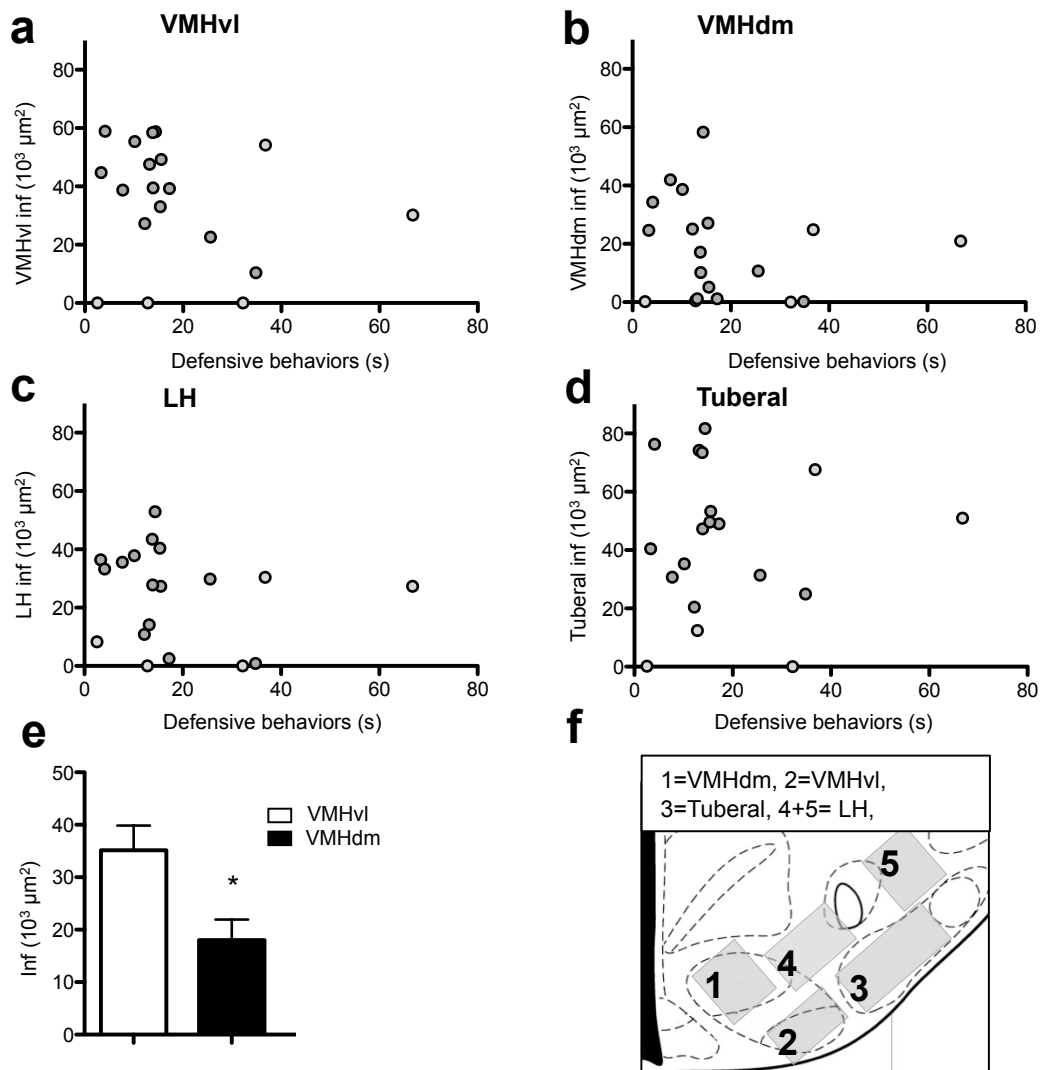
Supplementary Figure 3. (related to Fig. 2a-d). TomatoF expression in *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice (caudal part). Farnesylated tomato (tom-f, see Fig. 2a) expression was found in cell bodies in the VMHdm and in projections in a number of previously reported target brain regions [10]. Fluorescent images of rostral to caudal coronal brain sections from *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice are shown overlaid with the outlines of mouse brain structures deriving from a standard anatomical atlas [22]. Atlas outlines were morphed in some cases to better match the sections.
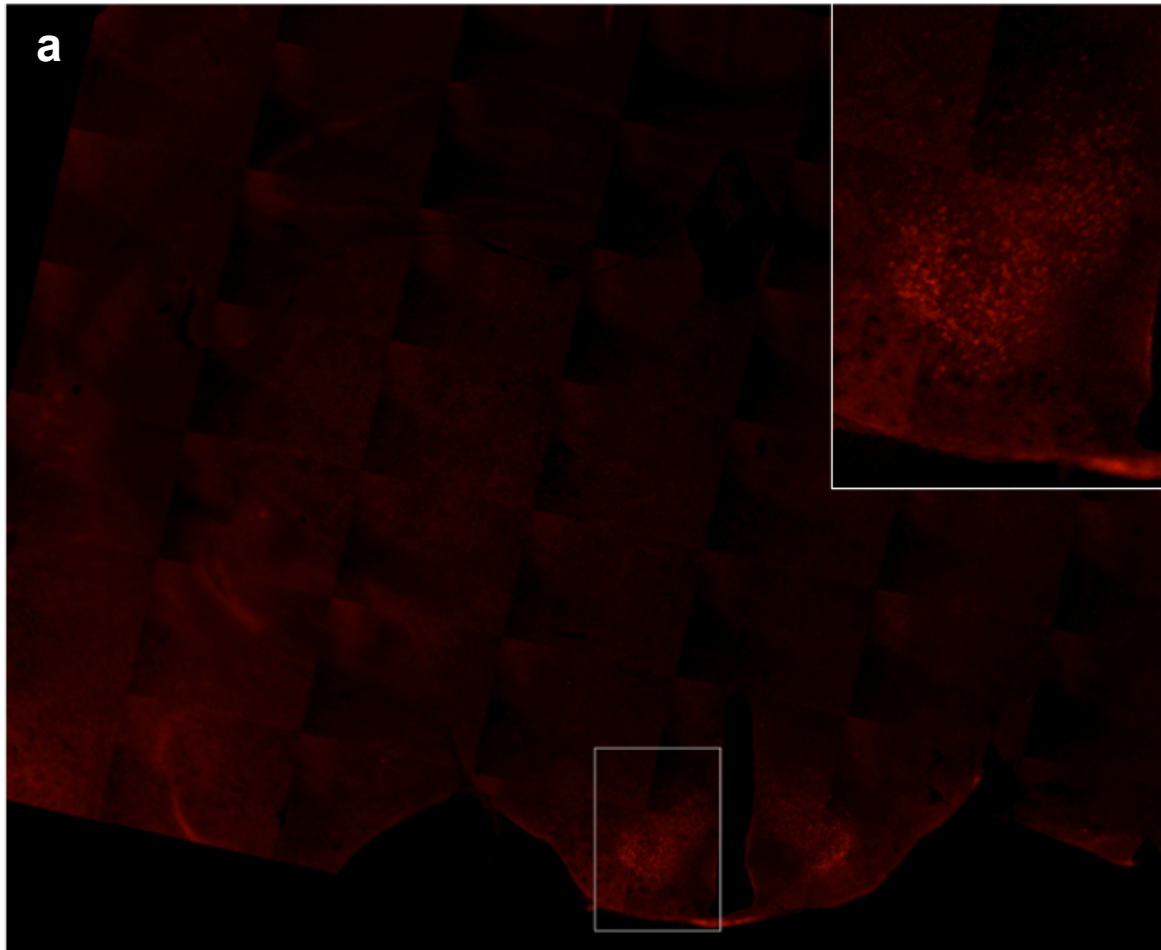
Supplementary Figure 4 (related to Fig. 2f). HA-hM4D is selectively expressed in VMHdm of *Nr5a1*::*hM4D*-2A-*tomatoF* mice. (**a**) Immunofluorecence with anti-HA antibodies in coronal brain sections of *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice revealed robust expression of HA-hM4D in the dorsal-medial and central portions of VMH. No detectable anti-HA staining was seen outside VMH.
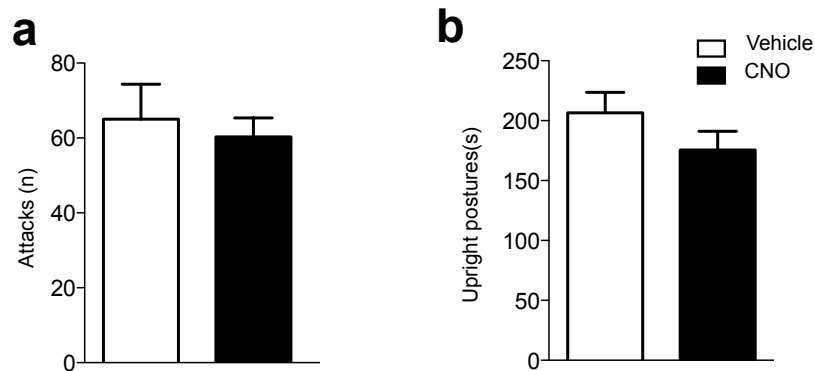
Supplementary Figure 5 (related to Fig. 3e-f). Extent of infection and its correlation with defensive behavior in mice locally injected with AAV-*Syn*::*Venus*-2A-*hM4D* in VMHvl. (**a**, **c**, **e**) Diagrams and (**b**, **d**, **f**) quantitative graphs of the extent of infection as estimated by Venus reporter gene expression in individuals from three groups (**ab**, **cd**, **ef**) of mice injected locally with AAV-*Syn*::*Venus*-2A-hM4D in the VMHvl and treated with CNO. Diagrams show the color-coded extent of infection superimposed on a coronal brain section from a standard atlas [22] (Bregma -1.82). Graphs show the total area of bilateral infection in posterior VMHvl (color-coding matches diagrams) plotted against the defensive behavior displayed by CNO treated animals in response to exposure to an aggressive conspecific.
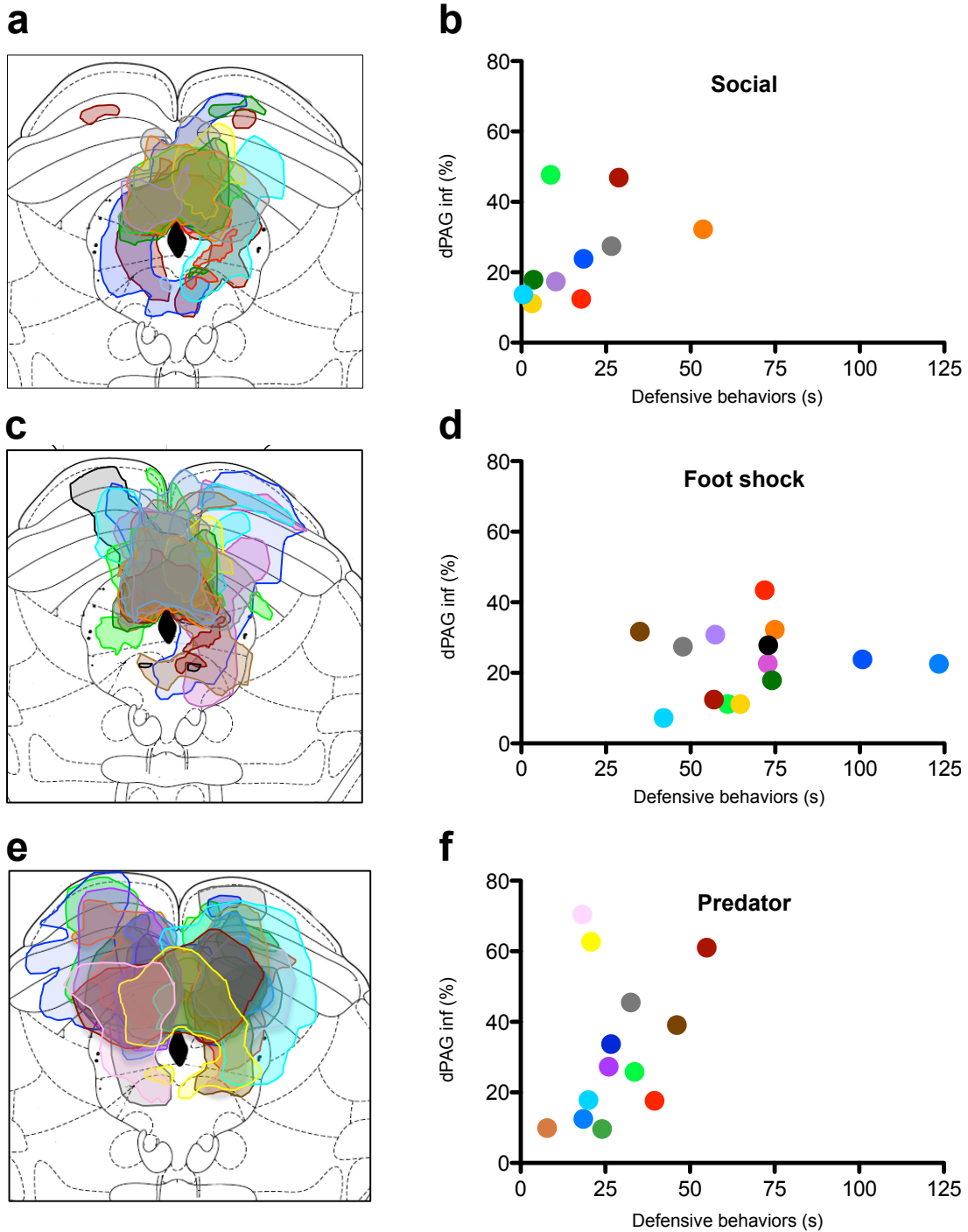
Supplementary Figure 6 (related to Fig. 3d-e). Correlation of extent of infection in different hypothalamic areas and defensive behaviors in mice locally injected with AAV-*Syn*::*Venus*-2A-*hM4D* in VMHvl. Extent of infection (total bilateral area) in (**a**) VMHvl, (**b**) VMHdm, (**c**) lateral hypothalamus (LH), and (**d**) tuberal nucleus plotted against the amount of defensive behaviors displayed by CNO treated animals in response to exposure to an aggressive conspecific. Correlation between the extent of infection and defensive behavior was calculated by MANCOVA (P = 0.032) followed by pairwise correlations with VMHvl (Pearson's r = – 0.708, P = 0.0046), VMHdm (Pearson's r = – 0.524, P = 0.0545), LH (Pearson's r = – 0.482, P = 0.0805), and tuberal (Pearson's r = – 0.315, P = 0.273) nuclei. For the statistical analysis outliers (reported in lighter grey) showing poor infection (< 20,000 μm² total infection, N = 3) or behavior (> 1.5 x IQR above third quartile, N = 2) were excluded.

(**e**) A significantly lower extent of infection was seen in VMHdm compared to VMHvl in these animals (N =19, * P < 0.035). (**f**) Scheme of the areas used for quantification of each region. Rectangular areas were matched on nuclei from a standard atlas [22]. For quantification in   the tuberal nucleus we considered the area located in the base of the tuberal region of the hypothalamus, just laterally to the VMHvl as described by [10].
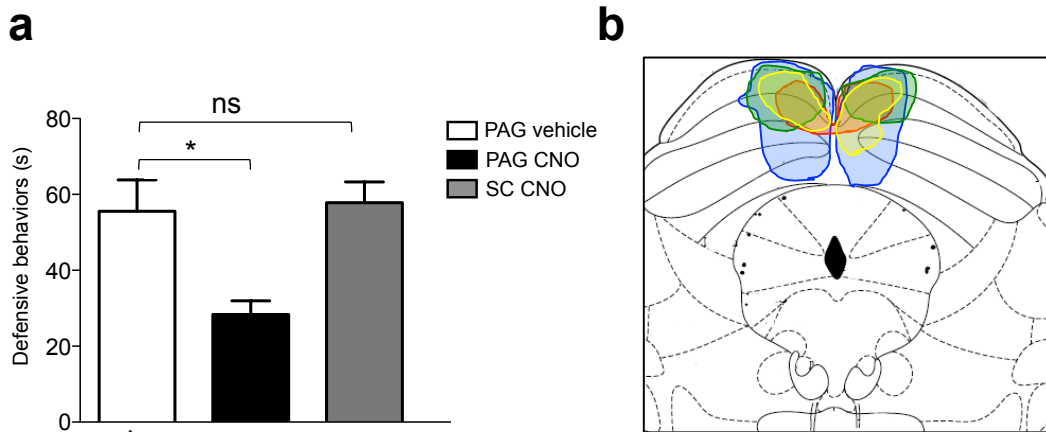
Supplementary Figure 7 (related to Fig. 3d-f). Attacks received and submissive behavior were not affected by pharmacogenetic inhibition of VMHvl. Number of (**b**) upright postures (P = 0.19) or (**a**) biting attacks (P = 0.58) received by the experimental mouse infected in VMHvl with AAV-*Syn*::*Venus*-2A-*hM4D* during the encounter with the aggressive stimulus mouse was not altered by CNO treatment when compared to vehicle treated control mice (VMHvl: N = 17-19, P > 0.05).
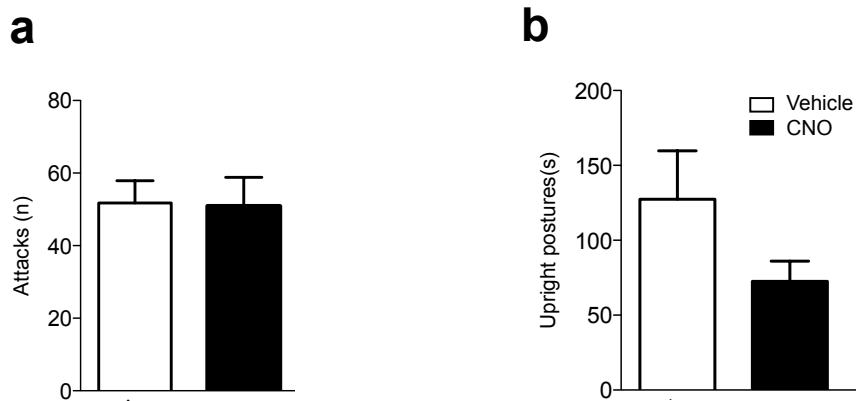
Supplementary Figure 8 (related to Fig. 3g-h). Extent of infection and its correlation with defensive behavior in mice locally injected with AAV-*Syn*::*Venus*-2A-*hM4D* in dPAG.

(**a**, **c**, **e**) Diagrams and (**b**, **d**, **f**) quantitative graphs of the extent of infection as estimated by Venus reporter gene expression in mice injected locally with AAV-*Syn*::*Venus*-2A-*hM4D* in the dPAG and treated with CNO. Diagrams show the color-coded extent of infection superimposed on a coronal brain section from a standard atlas [22] (Bregma -4.36). Graphs show the average percentage of bilateral infection in dPAG (color-coding matches diagrams) plotted against the defensive behavior displayed by CNO-treated animals in response to exposure to an aggressive conspecific (**ab**, Social; **cd**, Foot shock; **ef**, predator).

a

stretch postures Data 2

b

Supplementary Figure 9 (related to Fig. 3g-h). Mice injected with AAV-*Syn*::*Venus*-2A-*hM4D* in superior colliculus (SC) and treated with CNO do not show decreased defensive behavior to a predatory rat. (**a**) CNO-treated mice locally injected with AAV-*Syn*::*Venus*-2A-*hM4D* in dPAG (N = 13, P = 0.03), but not SC (N = 4, P = 0.81) showed a decrease in defensive behaviors to a predatory rat compared to similarly infected vehicle-treated mice (N = 5). (**b**) Diagram showing the extent of infection superimposed on a coronal brain section from a standard atlas [22] (Bregma -4.36) in individual CNO-treated mice injected with AAV-*Syn*::*Venus*-2A-*hM4D* in SC.

a

interactiontCamilla's vertical

b

immobility

Supplementary Figure 10 (related to Fig. 3g-h). Attacks received and submissive behaviors following pharmacogenetic inhibition of dPAG. CNO treatment did not change the (**a**) number of attacks received (P = 0.93) by mice infected in dPAG with AAV-*Syn*::*Venus*-2A-*hM4D*. (**b**) Time spent in upright postures (P = 0.19) during the encounter with the aggressive stimulus mouse was decreased by CNO treatment when compared to vehicle treated control mice (N = 9-10).