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Paula Gomez Sotres

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École Doctorale des Sciences de la Vie et de Santé

Spécialité – Neurosciences

Par **Paula Gomez Sotres**

Le rôle des cellules astrogliales dans la transmission sociale du stress

The role of astrocytes in the social transmission of stress

Sous la direction de Giovanni MARSICANO et Jaideep BAINS

Soutenue le 26/01/2024

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RÉSUMÉ

Les odeurs sociales transmettent des émotions et modifient le comportement. Par exemple, les informations olfactives provenant d'individus stressés peuvent induire des changements physiologiques et synaptiques similaires au stress chez des partenaires naïfs. L'expérience directe du stress altère la cognition, mais on ne sait pas' encore si le stress transmis socialement peut également modifier les processus de mémorisation. Nous montrons ici que l'investigation sociale d'un individu stressé, ou l'exposition à des signaux olfactifs spécifiques de cet individu, est suffisante pour altérer la reconnaissance d'objets nouveaux (NOR) chez des souris mâles non stressées. Les récepteurs cannabinoïdes de type 1 participent au stress, à la mémoire et aux interactions sociales, mais leur contribution à la transmission sociale du stress n'a pas encore été étudiée. Dans ce travail de thèse, j'ai utilisé le comportement, des manipulations génétiques de l'expression de CB1 dans différentes populations cellulaires et subcellulaires et des enregistrements in vivo de l'activité calcique pour étudier le rôle des récepteurs CB1 dans la transmission sociale du stress et ses adaptations cognitives. Les résultats montrent que les adaptations cognitives induites par la transmission sociale du stress nécessitent des récepteurs cannabinoïdes de type 1 associés à la mitochondrie (mtCB1) dans les astrocytes du bulbe olfactif. L'exposition de souris à des odeurs de stress augmente les niveaux de calcium mitochondrial de manière dépendante des récepteurs mtCB1. En accord, le blocage de l'activité de l'uniporteur de calcium mitochondrial (MCU) dans les astrocytes du bulbe olfactif supprime à la fois l'exploration des partenaires stressés et ses conséquences cognitives. Ces données sont cohérentes avec l'idée que la régulation du calcium mitochondrial astrocytaire du bulbe olfactif par les récepteurs mtCB1 est nécessaire pour déterminer la saillance des odeurs provenant de partenaires stressés et pour définir leurs conséquences cognitives. Ainsi, certaines odeurs sociales peuvent avoir un impact sur des processus cognitifs non liés, et le traitement astrocytaire représente une étape clé pour détecter leur pertinence et leur signification.

Mots-clés: astrocytes, olfaction, mitochondries, endocannabinoïdes, stress, social, cognition

ABSTRACT

Social odors transmit emotions and alter behavior. For instance, olfactory information from stressed individuals can induce stress-like physiological and synaptic changes in naïve partners. Direct stress experience alters cognition, but whether socially transmitted stress can also alter memory processes is currently unknown. Here we show that social investigation of a stressed individual, or exposure to specific olfactory signals from that individual, is sufficient to impair novel object recognition (NOR) in unstressed male mice. Cannabinoid-type-1 receptors participate in stress, memory and social interactions, but their contribution to social transmission of stress has not been addressed yet. In this Thesis work, I used behavior, genetic manipulations of CB1 expression in different cellular and subcellular populations and *in vivo* recordings of calcium activity to study the role of CB1 receptors in social transmission of stress and its cognitive adaptations. The results show that the cognitive adaptations induced by social transmission of stress require mitochondria-associated cannabinoid type-1 (mtCB1) receptors in astrocytes of the olfactory bulb. Exposure of mice to stress odors raises the levels of mitochondrial calcium in a mtCB1 receptor-dependent manner. In agreement, blockade of the Mitochondrial Calcium Uniporter (MCU) activity in astrocytes of the olfactory bulb suppresses both exploration of stressed partners and its cognitive consequences. These data are consistent with the idea that olfactory bulb astrocytic mitochondrial calcium regulation by mtCB1 receptors is necessary to determine the salience of odors coming from stressed partners and to define their cognitive consequences. Thus, certain social odors can impact unrelated cognitive processes, and astrocytic processing represents a key step to detect their relevance and meaning.

Keywords: astrocytes, olfaction, mitochondria, endocannabinoids, stress, social, cognition

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- **Olfaction and Neuroscience day** (Bordeaux, France), October 2023 –*Olfactory bulb astrocytes control social transmission of stress and its cognitive consequences*
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CB1 receptors are necessary for the amnesic effects of socially transmitted stress. Gomez-Sotres, P., Skupio, U., Cannich, A., Kaladzic, F.J., Guisquet, D., Busquets-Garcia, A., Bains, J.S, & Marsicano, G.

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1 (CB1) receptors control complex social emotional behaviors. Gomez-Sotres, P., Busquets-Garcia, A., Bains, J.S, & Marsicano, G.

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LIST OF ABBREVIATIONS

2-SBT: 2-sec-butyl-4,5-dihydrothiazole
2-AG: 2-Arachidonoylglycerol
ACo: anterior cortical amygdaloid nucleus
AEA: N-Arachidonylethanolamine
AG: anogenital investigation
AKT: Protein kinase B
ALDH1L1: Aldehyde dehydrogenase 1 family member L1
ANLS: Astrocyte-neuron lactate shuttle
ANOVA: Analysis of variance
AOB: Accessory olfactory bulb
AON: Anterior olfactory nucleus
AOS: Accessory olfactory system
AmPir: Amygdaloid piriform transition area
aPC: anterior piriform cortex
ASD: Autism spectrum disorder
ATP: Adenosine triphosphate
AUC: Area under the curve
BAOT: Bed nucleus of the accessory olfactory tract
BLA: Basolateral amygdala
BNST: Bed nucleus of the stria terminalis
BO: Body exploration
BSNT: Bed nucleus of the stria terminalis
BTBR: The BTBR T+tf/J mouse strain
CA: Cornu Ammonis (part of the hippocampus)
cAMP: Cyclic adenosine monophosphate
CAG: Cytosine-adenine-guanine (DNA sequence)
CB1: Cannabinoid-type-1 receptors
CB2: Cannabinoid-type-2 receptors
CBD: Cannabidiol
CBDV: Cannabidivarin
CCK: Cholecystokinin
CFF: Centrifugal fibers
CMV: Cytomegalovirus
CNS: Central nervous system
CORT: Corticosterone
COVID: Coronavirus Disease
CRE: Cre recombinase
CREB: cAMP response element-binding protein
CRH: Corticotropin-releasing hormone

DAGL: Diacylglycerol lipase
DAPI: 4',6-diamidino-2-phenylindole (fluorescent dye)
DEM: Demonstrator
DIO: Double-floxed inverted open reading frame (virus)
DSE: Depolarization-induced suppression of excitation
DSI: Depolarization-induced suppression of inhibition
DSM: Diagnostic and Statistical Manual of Mental Disorders
EAAC: Excitatory amino acid carrier
ECS: Endocannabinoid system
eCBs: endocannabinoids
EGFP: Enhanced green fluorescent protein
EPL: External plexiform layer
EPSC: Excitatory postsynaptic current
ER: Endoplasmic reticulum
FAAH: Fatty acid amide hydrolase
FMR: Fragile X mental retardation
FMRP: Fragile X mental retardation protein
FS: Foot-shock
FXS: Fragile X syndrome
GABA: Gamma-aminobutyric acid
GAD: Glutamate decarboxylase
GCL: Granule cell layer
GFAP: Glial fibrillary acidic protein
GFP: Green fluorescent protein
GG: Grueneberg ganglion
GL: Glomerular layer
GLAST: Glutamate aspartate transporter
GLT: Glutamate transporter
GPCR: G protein-coupled receptor
HA: Hemagglutinin
HIF: Hypoxia-inducible factor
HPA: Hypothalamic-pituitary-adrenal axis
HPC: Hippocampus
HRP: Horse radish peroxidase
HYP: Hypothalamus
IC: Insular cortex
IPL: Inner plexiform layer
IPSC: Inhibitory postsynaptic current
KI: Knock-in
KO: Knockout
LEnt: Lateral entorhinal cortex
LFPS: Local field potentials
LSP: Lateral synaptic potentiation
LTD: Long-term depression

LTP: Long-term potentiation
M/T: Mitral/tufted cells
MAGL: Monoacylglycerol lipase
MCL: Mitral cell layer
MCU: Mitochondrial Ca²⁺ uniporter
MeA: Medial amygdala
MERC: Mitochondria-ER contact sites
MHC: Major histocompatibility complex
MICU1: Mitochondrial calcium uniporter complex subunit 1
MOB: Main olfactory bulb
MOE: Main olfactory epithelium
MOS: Main olfactory system
mPFC: medial prefrontal cortex
MPOA: Medial preoptic area
MSN: Medium spiny neuron
mtCB1: mitochondrial CB1 receptors
MTMT: (methyl-thio)methanethiol
MUP: Major urinary protein
NA: Noradrenaline
NADH: Nicotinamide adenine dinucleotide (reduced)
NAPE: N-Arachidonoyl phosphatidylethanolamine
NCX: Sodium-calcium exchanger
NDFUS: Neurodegenerative disease-related protein fus
NMDA: N-Methyl-D-aspartate
NOR: Novel object recognition
NAc: nucleus accumbens
OB: Olfactory bulb
OBS: Observer
OEC: Olfactory ensheathing cell
OR: Olfactory receptor
OSN: Olfactory sensory neuron
OT: Oxytocin
OXT: Oxytocin receptor
PBS: Phosphate-buffered saline
PBST: Phosphate-buffered saline with Tween
pCoa: posterior cortical amygdaloid nucleus
PEA: Phenyl Acetaldehyde
PFC: Prefrontal cortex
PG: Periglomerular cell
PKA: Protein kinase A
PLCo: posterolateral cortical amygdaloid nucleus
PMCo: posteromedialcortical amygdaloid nucleus
pPC: posterior piriform cortex
PVN: Paraventricular nucleus

RAB: Ras-related protein Rab
RIM: Rimonabant
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RS: Rescue
sAC: soluble adenylyl cyclase
SEM: Standard error of the mean
SERCA: Sarco/endoplasmic reticulum Ca²⁺-ATPase
SHANK: SH3 and multiple ankyrin repeat domains
SOM: Septal organ of Masera
STS: Social transmission of stress
TAAR: Trace amine-associated receptor
THC: Tetrahydrocannabinol
TMT: Trimethylthiazoline
TRPV: Transient receptor potential cation channel subfamily V
USV: Ultrasonic vocalization
VMH: Ventromedial hypothalamus
VNO: Vomeronasal organ
VPA: Valproic acid
VTA: Ventral tegmental area
WIN: WIN 55,212-2 (synthetic cannabinoid)
WT: Wild-type

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I. INTRODUCTION

Chapter 1: Social behaviors

1.1. Why are we social?

“Man is by nature a social animal; an individual who is unsocial naturally and not accidentally is either beneath our notice or more than human. Society is something that precedes the individual.” - Aristotle, *Politics*.

As many other animals, humans are inherently social. We share social behaviors like affiliation, aggression, or the establishing of a hierarchical system in our social groups. We share this with different animals, including those with much more “simpler” brains than ours. Even some bacteria exhibit types of social recognition of others to allow them to form colonies of individuals of the same strain (Gibbs et al., 2008). But, if so many animals are social, the bigger question is, why? In other words, what is the adaptive value of sociability?

If we think about the classical view of evolution (Spencer, 1864), one would expect that sociability will positively affect fitness (Hamilton, 1963). Indeed, living in groups can offer protection from predators, increase access to conspecific reproductive mates, improve the physiological responses to certain situations and cooperation to find or defend food or territory from others (van den Bergh, 2018; Krause and Ruxton, 2002). However, being social can also come with a cost: social groups have higher intraspecific competition for reproduction and resource sharing, aggression and disease transmission (Krause and Ruxton, 2002).

According to behavioral ecology theory, sociability evolves when the benefits of interactions with conspecifics outweigh the costs (Krause and Ruxton, 2002). This balance depends on the species and habitats in which they live (Bourke, 2014). For example, in humans the main costs of being social are increased competition for resources and pathogen exposure. However, the benefits are far greater: cooperation in an infinite number of ways, psychological support, better chance at finding a mate, and protection are some of those (Shen et al., 2014).

Indeed, pathologies like autism or antisocial personality disorder are just examples on how not being able to socialize in a healthy manner with individuals around us can have a deep impact in our daily functioning. On the other hand, restriction of social interactions (self-isolation) can lead to detrimental consequences that can also affect our wellbeing. The COVID-19 pandemic is an example of this: rates of anxiety and depressive disorders rose 25% globally after the first year of the pandemic (WHO; (Kupcova et al., 2023), showing how lack of daily social interactions can have a deep effect on our mental health.

Sociability is, for its value as an evolutionary drive force, an example of convergent evolution across many taxa (Fischer et al., 2019). In other words, similar social behaviors relying on similar mechanism have arisen in many species, that share or not a common ancestor. Understanding how the social brain works, using the similarities across the evolutionary scale, is a key step to understand the current social world we live in, and although much is left to know, accumulating evidence has started to allow us to help those who are pathologically unsocial. Despite the fact that, as mentioned, even very simple animals display social interactions, mammalian social behaviors are the subject of my thesis work, and they will be mainly described here.

1.2. The social brain: circuits underlying the different phases of social behaviors

The social brain, term coined in 1990 by the American psychiatrist and neuroscientist Leslie Brothers after her studies in amygdala and orbitofrontal cortex-lesioned monkeys, engulfs all those brain structures in charge of establishing social interactions (Brothers, 1990). The study of the social brain resulted in the creation of the social neuroscience field that tries to unravel the underlying neural basis of social interactions, at all cognitive levels: from the simple social recognition to the most advanced levels of empathy (Insel and Fernald, 2004). Because of the conservation of social behaviors across taxa, we can use animal models to study which brain regions contribute to the social brain (Panksepp and Panksepp, 2013).

Ethologists have been trying to differentiate distinct components of social behaviors since the early 20th century (Sherrington, 1906; Lorenz, 1950; Tinbergen, 1951). Whereas these classifications sometimes lack validity due to the complex nature of behavior, they are still conceptually useful to simplify the complexity arising from generalizing social brain functioning (Ball & Balthazart, 2007). Separating it into an appetitive and a consummatory phase, Lorenz and Tinbergen provided the first distinction of social behaviors (Lorenz, 1981; Tinbergen, 1951). Based on this first categorization, authors like Wei *et al.* recently proposed a further classification based on four phases, which could better explain the transition points that define the progression of a social behavior (Wei *et al.*, 2021). The first three phases focus on the identification phase that aims to collect information and bring the animal to close proximity to be able to take the appropriate behavioral actions, that supposes the last phase. Thus, following this classification, most social behaviors can be described as follows (**Figure 1**): (1) a first phase is social detection, in which the animals perceive the presence of social cues. (2) A second phase is approach/avoidance, where animals activate locomotor functions to get closer or farther from the social stimulus. In case of approach, (3) the third phase is investigation, in which animals physically interact exchanging additional social information. Finally (4), the fourth phase is given by social consummation, during which animals exert the type of social behaviors triggered by the previous phases (fight, mating, playing, etc.). These phases are shortly described below.

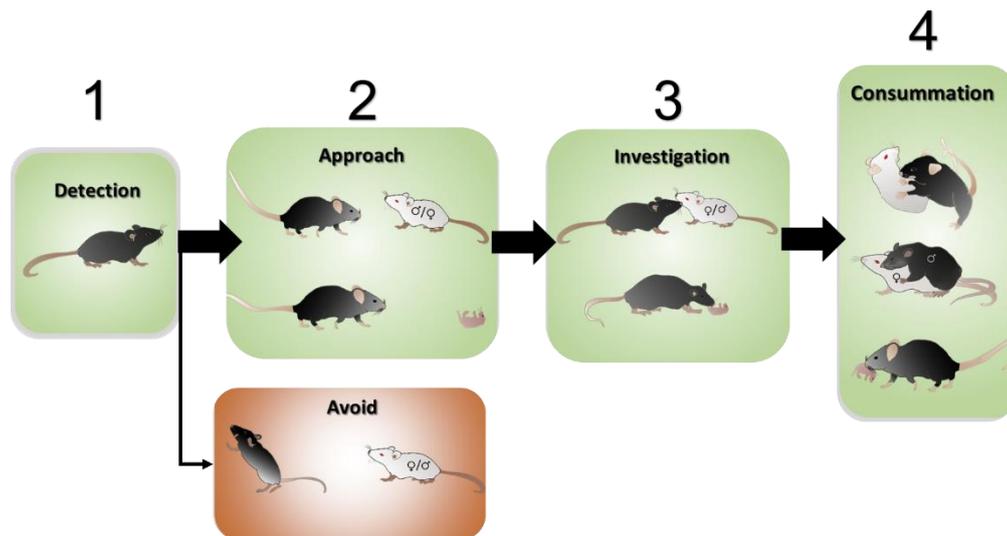


Figure 1: (adapted from Wei et al., 2021): Different phases of innate social behaviors that have associated characterized circuits.

1.2.1. Phase 1: Distant detection of social stimuli

The start of a social interaction generally occurs when two or more individuals of the same species are close enough to exchange auditory, visual and olfactory signals, without being in physical contact yet. In such situations, the first thing an individual aims for are the identification of the presence, location and nature of the social target(s).

Regarding audition, simple vocalizations can be used as attractive or aversive signals that can help the identification of the target, while the development of language is the most complex level of auditory communication associating complex meanings to the identity of the target. Indeed, vocal cues are used in many species to attract or detect a potential partner, including mice (Tschida et al., 2019) birds (Loning et al., 2023), frogs (Tobias et al., 2004) and humans (Hughes and Puts, 2021), just to name a few. Interestingly, vocalizations related with appetitive or aversive emotional states in rats have been seen to alter the behavior of surrounding rats (Kim et al., 2010; Saito et al., 2016; Seffer et al., 2014). In mammals, the auditory information goes from the periphery to auditory subcortical areas, including the medial geniculate nucleus, and arrives at the auditory cortex (Pollak et al., 2003). The auditory cortex encodes the temporal statistics and identity of vocalizations in rodents (Carruthers et al., 2013; Paraouty et al., 2023) and humans (Hosaka et al., 2021).

Vision is also used to locate and identify a social target, providing information such as distance, facingness or contingent motion (McMahon and Isik, 2023). For example, zebra fishes usually shoal with other conspecifics, but replacing real zebra fish images by dots of similar size with the same kinetics was sufficient for zebra fish to show the recognition of those as potential shoal partners (Larsch and Baier, 2018). The identification of social targets is a fast and automatic response that depends on the visual cortex. However, visual detection of social targets offers more information than simple space reference of a social target. The visual cortex can also encode specific core components of social interactions, particularly in groups of individuals (McMahon and Isik, 2023). For example, in humans, the extra striate body area of the visual cortex shows selective responses to dyads of bodies instead of individual bodies, faces or objects, suggesting a specific early visual detection of social cues (Abassi and Papeo, 2020). Lack of visual function can deeply impair social detection, resulting in decreased social interactions in humans and mice, despite the compensatory potentiation of other sensory modalities (Bouguiyoud et al., 2022; Klauke et al., 2023).

Olfaction is critical for mammalian social behavior, particularly in rodents (Contestabile et al., 2021; Ryan et al., 2008). When a rodent detects an odor, it moves its nose and vibrissae up and down during the inspiration/expiration cycles (Kurnikova et al., 2017) and it adopts a strategy to locate the odor source by performing serial-sniffing (sampling the cue, moving, and sampling again) (Liu et al., 2020). Humans also show olfactory exploration behaviors for conscious odor detection, like active smelling (Han et al., 2022). However, due to the particular neuroanatomical organization of olfactory circuits (see below), most of the odors we detect escape conscious recognition, reason by which it was previously thought that humans were “bad sniffers”(McGann, 2017). However, humans can detect up to 1 trillion odors including social odors (Buschdid et al., 2014a; de Groot et al., 2012), supporting the key role of olfactory cues in human’s every day social interactions. Odors are the only sensory cues that can be detected and encoded in the brain without a thalamic relay, in contrast with auditory, tactile, gustatory or visual cues. Social odors coming from conspecific, also called **chemosignals**, are detected by olfactory sensory organs located in the nasal cavity (Dulac and

Wagner, 2006; Lübke and Pause, 2015). In rodents, four main systems have been related to the detection and processing of chemosignals: the main olfactory system (Lin et al., 2005) (MOS), the accessory olfactory system (Dulac and Wagner, 2006) (AOS), Grunenberg ganglion (Brechtbühl et al., 2008) (GG) and septal organ of Masera (Ma et al., 2003) (SO). These project to brain regions like the piriform cortex, cortical amygdala, medial amygdala, lateral entorhinal cortex and olfactory tubercle among others (Martinez-Marcos, 2009). Olfactory circuits will be discussed in depth in Chapter 3 of this Thesis.

1.2.2. Phase 2: Approach/avoidance

The approach/avoidance phase arises from the integration of social information with intrinsic social motivation and disposition of individuals, resulting in the actual locomotor behavior that changes the distance to the social stimulus. Specific mechanisms related to arousal and reward/aversion have been related to this phase of social behaviors across social species. For example, in *Drosophila*, social cues converge in a specific subpopulation of mushroom body serotonergic cells, whose inactivation impairs social approach, but these neurons don't appear to be involved in other locomotor behaviors or vision/olfaction (Sun et al., 2020). Social interactions are inherently rewarding for rodents (Trezza et al., 2011). Two brain regions are critical for choice between social approach/avoidance: the ventral tegmental area (VTA), and the nucleus accumbens (NAc). Different social sensory cues activate VTA neurons (Contestabile et al., 2021). Projections to VTA from the superior colliculus, a brain region involved in sensory information processing and spatial attention (Dean et al., 1989) are active during the orientation towards a social stimuli (Solié et al., 2022). The VTA neurons that modulate this function target mainly the dorsolateral striatum (DLS), and their inhibition induces increased social interaction (Solié et al., 2022). Interestingly, VTA to NAc projections activation results in an increased sociability but not in exploration of non-social stimuli (Gunaydin et al., 2014; Solié et al., 2022). This effect is mediated by D1-positive medium spiny neurons (MSNs) in the NAc (Gunaydin et al., 2014). Interestingly, the NAc also mediates specific approach behaviors towards social stimuli that are emotionally aroused. In rats, the stress of a juvenile partner can be detected through olfactory (Inagaki

et al., 2014) or auditory cues (Parsana et al., 2012) leading to a social approach of this stimulus instead of a naïve one (Rogers-Carter et al., 2018a). This is mediated by insular-to-NAc projections (Rogers-Carter et al., 2019), and suggests an integration of not only internal social reward in the NAc but also of emotion. By modulating the brain stem motor circuitry, the output afferents of both VTA and NAc probably mediate locomotor responses (Wei et al., 2021).

1.2.3. Phase 3: Investigation

In case that previous phases generated an approach and once two or more individuals are in close proximity, the social targets would investigate each other in order to acquire more information concerning familiarity (Brennan and Kendrick, 2006), social rank (Wesson, 2013), emotional state (Kiyokawa et al., 2004) or reproductive condition of the other(s) (Takenawa et al., 2023). The recognition of the identity of the partner is determined by the sensory cues emitted, but also by the previous experience of the investigating animal, such as cognitive processes or other specific internal state. For example, mice are able to recognize other familiar mice in pain by visual cues but only if they have also experienced the same type of pain (Langford et al., 2006).

In close proximity, non-volatile chemosignals present around the face and anogenital areas of a rodent can be detected by the vomeronasal organ, which projects to the AOB and then to limbic areas including the medial amygdala (MeA), the posterior part of the bed nucleus of the stria terminalis (BSNT) and the posterior cortical amygdala (CoApm) (Scott et al., 2023; Spehr et al., 2006a). Moreover, other nonvolatile olfactory cues are also being processed by the MOB, and converge onto the MeA (Martinez-Marcos, 2009) either directly or indirectly through the cortical amygdala (Fig. 3). This brain region receives inputs from brain regions like the hypothalamus (Dwyer et al., 2022; Ferguson et al., 2001), hippocampus (Dwyer et al., 2022), ventral tegmental area (Dwyer et al., 2022) and medial prefrontal cortex (Sotoudeh et al., 2022) among others. Different GABAergic MeA subpopulations are active during investigation of same-sex, opposite-sex, pups or predator cues and they modulate approach/avoidance responses (Li et al., 2017a; Lischinsky et al., 2023; Miller et al., 2019). Interestingly, optogenetic activation of MeA posterior GABAergic neurons induces

different effects depending on intensity. Whereas low intensity stimulations increase social grooming, stronger ones induce attack and aggression between male mice (Hong et al., 2014). Together, these data support the essential role of the MeA in the integration of investigatory information preceding the consummatory phase of the social behavior (Figure 3).

1.2.4. Phase 4: Consummation

Consummatory behaviors are defined as the resulting actions deriving from motivation (Wei et al., 2021). The hypothalamus can be considered the output region of the social brain, as most studied social behaviors until now rely in a way or another on this brain region (Ferguson et al., 2002; Mei et al., 2023; Remedios et al., 2017a). The hypothalamus is a complex area, where different nuclei interact with each other and with other brain regions. The population activity in the neurons of the hypothalamus can be used to decode social behavior (Remedios et al., 2017). Particularly, nuclei like the ventromedial hypothalamus (VMH), the medial preoptic area (MPOA) and the premamillary nucleus (PMv) are activated during aggressive behaviors and mating responses (Guo et al., 2023b; Hashikawa et al., 2017; Mei et al., 2023; Miller et al., 2019).

One the other hand, the hypothalamus is controls other social behaviors *via* neuropeptides. Oxytocin is produced mainly by neurons of the paraventricular nucleus of the hypothalamus (PVN) and of the supraoptic nucleus (Ferguson et al., 2002). These neurons are involved in social transmission of maternal care in female mice (Carcea et al., 2021), social grooming (Matsumoto et al., 2021), pair bonding (Marlin and Froemke, 2017) and social emotional discrimination (Ferretti et al., 2019). Vasopressin-producing neurons in the PVN and in the suprachiasmatic nucleus (Caldwell, 2012) are involved in affiliation, pair bonding, aggression, parental behavior and social recognition (Ferguson et al., 2002; Rigney et al., 2023). Lastly, neurons using the corticotropin-releasing factor (CRF/CRH) in the PVN mediate stress response in the brain by activating the hypothalamic-pituitary-adrenal axis (Herman et al., 2016), but they have also been linked to the expression of social behaviors (Bagosi et al., 2023). For instance, CRH-positive neurons in the PVN are active during aggression (Kim et al., 2019), pup interaction (Kim et al., 2019), and during interaction with a stressed

congener (Sterley et al., 2018). CRH1 and CRH2 receptors are expressed in multiple brain regions (**Figure 2**), including the periaqueductal gray and the cerebellum, which could be the motor outputs of this hypothalamic system (Bagosi et al., 2023).

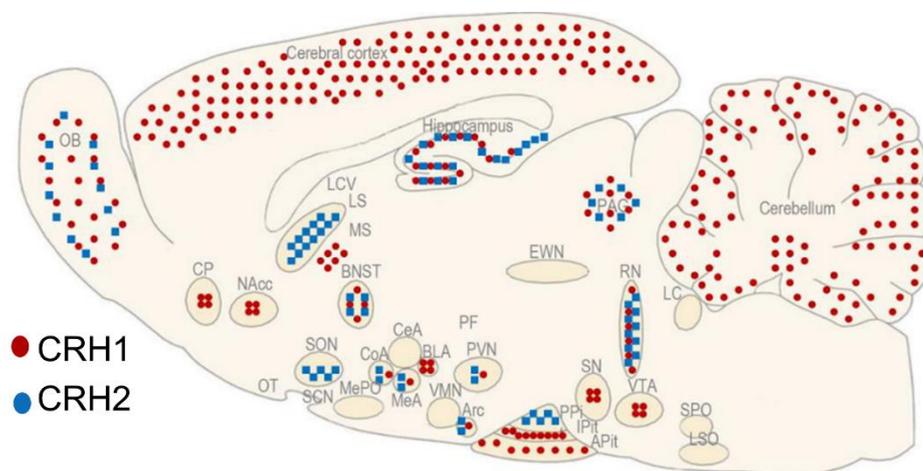


Figure 2: (adapted from Bagosi et al., 2023): CRH1 and CRH2 receptor expression in the mouse brain.

Chapter 2: Social transmission of emotions

Emotions are mental states that arise from significant situations affecting the neurophysiological state of an individual, accompanied with thoughts and behavioral responses with a degree in pleasure or displeasure (Damasio, 1998). In animals, science has mostly focused on understanding the neurobiology of affective states, meaning the physiological and behavioral responses to an emotion-triggering stimulus (LeDoux, 2021; Tinbergen, 1951). Emotions are generally expressed through emotional cues like odors (Kiyokawa et al., 2004; Sterley et al., 2018), vocalizations (Rogers-Carter et al., 2018a; Saito et al., 2016) or specific behaviors (Ferretti et al., 2019; Kavaliers et al., 2001; Langford et al., 2006) that other conspecifics can detect and react to. The detection of these cues helps individuals to get an intuitive sense of the identity and state of surrounding partners and, in turn, it modulates social interactions (Sterley and Bains, 2021). Social interactions can be used to obtain a specific benefit from the partner, like resource sharing or mating (Fischer et al., 2019), but they also help choosing adaptive behaviors to specific situations that were not directly experienced (Puścian et al., 2022). Thus, by enabling individuals to recognize and share aspects of the partners' affective state (de Waal and Preston, 2017), such transmission of emotional information can help faster adapting to different situations.

2.1 Emotional contagion and empathy

Empathy, the psychological construct of understanding someone else emotions (de Waal, 2008), includes sharing the affective state of the other person as well as understanding why they are feeling like that and adopting their perspective (de Waal, 2008). Empathy plays a key role in keeping healthy human societies (Fuchsman, 2015) and the study of this function has focused until the past few years in humans and non-human primates (Panksepp and Panksepp, 2013). However, many other animals have emotions and express them, like Darwin proposed in 1872 in his book *The expression of emotions in man and*

animals (Darwin and Ekman, 1998). Animals from many species react differently in response to an emotional conspecific when socializing with a neutral one (Pérez-Manrique and Gomila, 2022). This suggests that the communication of specific information associated to the emotional state of the partner can induce relevant change in the behavior of the receiver also in non-human primates.

Since social animals depend on interactions with one another to survive, empathic-like behaviors have been conserved across species likely because they offer an evolutionary advantage (Keysers and Gazzola, 2016; Panksepp and Panksepp, 2013). However, empathy does not look the same in all animals, as it depends on the cognitive capability of each species (Panksepp and Panksepp, 2013). At the most basic level, we can talk about **emotional contagion**, defined as sharing of the affective states between individuals (Meyza et al., 2017). The emotional contagion of an affective state involves some type of state-matching that can result in a copy of the physiological or behavioral responses observed in the emotional congener (Panksepp and Panksepp, 2013). In this context, studies with rodents have provided with evidence that both rats and mice can display emotional contagion of affective states. For example, rodents show fear responses when their partners are in distress (Carrillo et al., 2019; Jeon et al., 2010) or copy pain related behaviors when their partners are in pain (Church, 1959; Langford et al., 2010). The use of animal models has become a useful tool to facilitate an empirical approach to study the most basic form of empathy, emotional contagion, with manipulations of the neurobiological and behavioral substrates that cannot be easily achieved in humans.

2.1.1. The use of rodent models to understand emotional contagion

On the search for better animal models of empathic-like behaviors, the biggest advance has been made in rodent models because of their availability, inexpensiveness and easy handling and manipulation. Rodents are highly social animals that live in hierarchical societies and show high levels of cognitive flexibility, thus supporting their use to study multiple levels of empathy.

During the last 60 years, large evidence accumulated supporting the existence of social transmission of emotions in rats and mice. The study of social modulation of emotional behavior started in 1939, when Anderson (Anderson,

1939) tested pairs of rats together in an open field observing that the presence of the other rat affected the number of fecal boluses, likely as a consequence of emotionality (1939). On the same line of thought, Davitz & Mason (1955) presented results from an experiment in which rats that were fear conditioned to a light cue had a reduction of their freezing response if tested with another rat. Moving on further, Church (1959) published an experiment in which rats were fear conditioned in pairs, and afterward one of them was trained to push a lever that would shock their conspecific in exchange of a food pellet after a 22-h food deprivation. This study showed a strong decrease in lever pressing by the rats that were conditioned together as compared to rats that observed a stranger conspecific receive the shock. The author hypothesized that this reduction in lever pressing (buffering) was due to "fear for their partner", even in expense of their own need of food. Whereas the first two articles suggested that the presence of a partner can modulate the affective state of rats, Church's findings added that rats are also able to distinguish themselves from their partners. Thus, these data suggested for the first time that the effects of emotional contagion are not based solely on pure autonomic imitation, but it involve higher cognitive processing.

Since those seminal studies, the interest in rodent emotional contagion has increased consistently. The majority of the published articles on rodent models to study empathic-like behaviors have focused on social transmission of negative affective states, such as fear, pain and stress. The reason of this preference likely relies on the fact that the measurement of well characterized behavioral responses associated with negative emotions, such as freezing or avoidance behaviors, is easier than less evident responses to positive affective states (Blanchard and Blanchard, 1969). However, in the most recent years the increase on the interest about positive emotions in animals reverberated as well on the study of emotional contagion of affective states associated with rewarding or "appetitive" stimuli. The most used appetitive stimulus are 50Hz ultrasonic vocalizations, associated with social joy, rough-and-tumble play behaviors and anticipation to sexual behavior (Seffer et al., 2014), but the behavioral read out resulting from the transmission of positive affective states is more complex than with negative ones, which results in a considerably smaller number of published articles.

Interestingly, these rodent paradigms have begun to provide some clinically relevant information about one of the core symptoms of social pathologies like autism spectrum disorders (ASD): social-emotional reciprocity (DSM-V). BTBR T+Itpr3tf/J (BTBR) inbred mice, considered a mouse model for idiopathic ASD, display the core behavioral features of this pathology (Blanchard et al., 2012) and differences while interacting with a stressed partner both behaviorally and at the level of brain c-fos activation in the prefrontal cortex and in the amygdala in comparison with C57/BL6 mice (Meyza et al., 2015). Moreover, both BTBR and FMR1 KO mice (a genetic model for ASD) were tested for emotional contagion of pain, showing no response in comparison to wildtype C57/BL6 mice (Martin et al., 2022). The use of rodent models to further understand the neurobiological basis of social emotional reciprocity could provide key contributions to find treatments to specific deficits associated with these types of complex pathologies.

2.2. Socializing with emotional congeners

Rodents, like humans, can send and receive social cues which transmit affective information between conspecifics (Sterley and Bains, 2021b). This transmission determines behavioral responses in the partners, and, in some cases, it can lead to the emotional contagion of their original affective state (Sterley and Bains, 2021b). In other words, independently from our sharing of others' emotions, their detection determines specific social behavior responses (e.g. approaching, avoiding, etc.). However, when emotional contagion occurs, we do not only change our social behaviors, but we use this information to adapt our responses to the type of environment "described" by our partner's emotions.

2.2.1. Communication of negative affective states

Negative emotions arise as part of the adaptive responses to wellbeing-threatening situations (LeDoux, 2021). They come together with specific body changes favoring adaptive behaviors, like the urge to escape, attack, hide and others (Lazarus, 1991). Animals that experienced certain extreme negative emotions, such as the ones caused by a life-threatening last-second escape from a predator, can be in an state of arousal for even days after the

encounter(Adamec and Shallow, 1993). Thus, the interaction with a stressed conspecific can carry information about spatially and temporally distant events, which in turn modify the behavior of the receiver (Andraka et al., 2021). Thus, the responses of an observer animal are different whether the partner is being threatened at the moment or it has experienced the threat in the past (Andraka et al., 2021). Two different types of social transmission of aversive affective states are majorly studied, which depend on the time in which the emotionally arousing stimulus is delivered to the demonstrator: **observational aversive learning** and **social transmission of stress (Figure 3)**. In observational aversive learning, the observer witnesses a demonstrator receive a stressor directly (Keum and Shin, 2019). In the case of social transmission of stress, the observer interacts with a demonstrator that has received a stressor in the past (Panksepp and Panksepp, 2013). The next two sections focus on the description of these two types of social communication, together with the rodent brain circuits associated to them.

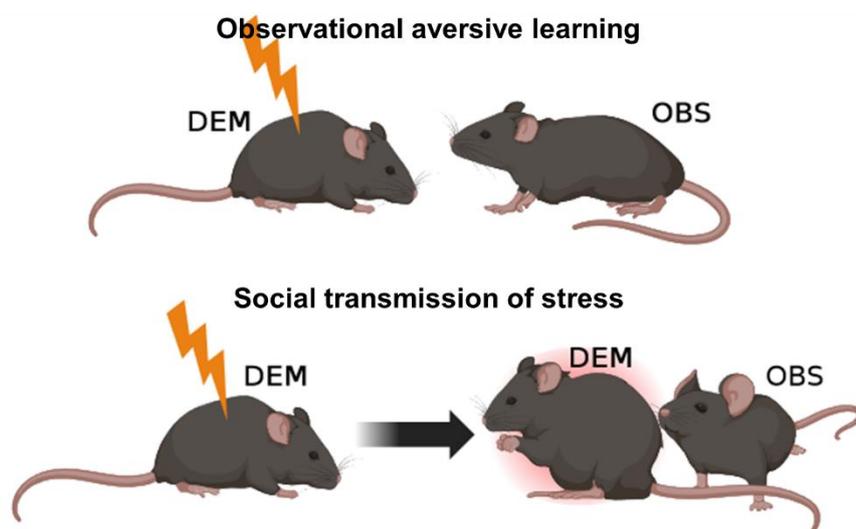


Figure 3: Observational aversive learning and social transmission of stress.

2.2.1.1. Observational aversive learning

Observational fear phenocopying by **social transmission of fear information**, or vicarious fear, has been observed in primates including humans (reviewed in Olsson and Phelps, 2007). Rodents also react to the fear of others under a direct threat, and this phenomenon has been studied using paradigms that trigger innate behavioral responses (Keum and Shin, 2019). In the most common of these paradigms, an observer witnessing the punishment of a

demonstrator with foot-shocks displayed passive aversive responses like freezing, similar to the ones adopted by the demonstrator (Andraka et al., 2021; Atsak et al., 2011; Carrillo et al., 2019; Chen et al., 2009; Gonzalez-Liencre et al., 2014; Jeon et al., 2010; Kim et al., 2014). Interestingly, this phenocopy was delayed in time, suggesting that observers did not merely mirror the freezing of the demonstrators, but they instead initiated similar behaviors independently (Andraka et al., 2021). Furthermore, when observers were presented with conditioned stimuli previously paired with foot-shocks to the demonstrator, they also displayed freezing (Allsop et al., 2018; Chen et al., 2009; Jeon et al., 2010), indicating that they likely learned from the negative affective states of their partners. In this paradigm, observers are probably responding to both the fear and the pain of the foot-shocked demonstrators. Indeed, the circuitry behind the modulation of observational freezing learning involves both fear- and pain-associated brain regions, such as the anterior cingulate cortex (ACC), lateral amygdala and several thalamic nuclei system (Carrillo et al., 2019; Jeon et al., 2010; Kim et al., 2014). The central amygdala (CeA) also participates in vicarious freezing: optogenetic inactivation of neurons in this brain region recruited during observation of a foot-shocked partner, led to a decrease of freezing in favor of exploration (Andraka et al., 2021). Visual inputs to the cortex and amygdala are thought to deliver visual information related to vicarious freezing responses, where the information integrates with the individuals own fear circuits (Olsson and Phelps, 2007). For instance, observers orient their heads towards the emotionally-altered demonstrator (Carrillo et al., 2019; Chen et al., 2009), and using an opaque screen inhibits the expression of vicarious fear (Jeon et al., 2010), proving that the ability to display observational freezing depends specifically on vision.

Humans also learn vicariously from the pain of others. For example, children displayed lower pain thresholds to painful cold water after observing their mothers exaggerating their response to the same stimulus, and reduced their pain facial expressions if their mothers did so (Goodman and McGrath, 2003). Mice also react to pain of other conspecifics (Langford et al., 2006; Smith et al., 2016). Pain-associated behaviors such as writhing (stretching until the abdomen touches the floor) caused by chemical injections are facilitated in the presence of

a demonstrator in pain (Langford et al., 2006). The phenocopy of the writhing behavior relies exclusively on visual cues, as mice separated from the demonstrator by an opaque screen did not display any facilitated response (Langford et al., 2006).

Phenocopying of negative affective states is strengthened by previous experience of the specific distress. Indeed, a pre-exposure to foot-shock in mice (Chen et al., 2009) and rats (Atsak et al., 2011) strongly potentiates the freezing response of observers exposed to a demonstrator receiving a foot-shock

2.2.1.2. Social transmission of stress

Environmental threats induce an arousal in animals that leads to the activation of the stress response, a state of alarm that triggers a range of automatic and hormonal changes to help survival (Rodrigues et al., 2009). These changes can last for minutes to hours (Holloway et al., 2023) and include the release of glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla and sympathetic nerves, modulating the hypothalamic-pituitary-adrenal (HPA) axis (McEwen et al., 2015). Socializing with conspecifics after a threat-induced affective state therefore involves socializing with stressed conspecifics.

When an individual socializes with a conspecific that has experienced a stressor, it typically tends to spend more time socializing with it than a neutral one (Ferretti et al., 2019; Sterley et al., 2018; Wu et al., 2021). In 2018, Sterley and colleagues started determining the neurobiological bases of social transmission of stress, paving the way to the present Thesis work. The PVN has been linked to social behaviors (see sections above), but it is also a key brain region to control stress responses and to regulate defensive behaviors towards a threat (Daviu et al., 2020; Füzési et al., 2016a). Mice that interact with familiar stressed partners display aspects of state-matching to them after the interaction. The activity of CRH-positive neurons in the PVN (PVN^{CRH}) is potentiated by foot-shock experience. Interestingly, this effect is also observed in naive partners socializing with previously foot-shocked demonstrators (Sterley et al., 2018). Interestingly, photoinhibition of these cells in the observer prevented social investigation of a stressed demonstrator (Sterley et al., 2018). Moreover, the plasmatic levels of

corticosterone in observer mice correlated to those of their stressed demonstrators (Sterley et al., 2018). This agrees with human data showing that plasma cortisol and other physiological stress markers like cardiac activity increased when individuals were paired with participants showing signs of stress (Buchanan et al., 2012; Dimitroff et al., 2017a; Engert et al., 2014). In mice, the PVN^{CRH}-mediated social approach and physiological phenocopy is inhibited by placing a barrier with holes in between the observer and demonstrator and it can be induced by the mere presentation of a cotton swab with secretions from this area (Sterley et al., 2018). This suggests that this type of social transmission of stress is largely mediated by detection of non-volatile olfactory chemosignals present in the anogenital area of stressed partners (Sterley et al., 2018). Another electrophysiological consequence of social transmission of stress has been recently described in the hippocampus. CA1 hippocampal LTD was observed both in demonstrators after tail-shock stress and in observers after social interaction, correlating with the time spent in anogenital sniffing (Lee et al., 2021).

The study of social transmission of stress in rats offers more information about the contribution of other brain regions to this phenomenon (Rogers-Carter et al., 2018a). By modulating synaptic activity of glutamatergic neurons that co-express CRH1 and cannabinoid-type-1 receptors (CB1), CRH signaling in the insula of male rats increased the social investigation of stressed unfamiliar juvenile partners (Rieger et al., 2022). However, a stressed unfamiliar adult was avoided, inducing self-grooming (Rogers-Carter et al., 2018), a behavior that has been related with expression of stress (Füzesi et al., 2016). More recent evidence indicates that the insular neurons involved in social approach towards stressed juvenile demonstrators project to the nucleus accumbens (Rogers-Carter et al., 2019). Interestingly, inhibition of BLA to posterior insular cortex in rats eliminated approach to stressed juveniles (Djerdjaj et al., 2022), suggesting a putative circuit of BLA-IC-NAc involved in this behavior in rats. Furthermore, stress-related vocalizations recorded during the interaction between demonstrators and observers might play a role in the transmission of stress information in rats, although their appetitive or aversive nature is still to be determined (Andraka et al., 2021; Rogers-Carter et al., 2018a). On the other hand, like in mice, odors were shown to be sufficient to trigger stress transmission in rats. Chemosignals

originating from different parts of the body of stressed demonstrators induced different effects on observers: whereas odors from the whisker pad increased social exploration of partners, odors from the anogenital area resulted in stress-induced hyperthermia (Kiyokawa et al., 2004). This suggests that different types of stress chemosignals could play distinct roles in the different outcomes of social transmission of stress.

By modulating amygdala (Ferretti et al., 2019) and prefrontal cortex (Scheggia et al., 2019) activity, PVN oxytocinergic neurons (PVN^{OXT}) are also involved in the discrimination of a stressed partner from a neutral one. The inhibition of PVN^{OXT} projections to CeA did not alter the social interaction with stressed or neutral partners independently, but it specifically affected the preference for the stressed one in a choice situation. The fact that both CRH (Andraka et al., 2021) and OXT signaling (Ferretti et al., 2019) in the CeA are involved in social interactions with stressed partners suggests that this brain region might represent an integration spot for social discrimination and transmission of stress.

The consummatory phase of social transmission of stress is not always clear. Most of the studies in rodents measure locomotor approach or avoidance as a behavioral consequence of the processing of the stress information (Meyza et al., 2015; Rogers-Carter et al., 2018a; Scheggia et al., 2019; Sterley et al., 2018). However, other behaviors can be triggered, such as sniffing and allogrooming (Matsumoto et al., 2021; Sterley et al., 2018; Wu et al., 2021). Whereas sniffing is likely performed to gather further information from the stressed partner, allogrooming has been proposed to be an affiliative pro-social behavior that is important for the formation and maintenance of social groups (Wu et al., 2021b), but also for social stress buffering (Wu et al., 2021).

The transmission of stress information allows an animal to acquire information about dangerous situations in order to adapt its behavior without having to experience it directly (Sterley and Bains, 2021). Because observers match the physiological state of the stressed demonstrators, one could hypothesize that the behavioral consequences of stress should be similar between demonstrators and observers. This aspect remains to be further studied and represents the main objective of the present work.

2.2.2. Transmission of positive affective states

Being happy, in a positive affective state, has been suggested to increase longevity and improve health (Seligman and Csikszentmihalyi, 2000). Living in groups, social animals have been proposed to share not only negative states, but also positive ones (Michon et al., 2023). Despite the fact that determining what is a positive emotional state is a challenge in rodent models, parallels can be proposed between human "happiness" and rodent positive affective states (Webb et al., 2019). The study of positive emotions in humans makes use of self-reported descriptions of the emotional state, associated with the recording of facial expressions (Burgdorf and Panksepp, 2006; Ekman et al., 1990). Similar facial expressions in response to ingestion of palatable sweet solutions can be observed in humans (Greimel et al., 2006) and other primates (Berridge, 2000), and analogous responses have been described in rodents (Peciña et al., 2003). Comparable to human laugh, rats can emit specific vocalizations that arise from anticipation of reward or from prosocial behaviors, like conspecific reunion or social play (Brudzynski, 2013; Wöhr and Schwarting, 2013). Therefore, it is possible to study these types of positive emotional states in animals and to investigate whether social interactions imply exchange of positive affective information (Jirkof et al., 2019).

Vocalizations of rats emitted ultra-sound vocalizations (USVs) at 50Hz (Seffer et al., 2014), usually produced during social interactions, juvenile play and tickling (Gloveli et al., 2023), elicit approach responses towards the source of the sound (Wöhr and Schwarting, 2007). Moreover, these experiments showed that exposure to such vocalizations triggers an increase of exploratory behavior, indicating for the first time that social transmission of positive affective states has the potential to modify unrelated behaviors of observers (Wöhr and Schwarting, 2007). More recent evidence showed that rats exposed to the 50Hz USVs had the tendency to assign positive value to ambiguous stimuli, suggesting the existence of a sort of socially-transmitted "optimistic" affective state (Saito et al., 2016). In agreement, 50Hz USVs induce a phasic dopamine release in the

nucleus accumbens of listening rats similar to the one of demonstrators (Willuhn et al., 2014).

Mice that are water-deprived for 23h and offered water *ad libitum* for 1h have been proposed to be in a positive (relieve) affective state, because they displayed reduced plasma CORT concentrations. Naive mice can discriminate between neutral and relieved partners, passing more time in closed proximity of the relieved mouse (Ferretti et al., 2019; Scheggia et al., 2019). Moreover, the mere exposure to a relieved mouse can induce conditioned place preference in naïve individuals (Ferretti et al., 2019). This type of positive state transmission was modulated by oxytocin projections from the PVN to the CeA and the mPFC, and it was conveyed by volatile olfactory cues (Ferretti et al., 2019; Scheggia et al., 2019). Interestingly, humans also seem to respond to odors associated with positive states with specific facial responses (de Groot et al., 2015a).

Overall, these data show that animals are capable to react to social cues induced by positive affective states of others, and to adapt their behavior in response to them. Further research will be needed to understand the extend of neurobiological similarity between observers and demonstrators of positive affective states.

Chapter 3: Olfactory chemosignaling

Chemosignals are chemical odorants that animals produce and can be detected by others as social cues. Olfactory chemosignaling, the social communication through scent cues, plays a pivotal role in various aspects of both human and animal behavior. Whereas all pheromones are chemosignals, not all chemosignals can be considered pheromones. This is due to the very strict definition of pheromone, as chemosignals that induce highly stereotyped behaviors (Doty, 2010). For this reason, I will use the wider term chemosignal, that has less restrictions than pheromone (Doty, 2010).

3.1. The olfactory system: focus on the olfactory bulb and its connectivity

In order to understand how chemosignals are detected, it is necessary to have a general overview of the olfactory system and general odor processing. In this section, I will describe the olfactory system of the mouse, how odors get detected and processed, focusing specifically on the olfactory bulb and its functional connectivity. The reader interested in more details on the global olfactory system is referred to exhaustive reviews on the subject (Grabe and Sachse, 2018; Mori and Sakano, 2021; Murthy, 2011)

3.1.1. Odor processing: the Main and the Accessory Olfactory Bulb

Odorant molecules can arrive to the nasal cavity *via* two pathways: orthonasal and retronasal (Pierce and Halpern, 1996). In the orthonasal pathway, odorant molecules are inhaled through the nostrils while smelling, and the amount of molecules that enters into the nasal cavity is determined by the sniffing pattern (Youngentob et al., 1987). In contrast, the retronasal pathway is activated when a food or beverage is in the mouth, and volatile molecules are released during mastication and expiration, providing information about ingested items. In this case, the modulation of molecule intake is influenced by the dynamics of mastication and expiration (Burdach and Doty, 1987).

Once in the nasal cavity, odorants can be detected by olfactory sensory neurons (OSN) present in one of four olfactory systems (**Figure 3a**): the main olfactory system (Lin et al., 2005) (MOS) which includes the main olfactory epithelium (MOE) and main olfactory bulb (MOB), the accessory olfactory system

(AOS) which includes the vomeronasal organ (VNO) and accessory olfactory bulb (AOB)(Dulac and Wagner, 2006), the Gruenenberg ganglion (Brechtbühl et al., 2008) (GG) and the septal organ of Masera (Ma et al., 2003) (SOM).

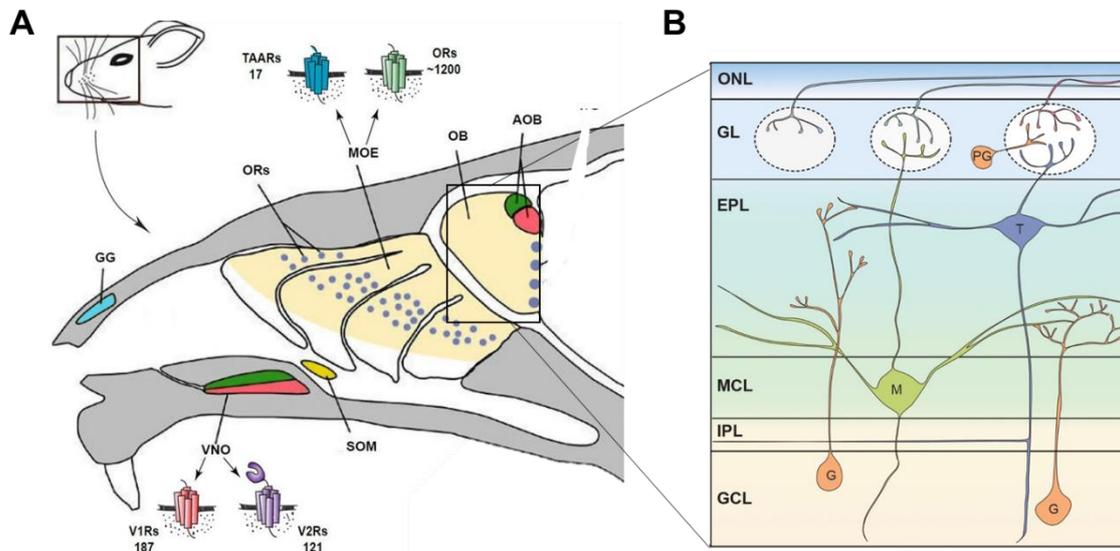


Figure 3: Adapted from (Imamura et al., 2020) and (Hussain, 2011). A) The different olfactory sensory systems located in the nasal cavity of mice together with their most expressed receptors and targets: VNO, vomeronasal organ, MOE; main olfactory epithelium; SOM, septal organ of masera; GG, Grueneberg ganglion; OB, main olfactory bulb; AOB, accessory olfactory bulb B) Distribution of the MOB; OSN axons tangentially run at the surface of the MOB within the olfactory nerve layer (ONL), before entering the glomeruli. Mitral cells (M, with somas located in the mitral cell layer, MCL) and tufted cells (T, with somas located in the external plexiform layer, EPL) send a single dendrite to their corresponding glomerular structure and several extending on the EPL, situated beneath the glomerular layer (GL). Within the EPL, mitral and tufted cells form dendrodendritic synapses with granule cells, another type of interneuron. Tufted cell somata are mainly located in the EPL, while mitral cell somata form a thin layer known as the mitral cell layer (MCL) just below the EPL. The granule cell layer (GCL) lies beneath the MCL and constitutes the largest layer in the olfactory bulb (OB), primarily composed of granule cell somata, which are GABAergic interneurons. An additional layer, the internal plexiform layer (IPL), is present between the MCL and GCL and contains axon collaterals from tufted cells.

The main olfactory system is the biggest of these four systems. OSNs in the MOE have cilia covered by mucus, and detect odorants that bind to their olfactory receptors (ORs, around 1000 genes in the mouse (Bear et al., 2016))

located close to the apical dendrites (Freitag et al., 1998). Neurons that express the same ORs are scattered in four main zones of the MOE (Ressler et al., 1993). However, their axons reach the main olfactory bulb (MOB) converging into specific structures called glomeruli, which follow a topographical organization (Mori et al., 2006). Each OR can be activated by specific subsets of similar odorant molecules, but a single odorant can activate to variable extent different receptors. The combined activation of ORs, OSNs and glomeruli provide the information on the specific nature of an odor or of a blend (Malnic et al., 1999). In the glomerular layer (**Figure 3b**), OSNs form dendro-dendritic synapses with the olfactory bulb projection neurons, called mitral (M) and tufted (T) cells together with periglomerular cells (PG) (Bear et al., 2016; Lohr et al., 2014). The modulation of the activity of M/T neurons happens at several levels within the olfactory bulb. At the glomeruli layer, M/T cell plasticity is controlled by GABAergic inhibition via periglomerular and short-axon cells but also by modulation of periglomerular astrocytic network coupling (Roux et al., 2011a). Moreover, granule GABAergic cells, the most abundant type of neurons in the MOB, are activated by M/T cells and they are responsible for lateral inhibition of the same cells. Granule cells are also the target of centrifugal inputs that arrive from different brain areas and alter their activity (reviewed in (Imai, 2014)). Therefore, the connection between OSN and M/T cells and their modulation within the MOB represent the first central processing station of olfactory information.

A minority of OSNs of the MOE express trace amine-associated receptors (TAARs, 15 genes in the mouse; **Figure 3a**), a still not fully characterized family of ORs detecting amines deriving from natural sources like urine, predators or decomposing flesh (Guo et al., 2023a). The OSNs expressing TAARs project mainly to a discrete group of glomeruli in the dorsal part of the MOB (Liberles, 2015), where they regulate selectively the detection of low concentration of amines (Dewan et al., 2018).

In addition to the MOS, water-soluble chemosignals and other labile chemicals can be detected by the vomeronasal organ (VNO) in rodents. This is a separate epithelium in which the sensory neurons express vomeronasal receptors type 1 and 2 (V1R and V2R). These neurons project to the AOB in glomerular structures, similar to the MOB glomeruli. However, in contrast to the ones of the

MOB, the mitral cells of the AOB send projections to more than one glomeruli, (although often corresponding to the same vomeronasal receptor)(Bear et al., 2016). While the general layer structure of the AOB is identical to that of the MOB, their output connections are largely different.

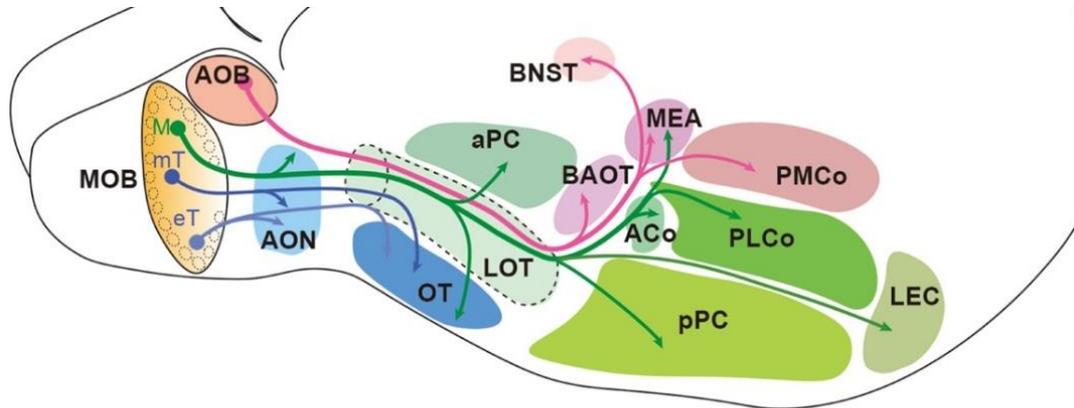


Figure 4: Innervation patterns of olfactory bulb projection neurons in the rodent brain. Mitral cells in the accessory olfactory bulb (AOB) project their axons to the bed nucleus of the accessory olfactory tract (BAOT), the bed nucleus of stria terminalis (BNST), the medial amygdaloid nucleus (MEA), and the posteromedial cortical amygdaloid nucleus (PMCo). Mitral cells (M) in the main olfactory bulb (MOB) innervate the anterior olfactory nucleus (AON), the anterior and posterior piriform cortex (aPC and pPC), the olfactory tubercle (OT), the lateral entorhinal cortex (LEC), the MEA, and the anterior and posterolateral cortical amygdaloid nucleus (ACo and PLCo). However, the axons of tufted cells (mT and eT) project only to the anterior portion of the olfactory cortex including the pars externa of the AON and the anterolateral OT.

The other two olfactory systems, the GG and the SOM, are less explored. The GG is present at the tip of the nose, and is composed by rounded cells that lack the typical microvilli of OSNs, but have primary cilia and are ensheathed by glia (Brechtbühl et al., 2008). Moreover, they express the typical receptors of other olfactory systems, such as the VNO (V2R83 receptor) and MOE (several subtypes of TAARs)(Fleischer et al., 2007). These cells send a single axon to a necklace complex in the MOB, and are activated by alarm signals (Fuss et al., 2005). The SOM sensory neurons, on the other hand, resemble typical OSNs and project to the MOB, but little is known about their physiological functions (Ma et al., 2003).

3.1.2. Main functional connectivity of the olfactory bulb

The target regions of AOB and MOB mitral and tufted cells exhibit minimal overlap, as illustrated in **Figure 4** (reviewed in (Imamura et al., 2020; Martinez-Marcos, 2009)). AOB mitral cells convey information from the vomeronasal organ to various regions such as the bed nuclei of the accessory olfactory tract (BAOT) and of the stria terminalis (BNST), the medial amygdaloid nucleus (MEA), and the posteromedial cortical amygdaloid nucleus (PMCo). On the other hand, MOB mitral and tufted cells innervate the olfactory cortex, including the anterior olfactory nucleus (AON), anterior and posterior piriform cortex (aPC and pPC), olfactory tubercle (OT), lateral entorhinal cortex (LEnt), MEA, and anterior and posterolateral cortical amygdaloid nucleus (ACo and PLCo). Studies indicate that within the MEA, AOB mitral cell axons terminate in the deep region, while MOB mitral cell axons are confined to the superficial layer without overlapping. Single mitral cells may innervate the entire olfactory cortex, whereas tufted cells selectively project to specific areas within the AON and OT (Fig. 2). Notably, external tufted cells, previously uncertain in their axonal projections outside the olfactory bulb (OB), have been shown by recent research to target the anterolateral edge of the OT and the pars externa of the AON. However, even though olfactory processing seems to be so different between the AOB and the MOB, certain odors are processed by both systems in parallel, particularly for specific social chemosignals (discussed below)(Spehr et al., 2006a).

3.1.3. Smelling: a multifaceted process

When we smell an orange, we do not only detect the smell of the orange but also discriminate the identity of the orange from, for example, other fruits. We might even become happy if we usually like oranges or we are hungry. The perception of olfactory stimuli is a complex process that involves the interplay of several factors, including the intensity, the identity and the affective value, that collectively form the so called olfactory percept. The olfactory percept is the result of several processes across the olfactory systems and many other brain regions. However, the olfactory bulb is the first region where meaning start to be assigned to the neuronal signals triggered by odorants (Malnic et al., 1999).

The **detection** of odorants depends on specific ORs (Niimura, 2012). In fact, a single mutant in one of the genes encoding for these receptors for a certain

odorant can lead to specific anosmia, or the inability to detect a particular odorant (Amoore, 1967; Griff and Reed, 1995). Odor detection thresholds, that can be defined as the minimal concentration that leads to the percept of an odor, can be assessed by presenting a single odorant at increasing concentrations (Dewan et al., 2018). At higher odorant concentrations, the map of activation of the glomeruli becomes bigger in magnitude but smaller in latency of activation (Spors et al., 2006). Moreover, spike activity both in individual OSNs (Rospars et al., 2000) and mitral cells (Cang and Isaacson, 2003) depends on the odorant intensity.

Odorant **discrimination** is the ability to tell odors apart, and it depends on the differences in the receptive ranges of the ORs that are activated by specific odorant molecules (Kajiya et al., 2001), but also in M/T activity which can be modulated by centrifugal projections and surrounding interneurons (Bolding and Franks, 2017; Li et al., 2020).

Lastly the **affective value** of an odor indicates whether an odor is positive or negative, and it is also at least partially encoded in the olfactory bulb. M/T cells increase their synchronized firing when responding to odors previously associated with rewards, and decrease in the opposite condition, independent of the odor identity (Doucette et al., 2011). M/T cells project as well to regions like the olfactory tubercle (Gadziola et al., 2020) and posterior cortical amygdala, that further encode odor values and mediate valence-driven behaviors like approach or avoidance (Iurilli and Robert Datta, 2017).

3.2. Human versus rodent chemosignaling

It is widely believed the human sense of smell is way inferior than any other mammal, particularly in comparison with rodents. This belief can be traced back to the classification system created by the 19th-century neuroanatomist Paul Broca. Broca categorized animals into "osmatic," relying on olfaction for behavior, and "anosmatic," including humans, which do not heavily depend on their sense of smell (McGann, 2017). However, although there are major anatomical differences between rodents and humans' olfactory systems, accumulating evidence has shown that olfaction in humans, and particularly chemosignaling,

contributes to a wide variety of behaviors in health and pathology (Stevenson, 2010).

Adult humans don't have an accessory olfactory bulb (Boehm and Gasser, 1993) and their main olfactory bulb represents 0.01% of total brain size (Kavoi and Jameela, 2011), in comparison with the 2% of the mouse brain (G et al., 1988). Humans also have less genes encoding for olfactory receptors, around 390 functional characterized ones (Glusman et al., 2001), in comparison with the 1,100 in the mouse (Niimura, 2012). However, we are able to detect odors with the size of an atom or two, and can discriminate up to 1 trillion olfactory stimuli (Bushdid et al., 2014b). Indeed, we have plenty of evidence that human behavior is strongly influenced by odors, in contrast with the classical 19th and 20th century assumptions. Environmental odors can lead to the consolidation and retrieval of particular memories in humans (Glachet and El Haj, 2021; Herz, 2016), decrease stress and other negative mood states (Herz, 2016; Jousain et al., 2014), and prompt approach and avoidance behavior (He et al., 2014). Furthermore, the human olfactory system plays an unconscious key role in shaping social interactions. We tend to smell our hands much more after shaking hands with others without realizing, a sign that there might be some type of olfactory sampling in this social interaction (Frumin et al., 2015). Studies show that healthy subjects trust a person more when certain odors are present in her body odor cocktail, like compounds resembling the structure of lavender (van Nieuwenburg et al., 2019). Conversely, humans do the opposite when they detect odors related to fear (Endevelt-Shapira et al., 2018). While the existence of human pheromones is debated (Hare et al., 2017; Preti et al., 2003), these cases are just a few examples that the detection of social chemosignals in humans strongly impacts social behavior.

Our own body odor cocktail reflects our internal state, including age, diet (Havlicek and Lenochova, 2006), health (Olsson et al., 2014) and even immune identity (Havlíček et al., 2020). In addition, more transient states like those related to emotions such as fear (de Groot et al., 2015, 2021), anxiety (de Groot et al., 2012) or even happiness (de Groot et al., 2015; Smeets et al., 2020) can be reflected in bodily odors (Smeets et al., 2020). These odors can be found in secretions coming from different body parts, like the axillae or in tears (Gelstein et al., 2011), and appear to be mostly volatile (Lübke and Pause, 2015).

Furthermore, social chemosignal processing is altered in humans with social pathologies like autism (Endevelt-Shapira et al., 2018), something also observed in mouse models of this pathology (Wöhr, 2015). While odor communication could differ across species, the basis of social chemosignal communication is present in all mammals and supports the use of rodent models to further understand the underlying neurobiological basis of this process.

3.3. Social odors: what are they?

Social odors, or chemosignals, can be defined as chemical stimuli naturally produced by an organism that serve to communicate with other organisms to elicit some specific reaction (Monfils and Agee, 2019). Several types of chemicals have been detected in social odors, associated with the identity of an individual, emotional or reproductive state. In this part, I will briefly summarize what is known about the nature of social odors in rodents, focusing on those known to be associated to communication of emotional states.

The most accepted idea is that, in mammals, the main olfactory bulb (MOB) detects airborne chemicals via the olfactory epithelium, while the vomeronasal organ (VNO) and accessory olfactory bulb (AOB) detect aqueous-phase chemosignals— via microvillar sensory neurons (Dulac and Wagner, 2006). The MOB is not tuned to specific ligands, but it detects the molecular features of odorant blends (Malnic et al., 1999). On the other hand, the VNO mediates identical odor responses independent of whether the odorant is in a blend or by itself. In other words, it mediates fixed responses to specialized ligands (Kimoto et al., 2005). Mouse urine is a blend of both volatile and nonvolatile compounds, and it is the main source of social odors in these animals (Lin et al., 2005), although secretions containing social odors have also been detected in other regions like the orofacial area (Shi et al., 1989). Smelling urine leads to activation of cells in the mouse MOB but also in the VNO (Lin et al., 2005), showing that there is at least a partial overlap between the two systems in the processing of social signals (Spehr et al., 2006a).

One of the main components in terms of chemosignals of the mouse urine are major urinary proteins (MUPs), which play a role in concentrating and

stabilizing volatile hydrophobic chemosignals and other lipophilic molecules, influencing their transport to the VNO (Kaur et al., 2014; Zhou and Rui, 2010). Bound to MUPs, chemosignals are detected by the VSNs neurons in the VNO (Dulac and Wagner, 2006). MUPs mediate behaviors like aggression (Chamero et al., 2007), sex partner recognition (Roberts et al., 2010), and can convey directly identity information to conspecifics (Chamero et al., 2007; Hurst et al., 2001; Sturm et al., 2013). They are secreted mainly by the liver into the urine (Zhou and Rui, 2010), but also by other secretory tissues including the submaxillary gland (Shi et al., 1989). As part of the lipocalins family (Charkoftaki et al., 2019), MUPs have a β -strand that creates a pocket for the hydrophobic molecules to bind, increasing their half-life in the secretory liquids (Zhou and Rui, 2010).

One example of chemosignals processed in parallel by the MOB and the AOB are major histocompatibility complex (MHC)-associated chemical cues, which are nonvolatile small peptides. Mice react differently to partners depending on their MHC (Hurst et al., 2005; Leinders-Zufall et al., 2004). The peptide ligands of the MHC activate the VNO (Leinders-Zufall et al., 2004) when detected in direct contact with the mouse urine, but they can also activate directly the OSNs in the main olfactory epithelium (Spehr et al., 2006b). Gene polymorphisms in MHC are reflected in variation of urinary peptides that can be detected and discriminated by the conspecifics, which represent a real sampling of the genome of other individuals (Sturm et al., 2013). Interestingly, humans unconsciously prefer their own “self” MHC odor peptides than the ones of other people’s, showing that they are also able to evaluate these peptides for individual recognition (Milinski et al., 2013).

Volatile cues can also function as chemosignals, and are processed by both MOB and VNO. Some of these chemosignals are sex-specific, such as 2-heptanone and 2,5-dimethyl pyrazine (DMP), present in the female urine or (methylthio)methanethiol (MTMT) present in male urine (Lin et al., 2005, 2007a). Among the volatile compounds produced by a stressed mouse (Brechtbühl et al., 2013), a chemical called 2-sec-butyl-4,5-dihydrothiazole (2-SBT) was described as a putative volatile alarm chemosignal transiently produced by both male and female stressed mice (Brechtbühl et al., 2008). This chemosignal resembles in

structure to the sulfur-containing volatiles in predator scents (Brechtbühl et al., 2013). Interestingly, if 2-SBT is not linked to MUPs, it is detected by the Grueneberg Ganglion (GG) (Brechtbühl et al., 2013) and it also directly activates glomeruli in the MOB, as well as associated mitral/tufted and granule cell activity in the MOB (Matsuo et al., 2015a). Other chemical compounds that have been associated with alarm chemosignaling are aldehydes, like 4-methylpentanal and, hexanal and 2-heptanone (Gutiérrez-García et al., 2007; Inagaki et al., 2014).

Other chemosignals are exclusively processed in the MOB. These are mostly types of aldehydes, extremely hydrophobic, that have been detected in the body odor of mice and rats, not in the urine but in other body regions like around the whisker area (Bautze et al., 2012; Kiyokawa et al., 2007; Klein et al., 2015). These molecules, which include pentadecanal, hexadecanal and heptadecanal, are detected by specific OSNs that express OR37 family of receptors, projecting to specific glomeruli in the MOB. M/T cells receiving information from these glomeruli have direct projections to the hypothalamus (Bader et al., 2012), and appear to be linked to the buffering of emotional responses (Kiyokawa et al., 2009; Klein et al., 2015).

Interestingly, similar compounds to those seen in rodents can be found in emotionally altered humans (Smeets et al., 2020). For example, aldehydes are high in sweat samples collected from fearful subjects in comparison with neutral ones (Smeets et al., 2020). Sulfur-containing compounds are also present in body odor (De Lacy Costello et al., 2014), suggesting that similarity in the structure of the putative chemosignals mediating social communication of emotional states between rodents and humans exists.

3.4. Olfaction and stress

Stress-related chemosignals can help humans and animals adapting their behaviors to their environment in order to avoid exposing themselves to dangers. Whether they are directly produced by a threat (e.g. predator odors) or by a congener that has experienced the threat, these sensory cues are processed by a mixture of bottom-up and top-down parallel brain circuits that translate into behavioral decisions (Matsukawa et al., 2022). From the functional anatomic point of view, the glomeruli associated with aversive environmental odors are

located in the dorsal domain of the OB (Kobayakawa et al., 2007). Mitral cells from these glomeruli project to the AON where they maintain the same topography, and they can reach the cortical amygdala and piriform cortex (Miyamichi et al., 2010).

Chemosignals from predators, called kairomones, trigger innate aversive responses in rodents (Pérez-Gómez et al., 2015). Volatile sulfur containing compounds are the metabolic product of meat eating and have been identified in the body secretions of mammalian predators (Apps et al., 2015). The chemical isolation of some of these compounds, such as the one present in fox urine (2,5-dihydro-2,4,5-trimethylthiazoline, TMT), has largely contributed the dissection of the neurobiological basis of innate fear responses (Rosen et al., 2015). Exposure of mice to TMT activates the OR19 receptor located in the posterior dorsal part of the OB (Saito et al., 2017), and not yet identified targets in the Grueneberg ganglion (Brechtbühl et al., 2020), thereby producing a robust and persistent freezing response. However, other similar predator odors, like cat, rat or snake odors, are mainly detected by the vomeronasal organ and processed by the AOB (Papes et al., 2010; Staples et al., 2008). PEA (b-phenylethyl-amine), which is found in cat urine, activates TAAR4, a specific subset of TAAR receptors in the MOB (Dewan et al., 2013). These odors generally induce passive fear responses, but they can also trigger other types of defensive behaviors (Pérez-Gómez et al., 2015). Thus, despite being detected and processed by different olfactory systems, these odors trigger similar innate responses, suggesting that converging brain circuits might exist. Using c-fos activity mapping of the brain after predator odor exposure, Pérez-Gómez et al., 2015 showed that the aversive information associated with different kairomones converge into the MeA posteroventral division, which then projects to the ventral hypothalamus. Other brain regions involved in the innate reaction to these stimuli are the BLA, CeA, BNST and medial hypothalamic nuclei (Takahashi, 2014).

Chemosignals from stressed conspecifics, induce a stress response on the body of the receiver that is mediated by CRH neurons in the PVN (Sterley et al., 2018). There are several polysynaptic and monosynaptic connections from the olfactory system that might convey the information towards CRH neurons

(**Figure 5**). For instance higher olfactory areas, such as the lateral entorhinal cortex (LEn), the medial amygdala (MeA), the posterior cortical amygdala (PCo), the posterior piriform cortex (pPir) and the amygdaloid piriform transition area (AmPir) are mono- or poly-synaptically connected to CRH neurons (Kondoh et al., 2016). Interestingly, the AmPir of mice can be activated by fox and bobcat urine, and the chemogenetic inhibition of this region reduced the CRH-mediated hormonal response (Kondoh et al., 2016).

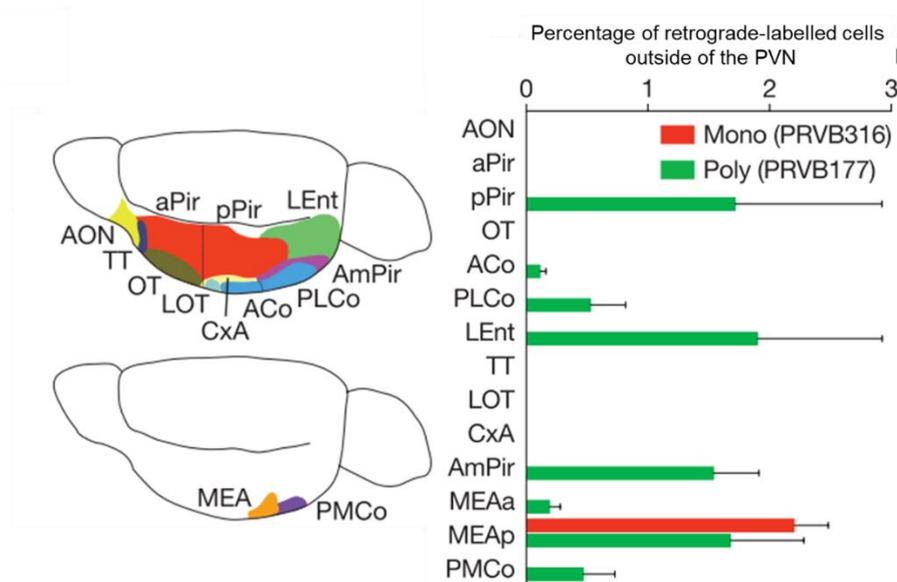


Figure 5: Adapted from (Kondoh et al., 2016) Higher olfactory areas contain neurons upstream of CRH neurons (labelled with poly or monosynaptic retrograde tracers from the PVN)

Alarm chemosignals arise from bodily secretions of conspecifics and elicit fear responses, which can be distinguished in behavioral and physiological consequences (Morozov and Ito, 2018). For instance, whereas foot shock-induced alarm chemosignals secreted by the perianal region of rats produced hyperthermia in naive conspecifics, odors from the whisker pad area determined their behavioral adaptations (sniffing, rearing and increased approach) (Kiyokawa et al., 2004). These types of stress chemosignals, such as 2-SBT (Brechtbühl et al., 2008), 2-heptanone (Gutiérrez-García et al., 2007), 4-methylpentanal and hexanal (Inagaki et al., 2014) are detected by three olfactory systems: the MOE, the VNO and the GG. C-fos activation induced by exposure to these signals in rats revealed the potential involvement of BNST, basolateral and medial

amygdala, PVN and dorsomedial hypothalamus, and locus coeruleus (Kiyokawa et al., 2005). Interestingly, some of these brain regions overlap with those activated by predator odors, which lead to the hypothesis of a similar circuit to process aversive odors coming from others.

Chapter 4: Astrocytes and control of behavior

Over the last ten years, an ever-growing collection of data has begun to shift the neurocentric approach that neuroscience had to the brain. Glia, and particularly astrocytes, have appear as emerging stars in the regulation of many functions(Oliveira et al., 2015), moving away from classical view of these cells as neuronal support. Being non-excitabile cells, the study of astrocytes was put aside in favor of neurons for decades. However, technical advancements in the ability to observe and quantify astrocyte calcium dynamics through imaging techniques(Yu et al., 2020) has revealed their involvement in maintaining brain homeostasis and regulating neuronal activity, which ultimately impacts cognitive brain functioning(Oberheim et al., 2012; Oliveira et al., 2015).

The view of astrocytes as “silent” cells begun to change in the 1970s, when it was discovered that glia exhibited a large number of GPCR-coupled signaling cascades (Agulhon et al., 2008; Van Calker et al., 1978; Porter and McCarthy, 1997). In the 90s, the use of fluorescent calcium indicators in cultured astrocytes revealed that these cells responded to glutamate, which was inducing calcium flushes (Charles et al., 1991; Cornell-Bell et al., 1990). These calcium events are homeostatically regulated by a variety of mechanisms, and contribute to the “excitability” of astrocytes (**Figure 6**; Agulhon et al., 2008). Since then, astrocytes have been found to take part in a plethora of functions. First, they play crucial roles in brain homeostasis, extending their influence to the blood-brain barrier and the regulation of extracellular ions and neurotransmitters (Parpura and Verkhratsky, 2012). Second, astrocytes maintain close physical contact with synapses, neurons, other glial cells, and vascular structures within the brain(Preston et al., 2019). Third, they express various functional neurotransmitter receptors, allowing them to sense the surrounding neuronal

activity(Araque et al., 2014). Fourth, astrocytes exhibit intracellular calcium-based excitability with complex temporal and spatial properties, triggering paracrine signaling to neighboring astrocytes(Araque et al., 2014). Finally, they have the ability to release neuro- and vasoactive substances, such as glutamate, D-serine, ATP, GABA, TNF- α , prostaglandins, or peptides, in a process known as gliotransmission (Araque et al., 2014). This gliotransmission, in turn, can modulate synaptic function, blood flow, and metabolism.

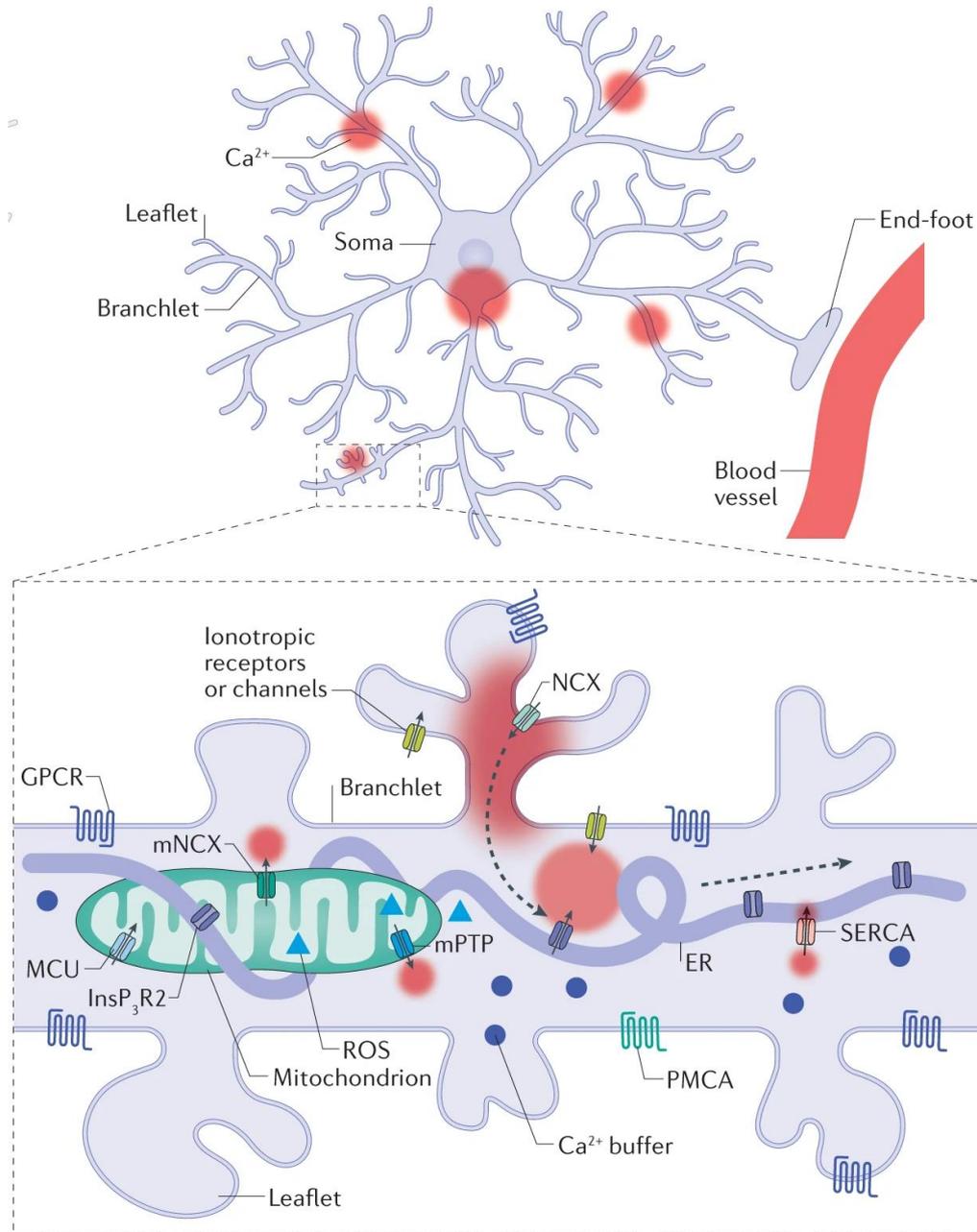


Figure 6 from (Semyanov et al., 2020): Astrocytic calcium modulation in microdomains. Astrocytes' soma is not a central signaling hub; instead, dispersed

calcium signals initiate localized cascades, modifying nearby neuronal signaling. Astrocytic processes include primary branches, higher-order branchlets, terminal leaflets, and end-feet contacting blood vessels. Calcium entry through the plasma membrane, facilitated by the sodium/calcium exchanger (NCX) following an increase in Na⁺ levels during neurotransmitter uptake and other receptor or channel activities, generates transients, propagated into branchlets and intensified through inositol 1,4,5-trisphosphate receptors. Moreover, G-protein-coupled receptors trigger astrocytic calcium signaling by producing inositol 1,4,5-trisphosphate. In the cytosol, calcium is buffered and eliminated by the plasma membrane calcium ATPase (PCMA) or transported to the ER (SERCA). Astrocytic branchlets with mitochondria participate in local calcium dynamics, releasing (mPTP and mNCX) and sequestering (MCU) calcium. Mitochondria also serve as a source of reactive oxygen species, regulating astrocytic calcium activity.

These processes converge in the concept of the tripartite synapse (Pérez-Alvarez and Araque, 2013). In the tripartite synapse, astrocytes respond to synaptic activity through calcium signaling, subsequently regulating neuronal activity and synaptic strength through the release of gliotransmitters. A recent proposition suggests that astrocytes integrate information both inside and outside synapses, processing signals in a scaled manner with temporal and spatial dimensions distinct from those of neurons (Araque et al., 2014).

Although progress has been made in understanding how astrocytes modulate synaptic transmission, the exploration of their impact on information processing at higher levels, such as neural networks and animal behavior, is a nascent field. In this section, I will summarize the evidence on the key role of astrocytes in the regulation of olfactory functions and social behaviors in mice.

4.1. Astrocytic modulation of olfactory bulb processing

The structural layout of the rodent olfactory system displays distinct anatomical features, characterized by the abundant presence of astrocytes in regions of the olfactory bulb that are densely populated with synapses. These areas within the OB serve as crucial sites for the initial stages of sensory processing. Glial cells have been observed ensheathing OSNs (olfactory ensheathing cells, OEC), surrounding the glomerular structures (periglomerular

astrocytes, pAC), and regulating synapses between M/T cells and interneurons (AC) (Figure 7)(Lohr et al., 2014).

Olfactory bulb astrocytes are scarce and heterogenous. If we take into account that astrocytes make around 20% of the total glia (according to data from postmortem human brains(Pelvig et al., 2008) and rodents(Sun et al., 2017)), in the olfactory bulb of mice this percentage only reaches 3-5%(Sun et al., 2017). Moreover, they express distinct markers that sometimes don't overlap. For example, the co-localization between the GFAP and the S100 β astrocytic

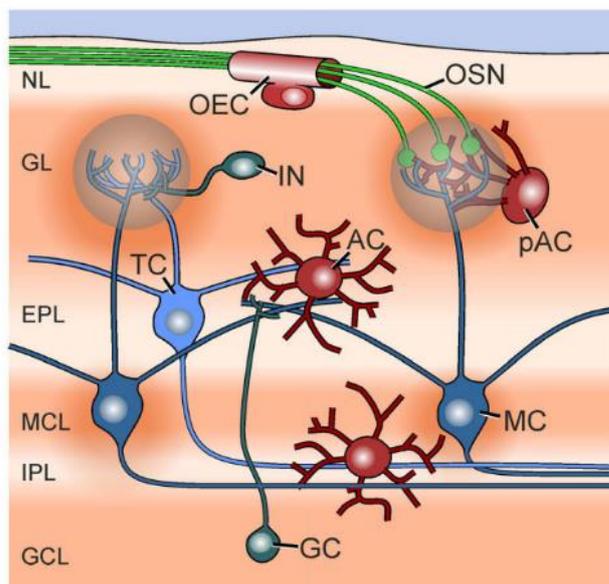


Figure 7: Astrocytes in the main olfactory bulb. Periglomerular astrocytes (pAC) extend processes into the neuropil of a single glomerulus, comprising synapses between axons of olfactory sensory neurons (OSN), mitral cells (MC), tufted cells (TC) and periglomerular interneurons (IN). Bundles of OSN axons are ensheathed by olfactory ensheathing cells (OEC). In the external plexiform layer (EPL), synapses between mitral/tufted cells and granule cells (GC) are accompanied by processes of astrocytes (AC). NL nerve layer, GL glomerular layer, MCL mitral cell layer, IPL internal plexiform layer, GCL granule cell layer

markers varies across layers: in the GL reaches 90%, in the EPL of about 75% and in the GCL is only of about 60%(Su et al., 2021). Cluster analysis reports at least 4 different astrocytic subpopulations in the olfactory bulb(Ung et al., 2021). From those, only 3 express GFAP and only 1 expresses ALDH1/1(Ung et al., 2021), which are the currently most used astrocytic reporters(Preston et al., 2019).

Adapted from Lohr et al, 2014

Periglomerular astrocytes are located around the glomeruli, representing less than 7% of the juxtglomerular cells, forming specialized compartments that engulf and protect the neuropil(Chao et al., 1997). Each astrocyte projects

processes into a single glomerulus, but they are connected with adjacent astrocytes since labelling them with biocytin also labelled surrounding cells(De Saint Jan and Westbrook, 2005).

Periglomerular astrocytes express high levels of glutamate transporters, suggesting their role in the prevention of synaptic crosstalk by glutamate reuptake(Utsumi et al., 2001). Upon olfactory nerve stimulation, astrocytes respond with an inward current, attributed to the accumulation of K⁺ in these cells. This K⁺ current reflects the time course of M/T cell depolarization, and was dependent on AMPA/NMDA and mGluR1 receptors in these cells, supporting the role of periglomerular astrocytes in the regulation on within-glomeruli communication(De Saint Jan and Westbrook, 2005). However, as mentioned, astrocytes in these regions are connected between each other, forming within-glomeruli networks, that express high levels of two connexins, connexin 43 (CX43) and connexin 30 (Cx30;Roux et al., 2011, 2015). The coupling of these networks depends on connexin 30, whose expression is modulated by sensory deprivation and, more dynamically, by extracellular potassium levels generated by neuronal activity(Roux et al., 2011b). Moreover, astrocytic coupling *via* CX43 also mediates oscillations generated by the spontaneous activity of the mitral cells at the glomerular layer. This coupling controls extracellular ATP levels and modulates mitral cells via adenosine 1 receptors(Roux et al., 2015). In another study, Sox9, a transcription factor present in some olfactory bulb astrocytes, was manipulated to study the contribution of astrocytes to glomerular and mitral cell activity(Ung et al., 2021). The KO of this gene in the OB decreased olfactory detection thresholds and discrimination in mice in response to different odors, and created an aberrant odorant map(Ung et al., 2021). Furthermore, it led to an alteration of M/T synaptic properties that could be recapitulated by the knock-down of GLT-1(Ung et al., 2021), adding to the previous data that underscores the function of OB astrocytes in capturing glutamate.

Astrocytes also partake in the synaptic modulation of olfactory bulb activity at non-glomerular synapses. Oscillatory activity at the gamma and beta frequency is thought to be important for odor-evoked information processing(Bathellier et al., 2006; Lepousez and Lledo, 2013; Ravel et al., 2003). These oscillations are thought to emerge from M/T cells to granule cell

interactions(Bathellier et al., 2006; Schoppa, 2006). In GLAST KO mice, lacking the glutamate-aspartate transporter, this oscillatory activity was affected, together with a change on evoked LFPS at the granule cell layer(Martin et al., 2012a). Authors propose a mechanism of action in which a lack of glutamate transport will accumulate glutamate from the mitral cell at the synapse with granule cell layers, leading to a power depression of oscillations caused by over-activation of GABAergic control of granule cells over M/T (Fig 3)(Martin et al., 2012a).

Aside from their described role as glutamate buffers, olfactory bulb astrocytes also control neuronal circuit function through Ca^{2+} signals. Manipulation of astrocytic calcium responses through Gq (hM3Dqi) or Gi/o pathways (hM4Di)(Durkee et al., 2019) led to opposite results: while the Gq-mediated calcium increase led to an inhibition of odor-evoked neuronal responses and an increase in odor detection thresholds, the Gi/o-mediated increase has opposite effects. Physiologically, astrocytic calcium signals showed similar time and space constraints as M/T cell activity. Together, this data suggests that astrocytic calcium modulates M/T activity, most probably through gliotransmission (Ung et al., 2020).

Several neuromodulators induce astrocytic calcium responses in olfactory bulb astrocytes, with an unknown role in olfactory processing. Short axon cells and a subpopulation of the external tufted cells produce dopamine in the olfactory bulb, and are mainly located in the glomerular layer(Fischer et al., 2020). Astrocytes in this layer released calcium from internal stores upon dopamine administration, showing that they are probably regulated physiologically by these dopaminergic sources(Fischer et al., 2020). Another example is noradrenergic modulation of olfactory processing, that originates from the centrifugal fibers from the locus coeruleus arriving to the olfactory bulb(Linster et al., 2020; Manella et al., 2013). Astrocytes respond to norepinephrine via $\alpha 1$ - and $\alpha 2$ -adrenergic receptors, which leads to a double phase cytosolic calcium response, with a first part mediated by IP3-dependent pathways, and a late part by store-operated calcium entry(Fischer et al., 2021).

Astrocytes control olfactory processing in the main olfactory bulb at different stages, impacting olfactory functions. However, whether they have

specific functions depending on the stimuli, as well as the underlying mechanisms behind their contributions remain mostly unknown.

4.2. Astrocytic control of social behaviors

The physiological role of astrocytes in the modulation of social behaviors is a nascent research area, but there are exciting clues about the region-specific role of these cells in regulating social phenotypes. Indeed, disrupting the glutamate transporter type 1 (GLT-1) in astrocytes in the amygdala reduces social interaction in rats (Lee et al., 2007), an effect also observed in full brain GLT-1-KO mice (Aida et al., 2015). Dorsomedial prefrontal astrocytes are involved in the expression of social behaviors that determine social rank in male mice, by determining the excitatory/inhibitory balance in that brain region (Noh et al., 2023). Astrocytic calcium levels change in the cerebellum during attack bouts in a social intruder paradigm, and the photoactivation of astrocytes triggers the onset of an attack (Asano et al., 2023). Social impairment induces a change in astrocytic lactate production with long term behavioral consequences (Sun et al., 2021) and social deficits caused by cannabinoid administration are linked to astrocytic glucose metabolism impairments (Jimenez-Blasco et al., 2020). These examples illustrate the different contribution of astrocytes to social behavior through their main four functions: synaptic homeostasis, modulation of neuronal activity, Ca²⁺ regulation and metabolic control.

The newly characterized functions of astrocytes as synaptic regulators have prompted a reconsideration of their role in physiology and disease. Particularly in synaptopathologies, like autism spectrum disorders (ASD) which are the most common (Zeidán-Chuliá et al., 2014). ASD are characterized for an impairment in normal social functioning, including social communication, and developing and maintaining social relationships (DSM-V). In this context, the use of mouse models recreating this social phenotype has begun to unravel the contribution of astrocytes to ASD-associated social impairments. For example, in vitro co-culture studies have demonstrated possible roles for astrocytes in Fragile X syndrome (FXS), associated with the mutation of the FMR1 gene. Specifically, the unique mutation causing FXS is crucial for the normal functioning of astrocytes and their impact on normal neuronal development (Jacobs et al., 2010). Moreover, GLT-1 expression was reduced in a mouse model of FXS (FMR1 KO mice) suggesting the critical involvement of glutamate reuptake by

astrocytes in the phenotypes associated with that mutation (Higashimori et al., 2013). Furthermore, in the environmental ASD-like animal model caused by prenatal VPA exposure, astroglial cells (particularly in the prefrontal cortex and hippocampus) show gene transcriptional modifications that correlate with decreased sociability and altered ultrasonic vocalizations (Bronzuoli et al., 2018). Understanding the physiological contribution of astrocytes to specific circuits regulating sociability could be the key to find new therapies to sociopathologies, now underexplored.

Chapter 5: The endocannabinoid system

5.1. General overview

Cannabis sativa, commonly known as marijuana, cannabis or hemp, has been utilized for centuries due to its therapeutic and recreational properties, with first medical uses dating from 2700 B.C. in China (Zuardi, 2006). Nowadays, marijuana remains the most consumed drug in the world, with about 2.5% of the total world population as consumers (WHO).

The discovery of a G-protein coupled receptors activated by Δ^9 -tetrahydrocannabinol (THC; Gaoni and Mechoulam, 1964), the main active compound in marijuana, during the 20th century consolidated the basis of cannabinoid research. Cannabinoid-type-1 receptors (CB1) were first discovered in the rat brain in 1988 (Devane et al., 1988), two decades after the characterization of the chemical structure of THC (Gaoni and Mechoulam, 1964). This discovery was quickly followed by the characterization of the 7-transmembrane receptor, whose activation led to a G_i -mediated inhibition of adenylyl cyclase activity in certain cells (Howlett et al., 1990), and resulted in analgesic effects in several rodent models (Melvin et al., 1993). Subsequently, another receptor known as the cannabinoid-type 2 (CB2) receptor was identified in the periphery (Munro et al., 1993) by sequence homology. Following the characterization of these receptors the presence of endogenous cannabinoid ligands (eCBs) was revealed. Two compounds, namely anandamide (AEA; Devane et al., 1992) and 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995) were identified and reported to cause the typical tetrad of effects that was produced by THC: antinociception, catalepsy, reduction of spontaneous activity and reduction of body temperature (Pertwee, 2006). The discovery of endogenous ligands prompted the research for the biochemical processes responsible for their synthesis and degradation, leading to the discovery of the synthesis enzymes DAG lipase- α and lipase- β (DAGL), NAPE-selective phospholipase D (NAPE-PLD), and degradation enzymes fatty acid amide

hydrolase (FAAH) and monoacylglycerol lipase (MAGL)(Mechoulam et al., 2014). More recently, the enzyme serine hydrolase alpha-beta-hydrolase domain 6 (ABDH6) was found to partake in the accumulation and efficacy of 2-AG in cannabinoid receptors(Cao et al., 2019). The concept of endocannabinoid system (ECS) arise from the accumulation of this knowledge, and can be defined widely distribute and polyfunctional neuromodulatory system that is virtually involved in all brain functions (Fride, 2005).

Although CB1 and CB2 are well known and characterized, numerous pharmacological studies suggest the existence of additional metabotropic and ionotropic cannabinoid receptors able to respond to the endogenous agonists (for review see(Brown, 2007)). Among these, the transient receptor potential vanilloid type 1 (TRPV1) ion channel, which was found to bind some cannabinoid ligands, G-protein-coupled receptor 55 (GPR55) and G-protein-coupled receptor 119 (GPR119) as novel potential cannabinoid receptors (Brown, 2007). However, considering the scope of this thesis, I will focus the following sections on CB1 receptors.

5.2. Distribution of CB1 receptors: anatomical and cellular localizations

CB1 receptors are the most abundant GPCRs expressed in the brain, with protein levels comparable to NMDA and GABA_A receptors(Busquets-Garcia et al., 2018a). They can be found virtually in all brain regions, but are particularly abundant in the neocortex, basal ganglia, amygdala, hippocampus, hypothalamus and cerebellum (Piomelli, 2003). The generation of mice with a CB1^{-/-} mutation (CB1-KO)(Marsicano et al., 2002) set the path for the dissection of the functional contribution of CB1 receptors to different behaviors, starting from the control of fear memory in the amygdala (Marsicano et al., 2002), and expanded, but not restricted, to motivation (Muguruza et al., 2019), memory (Busquets-Garcia et al., 2018b), stress (Hillard, 2014a), anxiety (Lafenêtre et al., 2007), locomotion (Soria-Gomez et al., 2021a), social behaviors (Håring et al., 2011), food intake (Bellocchio et al., 2010), pain (Monory et al., 2007) and sensory perception (Hutch et al., 2015).

The expression of these receptors has been reported in many cell types. GABAergic cells contain the highest levels of CB1 protein, although this depends on the brain region and specific neuronal subtypes (Marsicano and Lutz, 1999). Notably, amongst inhibitory neurons, most CCK positive ones (Pelkey et al., 2017) express CB₁ receptors, whereas only a little percentage of PV interneurons do (Katona et al., 1999). Glutamatergic cells, albeit at lower levels, also present CB1 receptors, which were identified first *via* protein analysis (Marsicano and Lutz, 1999) and later thanks to the development of one of the critical tools in CB1's research field, CB1-floxed mice (Marsicano et al., 2003). This mouse line carries a modified version of the CB1 gene that replaces the wildtype version and it's flanked by two LoxP sites, allowing for the manipulation of CB1 expression via Cre-mediated recombination (Marsicano et al., 2003). The introduction of novel conditional mutants lacking CB1 receptors in specific neuronal populations (Monory et al., 2006) using this method deepened the functional and anatomical understanding of CB1-regulation of neuronal functions and behavior. For example, the use of GABA-CB1-KO (generated by crossing CB1-floxed mice Dlx5/6 Cre mice ; Monory et al., 2006) and Glu-CB1-KO (crossed with Nex-Cre mice ; Kleppisch et al., 2003) revealed that, even though glutamatergic neurons express less CB1 in the hippocampus, CB1-mediated G-protein activation is much stronger in these neurons than in GABAergic interneurons, suggesting a higher efficacy of these receptors in glutamatergic cell subpopulations. In the synapse (**Figure 8**), CB1 receptors are classically located in the pre-synapse where, depending on the neuronal type, mediate distinct types of synaptic plasticity induced by the activity-mediated release of eCBs (Castillo et al., 2012). Moreover, postsynaptic effects of CB1-mediated neuronal modulation have been reported in the hippocampus (Neu et al., 2007) or in the neocortex, where they modulate somato-dendritic inhibition of inhibitory neurons (Marinelli et al., 2009), although no anatomical evidence of their membrane localization in the brain (Busquets-Garcia et al., 2018).

Interestingly, not only neurons express CB1, but also glial cells. Astrocytes have been seen to express low but significant levels of CB1 in different brain regions, that can only be detected through immunogold staining both in the plasmalemma (Han et al., 2012a) and in the mitochondria (Gutiérrez-Rodríguez

et al., 2018a). Although a bit more controversial, the presence of low levels of CB1 receptors have also been reported in cultured rodent microglia(Waksman et al., 1999).

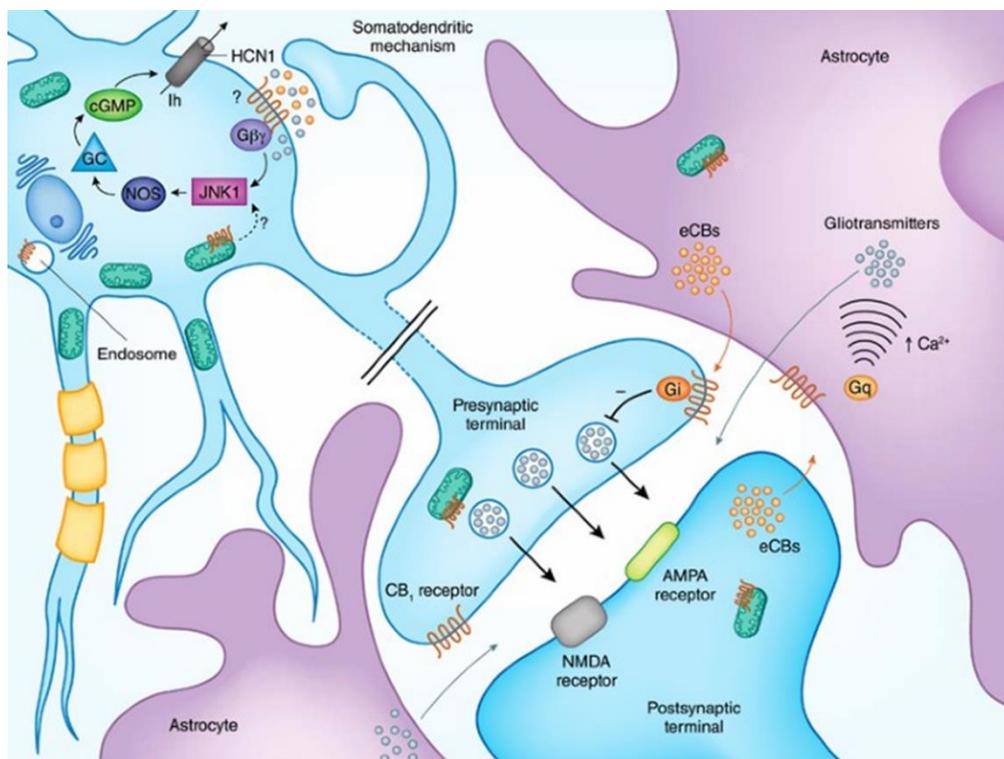


Figure 8: from Busquets-Garcia et al, 2018. Potential sites of CB1 receptor localization within the synapse. CB1 receptors are situated in both presynaptic terminals and postsynaptic regions of neurons, as well as on astrocytes, each exerting distinct effects in the tripartite synapse. While the presence of CB1 receptors in the presynaptic plasma membrane is well-established, recent findings suggest their existence in the mitochondrial membranes of both presynaptic and somatodendritic neuron compartments. However, the specific functions of these mitochondrial CB1 receptors remain incompletely understood. While the likelihood of CB1 presence in postsynaptic plasma membranes is conceivable, direct anatomical evidence is currently lacking. Some studies propose the expression of CB1 receptors in endosomes. Astrocytes also host CB1 and mtCB1 receptors, regulating synaptic functions within the astroglial context. Comprehensive details can be found in the main text for further clarification.

In physiological conditions, CB1 receptors are mostly observed at the plasma membrane level, but some evidence showed that there was CB1 staining at intracellular localizations (Bénard et al., 2012; Thibault et al., 2013). First, CB1

was observed in endosomal membranes in the rat forebrain, likely due to its internalization and retrograde transport(Thibault et al., 2013). More recently, the observation of immunogold particles close to mitochondrial structures in hippocampal preparations led to the discovery of mitochondrial CB1 receptors (mtCB1), both in GABAergic and glutamatergic neurons (Bénard et al., 2012) and later in astrocytes (Jimenez-Blasco et al., 2020). The dissection of the role of mtCB1 in the brain has begun to provide information about the specific contribution of CB1 receptor subcellular pools to given brain functions, not only in the hippocampus (Hebert-Chatelain et al., 2016a; Jimenez-Blasco et al., 2020; Serrat et al., 2021) but also, for example, in the basal ganglia (Soria-Gomez et al., 2021a).

5.3. CB1 receptors in synaptic plasticity

The discovery of retrograde endocannabinoid (eCB) signaling, inhibiting neurotransmitter release, originated from their role in short-term synaptic plasticity at GABAergic and glutamatergic synapses, known as depolarization-induced suppression of inhibition (DSI) (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) and depolarization-induced suppression of excitation (DSE)(Kreitzer and Regehr, 2001). Additionally, eCBs contribute to presynaptic long-term potentiation (Silva-Cruz et al., 2017) long-term depression (eCB-LTD) at both excitatory(Gerdeman et al., 2002; Robbe et al., 2001) and inhibitory synapses (Chevaleyre and Castillo, 2003; Marsicano et al., 2002). Overall, eCBs are key retrograde messengers regulating synaptic transmission in the short and long term (Castillo et al., 2012).

DSI and DSE, two distinct forms of short-term plasticity involving presynaptic inhibition of transmitter release by endocannabinoids, are associated with GABA or glutamate transmission, respectively: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE)(Diana and Marty, 2004; Wilson and Nicoll, 2002). Wilson and Nicoll demonstrated the involvement of endocannabinoids in hippocampal DSI post synaptic stimulation, revealing its CB1 receptor dependence by pharmacology (Wilson and Nicoll, 2002) . This phenomenon extends to the cerebellum (Diana et al., 2002) and inhibitory synapses between CCK-positive

Schaffer collateral associated interneurons in the stratum radiatum (Ali, 2007). Notably, studies indicate that DSI in hippocampal pyramidal neurons can also result from metabotropic glutamate receptors (mGluRs) (Varma et al., 2001) or muscarinic acetylcholine receptors activation (Kim et al., 2002), likely influencing postsynaptic neurons to induce endocannabinoid formation and release. In parallel, endocannabinoids modulate DSE in the cerebellum and hippocampus, influencing glutamatergic excitatory synapses (Kreitzer and Regehr, 2001; Maejima et al., 2001; Ohno-Shosaku et al., 2001). These eCB-mediated effects on synaptic transmission are not limited to specific brain regions, encompassing areas like the amygdala, substantia nigra pars reticulata, neocortex, striatum, and hypothalamus (Castillo et al., 2012). In summary, endocannabinoid-induced DSI and DSE play a vital role in regulating neurotransmitter control across various physiological processes.

LTP and eCB-LTD: Endocannabinoids exert influence on synaptic plasticity through long-term potentiation (LTP) and long-term depression (LTD) (Lu and Mackie, 2016). Mechanistically diverse forms of endocannabinoid-mediated LTD, involving or not involving CB1 receptors (Chávez et al., 2010; Kellogg et al., 2009), have been identified. Notably, endocannabinoids induce both homosynaptic and heterosynaptic LTD (eLTD) (Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Robbe et al., 2001), requiring the inhibition of adenylyl cyclase (AC), PKA, and involvement of presynaptic proteins like RIM1 α and RAB3B (Chevaleyre et al., 2007; Tsetsenis et al., 2011). The endocannabinoid system's modulation at synaptic levels may involve glial cells; astrocytes in the hippocampus produce endocannabinoids to modulate currents between pyramidal neurons (Navarrete and Araque, 2008, 2010). Indeed, astrocytic CB1 receptors are crucial for d-serine release and subsequent hippocampal LTP (Robin et al., 2018).

Autocrine self-inhibition: Endocannabinoids autonomously induce sustained self-inhibition in GABAergic interneurons and neocortical pyramidal neurons, operating at the individual neuron level without retrograde signaling. This autocrine modulation occurs mainly at the somatodendritic level, shaping diverse synaptic plasticity forms (Bacci et al., 2004; Marinelli et al., 2009).

5.4. Mitochondrial CB1 receptors

As far back as the 1970s, various studies documented cannabinoid effects on mitochondrial physiology in tissues other than the brain, such as reduction in complex I or V activity and alterations in mitochondrial ultrastructure (Bino et al., 1972; Chari-Bitron and Bino, 1971; Mahoney and Harris, 1972). At first these effects, together with others reported in cultured neurons (Campbell, 2001), were associated to indirect modulation of mitochondrial activity either by THC-related lipids or membrane CB1-activation (Busquets-Garcia et al., 2018). However in 2012, immunogold staining coupled to electron microscopy revealed specific particles associated with mitochondria membranes in neurons of the hippocampus (Bénard et al., 2012). These mitochondrial CB1 receptors modulated cellular respiration and energy production in the neurons, by decreasing cyclic AMP concentrations, protein kinase A activity and complex I enzymatic activity upon activation by exogenous and endogenous cannabinoids (Bénard et al., 2012). The impact of mtCB1 in neuronal bioenergetics was mediated by $G\alpha_{i/o}$ protein activation and consequent inhibition of soluble-adenylyl cyclase (sAC), which resulted in a decrease of the phosphorylation of certain subunits of the mitochondrial electron transport system, namely Complex I NDUFS2, and a subsequent decrease of mitochondrial respiration (Hebert-Chatelain et al., 2016a). At the synaptic level, they contributed to one type of endocannabinoid-mediated short term plasticity, depolarization-induced-suppression of inhibition (DSI) at hippocampal synapses, which was blocked by the application of rotenone, a potent selective complex I inhibitor (Bénard et al., 2012).

In silico subcellular protein localization analysis of the CB1 receptor resulted in the discovery of a mutation that reduced its probability of targeting mitochondria from 40% to 3%: deletion of the last 22 N-terminal aminoacids of the protein led to the characterization of the DN22-CB1 expression (Hebert-Chatelain et al., 2016a), which lacked mtCB1 but maintained their other CB1 locations, but showed equal functional properties as wildtype CB1 (Hebert-Chatelain et al., 2016a; Soria-Gomez et al., 2021a). DN22-CB1 expression in hippocampal cultures led to an impairment of the cannabinoid-mediated effects

on mitochondrial respiration and mobility(Hebert-Chatelain et al., 2016a). Moreover, viral-mediated expression of the DN22-CB1 construct in the hippocampus of CB1-KO mice rescued the impairment of novel object recognition memory induced by WIN injection in mice(Hebert-Chatelain et al., 2016a).

The abovementioned studies begun to unravel the control of mtCB1 of higher brain functions such as memory through the modulation of intramitochondrial control of bioenergetics. More recent data has shown that the activation of mtCB1 receptors in striatonigral terminals mediates the cataleptic behavioral effects induced by THC through intra-mitochondrial sAC-PKA decrease of mitochondrial respiration(Soria-Gomez et al., 2021a). However, the THC antinociceptive effects were mediated by plasma membrane CB1 in striatopallidal terminals and cytosolic PKA activation(Soria-Gomez et al., 2021a). This study is an example of how ubiquitous CB1 receptors can control specific brain processes depending on their sub-cellular locations. Furthermore, mtCB1 receptors in astrocytes modulate the glucose metabolism alterations induced by THC, which result in social impairments, showing that not only neuronal but astrocytic mtCB1 receptors can alter behavior through bioenergetics (further discussed below)(Jimenez-Blasco et al., 2020).

Aside from controlling cell bioenergetics, mtCB1 receptors regulate mitochondrial calcium handling. In astrocytes, activation of these receptors determines the transfer of calcium from the endoplasmic reticulum to the mitochondria via an AKT-mediated regulation of the mitochondrial calcium uniporter (MCU), which in turn is required for a specific astrocytic-mediated synaptic plasticity in the hippocampus(Serrat et al., 2021). A similar mechanism has been reported in neurons: CORT-mediated impairments in consolidation and retrieval are controlled by mtCB1 in distinct neurons in the locus coeruleus and hippocampus, an effect for which mitochondrial calcium dynamic changes are required(Skupio et al., 2023a). This data concludes that mitochondrial CB1 receptors have proven to be ubiquitous but to be essential for the specific regulation of some of the mitochondrial functions that are crucial for the brain.

5.5. The endocannabinoid system in astrocytes: focus on mtCB1

Reports of CB1 receptor expression in astrocytes date from the early 2000s (Stella, 2004) although the idea of a possible control of astrocytic functions by endocannabinoids arose with a study published in 1995 (Venance et al., 1995). CB1 expression was first reported in the plasmalemma of the perivascular endfeet of astrocytes from the nucleus accumbens of Sprague Dawley rats by electron microscopy and immunostaining (Rodríguez et al., 2001), and later in cingulate cortex, medial forebrain bundle and amygdala of Wistar rats (Moldrich and Wenger, 2000; Salio et al., 2002). However, the first functional report of CB1 in astrocytes was published by the lab of Alfonso Araque, in the context of the hippocampal tripartite synapse, where local application of cannabinoids induced astroglial Ca^{2+} increases, an effect that was reverted by the CB1 selective antagonist AM251 (Navarrete and Araque, 2008). Furthermore, the use of CB1 mutant mice together with staining techniques has provided definite evidence of the expression of these receptors in astrocytes from different brain regions (Han et al., 2012a), but also in subcellular compartments like the mitochondria (Gutiérrez-Rodríguez et al., 2018). Astrocytes also express the machinery to create and degrade endocannabinoids (Eraso-Pichot et al., 2023; Stella, 2004), which shows that they are not just passive eCB receivers but participate in eCB metabolism. Endocannabinoid-mediated CB1 activation has been seen to regulate astrocytic metabolism, inflammation, and regulation of synaptic plasticity (reviewed in REF (Eraso-Pichot et al., 2023)). Among these functions, two have been also linked to mtCB1 receptors, metabolism and synaptic plasticity, which I will describe below.

Astrocytic mtCB1 regulation of bioenergetics

Astrocytes are mainly, but not exclusively, glycolytic cells, which mean they are able to perform aerobic glycolysis and release lactate that will be used by neighboring neurons as a metabolic or signaling molecule (Magistretti and Allaman, 2018). This astrocyte-neuron lactate shuttle (ANLS) is modulated by neuronal activity: the release of glutamate by nearby neurons induces an inhibition of astrocytic mitochondria in favor of glycolysis and lactate release (Barros, 2013; Magistretti, 2011). Astrocytes can also oxidize fatty acids (Eraso-Pichot et al., 2017), glutamate and take part of the glutamate-glutamine cycle (Schousboe et al., 1993). These are examples on how astrocytic

metabolism is flexible and can be adapted to the environmental conditions. Recent research findings have unveiled the connection between astrocytic mtCB1 receptors, astrocyte glucose metabolism, and THC-triggered abnormalities in social behavior (Jimenez-Blasco et al., 2020; **Figure 9**). Exposure to a high dose of THC led 24 after to a decline in the stability and function of complex I, ultimately resulting in reduced lactate production and impaired social behaviors. These metabolic shifts depended on mtCB1 receptors on the CA1 of the hippocampus and prefrontal cortex and arose from a reduction in the phosphorylation of the Ser 173 residue of NADH: ubiquinone oxidoreductase subunit S4 (NDUFS4) through intra-mitochondrial protein kinase A (PKA)/cAMP signaling. The alterations in mitochondrial complex I not only impacted mitochondrial respiration but also diminished astrocytic mitochondrial reactive oxygen species (mROS). Astrocytes generate a higher level of mROS compared to neurons, a factor crucial in the physiological regulation of glucose utilization and neuronal survival (Vicente-Gutierrez et al., 2019). The transcription factor hypoxia-inducible factor 1 (HIF-1) responds to mROS, governing glycolysis and influencing the transformation of glucose into lactate (Kim et al., 2006). THC administration led to a reduction in the expression of the alpha subunit of HIF-1, causing a decrease in lactate production both in cultured astrocytes and in vivo, in a manner dependent on mtCB1, thereby impacting social behaviors in mice. This study (Jimenez-Blasco et al., 2020) demonstrated how the induction of bioenergetic stress in astrocytes by cannabinoids administration directly influences neuronal functions, thus further providing evidence for a direct impact of mtCB1 on behavioral functions.

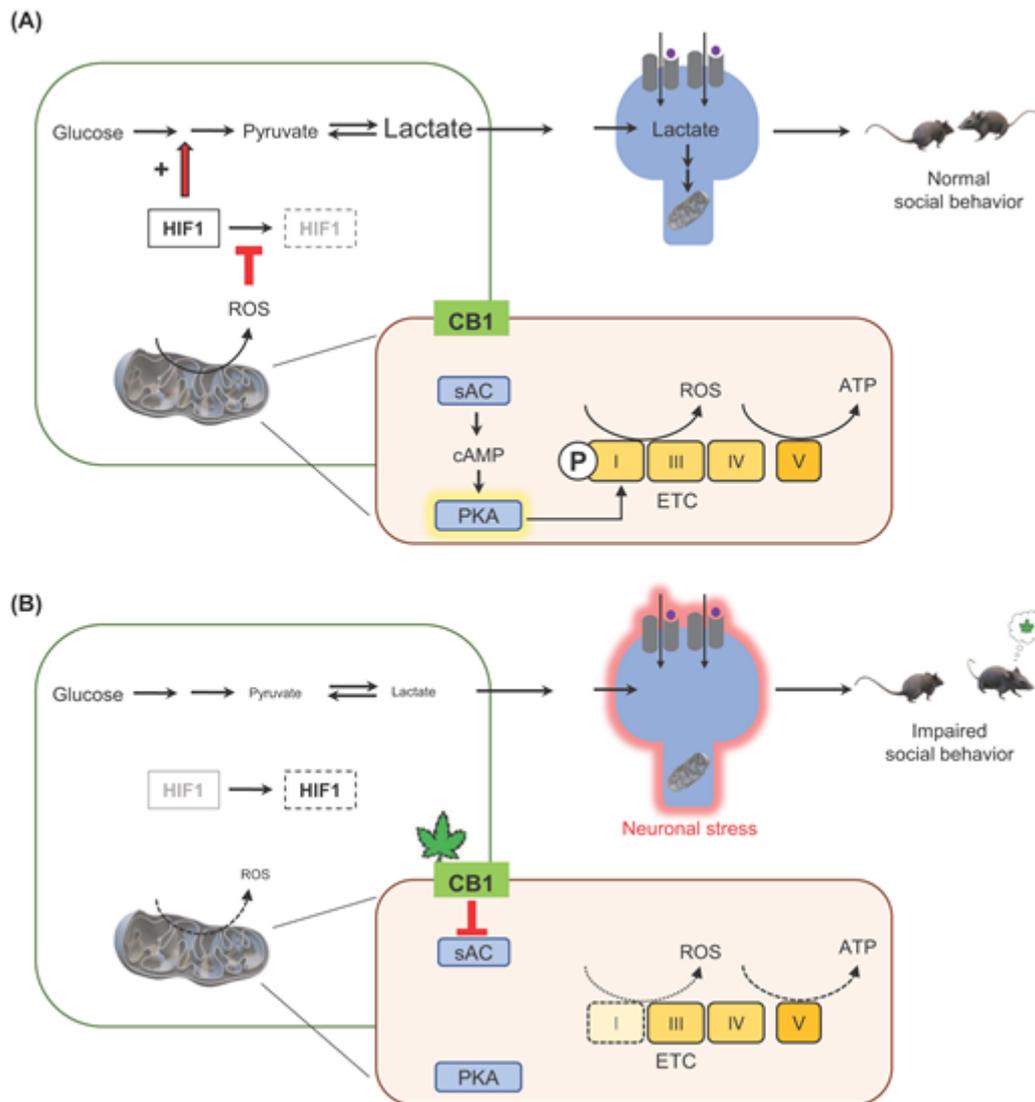


Figure 9 (from Fernández-Moncada and Marsicano, 2023): Astroglial mitochondrial CB1 receptors govern the metabolism of lactate and influence social behavior. (A) Under normal conditions, the sAC → cAMP → PKA pathway facilitates the assembly of complex I, mitochondrial respiration, and the generation of reactive oxygen species (ROS). This leads to the stabilization of HIF1 and the expression of various glycolytic enzymes, providing astrocytes with a heightened capacity for lactate production. This monocarboxylate, in turn, supports neuronal function and contributes to normal social behavior. (B) Prolonged activation (24 hours) of mtCB1 receptors with THC hinders the sAC → cAMP → PKA pathway, resulting in reduced stability of complex I, impaired mitochondrial respiration, and decreased ROS production. This diminished ROS level leads to the degradation of HIF1 and a subsequent decrease in lactate production capacity. The consequent reduction in lactate release results in neuronal oxidative stress, ultimately impairing social behavior. Abbreviations: ETC, electron transfer chain; V, adenosine triphosphate (ATP) synthetase-complex V.

Astrocytic mtCB1 regulation of calcium homeostasis and synaptic plasticity

Astroglial CB1 have so far been described to impact synaptic plasticity in different ways, both short and long term (reviewed extensively in Covelo et al., 2021; Navarrete et al., 2014; Oliveira da Cruz et al., 2016). For instance, their activation by exogenous cannabinoids can induce NMDAR-dependent hippocampal LTD (Han et al., 2012a). On the other hand, physiological ECS activation has been shown to induce heterosynaptic potentiation (**Figure 10**) in the hippocampus, amygdala, and striatum (Martín-Fernandez et al., 2017; Martín et al., 2015; Navarrete and Araque, 2008); spike-timing depression in the neocortex (Min and Nevian, 2012) or hippocampal LTP via D-serine release (Robin et al., 2018), depending on the experimental conditions. Most of these (if not all) require increase of intracellular calcium in the astrocytes (Navarrete et al., 2014; Oliveira da Cruz et al., 2016).

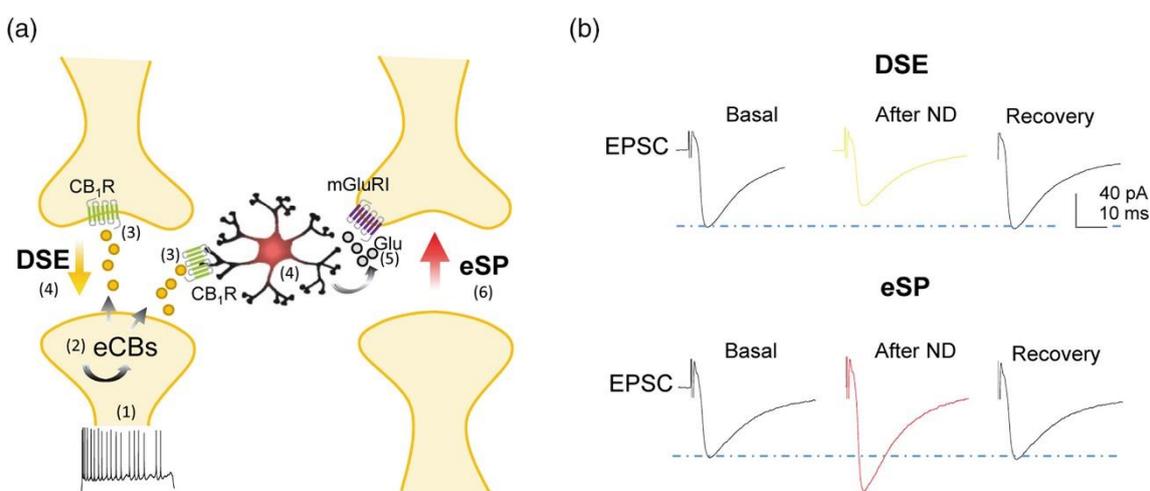


Figure 10: Endocannabinoids trigger depolarization-induced suppression of excitation (DSE) and excitatory short potentiation (eSP) signaling in neurons and astrocytes (from Noriega-Prieto et al., 2023). Specifically, (a) (1) heightened activity in the presynaptic terminal prompts the release of endocannabinoids (eCBs), (2) which then bind to presynaptic CB1 receptors (CB1R), leading to decreased glutamate release and the induction of DSE (3). Moreover, (3) when eCBs interact with astrocytic CB1 receptors (CB1R), (4) it enhances calcium mobilization from internal stores, resulting in the exocytosis of glutamate neurotransmitters and, consequently, (5) interaction with presynaptic metabotropic glutamate receptor I (mGluRI) in the heteroneuronal synapse, giving rise to excitatory short potentiation (eSP) (6). (b) Illustrated in representative

EPSC traces are instances before and after neuronal depolarization (ND), and during recovery, showcasing both DSE (upper traces) and eSP (bottom traces).

Indeed, astrocytic calcium handling is bidirectionally modulated by neuronal activity, with both ER (Göbel et al., 2020) and mitochondria (Agarwal et al., 2017) playing an active role. Interestingly, mtCB1 receptors in the astrocytes are involved in the modulation mitochondrial-mediated calcium dynamics (Serrat et al., 2021). Using a combination of calcium imaging in vitro and in vivo, Serrat et al showed that activation of mtCB1 triggers mitochondrial but not cytosolic calcium changes (Serrat et al., 2021). ER was the origin of the calcium source entering in the mitochondria through MERCS (mitochondrial-ER-contact sites) in a IP3-dependent manner (as shown by the blockade of both IP3 channels and SERCA transporters). Furthermore, the inhibition of the mitochondrial calcium uniporter (MCU), the main channel for entry of calcium in the mitochondrial matrix (Rizzuto et al., 2012), or the expression of a non-phosphorylatable form of the MICU1 subunit of the MCU were also shown to impede mtCB1-mediated calcium transfer. In other study, disruption of mitochondrial calcium in cultured astrocytes led to an increased in glutamate release, although this time accompanied by an increase on cytosolic calcium (Reyes and Parpura, 2008). These studies support the idea of a potential involvement of mtCB1-mediated mitochondrial calcium changes in different types of astrocytic mediated synaptic plasticity *via* glutamate release. This phenomenon undergoes the name of lateral synaptic potentiation (LSP) and was first described in the hippocampus (Gómez-Gonzalo et al., 2015). Indeed, eCBs released by one active pyramidal neuron act on pre-synaptic CB1 receptors on the synaptic terminals of homoneuronal synapses, inducing DSE. In addition, eCBs activate CB1 receptors in astrocytes inducing glutamate release which leads to LTP through mGluR receptors at heteroneuronal far synapses (Navarrete et al., 2014). Strikingly, this type of plasticity is absent in mice lacking mtCB1 in all the brain nor in animals that didn't express mtCB1 specifically in hippocampal astrocytes (which was induced by a double viral combination of Cre deletion and DN22-CB1 construct re-expression in CB1-flox mice)(Serrat et al., 2021).

In conclusion, cannabinoid receptors in astrocytes, particularly mitochondrial CB1 receptors in astrocytes, influences diverse functions, including metabolism and synaptic plasticity which set the base for a better understanding neurobehavioral outcome.

5.5. Endocannabinoid control of social behaviors in pathology and physiology

The first reports on the contribution to cannabinoids to social behaviors are from the 19th century psychiatrist Jacques Moureu, that compared the effects of hashish intoxication (containing high levels of THC) to the symptoms observed in patients with mental illness, highlighting a similarity in the aspect of social introversion of alienation(Moureau, 1845). Other later experiments reported opposite findings, with subjects that were usual cannabis consumers reporting a higher degree of empathy and social unity(Georgotas and Zeidenberg, 1979; Tart, 1970). While the data regarding in which direction cannabis affects social interactions is not clear, it strongly suggests that cannabinoids influence social interactions. Moreover, changes in cannabinoid receptor expression or endocannabinoid levels have been detected in patients of sociopathologies. Reduced CB1 receptor expression was observed in postmortem brains of individuals with autism spectrum disorders (ASD)(Purcell et al., 2001) and with schizophrenia(Wong et al., 2008). Furthermore, levels of circulating endocannabinoids, particularly AEA, are altered in children with autism(Aran et al., 2019). Together, this underwrites the idea that the endocannabinoid system is involved in the control of healthy social behaviors.

Manipulations on endocannabinoid levels in the brain support the physiological involvement of these messengers in social behaviors. Only one study assessed the contribution of 2-AG signaling to social interactions. In this, authors used DAGL α floxed mice to study the contribution of 2-AG to the 3-chamber social preference test. Crossing these mice with mouse lines carrying specific D1 or D2-MSNs promoters (Drd1a or Adora2a respectively), they observed that the deletion of the 2-AG degradation enzyme in D1-positive MSNs, animals spent more time exploring a compartment with a social stimulus than an empty one, and they showed repetitive behaviors in the form of self-grooming

g(Shonesy et al., 2018). In another set of experiments, they deleted the same enzyme but this time in specific regions of the basal ganglia, the dorsal striatum and nucleus accumbens, with a general viral approach not specific to neurons (AAV5-CMV-Cre). In this case, they observed that reduction of 2-AG levels in the accumbens induced self-grooming while in the dorsal striatum it abolished social preference (Shonesy et al., 2018). However, systemic pharmacological manipulations of 2-AG (with systemic injections of either JZL184, a MAGL inhibitor, or DO34, a DAGL inhibitor) didn't affect social preference in a 3-chamber test in naïve mice, although they managed to rescue social deficits observed in SHANK3 mutant mice, a rodent model for autism(Folkes et al., 2020a). More studies have assessed the contribution of AEA to social interactions, either by pharmacological or genetic manipulations. Systemic injection of the specific FAAH inhibitor URB597 induces an increase of social interaction in a variety of tests (reviewed in (Ahmed et al., 2022)). Furthermore, a total knockout of the FAAH gene was associated with increased direct social interaction, although this wasn't reflected in the 3-chamber test(Cassano et al., 2011). Lastly, the modulation of eCBS has been seen to revert social deficits in some mouse models of autism (**Table 1**), which adds to the idea of eCBS as possible therapeutic targets for sociopathies (Pietropaolo and Marsicano, 2022).

Mouse model	Origin	Molecular relation to the ECS	Mutation	Observed deficits	Therapeutic rescue	Refs
SHANK3-KO	Genetic	Couples mGlu5R/Homer complexes to PSD-95 (Tu et al., 1999) Required for eCB-LTD in striatum (Wang et al., 2017a)	SHANK3 B ^{-/-} (exons 13–16)	Change in social preference in 3-chamber test	Increase of 2-AG (JZL184 or DO34)	(Folkes et al., 2020a)
FMR1-KO	Genetic	FMRP binds to DAGL mRNA and traffics it to form DAGL-mGluR5 complexes (Jung et al., 2012; Maccarrone et al., 2010)	FMR1 full deletion	Change in social preference in 3-chamber test	Increase of AEA (URB597)	(Wei et al., 2016)
		Altered phasic 2-AG release in hippocampus		Change in preference between familiar and novel social	CBDV administration (a propyl analog of CBD)	(Premoli et al., 2023)

		(Jung et al., 2012)		stimuli in 3-chamber test		
Neurologin 3-KI	Genetic	Part of PSD95 complex(Irie et al., 1997) Altered tonic endocannabinoid signaling in hippocampus (Földy et al., 2013)	NL3 ^{R451C}	Increased aggression in adult	CB1 activation (WIN55,212-2)	(Hosie et al., 2018a)
BTBR	Idiopathic			Change in social preference in 3-chamber test	Increase of AEA (URB597) – abolished by CB1 inverse agonist AM251	(Wei et al., 2016)
Valproic acid	Idiopathic			Social play Change in social preference in 3-chamber test	Increase of AEA (URB597)	(Servadio et al., 2016)

Table 1: Animal models of ASD, their social phenotypes and their reported interactions with the eCB system, (adapted from Pietropaolo and Marsicano, 2022)

Cannabinoid receptors, particularly CB1, are expressed in key regions of “the social brain” including central and basolateral amygdala, prefrontal cortex, hippocampus, dorsolateral striatum, ventral tegmental area, and, to a lesser extent, the nucleus accumbens (Wei et al., 2017).

Data from different constitutive and conditional mutant mice lacking CB1 in different cell populations offer contradictory results, which strongly depends on the test conditions (summarized in **Table 2**) (Haller et al., 2004; Häring et al., 2011; Jacob et al., 2009). However, a majority of studies showed that CB1-KO and Glu-CB1-KO mice displayed fewer social interactions. Interestingly, one of these studies assesses that the difference in total time of exploration occurs in the first 5 min of the interaction, a time relevant for information gathering(Häring et al., 2011). But Glu-CB1-KO mutants present other alterations that could play a role in these social tests, namely problems with memory (Häring et al., 2011),

increase in spontaneous locomotion (De Giacomo et al., 2022; Häring et al., 2011; Jacob et al., 2009), general nonsocial exploratory deficits (Jacob et al., 2009).

Scarce evidence exists of the contribution of CB1 in astrocytes to social behaviors. Social preference was tested in an adaptation of the 3-chamber test where male GFAP-CB1-KO lacking astrocytic CB1 receptors in the whole brain were exposed to an arena with two chambers, one with a social stimulus (C57BL6 age-matched male) and the other one empty. Analyzing the time spent around the social stimulus versus the empty one showed no difference between GFAP-CB1-KO and littermate controls (Jimenez-Blasco et al., 2020). However, injecting 10mg/kg of THC in naïve mice leads to an impairment on social preference in the same test, an effect not present in GFAP-CB1-KO. This suggested that the deleterious effects of THC administration on social interaction depended on astrocytic CB1 receptors (Jimenez-Blasco et al., 2020). Indeed, as described in the previous section, high doses of THC induce in astrocytes a bioenergetic stress that results in an impairment of lactate production mediated by mtCB1. Systemic injection of lactate was able to rescue social deficits in naïve mice injected with THC both in the 2-chamber test and in a direct social interaction test, an effect that could be recapitulated by injecting a phosphomimetic form of NDUFS4 in the astrocytes of the hippocampus of mice before the THC treatment, linking THC-induced social deficits and astrocytic mtCB1-related molecular signaling (Jimenez-Blasco et al., 2020).

Moreover, endocannabinoid production by astrocytes also plays a potential role in social interactions. A study used GLAST-DAGLa-KO mice, with significantly higher levels of 2-AG in PFC and hypothalamus, to study the role of astrocytic eCBs in depression-related behaviors and maternal care. Interestingly, mutant mice were worse mothers, with latencies of pup retrieval that more than tripled those of wildtype moms and inability to properly make their nests. However, they did not differ in time spent with a cotton swab impregnated with pup odor and could see normally, suggesting that astrocytic eCBs could be involved in other aspects of maternal behavior not mediated by those senses (Schuele et al., 2021).

Few pharmacological manipulations of CB1 provided some information about the anatomical contribution of these receptors to social behaviors. CB1 receptors in the posterior piriform cortex are necessary for the dopaminergic control of this brain region during social interaction (Zenko et al., 2011a). Intra-hippocampal infusions of CB1 agonists in rats decreased social interaction, which was abolished by blocking associated dopaminergic transmission in the NAc (Loureiro et al., 2014, 2016). Increasing AEA levels in the BLA induces a CB1-mediated increase in social play in the basolateral amygdala of rats (Trezza et al., 2012).

This data points towards a physiological and pathological role of the ECS in social behaviors, but reflects the wide gaps in the knowledge about the biological basis of this contribution.

Genotype	Test	Age partner	Genotype partner	Environment	Housing	Effect	Ref
CB1 KO	Direct social interaction	Age-matched	Same genotype	New cage with red light	Single housed	No effect	(Terzian et al., 2014)
	3-chamber test	Age-matched	Wildtype mouse	Test arena with red light	Single housed	No effect	(Terzian et al., 2014)
	Direct social interaction	Younger (10% smaller)	Stranger with different genetic background	Home-cage (light NS)	Single housed 2 weeks before test	No effect	(Haller et al., 2004)
	Direct social interaction	Age-matched	Wildtype mouse	Neutral cage (light NS)	Single housed 2 weeks before test	Decrease	(Haller et al., 2004)
	Direct social interaction	Juvenile	Wildtype mouse	Home-cage with red light	Grouped housed	No effect	(Jacob et al., 2009a)
	Direct social interaction	Juvenile	Wildtype mouse	Home-cage with 500lux of white light	Grouped housed	Decrease	(Jacob et al., 2009a)
	Direct social interaction	Younger but adults	Wildtype mouse	Home-cage with red light	Single housed 1 week before the test	Decrease	(De Giacomo et al., 2022)
GLU-CB1-KO	Direct social interaction	Age-matched	Same genotype	New cage with red light	Single housed	No effect	(Terzian et al., 2014)

	Direct social interaction	Juvenile	Wildtype mouse	Home-cage with red light	Grouped housed	Decrease	(Jacob et al., 2009a)
	Direct social interaction	Juvenile	Wildtype mouse	Home-cage with 500lux of white light	Grouped housed	Decrease	(Jacob et al., 2009a)
	Direct social interaction	Younger but adults	Wildtype mouse	Home-cage with red light	Single housed 1 week before the test	Decrease	(Häring et al., 2011)
	3-chamber test	Younger but adults	Wildtype mouse	Test arena with red light	Single housed 1 week before the test	Decrease	(Häring et al., 2011)
GLU-CB1-RS	Direct social interaction	Younger but adults	Wildtype mouse	Home-cage with red light	Single housed 1 week before the test	Rescues	(De Giacomo et al., 2022)
	3-chamber test	Younger but adults	Wildtype mouse	Test arena with red light	Single housed 1 week before the test	Rescues	(De Giacomo et al., 2022)
GABA-CB1-KO	Direct social interaction	Age-matched	Same genotype	New cage with red light	Single housed	No effect	(Terzian et al., 2014)
	Direct social interaction	Younger but adults	Wildtype mouse	Home-cage with red light	Single housed 1 week before the test	Increase	(Häring et al., 2011)
	3-chamber test	Younger but adults	Wildtype mouse	Test arena with red light	Single housed 1 week before the test	Increase	(Häring et al., 2011)
GABA-CB1-RS	Direct social interaction	Younger but adults	Wildtype mouse	Home-cage with red light	Single housed 1 week before the test	Worsens	(De Giacomo et al., 2022)
	3-chamber test	Younger but adults	Wildtype mouse	Test arena with red light	Single housed 1 week before the test	Worsens	(De Giacomo et al., 2022)
D1-CB1-KO	Direct social interaction	Age-matched	Same genotype	New cage with red light	Single housed	Decrease	(Terzian et al., 2011)
	Direct social interaction	Age-matched	Same genotype	New cage with 500lux of white light	Single housed	No effect	(Terzian et al., 2011)

	Direct social interaction	Younger but adults	Wildtype mouse	Home-cage with red light	Single housed 1 week before the test	No effect	(Håring et al., 2011)
	3-chamber test	Younger but adults	Wildtype mouse	Test arena with red light	Single housed 1 week before the test	No effect	(Håring et al., 2011)
GFAP-CB1-KO	2 chamber test	Age-matched	Wildtype mouse	Test arena at 50lux	Single housed	No effect	(Jimenez-Blasco et al., 2020)

Table 2: Constitutive and conditional CB1 mutant mice in different social tests

5.6. Modulation of olfactory functions by the endocannabinoid system

The processing of olfactory information has been shown to be under control of the endocannabinoid system at several levels. Given the expression of cannabinoid receptors in the olfactory bulb (Herkenham et al., 1990) the processing of odors in this first relay of olfactory information in the brain also appears to be controlled by this system. Indeed, changes in neuronal activity of different olfactory bulb cell types induce endocannabinoid release which in turn activate CB1 receptors for controlling synaptic transmission and plasticity, finally leading in alterations of olfactory functions.

5.6.1 Odorant-mediated endocannabinoid release

Bulk measurements of eCBs concentration in the main olfactory bulb showed detectable levels of endocannabinoid-related lipids, with highest concentration of 2-AG than AEA (Soria-Gómez et al., 2014; Wang et al., 2019). Furthermore, enzymes involved in the biosynthesis and degradation of both 2-AG and AEA (e.g. NAPE-PLD, ABHD4, GDE1 and DGL α/β) were also present in this region (Wang et al., 2019).

Endocannabinoids in the olfactory bulb are most likely synthesized and released by postsynaptic neurons in result of excitation in response to following odor exposure. Indeed, olfactory bulb circuits are sites of endocannabinoid-modulated plasticity events, like DSI and DSE, which points towards an on-demand endocannabinoid synthesis and metabolism, although the specifics of these messengers haven't been characterized yet (Bhatia-Dey & Heinbockel,

2020). Moreover, characterization of cortical inputs to the olfactory bulb suggests a tonic endocannabinoid tone in this brain region, as AM-251 application alone, a CB1-specific antagonist, enhanced IPSCs in cortical GABAergic cells projecting to olfactory bulb granule cells (Zhou & Puche, 2021). Endocannabinoid production could originate from different cell sources in the olfactory bulb including external tufted cells in the glomerular cell layer, granule cells, deep short axon cells or mitral cells. Particularly, mitral neurons exhibit synchronized firing patterns during extended depolarization phases, initiating stimulation of both granule cells and deep short axon cells (Carlson et al., 2000; Schoppa & Westbrook, 2001).

This recurrent firing patterns of mitral/tufted neurons when interacting with granule cells may result in the tonic release of endocannabinoids, that could be modulating CB1-mediated plasticity in surrounding cells either in the glomerular layer or in the granule cell layer (Zhou & Puche, 2021). Indeed, in fasting conditions in mice, a decrease of cortical glutamatergic activity via CB1 receptors disinhibits mitral cell activity via a decrease of granule cell activity. This fasted state also leads to an increase in levels of AEA which could be associated to the increase in activity of mitral cells, supporting a role for endocannabinoid synthesis in modulating olfactory functions depending on the internal state of the animal (Soria-Gómez et al., 2014).

5.6.2. Cannabinoid-mediated synaptic plasticity

Endocannabinoids play a crucial role in modulating synaptic plasticity within the mammalian MOB both DSI and DSE. The endocannabinoid system was initially identified as a regulator of GABAergic transmission in the MOB through DSI (Wang et al., 2012). This regulation is mediated by CB1 receptors, which are known to modulate the firing patterns of periglomerular (PG) cells and external tufted cells (eTCs), both key components of the glomerular circuitry in the MOB. Activation of CB1 receptors on PG cells indirectly influences the activity of mitral and tufted cells by controlling inhibitory transmission (Wang et al., 2019a), potentially enhancing the sensitivity of the glomerulus to sensory inputs. Moreover, CB1 receptors are also present in glutamatergic corticofugal fibers (CFF) originating from higher-order olfactory areas, such as the anterior olfactory

nucleus and the anterior piriform cortex, targeting granule cells in the MOB (Soria-Gómez et al., 2014a). Notably, endocannabinoid levels in the MOB increase during fasting, resulting in the dampening of granule cell excitation. As GCs control mitral cell activity, CB1 receptor activation on CFFs leads to the disinhibition of mitral cells. This fasting-induced enhancement in olfactory sensitivity is associated with the amount of food ingested upon refeeding (Soria-Gómez et al., 2014a), suggesting that endocannabinoids play a pivotal role in regulating olfactory perception and food intake. The expression of CB1 receptors in CFF fibers also regulates downstream activity of synapses with deep short axon cells (dSAs) (Pouille and Schoppa, 2018). Depolarization of dSAs in the MOB triggers transient suppression of excitatory CFF inputs through CB1 receptors (DSE). This bidirectional effect can either control the synapses from dSAs to GCs or from GCs to mitral cells (Pouille and Schoppa, 2018), highlighting a complex control mechanism over olfactory bulb output neurons. Both of these mechanisms, DSI and DSE, allow individual neurons to disengage from their network, enhancing their capacity to encode information.

II. AIMS

AIMS

Social transmission of stress can be essential for the survival, allowing social animals to adapt their behavior according to the information relayed (Sterley et al., 2018). This process “copies” some of the consequences of direct stress experience, such as the induction of plasticity at hypothalamic synapses (Sterley et al., 2018). However, we do not know if other adaptations observed after direct stress exposure, such as specific cognitive impairment (Busquets-Garcia et al., 2016; Skupio et al., 2023), can also be observed after socially transmitted stress. Moreover the transmission of stress signals in rodents depends on olfactory processes to detect specific chemosignals (Sterley et al., 2018) that are generally firstly processed in the olfactory bulb to then impact behavior (Baum and Cherry, 2015). In this context, and as described in more detail in the Introduction section, the present PhD work is based on the following observations: (i) Both CB1 receptors and astrocytes in olfactory circuits have been independently associated to odor processing (Pouille and Schoppa, 2018; Soria-Gómez et al., 2014; Terral et al., 2019; Wang et al., 2019; Zenko et al., 2011); (ii) CB1 receptor signaling in neurons and/or astrocytes have been linked memory processing (Busquets-Garcia et al., 2018b; Hebert-Chatelain et al., 2016; Robin et al., 2018; Skupio et al., 2023) and the impact of stress on cognition (Busquets-Garcia et al., 2016; Hillard, 2014; Skupio et al., 2023); (iii) The recent discovery of mitochondrial CB1 receptors revealed new information linking mitochondria to synaptic and behavioral functions (Hebert-Chatelain et al., 2016b; Jimenez-Blasco et al., 2020; Skupio et al., 2023; Soria-Gomez et al., 2021); (iv) in astrocytes, mtCB1 receptors control cell calcium dynamics via MCU (mitochondrial calcium uniporter)(Serrat et al., 2021) and they are linked to social behavior (Jimenez-Blasco et al., 2020).

All these elements converged to define the main aim of the thesis: to **study the contribution of CB1 receptors and astrocytes to social transmission of stress and its cognitive consequences.**

To achieve this general aim, I divided my project into three specific objectives:

1. Cognitive consequences of socially transmitted stress in physiological conditions
2. Contribution of different subpopulations of CB1 receptors to this

phenomenon

3. Molecular mechanisms behind the role of CB1 receptors in social transmission of stress and its cognitive consequences

OBJECTIVE 1: *Cognitive consequences of socially transmitted stress in physiological conditions*

Social interactions with stressed partners allows individual to gather information about potential dangers without direct experience. Indeed, animals that either received a direct foot-shock stress or interacted with stressed partners (social transmission of stress, STS), present similar stress-induced alterations, such as for instance synaptic modulation of specific neurons (Lee et al., 2021; Sterley et al., 2018). This stress phenocopy is induced by social interactions mediated by specific odorant social cues (Sterley et al., 2018). One of the effects of direct stress is the impairment of some types of non-emotional memory, in the benefit of survival (Schwabe et al., 2022). However, no study to date has assessed what is the impact of social transmission of stress on cognitive adaptations.

Using behavioral analysis, I studied the dynamics of the social interactions between mice during STS, their impact on the non-emotional novel object recognition memory (NOR) test (Busquets-Garcia et al., 2013; Skupio et al., 2023b), and the sensory modality required for these effects.

OBJECTIVE 2: *Contribution of different subpopulations of CB1 receptors to this phenomenon*

Among other functions, CB1 receptors are involved in memory processes, olfaction, stress-induced amnesia and social interactions. During the last two decades, the use of constitutive and conditional mutant mice has allowed for the dissection of the contribution to behavior of distinct cellular and subcellular CB1 populations (Han et al., 2012; Marsicano et al., 2003; Monory et al., 2006; Skupio et al., 2023; Soria-Gomez et al., 2021). However, no study has assessed the role of specific subpopulations of CB1 receptors in STS. To this aim, I exposed several genetic mutant mouse lines lacking different cellular and subcellular populations of CB1 receptors to the STS protocol and analyzed the pattern of their social interactions. Social transmission of stress is mediated by olfactory

cues. Interestingly, CB1 receptors participate in olfactory processes (Soria-Gómez et al., 2014a). Therefore, I asked what could be the role of endocannabinoid signaling in olfactory circuits during STS. In this context, I used a combination of genetic manipulations to study the role of different cellular and subcellular CB1 receptor subpopulations in STS, and the subsequent cognitive adaptations. In particular, I focused on mitochondrial CB1 receptors in astrocytes of the olfactory bulb.

OBJECTIVE 3: Molecular mechanisms behind the role of CB1 receptors in social transmission of stress and its cognitive consequences.

CB1 receptors (mtCB1) control many different cell functions through a plethora of molecular mechanisms, including alterations of cytosolic and mitochondrial signaling (Hebert-Chatelain et al., 2016; Jimenez-Blasco et al., 2020) and calcium dynamics (Serrat et al., 2021). Therefore, I asked whether these functions of mtCB1 receptors are involved in STS and its cognitive consequences. For this, I used a combination of in vivo recordings (fiber photometry) and viral manipulations to explore the molecular underpinnings of the contribution of olfactory bulb CB1 receptors to STS.

The results of this thesis have been submitted as a publication by the time of the defense.

III. RESULTS: CHAPTER 1

Olfactory bulb astrocytes link social transmission of stress to cognitive adaptation

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ABSTRACT

Social odors transmit emotions and alter behavior. Indeed, olfactory information, transmitted from stressed individuals, induces stress-like physiological and synaptic changes in naïve partners. Direct stress experience alters cognition, but whether socially transmitted stress can also alter memory processes is currently unknown. Here we show that social investigation of a stressed individual, or exposure to specific olfactory signals from that individual, is sufficient to impair novel object recognition (NOR) in unstressed male mice. This requires mitochondria-associated cannabinoid type-1 (mtCB1) receptors and Mitochondrial Calcium Uniporter (MCU) activity. Targeted genetic manipulations, *in vivo* mitochondrial calcium imaging and behavioral analyses revealed that olfactory bulb astrocytic mitochondrial calcium regulation is necessary to determine the salience of odors coming from stressed partners and to define their cognitive consequences. Thus, astrocytic processing represents a key step to detect the meaning of social odors.

Keywords: astrocytes, olfaction, mitochondria, endocannabinoids, stress, social, cognition

INTRODUCTION

Understanding social signals from conspecifics that portend potential danger can determine the difference between life and death. In humans and animals, chemosignals associated with internal and affective states, such as disease (Olsson et al., 2014), fear (Chen et al., 2006; Mutic et al., 2017), stress (Dalton et al., 2013; de Groot et al., 2015b) or others (de Groot et al., 2015a; Lübke and Pause, 2015) can modulate how healthy individuals perceive and react to the environment. Consistently, alterations of chemosignal processing are present in subjects with social pathologies such as autism spectrum disorders (Endevelt-Shapira et al., 2018). In rodents, the interaction with an individual previously exposed to threat initiates specific investigative behaviors that enable observers to detect and process stress chemosignals (Kiyokawa et al., 2004; Meyza et al., 2015; Sterley et al., 2018). The processing of these stress chemosignals by an unstressed conspecific result in behavioral and synaptic changes that mirror those observed in the stressed individual. Direct stress also inhibits certain types of non-stress-related memory (Joëls et al., 2006), such as delayed alternation memory (Dorey et al., 2012) and novel object recognition (NOR) (Busquets-Garcia et al., 2016; Skupio et al., 2023b). Whether detecting and processing stress chemosignals also causes cognitive adaptations in unstressed conspecifics has yet to be explored.

The first central processing station of social chemosignals in mammals is the olfactory bulb (OB). Here, odor information is refined before being forwarded to limbic areas, eventually modulating a variety of behaviors. The activity of the olfactory bulb is under the control of many different local cell types, including astrocytes. These cells profoundly regulate general olfactory processes, such as oscillatory activity and odor detection and discrimination (Roux et al., 2011a, 2015; Ung et al., 2020, 2021). However, little is known about the contribution of these cells in funneling olfactory information into specific behavioral consequences.

Cannabinoid receptors, their endogenous lipid ligands (endocannabinoids) and the machinery for endocannabinoid synthesis and degradation compose the endocannabinoid system, which is involved in many different brain and body functions (Busquets-Garcia et al., 2015), including olfaction (Terral et al., 2020), stress processing (Busquets-Garcia et al., 2016;

Skupio et al., 2023) and cognition (Han et al., 2012a; Hebert-Chatelain et al., 2016a). Endocannabinoids regulate olfactory functions in the OB mainly through their action upon type-1 cannabinoid receptors (CB1) (Soria-Gómez et al., 2014a; Wang et al., 2012b, 2019c). CB1 receptors are expressed in several layers of the OB, but their functional characterization has been so far restricted to neurons (Heinbockel and Straiker, 2021). Astrocytes also express CB1 receptors that have important functional consequences (Covelo and Araque, 2016; Gutiérrez-Rodríguez et al., 2018a; Martin-Fernandez et al., 2017; Navarrete and Araque, 2008; Robin et al., 2018; Serrat et al., 2021). The recent discovery of CB1 receptors associated with mitochondria (mtCB1) (Bénard et al., 2012; Hebert-Chatelain et al., 2016a) and their presence in astrocytes (Gutiérrez-Rodríguez et al., 2018a; Jimenez-Blasco et al., 2020) has revealed new information linking mitochondria to synaptic and social functions (Jimenez-Blasco et al., 2020; Serrat et al., 2021). Moreover, astrocytic mtCB1 receptors control cellular calcium dynamics *via* modulation of the mitochondrial calcium uniporter complex (MCU) and its regulatory protein mitochondrial calcium uptake 1 subunit (MICU1) (Serrat et al., 2021). Thus, CB1 receptors are optimal tools to probe the functions of different cell types and organelles in social investigation, in the detection of affective state-related olfactory signals and eventually in the transmission of stress and its cognitive consequences.

In this study, we investigated the mechanisms and potential cognitive behavioral consequences of chemosignal-dependent social transmission of stress. Using mice, we found that detection of chemosignals from a stressed demonstrator leads to an impairment of retrieval of NOR in a naïve observer. Moreover, we show that subpopulations of CB1 receptors present in astrocytes and mitochondria of the observer are specifically required for these effects. Targeted deletion of mtCB1 in the astrocytes of the OB decreased social investigation of the stressed partner and abolished NOR impairment. Lastly, dysregulation of mitochondrial calcium handling in OB astrocytes induced the same impairment in social transmission of stress and its cognitive effects as the genetic deletion of astrocytic mtCB1 receptors. Thus, the regulation of calcium dynamics by astrocytic mitochondria in the OB plays an essential role in the cognitive alterations induced by social transmission of stress.

RESULTS

Social olfactory detection of stress impairs object recognition

Pairs of familiar mice housed together were separated for 5 min, when one (the demonstrator, DEM) was exposed to a footshock protocol (shock stress) or not (neutral) and the other (the observer, OBS) was left undisturbed in the home-cage (**Fig 1a**). After this, the pairs were reunited and allowed to freely interact for 5 minutes, during which 8 social and non-social behaviors of "stress" or "neutral" OBS mice were quantified and compared (**Extended Data 1a,b**). As expected (Sterley et al., 2018), OBS mice paired with stressed mice spent more time engaged in anogenital investigation, body exploration and allogrooming than those paired with neutral mice (**Fig 1b-g** and **Extended Data 1c**). The affective state of the demonstrator (stressed vs neutral) had no effect on several non-social behaviors (**Extended Data 1c**) or anxiety-like responses (**Extended Data 1d**) of OBS mice. These results confirm that interaction with a previously shocked partner triggers specific patterns of social responses that have been associated to social transmission of stress (Sterley et al., 2018). In order to investigate the behavioral relevance of these processes, we next addressed whether this type of social communication exerts a similar impact on cognitive performance as direct stress experience.

Since acute direct stress impairs both consolidation and retrieval of NOR in mice (Busquets-Garcia et al., 2016; Skupio et al., 2023b), we used this task to test the impact of socially-transmitted stress on cognition. Pairs of familiar mice were habituated and trained in the NOR test for two days. On day 3, the pairs underwent the social transmission of stress (STS) protocol 20 min before being tested for NOR retrieval (**Fig 1h**). As expected, stressed, but not neutral DEM mice, displayed impaired performance in the NOR without any alterations in total object exploration (**Fig 1i** and **Extended Data 1e, f**). This indicates that acute stress impairs the retrieval of object-specific information. Strikingly, impaired NOR retrieval was also evident in observers of stressed DEM, but not of neutral ones (**Fig 1j** and **Extended Data 1e, f**), demonstrating that the cognitive impact of socially-transmitted stress is similar to that of direct stress experience itself.

Chemosignals released from the anogenital region are necessary and sufficient for the synaptic changes triggered by socially-transmitted stress (Sterley et al., 2018). Thus, we asked whether these chemosignals were also sufficient to elicit the NOR impairment observed in mice after social interaction with stressed partners. Exposure to a cotton swab that was saturated with anogenital secretions of a stressed mouse was sufficient to impair NOR performance (**Fig 1k,l; Extended Data 1g,h**). In contrast, exposure to a cotton swab impregnated with the anogenital secretions of a naïve mouse did not alter NOR retrieval as compared to a cotton swab infused with saline (**Fig 1k,l; Extended Data 1g,h**). Importantly, the differential effects of the odorants were not linked to the time of exposure (**Extended Data 1i**), indicating that a short experience (2-3 seconds) of stress chemosignals is sufficient to impair NOR performance in mice. Together, these results show that anogenital investigation of stressed mice results in the detection of specific chemosignals that alter NOR retrieval, indicating that olfactory processes can link social emotional information to cognitive functions.

Astrocytic and mitochondrial CB1 receptors are specifically required for social anogenital investigation of a stressed partner

CB1 receptors are involved in memory processes, olfaction, stress-induced amnesia and social interactions (Busquets-Garcia et al., 2016; Jimenez-Blasco et al., 2020; Soria-Gómez et al., 2014a), suggesting the potential involvement of the endocannabinoid system in social olfaction resulting in the transmission of stress and its cognitive effects. To start addressing this hypothesis, we first used a battery of mutant mouse lines lacking CB1 receptors in different cellular and subcellular populations. Observer CB1 mutant mice were housed immediately after weaning with age-matched demonstrators (see Methods for more details). In comparison to wild-type littermates, OBS mice with a global deletion of CB1 receptors (CB1-KO mice; Marsicano et al., 2002) spent less time engaged in anogenital investigations of a stressed partner (**Fig 2a-c**). A similar phenotype was observed in OBS mice lacking CB1 in cortical glutamatergic neurons (Glu-CB1-KO; (Monory et al., 2006), astrocytes (GFAP-CB1-KO; Han et al., 2012) and in mitochondria (DN22-CB1-KI; Soria-Gomez et al., 2021) but not in forebrain GABAergic

neurons (GABA-CB1-KO; Monory et al., 2006) (**Fig 2a-c**). Some of these alterations did not appear to be specific for anogenital investigations. Global CB1-KO and Glu-CB1-KO displayed other changes in social behavior, such as a decrease in body exploration (**Fig 2d-f**) and/or allogrooming (**Extended Data 2a**). Conversely, the GFAP-CB1-KO and DN22-CB1-KI lines displayed a specific decrease of anogenital interactions with no other changes in social behavior (**Fig 2d-f** and **Extended Data 2a**). Importantly, no genotype effect was detected in the social behaviors during interactions with neutral DEM mice (**Extended Data 2b-d**), indicating that the mutations did not alter the basal ability of the mice to express these innate behaviors. Altogether, these data show that anogenital investigations specifically linked to the presence of a stressed partner are controlled by CB1 receptors located in astrocytes and mitochondria, suggesting that these cells and these organelles might be the sites where the endocannabinoid system controls social transmission of stress.

Mitochondrial CB1 receptors in astrocytes of the olfactory bulb are required for anogenital investigation and subsequent cognitive impairment

Social transmission of stress depends on olfactory cues (Sterley et al., 2018) and the data collected so far show that exposure to odors from the anogenital region of stressed mice is sufficient to impair NOR retrieval. As astrocytic and mitochondrial CB1 receptors appear to be necessary for anogenital, but not bodily, investigations, and considering that the first brain region devoted to the processing of chemosignals is the olfactory bulb (OB), we asked whether mitochondrial CB1 receptors in astrocytes of this brain region might be responsible for the social transmission of stress and its cognitive consequences. We used a double viral approach in CB1-flox mice (Marsicano et al., 2003) (**Fig 3a**), using Cre expression both to delete and to re-express wild-type or mutant CB1 receptors (Soria-Gomez et al., 2021c). Thus, we generated four groups (see Methods for details; **Fig 3b**): (i) control mice (Ctrl), expressing the CB1 receptor in a wild-type fashion, (ii) OB-GFAP-CB1-KO mice, lacking the receptor in OB astrocytes, (iii) OB-GFAP-CB1-RS (rescue) mice, carrying deletion of endogenous CB1 in OB astrocytes and re-expression of a wild-type form of the CB1 protein in the same cells, and (iv) OB-GFAP-DN22-

RS mice carrying deletion of endogenous CB1 in OB astrocytes and re-expression of the mutant DN22-CB1, lacking 22 aminoacids of the original CB1 gene in the same cells, thereby excluding mitochondrial association (Hebert-Chatelain et al., 2016a; Soria-Gomez et al., 2021c) (**Fig 3b**). In OB-GFAP-CB1-RS and OB-GFAP-DN22-RS, CB1 and DN22-CB1 constructs were respectively expressed to similar levels, mostly in the granule cell layer of the olfactory bulb (**Fig 3c**) and with astrocytic specificity of expression of around 82% for both constructs (calculated as the percentage of infected cells co-localizing with the astrocytic marker GFAP; **Fig 3d,e**).

First, we tested the behavior of these mutants in the NOR task under naïve or acute stress conditions (**Extended Data 3a,b**). OB-GFAP-CB1-KO, OB-GFAP-CB1-RS and OB-GFAP-DN22-RS mice displayed similar NOR performance as Ctrl animals (**Extended Data 3a,b**). They also showed the expected impairment in NOR following foot-shock (**Extended Data 3a,b**). To assess whether these genetic manipulations in the OB could alter general olfaction, we tested the mutant mice in a buried food test (Machado et al., 2018). We observed no differences between controls and OB-GFAP-CB1-KO mutant mice in this test, indicating that general olfactory perception was intact (**Extended Data 3c**). Moreover, no mutant group displayed any alteration in social interactions with an unfamiliar naive mouse (**Extended Data 3d**) or with a neutral DEM (**Extended Data 3e,f**). Thus, these genetic manipulations did not alter NOR performance, its impairment by direct stress, olfactory perception or social interactions in mice. This indicates that the approach is suitable to study the specific functions of astrocytic mtCB1 receptors in the OB during and after the social transmission of stress.

The deletion of CB1 receptors in OB astrocytes (OB-GFAP-CB1-KO) in observer mice interacting with stressed DEMs led to a decrease of anogenital investigation (**Fig 3f-h**), which was fully restored by the re-expression of wild-type CB1 in OB astrocytes (OB-GFAP-CB1-RS; **Fig 3f-h**). Notably, however, the re-expression of the mutant protein DN22-CB1 (OB-GFAP-DN22-RS) was not sufficient to rescue this phenotype (**Fig 3f-h**). During social transmission of stress, no mutant group displayed any other alteration in social (body exploration or allogrooming, **Extended Data 3g,h**) or non-social behaviors

(**Extended Data 3h**). Altogether, these data indicate that the anogenital exploration of a stressed partner, which is crucial for social transmission of stress, requires the presence of CB1 receptors associated with mitochondrial membranes in OB astrocytes.

Next, we asked whether this specific subpopulation of CB1 receptors also participates in the impairment of NOR retrieval following socially-transmitted stress. OB-GFAP-CB1-KO mice displayed normal NOR retrieval after interacting with a stressed demonstrator (**Fig 3i, Extended Data 3i**) or after either a short or long exposure to a swab impregnated with anogenital secretions from a stressed partner (**Extended Data 3j,k**). The effect of socially-transmitted stress was fully restored in OB-GFAP-CB1-RS (**Fig 3i, Extended Data 3i**). Strikingly, exclusion of CB1 receptors from astrocytic mitochondria in OB-GFAP-DN22-RS mice protected from the consequences of transmitted stress to a similar extent as the complete deletion of astrocytic CB1 in OB-GFAP-CB1-KO mice (**Fig 3i, Extended Data 3i**). The anatomical data shown in **Fig 3e** indicate that Cre-dependent recombination using AAV-GFAP-Cre mice in the OB involves about 20% of non-astrocyte cells, which are presumably neurons. Therefore, it is possible that the phenotypes of the mutant mice are linked to genetic alterations in these cells. To test this possibility, we injected a neuronal specific AAV (AAV-Syn-Cre) into the OB of CB1-flox mice (**Extended Data 4a**) and evaluated their social behavior and subsequent NOR performance. This manipulation did not alter anogenital investigation or body exploration (**Extended Data 4b-d**), nor did it affect impairment of NOR retrieval following stress transmission (**Extended Data 4f-h**). Collectively, these results indicate that mtCB1 receptors in OB astrocytes are necessary for specific social olfactory behaviors required for the transmission of stress and its impact on NOR.

Mitochondrial calcium handling in olfactory bulb astrocytes is required for social processing of stress chemosignals

Astrocytic mtCB1 receptors modulate calcium dynamics *via* regulation of ER-mitochondrial contact sites (Serrat et al., 2021). More specifically, they are involved in the activation of MICU1, a key regulator of the mitochondrial calcium uniporter (MCU) channel, eventually determining the impact of astrocytes on

synaptic functions through the modulation of their calcium handling capabilities (Serrat et al., 2021). Thus, we hypothesized that mitochondrial calcium handling in OB astrocytes might be mechanistically involved in social transmission of stress and its impact on NOR performance.

First, we tested whether the detection of a stress chemosignal was associated with a change in mitochondrial calcium in OB astrocytes. We expressed a mitochondrial genetic calcium indicator (mitoGcAMP6s; Serrat et al., 2021) in the astrocytes of the OB of naïve mice (**Fig 4a**). Using fiber photometry, we then recorded mitochondrial calcium signals of mice responding to a successive and counterbalanced exposure to saline and an anogenital stress secretions derived from a familiar individual (stress odor; **Fig 4b**). Olfactory bulb astrocytic mitochondria were more responsive to stress odor than to a saline one (**Fig 4c-e**), suggesting that mitochondrial calcium transients are involved in the processing of chemosignals. As mitochondrial CB1 receptors are required for the behavioral consequences of stress odor transmission, we used a combination of AAVs to test their role on stress-odor induced mitochondrial calcium changes in OB astrocytes (**Fig 4f**). We generated Ctrl and OB-GFAP-CB1-KO mice (**Fig 3b**) expressing mitoGcAMP6s in astrocytes. This allowed us evaluating mitochondrial calcium dynamics upon presentation of saline or stress odor in the presence or absence of astrocytic CB1 receptors (**Fig. 4g**). The response of OB-GFAP-CB1-KO mice to the saline swab was not different from Ctrl mice (**Fig 4h**). However, the mitochondrial calcium increase induced by the stress odor in OB astrocytes was blunted in the mutants (**Fig 4i**), resulting in a significant differential effect in the two genotypes (**Fig 4j**). These results show that astrocytic CB1 receptors are required for mitochondrial calcium responses determining the discrimination of relevant odors in the OB.

To establish the causal relationship between changes in mitochondrial calcium dynamics, social transmission of stress and its cognitive consequences, we used a viral approach to express a non-phosphorylatable dominant negative form of MICU1 (MICU^{S124A}; Ref. (Serrat et al., 2021), **Fig 5a**) in the astrocytes of the OB of wild-type mice, and tested them in the stress transmission-NOR protocol (**Fig 5b-d**). As compared to control littermates, mice injected with AAV-GFAP-MICU^{S124A} engaged in less anogenital contact with

their stressed partners (AAV-GFAP-MICU^{WT}) (**Fig 4e-h**), without any changes in body exploration (**Fig 4f-j**), allogrooming or other nonsocial behaviors (**Extended Data 5a**). Notably, mice carrying the dominant negative form of MICU1 in OB astrocytes lacked the transmitted stress-induced impairment of NOR retrieval (**Fig 4k and Extended Data 5b,c**). These mutant mice (i) displayed normal social behaviors during interactions with neutral DEM partners (**Extended Data 5d,f**); (ii) were not impaired in NOR performance after interaction with a neutral DEM mouse (**Extended Data 5h,i,j**); and (iii) were able to normally retrieve buried food pellets (**Extended Data 5k**). Thus, mitochondrial calcium handling by OB astrocytes is not involved in basal social interactions, cognitive performance and olfactory abilities, but it plays a specific causal role in social transmission of stress and its cognitive consequences.

DISCUSSION

This study presents unexpected results demonstrating functional and mechanistic links between social transmission of stress, olfactory processing and cognitive alterations. These links require the activation of CB1 receptors located in astrocytic mitochondria of the OB, and ultimately rely on the control of calcium signaling by these organelles. Briefly, we found that detection of stress-induced chemosignals by naïve observers impairs non-emotional and odor-independent object recognition to a similar extent as direct stress experience. Mitochondrial CB1 receptors in OB astrocytes are required for changes in mitochondrial handling of calcium leading to the impairment of cognitive performance induced by social transmission of stress. Thus, smelling certain odors has the ability to override unrelated cognitive processes.

Our results show that deletion of mtCB1 receptors in astrocytes of the OB impairs anogenital investigation of a shocked DEM and the subsequent NOR deficit. How can OB astrocytes have such a selective impact on the behavioral consequences of specific olfactory experiences? The first possibility is that deleting CB1 receptors in OB astrocytes might impair general olfactory processing. The mutation, however, did not affect buried food detection and none of the OB mutants used in this study showed any impairment in body investigation. Thus, these data suggest that mutant mice are able to detect odors, both social and non-social. There is still the possibility that deletion of astrocytic mtCB1 receptors in the OB induces specific anosmia for the anogenital chemosignal(s) necessary for social transmission of stress. Specific anosmia is the inability to perceive a single odor while general olfactory processing is unchanged (Niimura, 2012). This phenomenon mostly depends on the dysfunction of the glomeruli in charge of processing specific odors (Niimura, 2012). There are, indeed, glomeruli that have been selectively associated with the processing of stress chemosignals (Brechtbühl et al., 2008). It seems, however, unlikely that our viral manipulations widely affecting CB1 receptor expression or MICU1 functions in astrocytes of the OB could specifically alter the activity of a limited number of glomeruli. Another possibility related to olfaction is that our manipulations might increase odor detection thresholds (hyposmia), implying that if our mutants spent more time with the stressed partners they would

eventually show NOR impairment. However, no correlation was found between the time spent in anogenital exploration and NOR performance (data not shown). More importantly, our data indicate that, whereas a few seconds of odor exposure are sufficient to induce cognitive impairment in wild-type mice, even much longer forced contacts (> 20 sec) with impregnated cotton swabs do not display any effect in mutant mice. Therefore, although this possibility cannot be fully excluded at the moment, the data argue against hyposmia as a cause for the observed deficits. Thus, regulation of odor detection/perception seems unlikely to mediate the impact of OB astrocytic mtCB1 receptor signaling and calcium handling on social transmission of stress and its cognitive consequences. Interestingly, recent studies showed that the detection of stress chemosignals in humans is determined by mechanisms requiring only a minimal threshold exposure (de Groot et al., 2021b). This concept is in agreement with our observation that few seconds of exposure to a swab impregnated with anogenital secretions of a stressed partner are fully sufficient to impair cognitive performance. Therefore, animal and human data appear to point to the idea that social transmission of stress is not a quantitative event, but a qualitative "all-or-none mechanism for tagging fear above a minimal threshold" (de Groot et al., 2021b).

Astrocytes have been implicated in the processing of information in the OB *via* the modulation of mitral and tufted cell activity (Roux et al., 2015; Ung et al., 2021). This suggests that our manipulations of mtCB1 receptors and mitochondrial calcium handling in astrocytes could alter the integration of olfactory information in the OB, possibly because of dysregulation of astrocyte-dependent OB circuitry. In this sense, mtCB1 receptors and mitochondrial calcium signaling in OB astrocytes would not merely participate in the "detection" of the stress odor, but it would represent a step for its "interpretation". Thus, our data are compatible with a scenario in which astrocytic control of specific olfactory signals attributes salience to that odor. In other words, we propose that mitochondrial calcium modulation in OB astrocytes does not impact the perception of stress signals, but it might assign specific significance to them, thereby favoring the spreading of the associated information to other parts of the brain. Indeed, despite the fact that a short exposure to the odor is sufficient to trigger the cognitive impairment, mutant mice also display a decreased

exploration of the stressed partner, possibly indicating an inability to process the attractivity of the odors emitted. In summary, the present results suggest that the "salience assignment" putatively occurring in the OB and requiring control of astroglial mitochondrial calcium likely leads to at least two distinct effects: (i) stress-like impairment of cognition and (ii) positive reinforcement, motivating the observer to explore the partner and gather more information. Interestingly, recent work suggested that the insular cortex and its connection with the nucleus accumbens might be involved in the motivation to explore stressed congeners (Rogers-Carter et al., 2019), but no evidence exists whether these circuits are also involved in cognitive effects of odors. By identifying one of the earliest mechanisms potentially able to assign salience to specific olfactory stimuli, the present study paves the way to investigations exploring the differences and overlappings between the circuits linking olfaction to cognitive, motivational and other behavioral processes.

Mitochondrial CB1 receptors in astrocytes have been associated to two main functions: control of glucose bioenergetics (Jimenez-Blasco et al., 2020) and regulation of cellular Ca^{2+} handling *via* modulation of calcium transport through mitochondria/ER contact sites (MERCs) (Serrat et al., 2021). Whereas very little is known concerning astrocytic glucose metabolism in the OB (Martin et al., 2012b), solid evidence indicates that astrocytic functions in this brain region largely rely on calcium release from internal stores (Doengi et al., 2008; Petzold et al., 2008; Roux et al., 2015). Calcium transients in astrocytes modulate olfactory-driven chemotaxis in *Drosophila* (Ma et al., 2016) and oscillatory activity of mitral cells in mice *via* adenosine receptors (Roux et al., 2015) and GABA gliotransmission (Sardar et al., 2023). The exact role of MERC-dependent calcium signaling in OB astrocytes is unknown. However, mitochondria in astrocytes are often located close to glutamate transporters, regulating vesicular glutamate release (Reyes and Parpura, 2008) and brain bioenergetics (Robinson and Jackson, 2016). It is therefore possible that mtCB1 activation in astrocytes regulates Ca^{2+} -dependent gliotransmission in the OB, thereby modulating the synaptic transmission and plasticity required to process the specific olfactory information derived from social stress odors and eventually to impact cognition.

This study shows that the processing of stress-related chemosignals in the OB during social interaction with a stressed individual leads to an impairment of NOR, a hippocampal-dependent, non-aversive and non-olfactory cognitive test. This implies that relevant olfactory information in the OB can trigger alterations in distant circuits managing non-olfactory and non-emotional information. The nature of these circuits is not currently known, but anatomical and functional studies allow for speculation. The OB is indirectly connected with the hippocampus (HPC) through the entorhinal cortex (EC) (Gourévitch et al., 2010; Martin et al., 2007), and OB oscillations play a role in the synchronization of EC-HPC activity during cognitive functions (Gourévitch et al., 2010; Martin et al., 2007; Salimi et al., 2021, 2022). Interestingly, these types of OB-EC-HPC synchronous oscillations have been observed in association with specific social odors (Pena et al., 2017), and social transmission of stress has been recently shown to impact synaptic properties of CA1 hippocampal pyramidal neurons (Lee et al., 2021). Thus, functional connectivity and electrophysiological data support the possibility of an olfactory—hippocampal circuit mediating impairment of object recognition following the social transmission of stress. Moreover, corticotropin-releasing hormone (CRH) modulates anogenital investigation of shocked partners (Rogers-Carter et al., 2018b; Sterley et al., 2018), and other stress-associated behaviors (Füzesi et al., 2016a). CRH-positive neurons are spread throughout the brain, but they are particularly concentrated in the paraventricular nucleus of the hypothalamus (PVN) (Cui et al., 2013). The OB projects to the HYP either indirectly through areas like the amygdala (Lin et al., 2007b; Thompson et al., 2012) or even directly from specific glomeruli (Bader et al., 2012). In turn, the PVN sends and receives dorsal hippocampal projections (Cole et al., 2022). The connections between OB, HPC and PVN could explain how CB1-dependent control of astrocytic functions in the OB and the regulation of calcium signaling in these cells might participate in the integration of relevant olfactory cues with CRH activity and hippocampal cognitive processes.

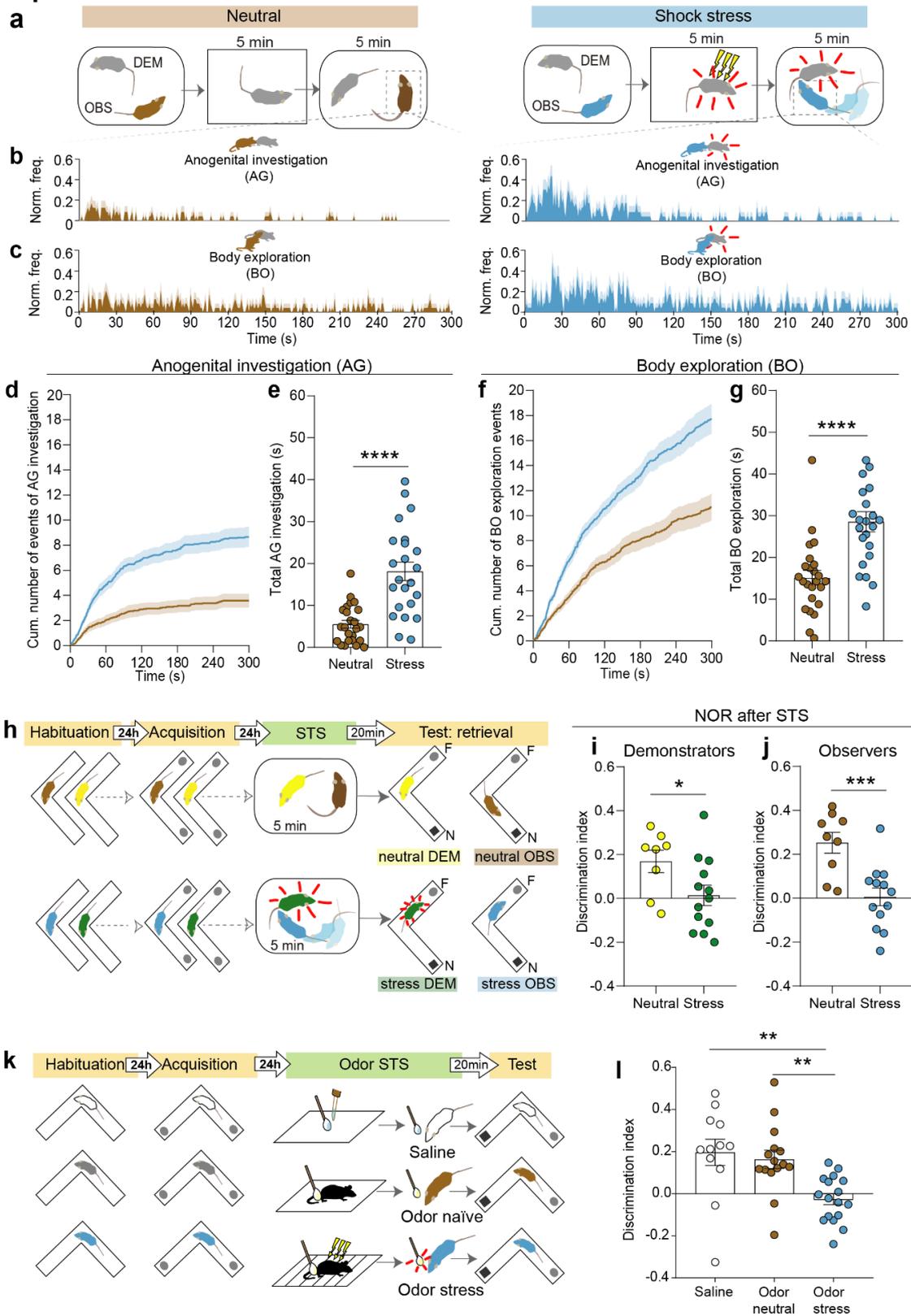
Interestingly, olfactory pathologies suggest that altered interpretation of odor signals could be even more debilitating than complete loss of smell, particularly in the frame of social interactions. Indeed, humans living with congenital social anosmia do often compensate their complete loss of perception

through other senses, such as vision and audition (Lemogne et al., 2015). However, those living with a distortion of social chemosignaling processing (i.e. altered interpretation of detected social odors, called social dysosmia) likely suffer from deeper social deficits (Endevelt-Shapira et al., 2018). Thus, OB astrocytic mtCB1 receptors and mitochondrial calcium signaling might contribute to an early specific step in the process leading from olfactory "percept" to mental "concept", resulting in the transformation of specific cues into vital pieces of information for the organism.

The transmission of stress information in mice appears to be mediated by alarm cues similar to the odor of predators (Brechtbühl et al., 2008, 2013) and, in humans, it seems that alarm- or fear-related chemosignals that are not detected within our conscious threshold are processed subconsciously, still requiring intact olfactory functions (Albrecht et al., 2011; Endevelt-Shapira et al., 2018; de Groot et al., 2012). Our results reveal a mechanistic link between social emotional odor communication and odor-unrelated cognitive consequences, thereby providing novel perspectives in the understanding of the impact of social odor processing on behavior and cognition. Since major mental conditions like autism spectrum disorders present impairments in social olfaction (Endevelt-Shapira et al., 2018) and in cognitive processing (Gaigg, 2012; Okumura et al., 2020), the present data might open novel conceptual frameworks to better tackle such conditions.

MAIN FIGURES

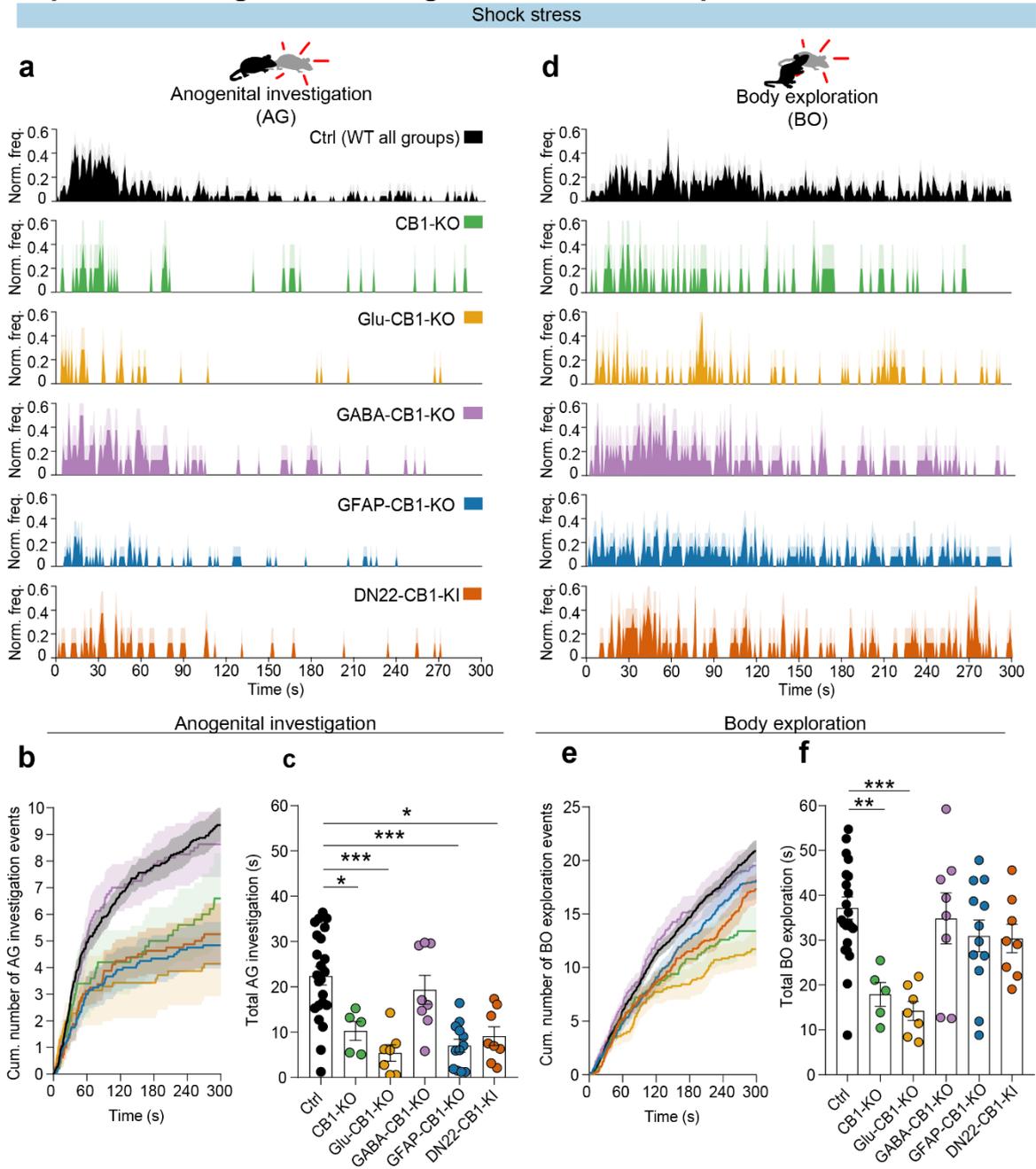
Fig 1. Social olfactory detection of stress impairs object recognition performance.



- a. Schematic representation of the protocol of social transmission of stress (STS, see Methods for details). Observer mice (OBS) socialize for 5 min with demonstrator familiar mice (DEM) after a neutral vs a stressful stimulus.
- b. Normalized frequency of anogenital investigation (AG) of observer mice over 5 min social interaction with neutral DEM (left, brown) or stress DEM (right, blue). $n_{(\text{neutral})} = 24$, $n_{(\text{shock stress})} = 23$.
- c. Normalized frequency of body exploration (BO) of observer mice over 5 min social interaction with neutral DEM (left, brown) or shocked stress DEM (right, blue). $n_{(\text{neutral})} = 24$, $n_{(\text{shock stress})} = 23$.
- d. Cumulative number of AG events of observer mice during 5 min social interaction with stress DEMs. $n_{(\text{neutral})} = 24$, $n_{(\text{shock stress})} = 23$.
- e. Total time of anogenital investigation of neutral (brown) or stress OBS (blue) mice during 5 min social interaction. Two tailed unpaired Student's t-test, $p < 0.0001$. $n_{(\text{neutral})} = 24$, $n_{(\text{shock stress})} = 23$.
- f. Cumulative number of BO events of observer mice during 5 min social interaction with stress DEMs. $n_{(\text{neutral})} = 24$, $n_{(\text{shock stress})} = 23$.
- g. Total time of body exploration of neutral (brown) or stress OBS (blue) mice during 5 min social interaction. Two tailed unpaired Student's t-test, $p < 0.0001$. $n_{(\text{neutral})} = 24$, $n_{(\text{shock stress})} = 23$.
- h. Schematic representation of novel object recognition protocol (NOR) coupled with STS (see Methods for details).
- i. Discrimination index in the NOR test of demonstrator mice after receiving a stress (green) or neutral (yellow) stimulus. Two tailed unpaired Student's t-test, $p = 0.0432$. $n_{(\text{neutral DEM})} = 8$, $n_{(\text{stress DEM})} = 13$.
- j. Discrimination index in the NOR test of observer mice after socializing with a stress DEM (blue) or neutral DEM (brown). Two tailed unpaired Student's t-test, $p = 0.0008$. $n_{(\text{neutral OBS})} = 9$, $n_{(\text{stress OBS})} = 13$.
- k. Schematic representation social odor transmission of stress and novel object recognition (see Methods for details).
- l. Discrimination index in the NOR test of naive mice after being exposed to a saline wet swab (saline, white), a swab impregnated with anogenital odor of a naive partner (odor naïve, grey) or the anogenital odor of a stressed partner (odor stress, blue). Ordinary one-way ANOVA, $p = 0.0002$. Bonferroni multiple comparisons, $p_{(\text{saline vs odor stress})} = 0.0025$, $p_{(\text{odor naive vs odor stress})} = 0.0066$, $n_{(\text{neutral DEM})} = 9$, $n_{(\text{stress DEM})} = 13$.

Data are expressed in mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$. For detailed statistical information, see Extended Data Table 1.

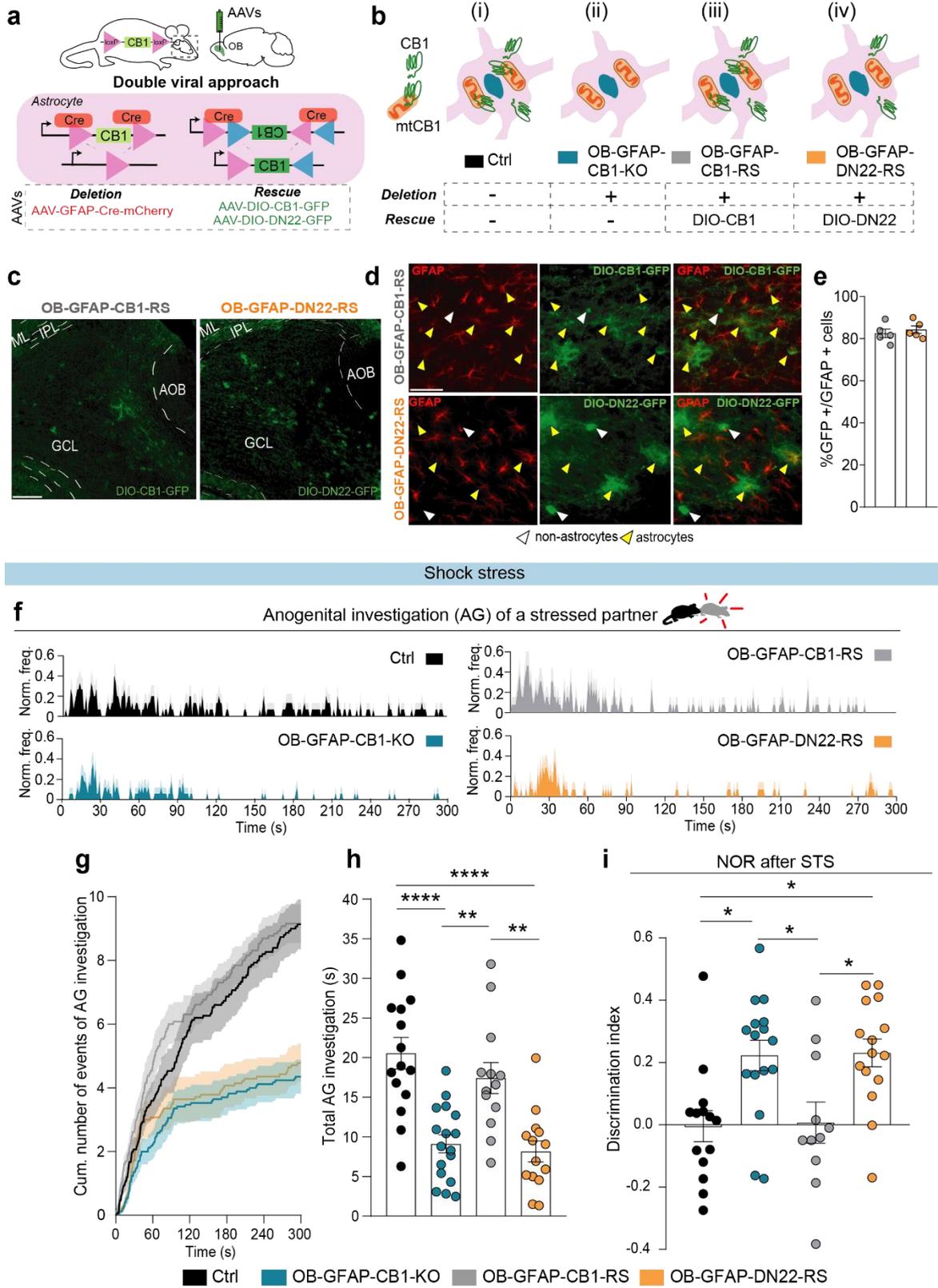
Fig 2. Astrocytic and mitochondrial CB1 receptors are specifically required for anogenital investigation of a stressed partner.



- a. Normalized frequency (norm. freq.) of anogenital investigation (AG) of global or conditional CB1 mutant observer mice over 5 min social interaction with a stressed partner. Ctrl mice include WT of all groups (not significantly different). Mutant mice are CB1-KO, Glu-CB1-KO, GABA-CB1-KO, GFAP-CB1- and DN22-CB1-KI
- b. Cumulative number of AG events of global or conditional CB1 mutant observer mice during 5 min social interaction with a stressed partner.
- c. Total time of anogenital investigation of a stressed partner by global or conditional CB1 mutant observer mice during 5 min social interaction. Ordinary one-way ANOVA, $p < 0.0001$. Bonferroni multiple comparisons, $p_{(WT \text{ vs } CB1-KO)} = 0.0111$, $p_{(WT \text{ vs } Glu-CB1-KO)} < 0.0001$, $p_{(WT \text{ vs } GFAP-CB1-KO)} < 0.0001$, $p_{(WT \text{ vs } DN22-CB1-KI)} = 0.0005$.
- d. Normalized frequency of body exploration (BO) of global or conditional CB1 mutant observer mice over 5 min social interaction with a stressed partner
- e. Cumulative number of BO events of global or conditional CB1 mutant observer mice during 5 min social interaction with a stressed partner.
- f. Total time of body exploration of a stressed partner by global or conditional CB1 mutant observer mice during 5 min social interaction. Ordinary one-way ANOVA, $p = 0.0002$. Bonferroni multiple comparisons, $p_{(WT \text{ vs } CB1-KO)} = 0.005$, $p_{(WT \text{ vs } Glu-CB1-KO)} < 0.0001$.

$n_{(WT)} = 23$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 7$, $n_{(GABA-CB1-KO)} = 8$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$. Data are expressed in mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. For detailed statistical information, see Extended Data Table 1.

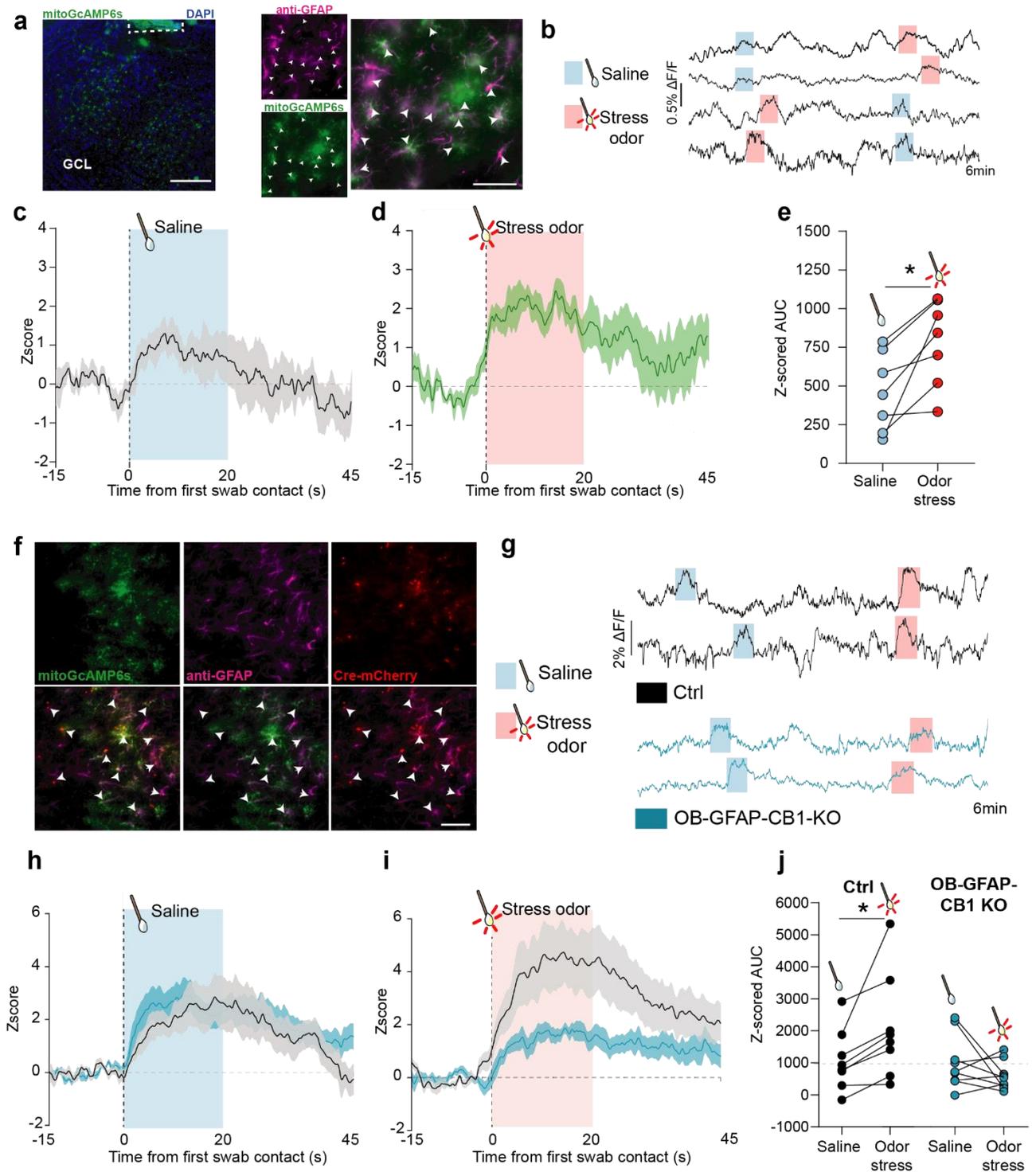
Fig 3. Mitochondrial CB1 receptors in astrocytes of the olfactory bulb are required for social processing of stress chemosignals.



- a. Schematic representation of the viral strategy to genetically manipulate specific subcellular CB1 receptor populations in astrocytes of the olfactory bulb (OB).
- b. With this approach, we generated four olfactory bulb mutant groups: Ctrl, OB-GFAP-CB1-KO, OB-GFAP-CB1-RS and OB-GFAP-DN22-RS (see Methods and main text for details).
- c. Representative low magnification micrographs showing the viral expression of endogenous fluorescence of the AAV-DIO-CB1-GFP and AAV-DIO-DN22-GFP (in green). Scale bar, 250 μ m.
- d. Representative high magnification micrographs of the granular cell layer of the main olfactory bulb showing immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP, red, left), endogenous fluorescence of the AAV-DIO-CB1-GFP (top) and AAV-DIO-DN22-GFP (bottom, green, center) and the merging of both stainings (right). Yellow arrowheads, cells co-expressing AAV and GFAP (presumably astrocytes); white arrowheads, cells expressing only the AAV (presumably not astrocytes).
- e. Quantifications of percentages of GFAP-positive cells expressing either AAVs (n = 5 mice for each AAV. Scale bar, 50 μ m).
- f. Normalized frequency of anogenital investigation (AG) of OB-GFAP-CB1 mutant observer mice over 5 min social interaction with a stressed partner. $n_{(Ctrl)} = 15$, $n_{(OB-GFAP-CB1-KO)} = 17$, $n_{(OB-GFAP-CB1-RS)} = 13$, $n_{(OB-GFAP-DN22-RS)} = 14$.
- g. Cumulative number of AG events of OB-GFAP-CB1 mutant observer mice during 5 min social interaction with a stressed partner. $n_{(Ctrl)} = 15$, $n_{(OB-GFAP-CB1-KO)} = 17$, $n_{(OB-GFAP-CB1-RS)} = 13$, $n_{(OB-GFAP-DN22-RS)} = 14$.
- h. Total time spent in anogenital investigation of a stressed partner by OB-GFAP-CB1 mutant observer mice during 5 min social interaction. Ordinary one-way ANOVA, $p < 0.0001$. Bonferroni multiple comparisons, $p_{(Ctrl \text{ vs } OB-GFAP-CB1-KO)} < 0.0001$, $p_{(Ctrl \text{ vs } OB-GFAP-DN22-RS)} < 0.0001$, $p_{(OB-GFAP-CB1-RS \text{ vs } OB-GFAP-CB1-KO)} = 0.0032$, $p_{(OB-GFAP-CB1-RS \text{ vs } OB-GFAP-DN22-RS)} = 0.0015$. $n_{(Ctrl)} = 15$, $n_{(OB-GFAP-CB1-KO)} = 17$, $n_{(OB-GFAP-CB1-RS)} = 13$, $n_{(OB-GFAP-DN22-RS)} = 14$.
- i. Discrimination index in the NOR test of OB-GFAP-CB1 mutant observer mice after socializing with a stress DEM. Ordinary one-way ANOVA, $p = 0.0009$. Bonferroni multiple comparisons, $p_{(Ctrl \text{ vs } OB-GFAP-CB1-KO)} = 0.0129$, $p_{(Ctrl \text{ vs } OB-GFAP-DN22-RS)} = 0.0109$, $p_{(OB-GFAP-CB1-RS \text{ vs } OB-GFAP-CB1-KO)} = 0.361$, $p_{(OB-GFAP-CB1-RS \text{ vs } OB-GFAP-DN22-RS)} = 0.0303$. $n_{(Ctrl)} = 14$, $n_{(OB-GFAP-CB1-KO)} = 16$, $n_{(OB-GFAP-CB1-RS)} = 11$, $n_{(OB-GFAP-DN22-RS)} = 15$.

Data are expressed in mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. For detailed statistical information, see Extended Data Table 1.

Fig 4. Mitochondrial calcium responses in OB astrocytes to stress chemosignals require astrocytic CB1 receptors.



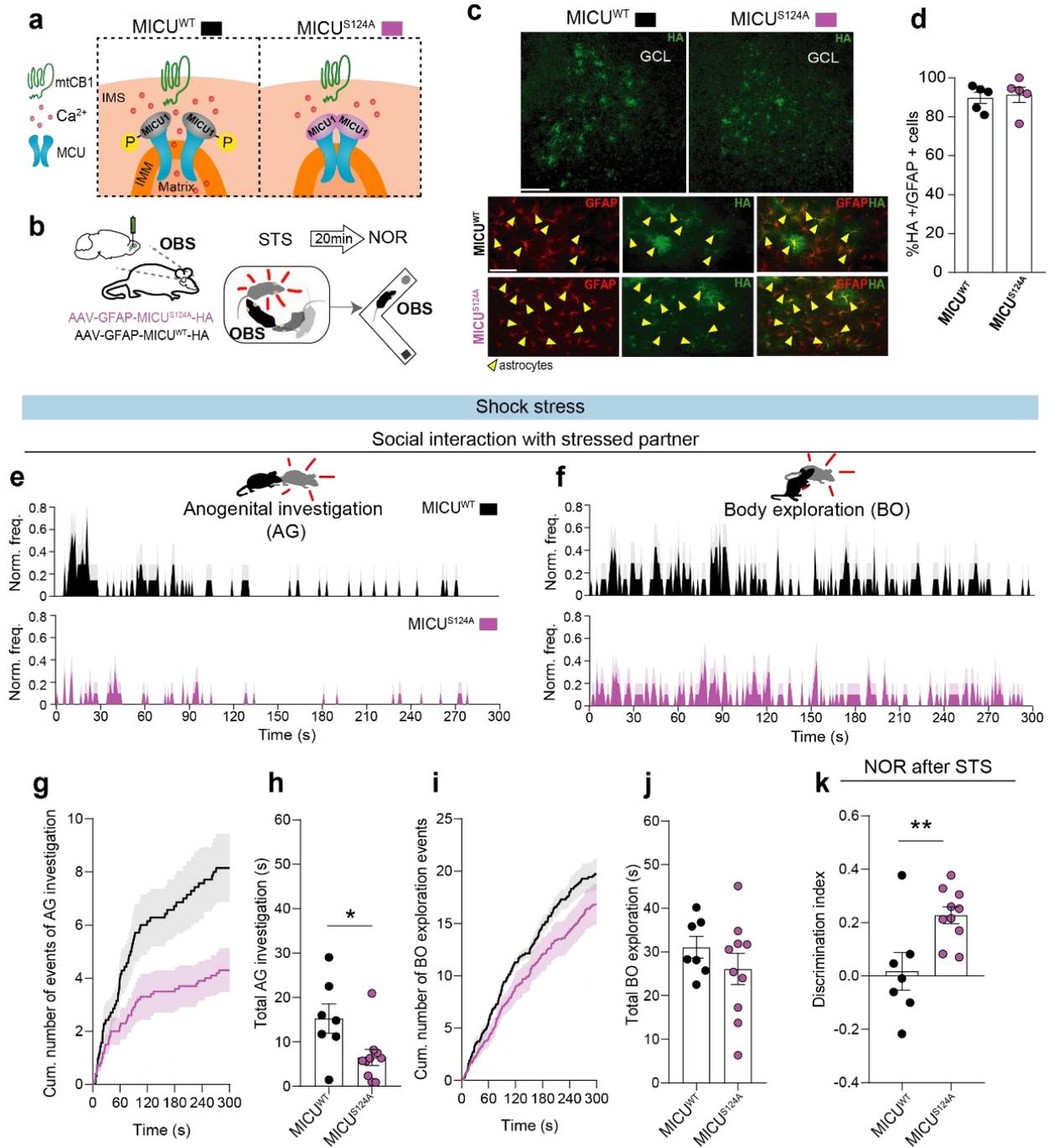
- a. Representative low magnification micrographs of the main olfactory bulb showing fiber placement (blue DAPI, green, mitoGcAMP6s), and high magnification micrographs showing mitoGcAMP6s endogenous fluorescence (green) and immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP, purple). Scale bar, 500 μ m (low, left), 50 μ m (high, right).
- b. Representative mitochondrial calcium signal traces of animals expressing mitoGcAMP6s in the astrocytes of the olfactory bulb in response to a saline-wet cotton swab (blue) and a cotton swab impregnated with secretions from a stressed partner (red). Scale is 0.5% $\Delta F/F$. n = 7.
- c. Z-scored $\Delta F/F$ mitochondrial calcium responses in astrocytes of the olfactory bulb of mice aligned to the onset of the first contact to a saline cotton swab exposure. Blue rectangle represents the time of exposure. n = 7. Scale is 0.5 % $\Delta F/F$.
- d. Z-scored $\Delta F/F$ mitochondrial calcium responses in astrocytes of the olfactory bulb of mice aligned to the onset of the first contact with a cotton swab impregnated with secretions from a stressed partner (stress odor). Red rectangle represents the time of exposure.
- e. Z-scored AUC from saline and stress odor exposures from the time corresponding to the time window covered by blue and orange rectangles of each mouse. Two tailed paired Student's t-test, p = 0.0137. n = 7.
- f. Representative low magnification micrographs of the main olfactory bulb showing fiber placement, and high magnification micrographs showing mitoGcAMP6s endogenous fluorescence (green), Cre endogenous fluorescence resulting from the AAV-GFAP-Cre-mCherry injection (red) and immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP, purple). Scale bar, 50 μ m.
- g. Representative mitochondrial calcium signal traces of animals expressing mitoGcAMP6s in the astrocytes of the olfactory bulb in response to a saline-wet cotton swab (blue) and a cotton swab impregnated with secretions from a stressed partner (orange). Black traces correspond to CB1 flox mice injected with a control virus (Ctrl) and blue traces correspond to CB1 flox animals injected with an AAV-GFAP-Cre virus in the olfactory bulb (OB-GFAP-CB1-KO). Scale is 2% $\Delta F/F$.
- h. Z-scored $\Delta F/F$ mitochondrial calcium responses in astrocytes of the olfactory bulb of mice aligned to the onset of the first contact to a saline cotton swab exposure. Blue rectangle represents the time of exposure. Black signal, Ctrl. Blue signal, OB-GFAP-CB1-KO. n_(Ctrl) = 8, n_(OB-GFAP-CB1) = 9.
- i. Z-scored $\Delta F/F$ mitochondrial calcium responses in astrocytes of the olfactory bulb of mice aligned to the onset of the first contact with a cotton swab impregnated with secretions from a stressed partner (stress odor). Orange rectangle represents the time

of exposure. Black signal, Ctrl. Blue signal, OB-GFAP-CB1-KO. $n_{(\text{Ctrl})} = 8$, $n_{(\text{OB-GFAP-CB1})} = 9$.

- j. Z-scored AUC from saline and stress odor exposures from the time corresponding to the time window covered by blue and orange rectangles both Ctrl and OB-GFAP-CB1-KO mice. Two tailed paired Student's t-test (Ctrl), $p = 0.0260$. $n_{(\text{Ctrl})} = 8$, $n_{(\text{OB-GFAP-CB1})} = 9$.

Data are mean \pm SEM. For detailed statistical information, see Extended Data Table 1

Fig 5. Mitochondrial calcium modulation via MICU1 in OB astrocytes is required for social transmission of stress and its cognitive consequences



- a. The astrocytic overexpression of an adenovirus carrying a mutated non-phosphorylatable form of MICU1 (MICU^{S124A}, left diagram) is expected to reduce mitochondrial calcium uniporter (MCU)-dependent calcium uptake by mitochondria. As a control, a virus overexpressing the wild-type form of MICU1 was used (MICU^{WT}, right diagram) as a control. mtCB1, mitochondrial CB1 receptor; Ca²⁺, calcium; MCU, mitochondrial calcium uniporter; IMS, intermembrane mitochondrial space; IMM, intramembrane mitochondrial space.
- b. Schematic representation of the viral strategy to manipulate mitochondrial calcium handling in astrocytes of the olfactory bulb of wild-type C57BL/6-N mice and behavioral protocol (see Methods for details).
- c. Top, low magnification representative micrographs of immunostaining of the HA tag of the injected viruses in the GCL of the main olfactory bulb. Bottom, high magnification representative micrographs of immunostaining of the granule cell layer (GCL) of the olfactory bulb showing GFAP (red) and the HA tag of the injected viruses (green). Scale bars are 150µm (top) and 50 µm (bottom).
- d. Percentage of HA-positive cells co-expressing GFAP (n=5 mice for each virus).
- e. Normalized frequency of anogenital investigation (AG) of MICU1 mutant observer mice over 5 min social interaction with a stressed partner.
- f. Normalized frequency of body exploration (BO) of MICU1 mutant observer mice over 5 min social interaction with a stressed partner.
- g. Cumulative number of AG events of MICU1 mutant observer mice during 5 min social interaction with a stressed partner.
- h. Total time spent in anogenital investigation of a stressed partner by MICU1 mutant observer mice during 5 min social interaction. Mann-Whitney test, p = 0.025.
- i. Cumulative number of BO events of MICU1 mutant observer mice during 5 min social interaction with a stressed partner.
- j. Total time spent in body exploration of a stressed partner by MICU1 mutant observer mice during 5 min social interaction.
- k. Discrimination index in the NOR test of MICU1 mutant observer mice after socializing with a stress DEM.

n(MICU^{WT}) = 7, n(MICU^{S124A}) = 10. Data are mean ± SEM. ** p < 0.01. For detailed statistical information, see Extended Data Table 1.

Extended Data 1: Statistical details of main figures

Figure	Condition	n (all groups)	Normality	Mean \pm SEM of group	Analysis (post hoc test) reported in figure	Factors analyzed	Test values	P values
Figure 1: Social olfactory detection of stress impairs object recognition performance								
1e	Anogenital investigation in neutral vs stress OBS	23-24	Yes	OBS neutral = 5.510 \pm 0.9294	two tailed unpaired Student's t-test	OBS Condition effect on social interaction	t = 5.358, df =45, CI = 7.887 to 17.39	<0.0001
				OBS stress = 18.15 \pm 2.206				
1g	Body exploration in OBS-neutral vs OBS-shock	23-24	Yes	OBS neutral = 15.10 \pm 1.710	two tailed unpaired Student's t-test	OBS Condition effect on social interaction	t = 4.504, df =45, CI = 7.446 to 19.49	<0.0001
				OBS stress = 28.57 \pm 2.419				
1i	Discrimination index in neutral DEM vs stress DEM	8-13	Yes	Neutral DEM = 0.1688 \pm 0.05103	two tailed unpaired Student's t-test	DEM Condition effect on NOR	t=2.167, df=19, CI = -0.3045 to -0.00525	0.0432
				stress DEM = -0.01388 \pm 0.046343				
1j	Discrimination index in OBS-neutral vs OBS stress after STS	8-13	Yes	OBS neutral = 0.2520 \pm 0.04743	two tailed unpaired Student's t-test	OBS Condition effect on NOR	t=3.951, df=20, CI = -0.3758 to -0.06225	0.0008
				OBS stress = 0.00601 \pm 0.04005				
1l	Discrimination index in Saline vs Odor naïve vs Odor shock	9-12	Yes	Saline = 0.1962 \pm 0.06245	Ordinary one-way ANOVA (Bonferroni post hoc comparisons)	Cotton swab exposure condition effect	F = 8.267, DFn = 2, DFd = 41	0.0010
				Odor naïve = 0.1628 \pm 0.04314		Saline vs Odor stress	t = 3.267, DF = 41, CI = -0.3763 to -0.0687	0.0025
				Odor stress = -0.02631 \pm 0.02637		Odor naïve vs Odor stress	t = 3.267, DF = 41, CI = 0.0446 to 0.3336	0.0066
						Saline vs Odor naïve	t = 0.5279, DF = 41, CI = -0.1914 to 0.1246	>0.9999

Figure 2: Astrocytic and mitochondrial CB1 receptors are specifically required for anogenital investigation of a stressed partner.

2c	Anogenital investigation in CB1 mutant lines	5-23	Yes	Ctrl = 22.42 ± 2.031	Ordinary one-way ANOVA (Bonferroni post hoc comparisons)	Genotype effect	F = 10.75, DFn = 5, DFd = 57	<0.0001
				CB1 KO = 10.25 ± 2.087		Ctrl vs CB1 KO	t = 3.193, DF = 57, CI = 2.014 to 22.33	0.0115
				Glu-CB1 KO = 5.401 ± 1.815		Ctrl vs Glu-CB1 KO	t = 5.103, DF = 57, CI = 8.131 to 25.90	0.0012
				GABA-CB1 KO = 19.34 ± 3.207		Ctrl vs GABA-CB1 KO	t = 0.9715, DF = 57, CI = -5.369 to 11.53	> 0.9999
				GFAP-CB1 KO = 6.996 ± 1.443		Ctrl vs GFAP-CB1 KO	t = 5.607, DF = 57, CI = 8.091 to 22.75	0.0021
				DN22-CB1 KI = 9.125 ± 2.056		Ctrl vs DN22-CB1-KI	t = 4.192, DF = 57, CI = 4.843 to 21.74	0.0005
2f	Body exploration in CB1 mutant lines	5-23	Yes	Ctrl = 37.16 ± 2.490	Ordinary one-way ANOVA (Bonferroni post hoc comparisons)	Genotype effect	F = 5.801, DFn = 5, DFd = 57	0.0002
				CB1 KO = 17.93 ± 2.649		Ctrl vs CB1 KO	t = 3.399, DF = 57, CI = 4.153 to 34.31	0.0062
				Glu-CB1 KO = 14.22 ± 2.120		Ctrl vs Glu-CB1 KO	t = 4.634, DF = 57, CI = 9.744 to 36.12	0.0001
				GABA-CB1 KO = 34.84 ± 5.683		Ctrl vs GABA-CB1 KO	t = 0.4930, DF = 57, CI = -10.22 to 14.86	>0.9999
				GFAP-CB1 KO = 30.92 ± 3.541		Ctrl vs GFAP-CB1 KO	t = 1.529, DF = 57, CI = -4.639 to 17.12	0.6594
				DN22-CB1 KO = 30.36 ± 3.170		Ctrl vs DN22-CB1 KI	t = 1.445, DF = 57, CI = -5.743 to 19.34	0.7704

Figure 3: Mitochondrial CB1 receptors in astrocytes of the olfactory bulb are required for social browsing and subsequent impairment of novel object recognition.

3e	Viral specificity of AAV-CB1-GFP and AAV-DN22-GFP constructs	5	Yes	DIO-CB1-GFP = 82.49 ± 2.036	two tailed unpaired Student's t-test	% GFP+/GFAP+ positive cells in DIO-CB1-GFP expressing mice vs DIO-DN22-GFP	t=0.6362, DF=8, CI = -4.549 to 8.015	0.5424
				DIO-DN22-GFP = 84.22 ± 1.811				
3h	Anogenital investigation	13-17	Yes		Ordinary one-way ANOVA	Genotype effect	F = 14.62, DFn = 3, DFd = 55	<0.0001

	in OB-CB1 mutants			Ctrl = 20.55 ± 1.987	(Bonferroni post hoc comparisons)	Ctrl vs OB-GFAP-CB1-KO	t = 5.231, DF = 55, CI = 5.455 to 17.43	<0.0001
				OB-GFAP-CB1-KO = 9.106 ± 1.138		Ctrl vs OB-GFAP-DN22-RS	t = 5.403, DF = 55, CI = 6.117 to 18.68	<0.0001
				OB-GFAP-CB1-RS = 17.41 ± 1.958		OB-GFAP-CB1-KO vs OB-GFAP-CB1-RS	t = 3.651, DF = 55, CI = -14.53 to -2.079	0.0035
				OB-GFAP-DN22-RS = 8.150 ± 1.319		OB-GFAP-DN22-RS vs OB-GFAP-CB1-RS	t = 3.894, DF = 55, CI = 2.752 to 15.77	0.0016
						Ctrl vs OB-GFAP-CB1-RS	t = 1.341, DF = 55, CI = -3.267 to 9.540	>0.9999
						OB-GFAP-CB1-KO vs OB-GFAP-DN22-RS	t = 0.429, DF = 55, CI = -5.143 to 7.054	>0.9999
3i	Discrimination index of OB-CB1 mutants after STS	12-16	Yes	Ctrl = -0.0047 ± 0.04982	Ordinary one-way ANOVA (Bonferroni post hoc comparisons)	Genotype effect	F = 6.379, DFn = 3, DFd = 52	0.0009
				OB-GFAP-CB1-KO = 0.2227 ± 0.04906		Ctrl vs OB-GFAP-CB1-KO	t = 3.259, DF = 53, CI = -0.4188 to -0.03618	0.0129
				OB-GFAP-CB1-RS = 0.0067 ± 0.06638		Ctrl vs OB-GFAP-DN22-RS	t = 3.319, DF = 53, CI = -0.4296 to -0.04100	0.0109
				OB-GFAP-DN22-RS = 0.2305 ± 0.0444		OB-GFAP-CB1-KO vs OB-GFAP-CB1-RS	t = 2.993, DF = 53, CI = 0.01838 to 0.4177	0.0361
						OB-GFAP-DN22-RS vs OB-GFAP-CB1-RS	t = 3.057, DF = 53, CI = -0.4283 to -0.02333	0.0303
						OB-GFAP-CB1-WT vs OB-GFAP-CB1-RS	t = 0.1263, DF = 53, CI = -0.2151 to 0.1962	>0.9999
						OB-GFAP-CB1-KO vs OB-GFAP-DN22-RS	t = 0.1135, DF = 53, CI = -0.1957 to 0.1801	>0.9999

Figure 4. Mitochondrial calcium responses in OB astrocytes to stress chemosignals require astrocytic CB1 receptors.

4e	Effect of odor exposure in mitochondrial calcium	7	Yes	Saline AUC = 458.9 ± 95.71	Two tailed paired t-test	Saline AUC vs stress AUC	t = 0.0137, DF = 6, CI = 93.91 to 553.2	0.0137
				Stress AUC				

				=782.5 ± 105.2				
4j	Effect of genotype in odor-induced mitochondrial calcium	7-9	Yes		Two-way ANOVA	Interaction odor x genotype	F (2, 10) = 6,645	0.0146
				Saline Ctrl = 962.2± 367.9	Two tailed paired t-test	Saline AUC vs Stress AUC Ctrl	t=5,864, df=6 CI = 391.7 to 952.6	0.0011
				Stress Ctrl = 1634± 403.5				
				Saline KO= 997.1± 274.6	Two tailed paired t-test	Saline AUC vs Stress AUC OB-GFAP-CB1-KO	t=1.417, df=8, CI = -1135 to 270,8	0.1941
Stress KO = 565.1± 135.3								

Figure 5: Mitochondrial calcium modulation via MICU1 in OB astrocytes is required for social transmission of stress and its cognitive consequences

5d	Viral specificity of AAV-MICU-WT and AAV-MICU-S1214A constructs	5	Yes	AAV-GFAP-MICU ^{WT} = 89.69 ± 2.920	two tailed unpaired Student's t-test	% GFP+/GFAP+ positive cells in AAV-GFAP-MICU ^{WT} and AAV-GFAP-MICU ^{S124A} expressing mice	t=0.0135, df=12, CI = -9.698 to 12.96	0.9894
				AAV-GFAP-MICU ^{S124A} = 91.32 ± 3.951				
4h	Anogenital investigation in AAV-MICU ^{WT} or AAV-MICU ^{S124A} OB injected mice	6-10	No	AAV-GFAP-MICU ^{WT} = 15.26 ± 33.18	two tailed unpaired Mann-Whitney test	AAV-GFAP-MICU ^{WT} vs AAV-GFAP-MICU ^{S124A}	U = 12	0.0250
				AAV-GFAP-MICU ^{S124A} = 6.507 ± 1.802				
4j	Body exploration in AAV-MICU ^{WT} or AAV-MICU ^{S124A} OB injected mice	6-10	Yes	AAV-GFAP-MICU ^{WT} = 31.09 ± 2.472	two tailed unpaired Student's t-test	AAV-GFAP-MICU ^{WT} vs AAV-GFAP-MICU ^{S124A}	t=1.049, df=15, CI = -15.16 to 5.158	0.3106
				AAV-GFAP-MICU ^{S124A} = 26.08 ± 3.570				

4k	Discrimination index in AAV-MICU ^{WT} or AAV-MICU ^{S124A} OB injected mice	6-10	Yes	AAV-GFAP-MICU ^{WT} = 0.0169 ± 0.0707	two tailed unpaired Student's t-test	AAV-GFAP-MICU ^{WT} vs AAV-GFAP-MICU ^{S124A}	t=3.018, df=15, CI = 0.06173 to 0.3585	0.0086
				AAV-GFAP-MICU ^{S124A} = 0.2270 ± 0.0315				

CI, 95% confidence interval. Df, degrees of freedom. SEM, standard error of the mean.

MATERIAL AND METHODS

Animals

All animal protocols were in accordance with the Guidelines for the Animal Care and Use and the European Communities Council Directive of September 22th 2010 (2010/63/EU, 74) and approved by the French Ministry of Agriculture and Education.

C57BL/6-N (Janvier, France) and inbred constitutive and conditional CB1 mutant (center's facility) male mice were used for the different experiments of this project. CB1 mutant mice included: *CB1^{f/f}* mice (CB1-flox) carrying a floxed version of the CB1 gene (Marsicano et al., 2003); CB1-knockout mouse line (CB1-KO) carrying a constitutive global deletion of the CB1 gene (Marsicano et al., 2002); NEX-CB1-knockout mouse line (Glu-CB1-KO) carrying a conditional deletion of the CB1 gene in forebrain glutamatergic neurons under the control of a Nex-Cre recombinase (Monory et al., 2006); DLX-CB1 knockout mouse line (GABA-CB1-KO) carrying a conditional deletion of the CB1 gene under the control of a *Dlx5/6*-Cre recombinase (Monory et al., 2006); GFAP-Ert2-CB1-knockout mouse line (GFAP-CB1-KO) carrying an inducible conditional deletion of the CB1 gene in GFAP-expressing cells (mostly astrocytes) under the control of a GFAP-Cre recombinase (Han et al., 2012a) and a knock-in mouse line replacing the wild-type CB1 gene by a truncated form of the CB1 gene lacking the first 22 amino acids that reduces its mitochondrial-associated expression (DN22-CB1-RS) (Hebert-Chatelain et al., 2016; Soria-Gomez et al., 2021). The respective wild-type littermates of all lines were used as controls for the behavioral experiments.

Constitutive and conditional CB1 mutant mice were used in behavioral experiments to assess social transmission of stress-driven behaviors. In the case of the GFAP-CB1-KO mice, they were injected with 8 daily injections of tamoxifen (Sigma, #T5648, 1 mg, i.p.), dissolved in 90% sesame oil, 10% ethanol to a final concentration of 10 mg/ml to induce the CreERT2 dependent CB1 gene locus excision 4 weeks before the beginning of the behavioral experiments. CB1-flox mice were used for surgical procedures to specifically assess the role of CB1 in the olfactory bulb. C57BL/6-N mice were used as demonstrators (DEM) in all

behavioral experiments using surgically induced mutant mice, and for surgical procedures to assess the role of mitochondrial calcium in astrocytes in the olfactory bulb.

Non-littermates C57BL/6-N mice coming from outside the facility and facility inbred mutant mice were housed together at 3 weeks old (directly post weaning) in collective cages of 6-8 in an animal facility with controlled temperature of $21\pm 2^{\circ}\text{C}$ in a 12h light/12h dark cycle (light starting at 7.00am) and with water and food *ad libitum*. Animals were used at 8-17 weeks of age for the surgical and behavioral procedures, and assigned semi-randomly to experimental procedures.

Adeno-associated viruses (AAV)

To generate a specific deletion on astrocytes of the OB, we used an AAV8-hGFAP-Cre-IRES-mCherry purchased from the University of North Carolina (UNC School of Medicine) and an AAV8-hGFAP-GFP or AAV8-GFAP-dsRed as a control. To generate the specific deletion of neurons in the olfactory bulb, we used AAV5-hSyn-Cre-GFP from Addgene (catalog number #105540), and its control AAV5-hSyn-GFP (Addgene, catalog number #105539). The AAV-CAG-Empty (used as control), AAV-CAG-DIO-CB1-GFP (expressing the wildtype CB1 construct) and AAV-CAG-DIO-DN22-GFP (expressing the DN22-CB1 construct excluding the mitochondrial associated location of the receptor) viruses were subcloned in AAV8-serotype-packing plasmids following lab standardized molecular cloning techniques described previously (Hebert-Chatelain et al., 2016a). The AAV-GFAP-mMICU1-S124A-HA-IRES-mRuby (expressing a mutated non-phosphorylatable form of the MICU1 subunit of the mitochondrial calcium transporter) and AAV-GFAP-mMICU1-WT-HA-IRES-mRuby (expressing the wildtype version of MICU1) were subcloned using the plasmids (Marchi et al., 2019) generously gifted by Prof. Paolo Pinton (University of Ferrara, Italy) in AAV8-serotype-packing plasmids (Serrat et al., 2021). The AAV-GFAP-mito-GcAMP6s for the fiber photometry experiments was subcloned in a AAV8-serotype-packing plasmid as previously published (Serrat et al., 2021). The titrations of all viruses were between 10^{10} and 10^{11} genomic copies per ml for all batches.

Surgery for viral injection and fiber implantation

Mice were injected intraperitoneally with buprenorphine (0.05mg/kg, Buprecare), sleep-induced using 5% isoflurane machine, and placed into a stereotaxic apparatus (Model 900, Kopf instruments, CA, USA) using 2% isoflurane for the duration of the surgery with mouse adaptor and lateral ear bars. Local analgesia with lidocaine (0.1ml at 0.5%, Lidor) was used under the skin of the head before incision. The viral injections were delivered bilaterally in the olfactory bulb through a glass pipette using a microinjector (NanoInject II, Drummond Scientific). In all surgeries, mice were injected bilaterally with two injections per site of a total volume of 0,45µl each in the following coordinates: AP + 4.1; ML ± 0.75; DV – 3 and - 2 at a speed of 5nl/s.

To assess the specific contribution of astrocytic CB1 receptors in the olfactory bulb to socially-transmitted stress-driven behaviors, CB1-flox mice were injected with a viral mix of two different viruses out of the following: AAV-GFAP-GFP/AAV-DIO-Empty (expressing GFP reporter protein in astrocytes as a control, Ctrl), AAV-GFAP-CRE-mCherry/AAV-DIO-Empty (generating a Cre-induced deletion of CB1 receptors in GFAP positive cells, OB-GFAP-CB1-KO), AAV-GFAP-CRE-mCherry/AAV-DIO-CB1-GFP (generating both a Cre-mediated deletion of CB1 receptors in astrocytes and a Cre-mediated re-expression of the wild-type construct of CB1, OB-GFAP-CB1-RS) and AAV-GFAP-CRE-mCherry/AAV-DIO-DN22-GFP (generating both a Cre-mediated deletion of CB1 receptors in astrocytes and a Cre-mediated re-expression of the DN22-CB1 construct, therefore re-expressing CB1 everywhere but in their mitochondrial-associated locations, OB-GFAP-DN22-RS). All viruses used were titered between $2-8 \cdot 10^{10}$ genomic copies/mL.

To exclude a neuronal contribution to impact on NOR of socially-transmitted stress, we injected CB1 Floxed mice with either an AAV-Syn-Cre-GFP or a AAV-Syn-EGFP (Ctrl) in the OB (titered $3,3 \cdot 10^{11}$ genomic copies/mL).

To measure mitochondrial calcium responses and the contribution to astrocytic CB1 receptors to this process, mice were injected in the OB with either only AAV-mitoGcAMP6s (C57BL/6-N mice) or in combination (CB1flox mice) with AAV-GFAP-CRE-mCherry (KO) or AAV-GFAP- dsRed (Ctrl). (titered $2-5 \cdot 10^{11}$ genomic copies/mL).

To assess the contribution of mitochondrial calcium in socially-transmitted stress, C57BL/6-N mice were injected with either AAV-GFAP-MICU^{WT} or AAV-GFAP-MICU^{S124A} in the OB (titered $3 \cdot 10^{11}$ genomic copies/mL).

For fiber photometry experiments, mice were injected with in the OB AAV-GFAP-mitoGcAMP6s and AAV-GFAP-CRE-mCherry or AAV-GFAP-dsRED in the experiments in which astrocytic CB1 receptors were manipulated (titered $2\text{-}6 \cdot 10^{11}$ genomic copies/mL). Then, the optical fiber (400 μm diameter, 0.5 NA) was placed 200 μm above the last injection site (at DV -2, therefore at -1.8) and fixed with dental cement (MajorRepair).

Following surgery, all mice received i.p. injection of 0.2 ml of saline solution and anti-inflammatory drug meloxicam (5 mg/kg, Metacam), that was continued for additional 2 days. Animals continued to be housed collectively and body weight was monitored daily during 4-5 days to assess recovery. Behavioral experiments were carried out 4-5 weeks after surgery and fiber photometry experiments 5-6 weeks after surgery.

Perfusion

AAV injected mice were deeply anesthetized with pentobarbital (400 mg/kg body weight), transcardially perfused first with 20ml of phosphate-buffered solution (PBS 0.1M, pH 7.4) following by 50 ml of cold 4% paraformaldehyde (Sigma, BO501128-4L). Brains were isolated and postfixed in the same fixative solution over night at 4°C and then transferred to a 30% (wt/vol) sucrose (Sigma, S0389) solution in PBS for cryopreservation. After, brains were frozen in isopentane (Sigma, M32631) and stored at -80°C. Free-floating frozen sagittal sections (30 μm) were cut out using a cryostat (Leica Biosystems, CM1950S). Mid olfactory bulb slices were stored in antifreeze solution at -20°C until further use.

Immunostaining

Immunostaining against GFAP - Sections were washed with PBST (0.3% Triton X-100 diluted in PBS 1X pH7.4) three times and then permeabilized 1 h at room temperature (RT) in a blocking solution [in PBS 1X: 10% donkey serum; 0.3% triton X-100]. Next, sections were incubated overnight at 4°C with polyclonal rabbit anti-GFAP (1:1000) (Agilent, DAKO Z0334) diluted in blocking solution.

After some washes with PBST, brain sections were incubated over 2h at RT with donkey anti-rabbit Alexa fluor 647 (1:500, Invitrogen) (polyc). Following some washes with PBST, section were stained with DAPI (1:20000; Invitrogen D3571), washed again with PBST and finally mounted and coversliped.

The sections were analyzed with an epifluorescence Leica DM6000 microscope (Leica, France) to check for the intrinsic fluorescence of the viruses and the identity of the infected cells. Mouse brains that didn't meet the expression requirements led to the exclusion of the mice from the experiments.

Immunostaining against GFAP and HA - Sections were washed with PBST (0.3% Triton X-100 diluted in PBS 1X pH7.4) three times and then incubated with 3% hydrogen peroxide diluted in PBST (Sigma, H1009-500ML) for 30 min . Following a step of permeabilization, that was carried out for 1h at RT in a blocking solution, sections were incubated overnight at 4°C with a mix of primary antibodies: polyclonal chicken anti-GFAP (1:1000) (USBiological #G2032-25F) and monoclonal rabbit anti HA (Cell Signaling, 3724) diluted in blocking solution. After some washes with PBST, brain sections were incubated over 2h at RT with a mix of secondary antibodies : goat anti rabbit IgG HRP linked antibody (Cell Signaling, 7074) and Rhodamine (TRITC) Conjugated affinipure donkey anti chicken (Jackson immunoresearch, 703-025-155) (1:500). Following some washes with PBST, section were incubated with TSA plus Fluorescein (1:250) (AKOYA biosciences, NEL741001KT). Afterward, cellular nucleus were stained with DAPI (1:20000; Invitrogen D3571), washed again with PBST and finally mounted and coversliped.

The sections were analyzed with an epifluorescence Leica DM6000 microscope (Leica, France) to check for the intrinsic fluorescence of the viruses and the identity of the infected cells. Mouse brains that didn't meet the expression requirements led to the exclusion of the mice from the experiments.

Social transmission of stress

Non-littermate animals were housed together at 3 weeks of age to establish a familiarity between them while avoiding dominance issues, and then moved to new cages in couples 1-2 days before the experiment. One of the members of the couple is the demonstrator (DEM, a C57BL/6-N mouse) while the other one

is the observer (OBS, depending on the experiment: [i] wild-type CB1 flox mice, [ii] CB1 mutant mice, [iii] operated CB1-flox mice, [iv] operated C57BL/6-N) (Fig 1a). As described previously (Sterley et al., 2018), the demonstrators were subjected to either a 5 min x 0.5mA/30s shock protocol (stress, foot-shock) in a clean fear conditioning chamber (stress DEMs), or to a 5-min exposure to a novel cage (neutral) similar to the home-cage but with clean bedding (neutral DEMs), and they immediately moved back to the home-cage where they were allowed full interaction with the observer (Fig 1a). Their behavior was recorded during 5 minutes and 8 different social and non-social behaviors are analyzed offline: anogenital exploration (snout toward the area of the congener), body exploration (all other snout contacts that aren't on or near the anogenital region), allogroom (grooming of the partner), self-groom, digging, rearing, walking, sitting and fighting.

Odor-dependent social transmission of stress

To test whether odors were sufficient to induce transmission of stress, DEMs were habituated for three days to being swabbed on the anogenital region before the test with a clean cotton swab for 3s, and a cotton swab was placed in the home-cage of each experimental couple for 2 days to avoid neophobia in the test. Demonstrators were swabbed with a humid cotton swab (wet with 1% saline solution) three times after the shock protocol (Odor stress), or after being removed from the home-cage (Odor neutral). A wet cotton swab was used as the control condition (Saline) (Fig 1k). Immediately after odor collection, the cotton swab was presented to the OBS in the home-cage, slightly touching their snout before dropping it on the cage bedding. Mice were allowed to interact with the cotton swab for 5 min, and then the cotton swab was removed from the cage. In the experiments of paired odor exposure and fiber photometry (Extended Data 3, and Fig 4), the cotton swab was lightly maintained in front of the snout of the mouse during either 2 or 20 seconds before removing it from the cage.

Novel object recognition memory task

An L-shaped maze of grey PVC with two perpendicular arms placed on a white background was used in this test. The test was performed under 50x lux

intensity with an overhung camera allowing the recording and later offline scoring of the maze exploration by the mouse.

The test consists in 3 daily phases as described previously (Cruz et al., 2020). On day 1, mice were habituated to the maze for 9 min before returning to the home-cage. On day 2, they were presented with two identical objects in each arm, and allowed to explore for 9 min to get familiar with them (acquisition phase). On day 3, either directly (naïve) or 20 min after stress manipulations (social transmission of stress or foot-shock), mice were exposed to the maze again where one of the familiar objects is replaced by a novel one, and allowed exploration for 9 min. Exploration of an object was counted when the animal had the nose on the object or facing the object in a distance less than 0.5cm. This phase tests the recognition performance of the animal by comparing the time spent in the novel versus the familiar objects. Object recognition capabilities are assessed by a discrimination index that is calculated by the time spent in the novel object minus the time spent in the familiar divided by the total time. Total exploration time and exploration of each object was also analyzed during the test. The position of the novel object and the associations of novel and familiar were randomized. All objects were previously tested to avoid biased preference. The apparatus as well as objects were cleaned with ethanol (70%) before experimental use and between each animal testing.

Buried food test

As described previously for this test (Machado et al., 2018), mice were habituated to a chocolate pellet for 3 days in the home-cage, and food deprived for 24h up until the test. Animals were moved to home-cage sized cages with 3-5 cm of clean bedding and allowed to roam for 10 minutes for habituation. Straight after, they were removed from the cages momentarily and a chocolate pellet was hidden below the bedding at a random corner. Mice were moved back to the cage and allowed to search for the pellet up until 5 minutes, by which the test was considered as failed. The time a mouse took until finding the pellet was counted offline blind to the genotype of the subject mice from the recorded videos.

Elevated plus maze test

The test was performed in an elevated plus maze consisting of 4 arms (height: 66cm) of 45-cm long and 10-cm wide disposed cross-shaped and connected by a central platform of 10cm x 10cm. The open arms had a light intensity of 75lux and the closed arms of 20lx. OBS mice were placed in the open platform 20 min after social interaction with demonstrators, and allowed to explore the maze for 5 min. The time spent in open and closed arms, and the number of times they enter in those, was analyzed offline by an experimenter blind to the condition.

Social interaction with a stranger test

Experimental animals were habituated in their home-cage for 10 min to the testing room. Straight after, a C57BL/6-N mouse of the same sex and age was introduced in the home-cage for 5 minutes, allowing full interaction between resident and stranger. Videos of the social interaction were recorded and 8 behaviors of the resident animals towards the partners were analyzed offline: anogenital exploration, body exploration, allogroom, self-groom, digging, rearing, walking, sitting and fighting). Animals that exhibited aggressive behaviors for more than 1min were excluded.

Fiber photometry experiments

Five to six weeks after surgery, freely-moving mitoGcAMP6s expressing mice were imaged using 470nm LED to excite the sensor, and 405nm for the isosbestic signal control. Observer mice with fiber implants were habituated to the connection during 3 days prior the test, in 10-minute sessions in which they were connected and allowed to roam in the home-cage with their familiar cage-mate. The fiber photometry set-up collected the emitted fluorescence with a sCMOS camera (Hamamatsu Orca Flash v3) through an optic fiber (core 400 μm , N.A 0.5) divided in 2 sections: a short fiber implanted in the brain of the mouse and a long fiber (modified patchcord), both connected through a ferrule-ferrule (1.25 mm) connection. To minimize the photobleaching effect of the recording and preserve a high signal to noise ratio, the light intensities in the tip of the patch cord were adjusted to $\sim 100 \mu\text{W}$ for the 470 nm channel and $\sim 50 \mu\text{W}$ for the 405 nm channel. A custom MATLAB script (Matlabworks) was used to synchronize video recording with fiber photometry, combined with a programmed

Arduino board. The sampling rate was settled at 20 Hz for both photometry (interleaved) and video recording.

On the test day, observer mice were separated from their partners for a habituation period of 5 min in which they stayed in the home-cage while their partners were shocked to collect the odor in a cotton swab. Observers were exposed to a cotton swab wet with saline or the cotton swab impregnated with the stressed partner chemosignals in an inter-individual alternated order (some mice had saline first, others stress odor first) with an interval of 4-5 minutes. Mice were only exposed to each odor one time, by establishing a close contact between the impregnated swab and the snout during an average time of 23.72 ± 2.59 seconds before removing the swab from the cage. Mitochondrial calcium signals were recorded during the duration of the test (20 min).

Raw calcium signals were pre-processed by removing the first minute of the recording to decrease the effect of the first exponential photobleaching, and by removing point artifacts. The 470nm signal was fitted to the isosbestic 405nm using linear regression (polynomial of first degree) and for each time point, $\Delta F/F$ was calculated as $(F_{470nm} - F_{405nm}(\text{fitted})) / F_{405nm}(\text{fitted})$. $\Delta F/F$ values were smoothed using a moving average of 0.5 seconds. Values during the time of swab presentation were extracted (20s) with a baseline period of 15s that was used to z-score the signals. These values were only extracted if they were accompanied by a significant calcium transient, detected by using a threshold of 2 times the mean absolute deviation of the peak prominence on a 2 min moving window. The area under the curve during the 20s of swab exposure of either saline or stress was calculated from the z-scored data. Calcium signals from saline and stress swabs were compared between mice.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data collection

Statistical methods to determine sample size were not used, but they were similar to those in the literature. Experimenters analyzing the raw videos were always blind to the condition of the subject. All mice were randomly assigned to experimental conditions. We used custom software to analyze the social behaviors and time spent in each arm during the novel object recognition. For the

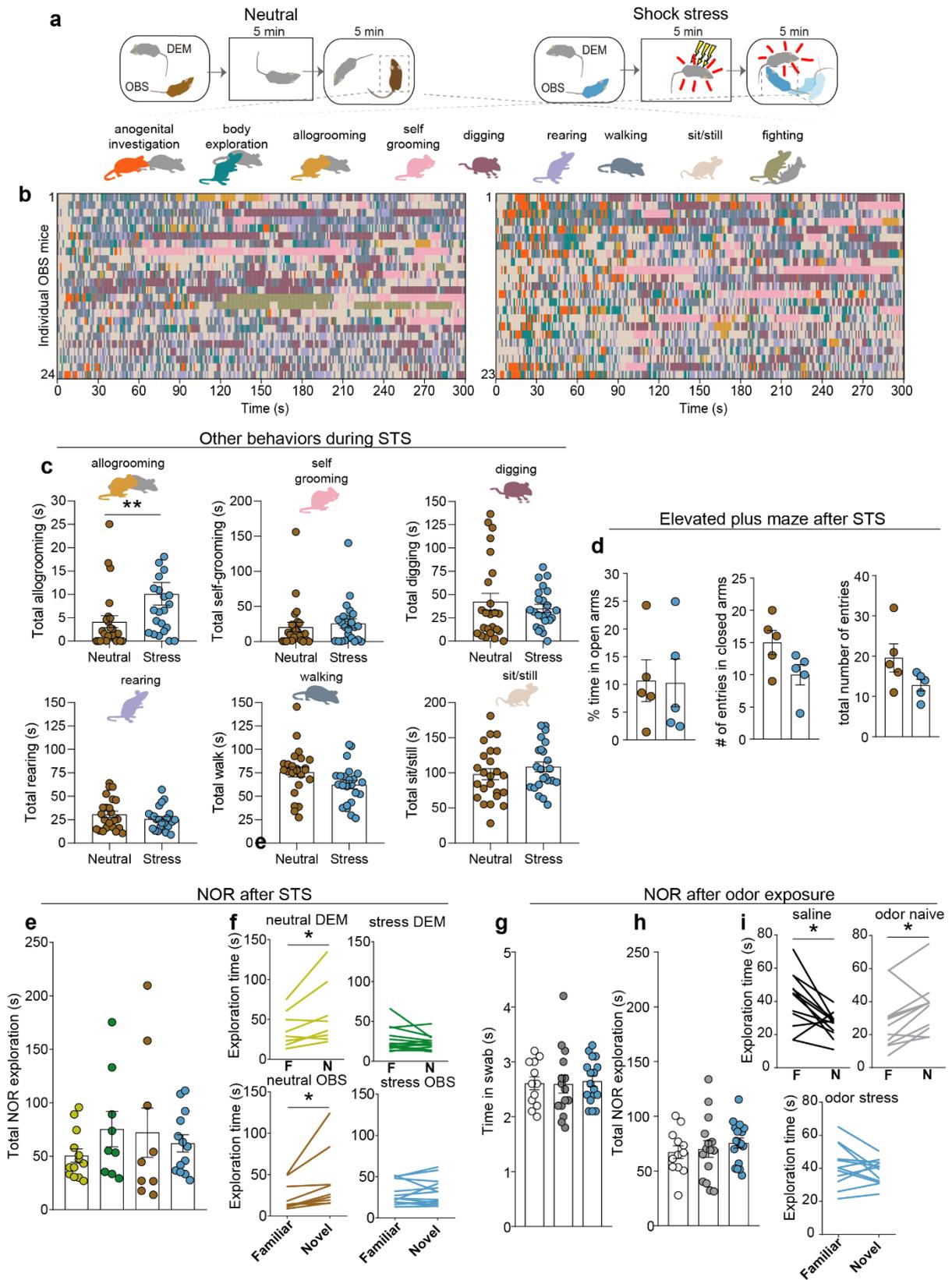
analysis of the immunostaining, we used Fiji. Raw data was processed and analyzed using Microsoft Excel 2020 and Graph Pad 8.0.

Statistical analysis

Graphs and statistical analysis were performed with Graph Pad 8.0. All data comes from distinct samples and is shown as independent data points per animal \pm standard error of the median (SEM). Normality of the data was assessed with the Kolmogorov-Smirnoff test for all sample sizes > 5 or Shapiro-Wilk test for sample sizes < 5 , and depending on the result, parametric (paired and unpaired Student's *t*-test, ordinary one-way ANOVA with Bonferroni post-hoc analysis, or ordinary two-way ANOVA when necessary) or non-parametric (unpaired Mann-Whitney test, Wilcoxon matched pairs signed rank test or Kruskal-Wallis test with Dunn's post-hoc analysis) were performed. Detailed statistical data for each experiment including exact mean \pm SEM values, test statistic with confidence intervals, group sizes, degrees of freedom and exact P value can be found in **Extended Table 1** (for main figures) and **Extended Table 2** (for extended data figures).

EXTENDED DATA FIGURES

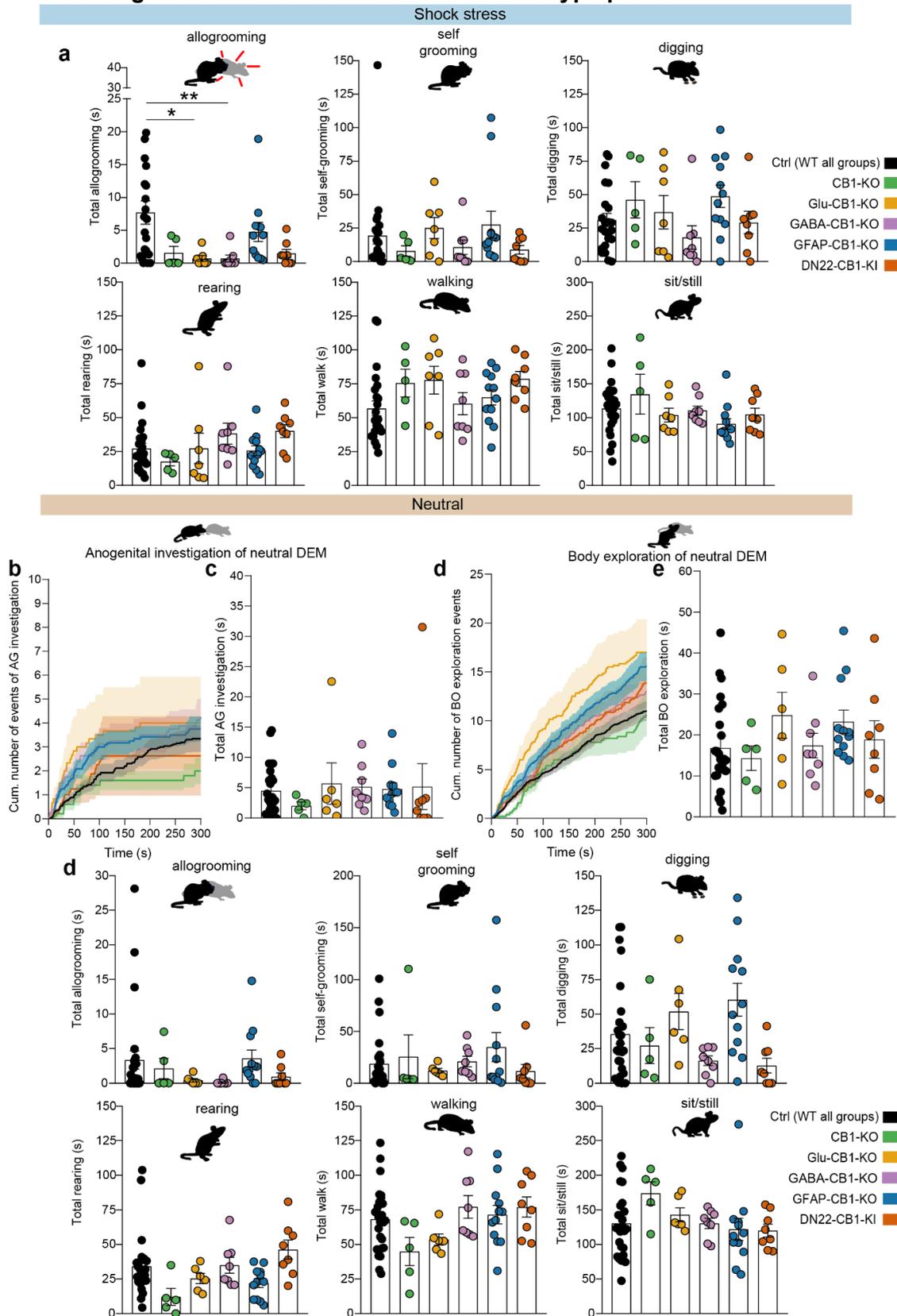
Extended Data 1. Behavioral analysis of animals exposed to stressed congeners or stress chemosignals.



- a. Behaviors of observed mice analyzed during the 5 min social interaction period with a neutral or shocked partner: anogenital investigation, body exploration, allogrooming (grooming of partner), self-grooming, digging, rearing, walking, sit/still and fighting.
- b. Rasterplot of behaviors of observers per second during 5 min social interaction with DEMs. Each line represents an individual mouse. $n_{(\text{neutral})} = 24$, $n_{(\text{stress})} = 23$.
- c. Total time spent on social (allogrooming) and nonsocial behaviors of neutral (brown) or stress OBS (blue) mice during 5 min social interaction. Two tailed unpaired Student's t-test or U-Mann Whitney. Mann-Whitney (allogrooming), $p = 0.0061$. $n_{(\text{neutral})} = 24$, $n_{(\text{stress})} = 23$.
- d. Percent of time spent in open arms, number of closed arm entries, and total arm entries of OBS-neutral and OBS-shock mice in an elevated plus maze test. Time in open arms: Two tailed unpaired Student's t-test. $n_{(\text{neutral})} = 5$, $n_{(\text{stress})} = 5$.
- e. Total object exploration of both DEMs and OBS in the NOR test after social interaction. Neutral DEM (yellow): 75.32 ± 16.62 . Ordinary one-way ANOVA. $n_{(\text{neutral DEM})} = 8$, $n_{(\text{stress DEM})} = 13$, $n_{(\text{neutral OBS})} = 9$, $n_{(\text{stress OBS})} = 13$.
- f. Total time spent in exploring novel (N) vs familiar (F) objects of DEMs and OBS in the NOR test after social interaction. Two tailed paired Student's t-test. $n_{(\text{neutral DEM})} = 8$, $n_{(\text{stress DEM})} = 13$, $n_{(\text{neutral OBS})} = 9$, $n_{(\text{stress OBS})} = 13$.
- g. Total time spent exploring the cotton swab impregnated with either saline, anogenital secretions of a naïve mouse (odor naïve) or AG secretions or a shocked mouse (odor stress) in the homepage of naïve mice. Saline: 2.593 ± 0.1631 . Ordinary one-way ANOVA. $n_{(\text{saline})} = 12$, $n_{(\text{odor naïve})} = 15$, $n_{(\text{odor stress})} = 17$.
- h. Total object exploration time (s) of mice exposed to a cotton swab impregnated with either saline, anogenital secretions of a naïve mouse (odor naïve) or AG secretions or a shocked mouse (odor shock) in the NOR test.
- i. Total exploration time spent in exploring novel (N) vs familiar (F) objects of mice exposed to a cotton swab impregnated with either saline, anogenital secretions of a naïve mouse (odor naïve) or anogenital secretions or a shocked mouse (odor stress) in the NOR test. Two tailed paired Student's t-test. $n_{(\text{saline})} = 12$, $n_{(\text{odor naïve})} = 15$, $n_{(\text{odor stress})} = 17$.

Data are expressed in mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

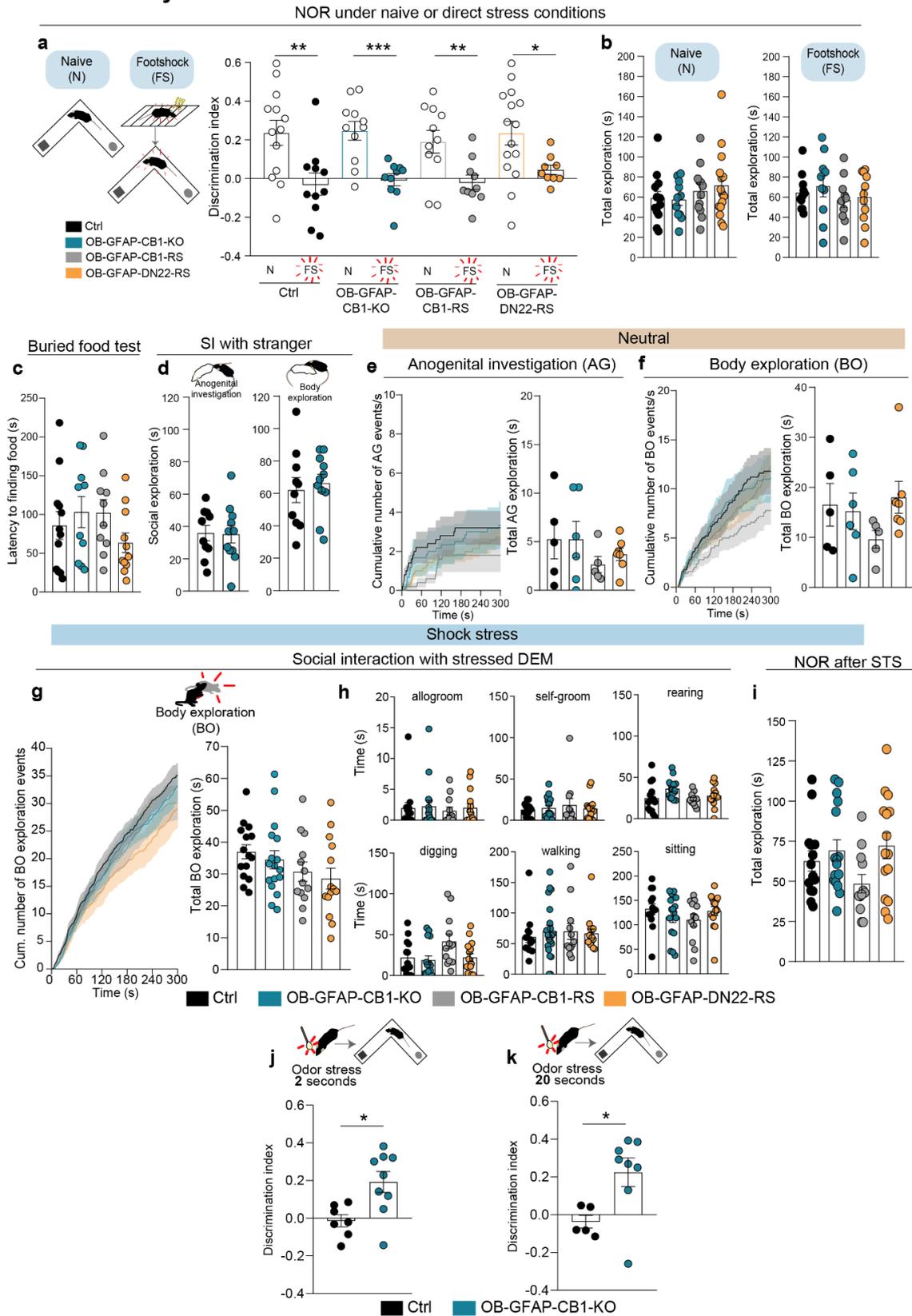
Extended Data 2. Behaviors of global and conditional CB1 receptor mutant mice in interacting with a shocked or neutral DEM wild-type partner.



- a. Total time spent on social (allogrooming) and nonsocial behaviors of global or conditional CB1 mutant observer mice during 5 min social interaction with a stressed DEM. Ordinary one-way ANOVA or Kruskal Wallis test. $n_{(Ctrl)} = 23$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 7$, $n_{(GABA-CB1-KO)} = 8$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$.
- b. Cumulative number of AG events of global or conditional CB1 mutant observer mice during 5 min social interaction with neutral DEMs. $n_{(Ctrl)} = 26$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 6$, $n_{(GABA-CB1-KO)} = 6$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$.
- c. Total time of anogenital investigation of global or conditional CB1 mutant observer mice during 5 min social interaction with a neutral DEM. Kruskal Wallis test. $n_{(Ctrl)} = 26$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 6$, $n_{(GABA-CB1-KO)} = 6$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$.
- d. Cumulative number of BO events of global or conditional CB1 mutant observer mice during 5 min social interaction with neutral DEMs. $n_{(Ctrl)} = 26$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 6$, $n_{(GABA-CB1-KO)} = 6$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$.
- e. Total time of body exploration of global or conditional CB1 mutant observer mice during 5 min social interaction with a neutral DEM. Kruskal Wallis test. $n_{(Ctrl)} = 26$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 6$, $n_{(GABA-CB1-KO)} = 6$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$.
- f. Total time spent on social (allogrooming) and nonsocial behaviors of global or conditional CB1 mutant observer mice during 5 min social interaction with a neutral DEM. $n_{(Ctrl)} = 26$, $n_{(CB1-KO)} = 5$, $n_{(Glu-CB1-KO)} = 6$, $n_{(GABA-CB1-KO)} = 6$, $n_{(GFAP-CB1-KO)} = 12$, $n_{(DN22-CB1-KI)} = 8$.

Data are expressed in mean \pm SEM. * $p < 0.05$ or ** $p < 0.01$.

Extended Data 3. Behavioral characterization of mice carrying deletions of CB1 in the olfactory bulb.

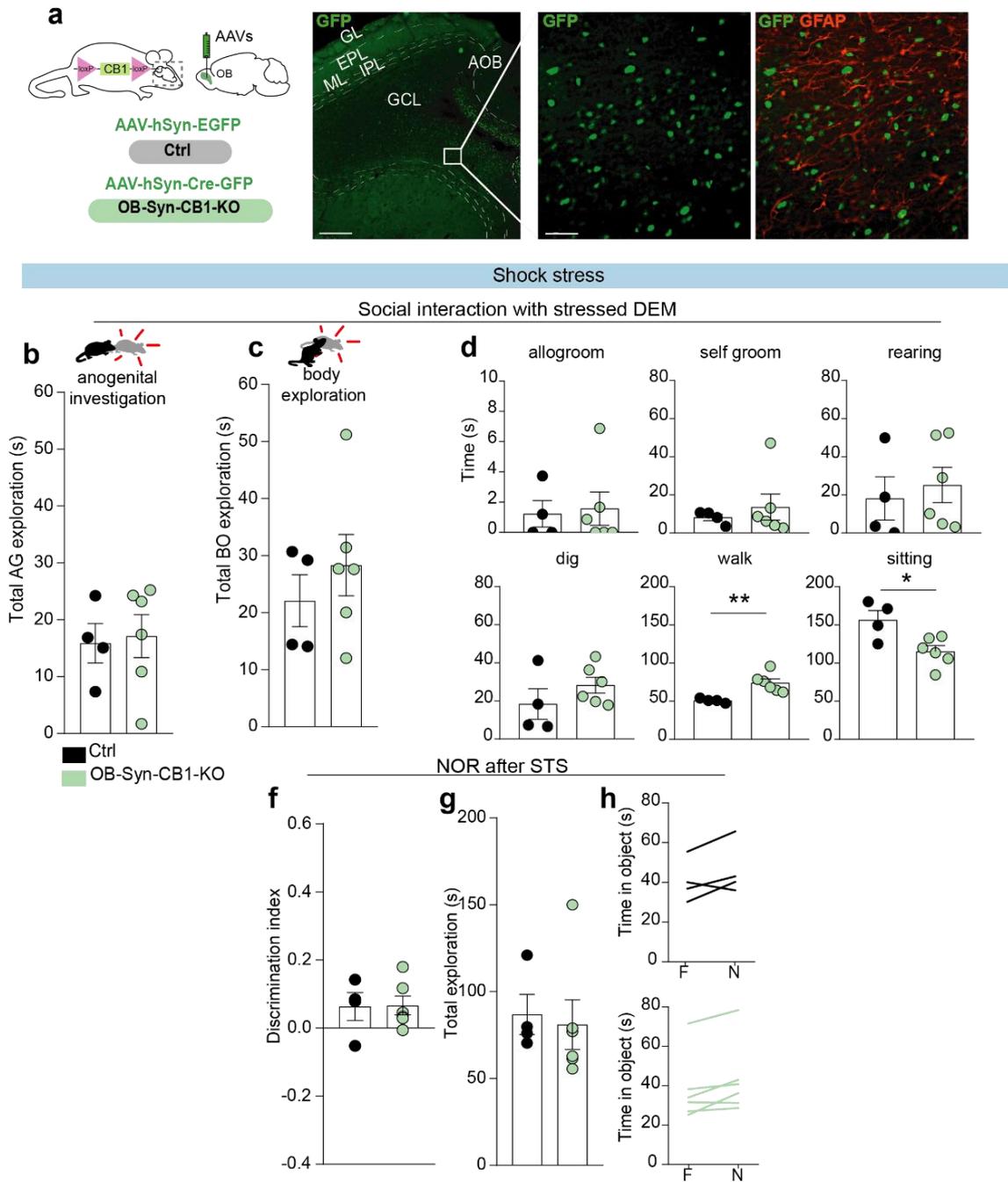


- a.** Discrimination index of OB-GFAP-CB1 mutants (Ctrl, OB-GFAP-CB1-KO, OB-GFAP-CB1-RS and OB-GFAP-DN22-RS) in the NOR either under naïve conditions (N) or after direct foot-shock (FS). Two-way ANOVA (genotype x NOR stress condition); two tailed unpaired Student's t-test, $p_{(N \text{ vs FS - Ctrl})} = 0.0065$, $p_{(N \text{ vs FS, OB-GFAP-CB1-KO})} = 0.0004$, $p_{(N \text{ vs FS, OB-GFAP-CB1-RS})} = 0.0086$, $p_{(N \text{ vs FS, OB-GFAP-DN22-RS})} = 0.03$. $n_{(Ctrl, N)} = 13$, $n_{(OB-GFAP-CB1-KO, N)} = 11$, $n_{(OB-GFAP-CB1-RS, N)} = 11$, $n_{(OB-GFAP-DN22-RS, N)} = 15$, $n_{(Ctrl, FS)} = 11$, $n_{(OB-GFAP-CB1-KO, FS)} = 10$, $n_{(OB-GFAP-CB1-RS, FS)} = 10$, $n_{(OB-GFAP-DN22-RS, FS)} = 9$.
- b.** Total time exploring the maze during NOR of OB-GFAP-CB1 mutant mice in naïve conditions or after footshock. $n_{(Ctrl, N)} = 13$, $n_{(OB-GFAP-CB1-KO, N)} = 11$, $n_{(OB-GFAP-CB1-RS, N)} = 11$, $n_{(OB-GFAP-DN22-RS, N)} = 15$, $n_{(Ctrl, FS)} = 11$, $n_{(OB-GFAP-CB1-KO, FS)} = 10$, $n_{(OB-GFAP-CB1-RS, FS)} = 10$, $n_{(OB-GFAP-DN22-RS, FS)} = 9$.
- c.** Latency of wild-type or OB-GFAP-CB1 mutant littermates to find a buried chocolate pellet under fresh bedding. $n_{(Ctrl.)} = 12$, $n_{(OB-GFAP-CB1-KO)} = 10$, $n_{(OB-GFAP-CB1-RS.)} = 10$, $n_{(OB-GFAP-DN22-RS)} = 11$.
- d.** Anogenital and body social exploration of an age-matched stranger partner by of wild-type or OB-GFAP-CB1-KO mutant littermates. $n_{(Ctrl.)} = 10$, $n_{(OB-GFAP-CB1-KO)} = 11$
- e.** Cumulative number of events per second (left) and total time (right) spent in body exploration of a neutral DEM by OB-GFAP-CB1 mutant mice during a 5 min interaction period. $n_{(Ctrl.)} = 5$, $n_{(OB-GFAP-CB1-KO)} = 6$, $n_{(OB-GFAP-CB1-RS.)} = 5$, $n_{(OB-GFAP-DN22-RS)} = 7$.
- f.** Cumulative number of events per second (left) and total time spent in body exploration (right) of a neutral DEM by OB-GFAP-CB1 mutant mice during a 5 min interaction period. $n_{(Ctrl.)} = 5$, $n_{(OB-GFAP-CB1-KO)} = 6$, $n_{(OB-GFAP-CB1-RS.)} = 5$, $n_{(OB-GFAP-DN22-RS)} = 7$.
- g.** Cumulative number of events per second (left) and total time spent in body exploration (right) of a stressed DEM by OB-GFAP-CB1 mutant mice during a 5 min interaction period. $n_{(Ctrl)} = 15$, $n_{(OB-GFAP-CB1-KO)} = 17$, $n_{(OB-GFAP-CB1-RS)} = 13$, $n_{(OB-GFAP-DN22-RS)} = 14$.
- h.** Social (allogrooming) and nonsocial behaviors of OB-GFAP-CB1 mutant OBS mice recorded during the 5 min social interaction with a stressed DEM. $n_{(Ctrl)} = 15$, $n_{(OB-GFAP-CB1-KO)} = 17$, $n_{(OB-GFAP-CB1-RS)} = 13$, $n_{(OB-GFAP-DN22-RS)} = 14$.
- i.** Total exploration of OB-GFAP-CB1 mutant OBS mice in the NOR test after social interaction with a stressed DEM.

- j.** Discrimination index in the NOR test of Ctrl and OB-GFAP-CB1 KO observer mice exposed for 2s to a swab with social stress odor. Two-tailed unpaired Student t-test, $p = 0.0106$, $n_{(Ctrl)} = 7$, $n_{(OB-GFAP-CB1-KO)} = 9$.
- k.** Discrimination index in the NOR test of Ctrl and OB-GFAP-CB1 KO observer mice exposed for 20s to a swab with social stress odor, U-Mann Whitney test, $p = 0.0295$, $n_{(Ctrl)} = 5$, $n_{(OB-GFAP-CB1-KO)} = 8$.

Data are expressed in mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

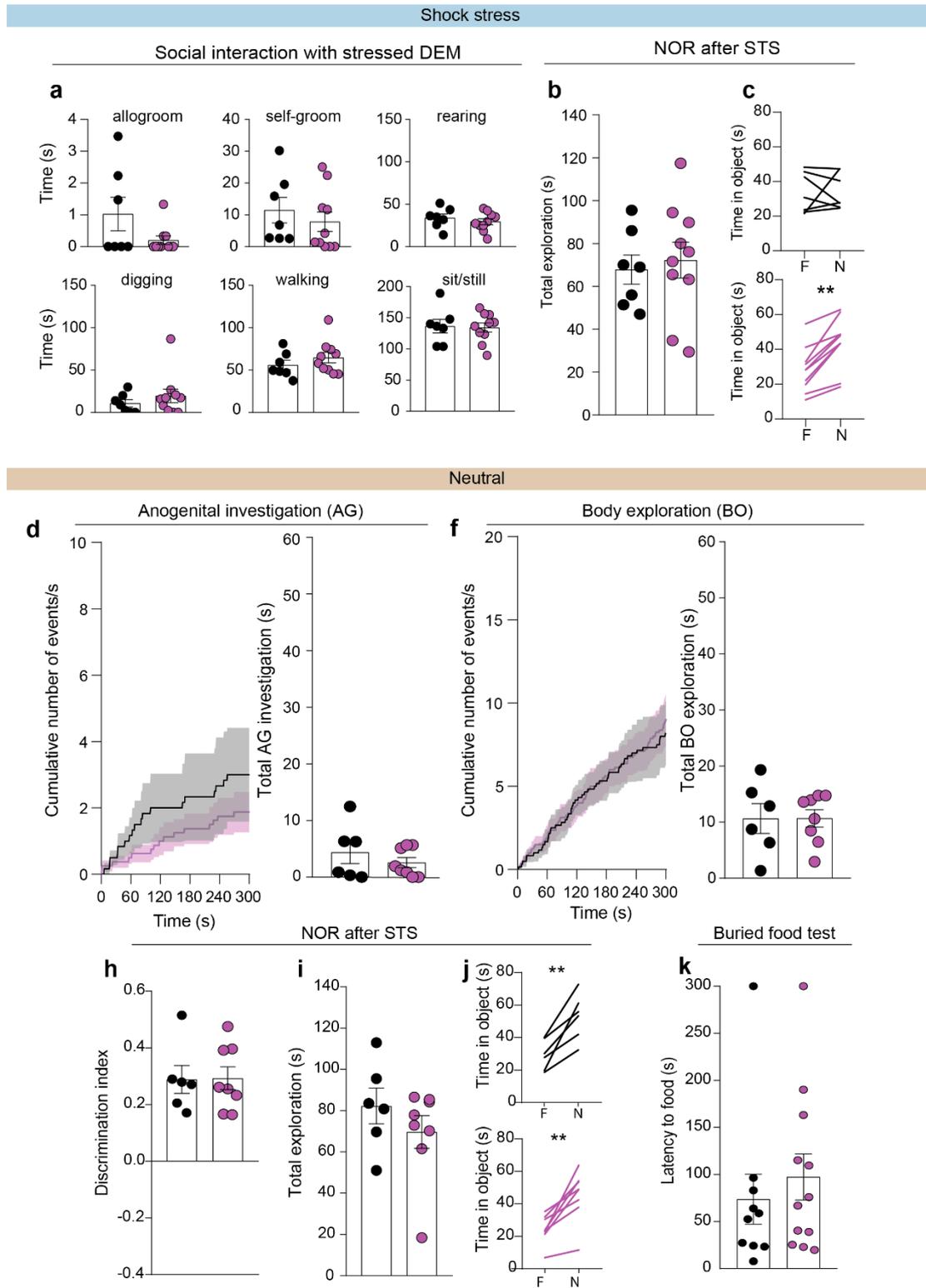
Extended Data 4. Deletion of CB1 in OB neurons doesn't affect social exploration of shocked DEM or socially transmitted stress-induced NOR impairment.



- a. Viral approach to manipulate mitochondrial calcium in astrocytes of the olfactory bulb (Methods) Immunostaining in sagittal slices of the main olfactory bulb (GCL = granule cell layer, ML = mitral layer, IPL = internal plexiform layer, AOB = accessory olfactory bulb) against GFAP (in red) and viral expression of endogenous fluorescence of the AAV-Syn-GFP-Cre virus injected in CB1 Flox mice to generate OB-Syn-CB1-KO mice. Scale bars are 500 μ m and 50 μ m.
- b. Total time of anogenital investigation of a stressed DEM by OB-Syn-CB1 mutant OBS during a 5 min social interaction test.
- c. Total time of body exploration of a stressed DEM by OB-Syn-CB1 mutant OBS during a 5 min social interaction test.
- d. Social (allogrooming) and nonsocial behaviors of OB-Syn-CB1 mutant OBS in the NOR after social interaction with a stressed DEM. Two-tailed unpaired Student t-test. $p_{(\text{walk})} = 0.0069$, $p_{(\text{sitting})} = 0.0163$.
- e. Discrimination index of OB-Syn-CB1 KO mice in the test phase of the NOR after social interaction with a stressed DEM.
- f. Total exploration of OB-Syn-CB1 mutant OBS in the NOR after social interaction with a stressed DEM.
- g. Total time spent in novel (N) vs familiar (F) objects of OB-Syn-CB1 mutant OBS in the NOR after social interaction with a shocked DEM.

For all panels, $n_{(\text{OB-Syn-CB1-WT})} = 4$, $n_{(\text{OB-Syn-CB1-KO})} = 6$. Data are expressed in mean \pm SEM. Data is not significant unless specified with * $p < 0,05$ or ** $p < 0.01$.

Extended Data 5. Disruption of mitochondrial calcium handling in OB astrocytes does not affect basal social behaviors, cognitive performance and olfactory abilities.



- a. Social (allogrooming) and nonsocial behaviors of MICU1 observer mice in the NOR after social interaction with a shocked DEM. $n_{(\text{MICU}^{\text{WT}})} = 7$, $n_{(\text{MICU}^{\text{S124A}})} = 10$.
- b. Total exploration of MICU1 observer mice in the NOR after social interaction with a shocked DEM. $n_{(\text{MICU}^{\text{WT}})} = 7$, $n_{(\text{MICU}^{\text{S124A}})} = 10$
- c. Total time spent in novel (N) vs familiar (F) objects by MICU1 observer mice in the NOR after social interaction with a shocked DEM. Wilcoxon matched-pairs test, $n_{(\text{MICU}^{\text{WT}})} = 7$, $n_{(\text{MICU}^{\text{S124A}})} = 10$
- d. Cumulative number of events per second (left) and total time (right) spent in body exploration of a neutral DEM by MICU1 observer mice during a 5 min interaction period. $n_{(\text{MICU}^{\text{WT}})} = 6$, $n_{(\text{MICU}^{\text{S124A}})} = 8$
- e. Cumulative number of events per second (left) and total time spent in body exploration (right) of a neutral DEM by MICU1 observer mice during a 5 min interaction period. $n_{(\text{MICU}^{\text{WT}})} = 6$, $n_{(\text{MICU}^{\text{S124A}})} = 8$
- f. Discrimination index of MICU1 observer mice in the test phase of the NOR after social interaction with a neutral DEM. $n_{(\text{MICU}^{\text{WT}})} = 6$, $n_{(\text{MICU}^{\text{S124A}})} = 8$
- g. Total exploration of MICU1 observer mice in the NOR after social interaction with a neutral DEM. $n_{(\text{MICU}^{\text{WT}})} = 6$, $n_{(\text{MICU}^{\text{S124A}})} = 8$
- h. Total time spent in novel vs familiar objects by MICU1 observer mice in in the NOR after social interaction with a neutral DEM. Two tailed paired Student's t-test or Wilcoxon matched-pairs test, $p_{(\text{MICU}^{\text{WT}})} = 0.0037$, $p_{(\text{MICU}^{\text{S124A}})} = 0.0016$, $n_{(\text{MICU}^{\text{WT}})} = 6$, $n_{(\text{MICU}^{\text{S124A}})} = 8$
- i. Latency to find buried chocolate pellet under clean bedding of MICU1 observer mice (during a 5 min test). $n_{(\text{MICU}^{\text{WT}})} = 10$, $n_{(\text{MICU}^{\text{S124A}})} = 12$

Data are expressed in mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

III. RESULTS: CHAPTER 2

Astrocytes in olfactory processing

INTRODUCTION

Astrocytes refine processing of odors in different ways. Recent evidence indicates their involvement as active regulators of the activity of M/T cells, the output cells of the olfactory bulb (Gurden et al., 2006; Martin et al., 2012b; Roux et al., 2011a; De Saint Jan and Westbrook, 2005; Sardar et al., 2023). Moreover, several neuromodulators affect olfactory bulb astrocytic calcium activity, like serotonin (Sardar et al., 2023), dopamine (Fischer et al., 2020) or noradrenaline (Fischer et al., 2021). In turn, manipulation of astrocytes affects correct olfactory detection and discrimination (Sardar et al., 2023; Ung et al., 2020, 2021). Interestingly, the endocannabinoid system has been proposed as another neuromodulator of M/T activity. Levels of tonic endocannabinoids (eCBs) change depending on the internal state of a mouse (Soria-Gómez et al., 2014b), which, in turn, participate in the modulation of M/T cell activity impacting odor detection (Soria-Gómez et al., 2014). In addition, the results presented on this thesis suggest that mitochondrial CB1 receptors in the astrocytes of the main olfactory bulb (MOB) are required for the correct processing of social stress odors. This is dependent on astrocytic mitochondrial calcium transients upon social odor-presentation, suggesting that astrocytic mitochondria could be a site for eCBs modulation of olfactory processing. However, whether astrocytes modulate other types of olfactory processing through mitochondrial calcium processes is not known.

The present chapter represents a compilation of preliminary data that attempts to shine light on several questions contributing to understanding the role of astrocytes in the MOB. We used fiber photometry recordings of mitochondrial and cytosolic calcium signals in the astrocytes of the OB to study three main objectives: (i) are calcium signals in the astrocytes encoding the identity of an odor? (ii) are odors inducing phasic eCBs release? (iii) are astrocytic CB1 receptors regulating olfactory processing of other odors? The preliminary data presented here offers more information about the relationship between specific odors and astrocytic calcium signals, suggesting that astrocyte calcium

in different compartments could contribute to the encoding of the salience of odors. However, similar eCBs release was observed independently of the identity of the odor presented. Moreover, observed that a putative alarm 2-SBT induces mitochondrial calcium changes and specific astrocytic mtCB1 dependent changes in the NOR similar to the ones we observed with the stress odor, suggesting this chemosignal as a candidate to mediate STS-induced cognitive adaptations. Together, these results provide the technical and theoretical basis to further understand how astrocytes contribute to the olfactory representation of specific odors.

RESULTS

Astrocytes respond differently to the odor of a familiar stressed partner than to a saline swab

Astrocytes in the glomeruli show cytosolic calcium changes in response to neuronal activation upon odor stimulation (Petzold et al., 2008; Ung et al., 2020), that can be different depending on the odor (Petzold et al., 2008). Moreover, astrocytes in the granule cell layer show calcium changes in response to neuromodulators (Sardar et al., 2023). However, whether astrocytic calcium changes are representing odor identity, particularly at the granule cell layer, remains unknown.

To represent an odor precisely, it is essential for it to elicit a consistent neural response, and this neural response must distinctly differentiate between various odors. For example, calcium signals recorded from M/T during the presentation of different odors can be used to decode the identity of the odors, although worse than spike activity (Xu et al., 2021). To study whether calcium signals in astrocytes can contribute to the representation of an odor, we recorded cytosolic calcium changes of the astrocytes (with a genetic calcium indicator, GcAMP6f) in the granule cell layer during presentation of either a cotton swab impregnated with the odor of a stressed familiar conspecific, or wet with saline (**Fig 1a,b**). Upon odorant presentation, the increase in cytosolic calcium was higher when presented with a cotton swab with odors than a cotton swab with stress (**Fig 1c,d**). This difference did not depend on the order of the presentation of the stimulus.

Experiments in which the same odor is repeated several times could offer more information whether these responses are consistent across presentations, but the present data suggests that astrocytes could contribute to the encoding of the stress odor and saline representations in the granule layer of OB.

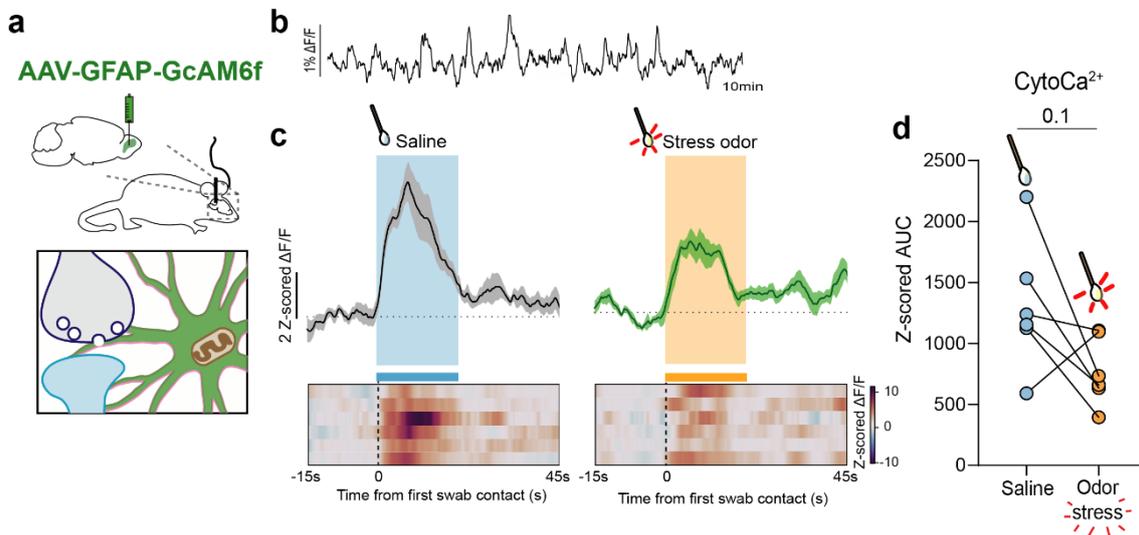


Fig. 1: Astrocytes respond differently to the odor of a familiar stressed partner than to a saline swab

a) A cytosolic calcium genetic indicator (GcAMP6f) was expressed in the astrocytes of the granule cell layer of the olfactory bulb of mice, that were exposed after recovery to a swab with saline or the odors of a familiar stressed partner to analyze their calcium responses using fiber photometry. b) Representative trace of the fiber photometry signal of a mouse expressing GcAMP6f in astrocytes of the OB. c) Z-scored $\Delta F/F$ from animals exposed to a swab with saline or with the secretions of a stressed familiar partner (stress odor). Dotted line represents the onset of the first contact with the swab. d) AUC during the time of the odor exposure (blue and orange rectangles) of animals exposed to saline or stress odor. Two tailed paired *Student* t-test, $p = 0.101$; $n = 6$. Data are represented by mean \pm SEM.

Astrocytic mitochondria show specific responses to different odors

The data presented in this Thesis work shows that astrocytic mitochondria higher calcium responses to the stress odor of a familiar conspecific in comparison to saline. To further study the meaning behind these different responses, that we propose that is related to the salience of the odor, we recorded

astrocytic mitochondrial responses in mice during the exposure of other social odors, like female anogenital odor (female odor) or the odor of a stranger male mouse of the same strain (stranger odor) (**Fig 2a**). While the odor of a stranger did not appear to affect mitochondrial calcium different than the saline (although the data shows individual variability), the female odor induced a higher response in all of the mice (**Fig 2b-e**). This data would support the hypothesis that mitochondrial calcium signals in the astrocytes contribute to the representation of specific relevant odors, or odorant blends, but not of others.

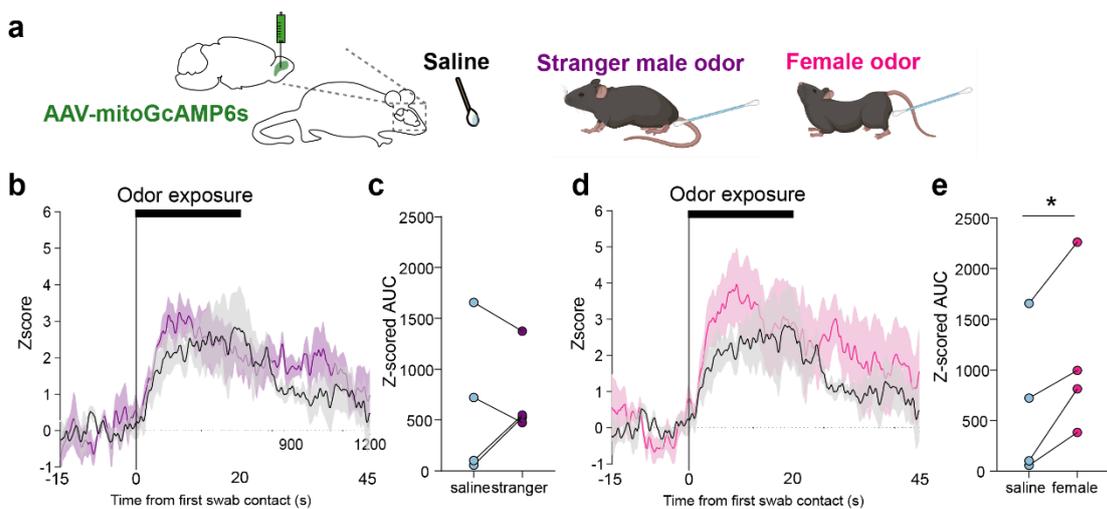


Fig. 2: Astrocytic mitochondria show specific responses to different odors

a) Mice expressing mitoGcAMP6s in the astrocytes of the olfactory bulb were exposed to saline, strange male odor and female anogenital odor consecutively, while the mitochondrial calcium responses were recorded. b) Z-scored $\Delta F/F$ from animals exposed to a swab with saline (black) or with the odor of a stranger male mouse (purple). Line represents the onset of the first contact with the swab, dark line the duration of the odor exposure. c) AUC during the time of the odor exposure animals exposed to saline or the odor of a stranger, Two tailed paired *Student t*-test, $p = 0.678$; $n = 4$ d) Z-scored $\Delta F/F$ from animals exposed to a swab with saline (black) or with the odor of the anogenital region of a female mouse (pink). Line represents the onset of the first contact with the swab, dark line the duration of the odor exposure. d) AUC during the time of the odor exposure of animals exposed to saline or female odor. Two tailed paired *Student t*-test, $p = 0.101$; $n = 4$. Data are represented by mean \pm SEM

Odor exposure induces phasic endocannabinoid release in the olfactory bulb

In the olfactory bulb, M/T cell activity is modulated by depolarization-induced suppression of inhibition (DSI) at the glomerular layer and depolarization-induced suppression of excitation (DSE) at the granule cell layer (Pouille and Schoppa, 2018; Soria-Gómez et al., 2014a). These two types of synaptic plasticity are modulated by eCBs (Terral et al., 2020), which suggest the idea that phasic endocannabinoid release modulates dynamic olfactory processes. Both neurons and astrocytes (Stella, 2004) have the machinery to produce eCBs. In this context, the use of novel sensors for eCBs *in vivo* can help to understand the interplay between activity-evoked endocannabinoid release and the modulation of brain functions by CB1 receptors. However, no research has been done on the contribution of phasic endocannabinoid modulation to olfactory functions.

Following the results presented in Chapter 1 of this Thesis, we hypothesize that neuronal activation upon odor-presentation would induce endocannabinoid release, that would in turn modulate astrocytic mtCB1 receptors. To this question, we used mass spectrometry to detect 2-AG and AEA levels in the brains of observers of neutral or stress partner, which were sacrificed after the social interaction (**Fig 3a**). We did not observe differences between the two groups, but our results were in line with the results published by Soria-Gomez and colleagues that observed higher levels of 2-AG than AEA in control mice (Soria-Gómez et al., 2014: **Fig 3b**). Olfactory processing occurs at microcircuits, that work together to generate an olfactory representation (Imai, 2014). It is likely that neuromodulation occurs locally at those microcircuits, and changes are not reflected in eCBs bulk measurements. Thus, we expressed the sensory GRAB_{eCB2.0}, which consists of a circular-permuted EGFP and the human CB1 cannabinoid receptor, in the neurons of the OB granule cell layer (using the Syn promoter) (**Fig 3c**). Signals recorded from the olfactory bulb reflected spontaneous endocannabinoid events, and evoked by odor presentation (**Fig 3c**). However, the exposure of saline or stress odor swabs to naïve male mice induced similar eCBs level change (**Fig 3d-e**) in the neurons of the OB. This

would suggest that, even though both stimuli induce endocannabinoid release, eCB-modulation of astrocytic mtCB1 receptors is specific for some odors.

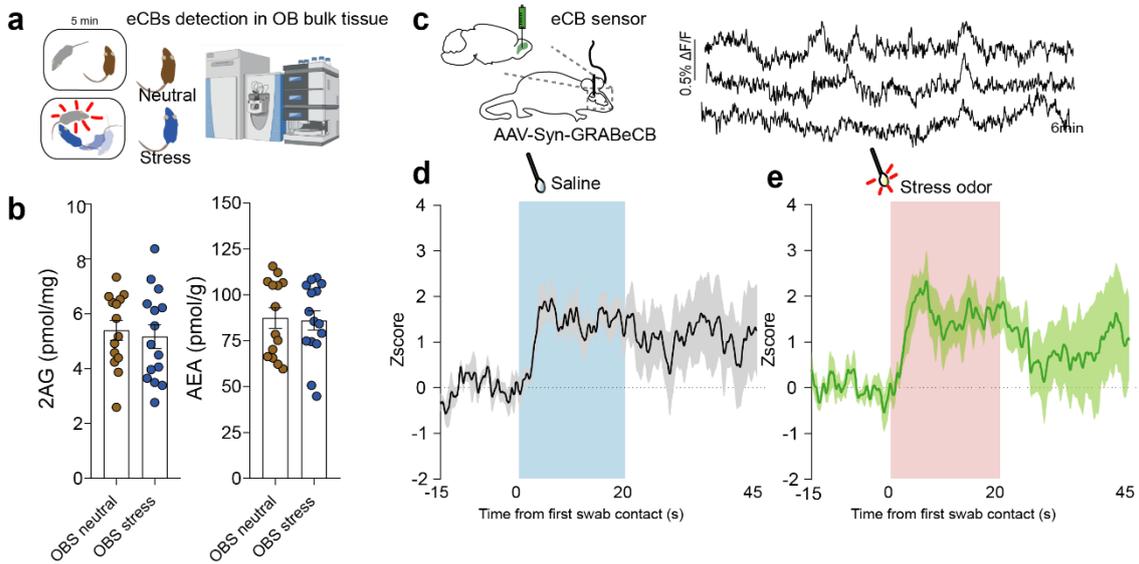


Fig. 3: Odor exposure induces phasic eCBs release in the olfactory bulb
 a) Brains of observers were shock-froze after STS, and their eCBs levels were checked in the olfactory bulb with mass spectrometry. b) 2-AG and AEA levels in the *bulk* tissue of the olfactory bulb in neutral or stress observers. n=18-20. c) Schematic representation of the viral strategy to record eCBs *in vivo* using AAV-Syn-GRABeCB, with representative traces (red stress odor presentation, blue saline presentation), d) Z-scored $\Delta F/F$ on eCBs signals upon presentation of a saline swab. n=5. e) Z-scored $\Delta F/F$ on eCBs signals upon presentation of a stress swab swab, n=5. Data are represented by mean \pm SEM

2-SBT, a putative alarm chemosignal mediating social transmission of stress.

Natural odors usually are composed by a complex mix of volatile and non-volatile chemicals, in which the identities and ratios of the constituents are important by odor-mediated behaviors. Based on the results of the first chapter of this Thesis, the odor emitted by a stressed mouse is most likely an odorant blend that induces some stress-like responses but also social interest, as anogenital contacts are persistent during the social interaction test. We hypothesize that 2-sec-butyl-4,5-dihydrothiazole (SBT), a chemical identified as

an innate alarm chemosignal in mice processed by the main olfactory bulb that induces aversive responses similar to predator odors and an increase in plasma corticosterone levels (Brechbühl et al., 2013; **Fig 4a**), could be part of the “stress odor” blend. To explore this idea, we exposed naïve mice expressing mitoGcAMP6s in the astrocytes of the main olfactory bulb to a cotton swab with saline (saline) and a cotton swab impregnated with 15uL of 1:100 2-SBT dilution in saline (Matsuo et al., 2015b), consecutively (**Fig 4b**). The two swabs induced different mitochondrial calcium responses in OB astrocytes (**Fig 4c-d**). Interestingly, the signals showed different dynamics, as 2-SBT induced consistently longer responses post-stimulation in comparison to saline (**Fig 4e-f**).

The stress odor coming from a shocked conspecific induced a cognitive impairment in the NOR test, mediated by astrocytic CB1 receptors. We hypothesize that 2-SBT could recapitulate the cognitive adaptations to stress of odors in the same way. To address this, we tested OB-GFAP-CB1-KO (mice lacking CB1 receptors in the astrocytes of the olfactory bulb) and control mice in the NOR test after exposure to a cotton swab impregnated with 2-SBT (**Fig 4f**). As expected, control mice were impaired in the NOR after 2-SBT exposure (**Fig 4g-h**). Moreover, OB-GFAP-CB1-KO were immune to the effects of 2-SBT in cognition (**Fig 4g-h**). These results suggest that 2-SBT could be a component in the stress odor inducing astrocytic CB1-mediated cognitive adaptations (Figure in next page).

The results presented in this Chapter will be further discussed in the general discussion, since they are preliminary and better understood in the context of the whole Thesis work. For details about methods, please refer to Chapter 1 – Fiber photometry and Odor-dependent social transmission of stress or the legends of the figures.

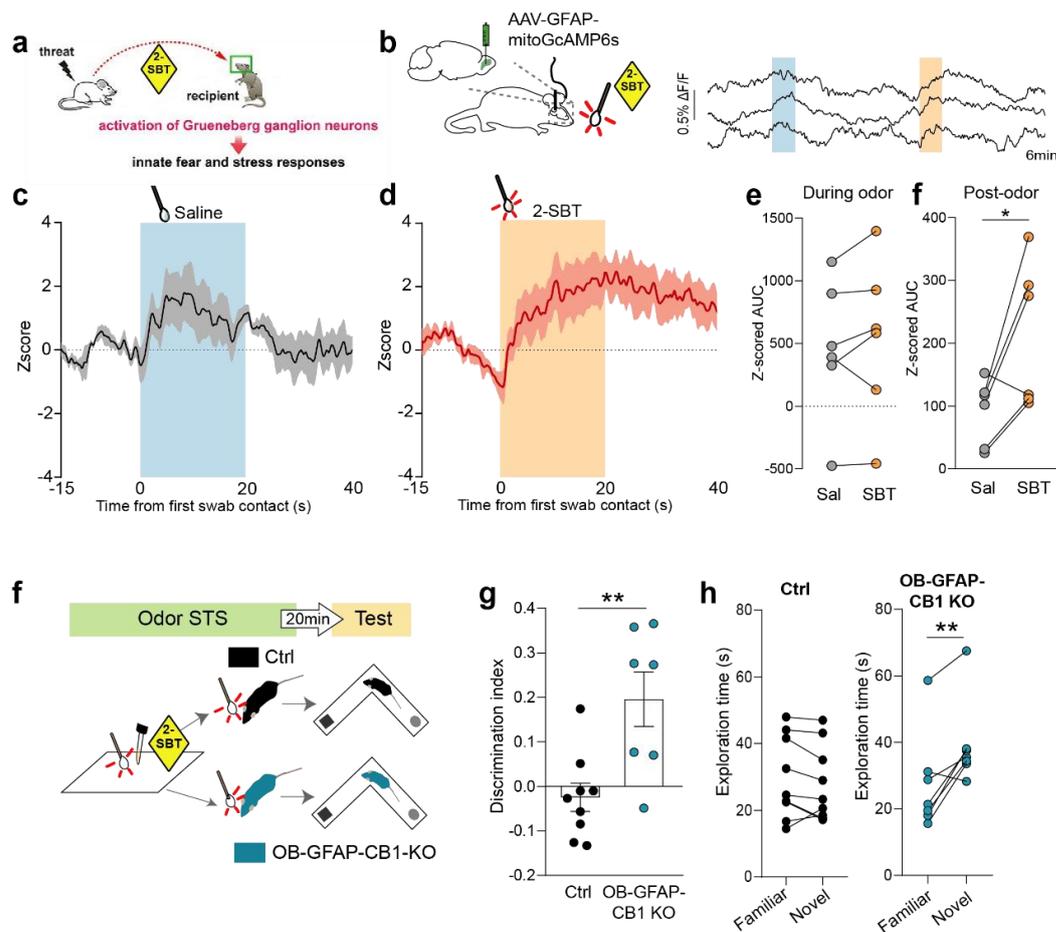


Fig. 4: 2-SBT induces mitochondrial calcium responses and astrocytic CB1-mediated cognitive impairment

a) 2-SBT is produced by mice in alarm conditions, detected by a specific olfactory system, the Grueneberg ganglion, and induces fear and stress responses b) Mice expressing mitoGcAMP6s in the astrocytes of the olfactory bulb were exposed to saline or 2-SBT 1:100 for 20 seconds, while the mitochondrial calcium responses were recorded. c) Z-scored $\Delta F/F$ on mitochondrial calcium signals upon presentation of a saline swab. d) Z-scored $\Delta F/F$ on mitochondrial calcium signals upon presentation of a swab impregnated with 15 μ L of 2-SBT 1:100 e) AUC of the odorant presentation period of saline and 2-SBT (0-20s) f) AUC of the period post-odorant presentation (20-40s) of the odorant presentation period of saline and 2-SBT. Two tailed paired *Student* t-test, $p = 0.04$, $n = 6$. f) Schematic diagram of the odor-mediated social transmission of stress by exposing mice to 2-SBT and then subsequent testing in the NOR. g) Discrimination index of control (Ctrl), and mice lacking CB1 receptors in the astrocytes of the bulb (OB-GFAP-CB1-KO) in the NOR after 2-SBT exposure. Two tailed unpaired *Student* t-test, $p = 0.0041$ h) Exploration time in seconds of either familiar or novel objects of both Ctrl

and OB-GFAP-CB1-KO mice in the NOR after 2-SBT exposure. Two tailed paired *Student t*-test (OB-GFAP-CB1-KO), $p = 0.0083$, n (Ctrl) = 9, n (OB-GFAP-CB1-KO) = 7.

IV. GENERAL DISCUSSION

DISCUSSION

In stressful situations, survival mechanisms override most other functions, including cognitive abilities not directly related to the danger itself. An individual's stress can be transmitted to others through social olfactory interactions. However, the consequences of this transmitted stress on cognitive functions and the underpinning cellular and molecular mechanisms were not addressed. The present Thesis work focused on unraveling the neurobiological basis of social transmission of stress and its cognitive adaptations. In this context, we observed that the consequences of social transmission of stress in a non-emotional memory test were similar to those observed after direct stress experience. Moreover, using genetic approaches, this work demonstrated that specific social behaviors necessary for the detection of stress olfactory signals and the subsequent cognitive adaptations require astrocytic mitochondrial CB1 receptors in the olfactory bulb. At the molecular level, these behaviors rely on calcium handling by astrocyte mitochondria. Thus, the detection and processing of social odors that signal the affective state of others can cause cognitive alterations, linking olfactory experience to non-odor-related mental functions. These data imply that smelling certain odors in absence of other stimuli can impact unrelated cognitive abilities. Moreover, they reveal a fully unexpected role of astrocytic mitochondria in the olfactory bulb to detect and process specific stress-related odorants, in order to modulate cognition.

1. The adaptive value of learning from others

Sociability evolved because it can provide advantages favoring survival and reproduction (Demetrius and Ziehe, 2007). Part of sociability is the capacity to recognize and comprehend the negative states of fellow beings. This "empathetic awareness" not only fortifies the bonds within a community, but it also enhances the overall adaptation of individuals towards the environment. In other words, observing and understanding the stress signals from others can serve as valuable source of information, helping individuals adapting their own behaviors to different situations. Both humans and rodents can react to the stress of others (Dalton et al., 2013; Dimitroff et al., 2017; Endevelt-Shapira et al., 2018; Ferretti et al., 2019; Rogers-Carter et al., 2018; Sterley et al., 2018). In mice, this social

transmission of stress modulates similar physiological functions in both stressed animals and partners interacting with them, like changes in synaptic plasticity of specific neurons (Lee et al., 2021; Sterley et al., 2018). Social odors associated with stress emotional states mediate this type of communication (Lee et al., 2021; Sterley et al., 2018). Our results show that, by reacting to stress odors from others, mice can adapt their behavior in a cognitive task unrelated to the odor itself, suggesting that stress signals carry valuable information to improve the survival of conspecifics.

Stress induces a whole-brain response that alters hormonal levels, neuronal functions and behavioral responses (Joëls and Baram, 2009). These changes can consist in the alterations of several cognitive functions, including those related to decision-making, learning and memory and emotional responses (Joëls and Baram, 2009). Acute stressors are accompanied with a rapid surge of neurotransmission, neuronal activation and hormone release, and their cognitive adaptations can last for days (Sandi, 2013). Among the effects of stress on cognition, recent research focused on different aspects of memory, such as its different phases (e.g. acquisition, consolidation, retrieval), its temporal features (e.g. short- or long-term memory) and its types (explicit, implicit, goal-directed, habits, etc.) (Joëls et al., 2006; Sandi, 2013). The classical idea is that memory is regulated by stress in an inverted-U shape fashion: both low and high levels impair memory performance (Sandi, 2013). This, however, seems to only apply for complex tasks that require undivided attention, such as working memory, decision making or multitasking (Diamond et al., 2007). However, emotional conditioned responses are linearly correlated with stress intensity: the higher the stressor, the better the performance (Sandi, 2013). Conversely, the performance of non-emotional tasks like consolidation and retrieval of novel object recognition (NOR) appear to be inversely correlated with stress (Busquets-Garcia et al., 2016; Skupio et al., 2023b). The NOR is a task that relies on mice natural exploratory behavior in the absence of external reinforced or conditioned stimuli, to investigate episodic non-emotional memory functions (Antunes and Biala, 2012). Despite the potential involvement of neocortical areas (Balderas et al., 2013), NOR induces synaptic plasticity in the hippocampus, and it is abolished by ablation of this brain region and by local injection of a NMDAR antagonist

(Laurie), indicating that hippocampal circuits are required for this type of memory. Our results show that social transmission of foot-shock stress impairs cognitive performance in the NOR, as observed in direct stress experience while social interaction with a “neutral” partner did not. This suggests that the detrimental effects on NOR induced by stress in foot-shocked male demonstrators are also phenocopied in observers after social interaction. However, corticosterone (CORT) levels are increased to the same level in stressed and neutral pairs of demonstrator/observer (Sterley et al., 2018), indicating that CORT levels are not predictive of social transmission of stress, but NOR performance is. This goes in line with the results showing that plasticity at PVN^{CRH} is only present in stressed observers (Sterley et al., 2018), as well as CA1 hippocampal LTD (Lee et al., 2021). Interestingly, in females, PVN^{CRH} synaptic plasticity was induced by socialization with neutral or stressed demonstrators (Sterley et al., 2018). This phenomenon could relate to the proposed higher sensitivity to stressors of females in comparison with males (Senst et al., 2016). This suggests that sexual hormonal states likely modulate both neuronal and cognitive effects of social transmission of stress. In this frame, the perceived levels of stress might determine different effects in observers. Future studies exposing male and female demonstrators to rising levels of stress will address this possibility. Indeed, similar mechanisms might contribute to choosing the most adequate behavioral response according to the level of danger communicated by the demonstrator.

The literature reviewed in the introduction showed examples on how the transfer of fear- or stress-related information is subserved by different circuits and sensory modalities, depending whether the observer interacts with the demonstrator at the moment of receiving the emotional triggering stimulus or later (Andraka et al., 2021; Jeon et al., 2010; Sterley et al., 2018). Thus, the interaction with an aroused conspecific can carry information about spatially and temporally distant events, which in turn modify in different ways the behavior of the receiver. For example, the observation of a partner receiving foot-shock in a shuttle box triggered passive aversive responses in rats whereas the pairing of rats with partners that recently received shocks (remote threat) increased in social exploration with barely any freezing (Andraka et al., 2021). The theory of predator imminence continuum postulates that an animal selects a specific defensive

response based on the proximity of the threat (Fanselow, 2022; Fanselow and Lester, 2020). In this frame, I propose that a similar theory might apply to the consequences of social transmission of negative affective states. While experiencing a threat or aversive stimulus, a demonstrator shows specific innate behaviors that observers can visually copy (Keum and Shin, 2019c). On the other hand, an observer interacting with a demonstrator that has been previously threatened (Fanselow, 2022) won't display threat-related behaviors, but other responses mostly triggered by chemosignaling (Brechtbühl et al., 2008; Kiyokawa et al., 2004; Sterley et al., 2018), such as approach/avoidance or prosocial/helping actions (Karakilic et al., 2018; Knapska et al., 2010; Meyza et al., 2015; Rogers-Carter et al., 2018; Wu et al., 2021). Interestingly, these responses generally follow different modalities and a temporal pattern: whereas approach/avoidance occur earlier, prosocial behaviors like allogrooming takes longer to appear, at a moment when the threat exposure is farther in time. Therefore, as proposed in **Figure 11**, it is possible that social transmission of stress is a graded experience for observers, with threat intensity that is diluted with time. Experiments using different delays between stress of demonstrator and social interactions will address this possibility.

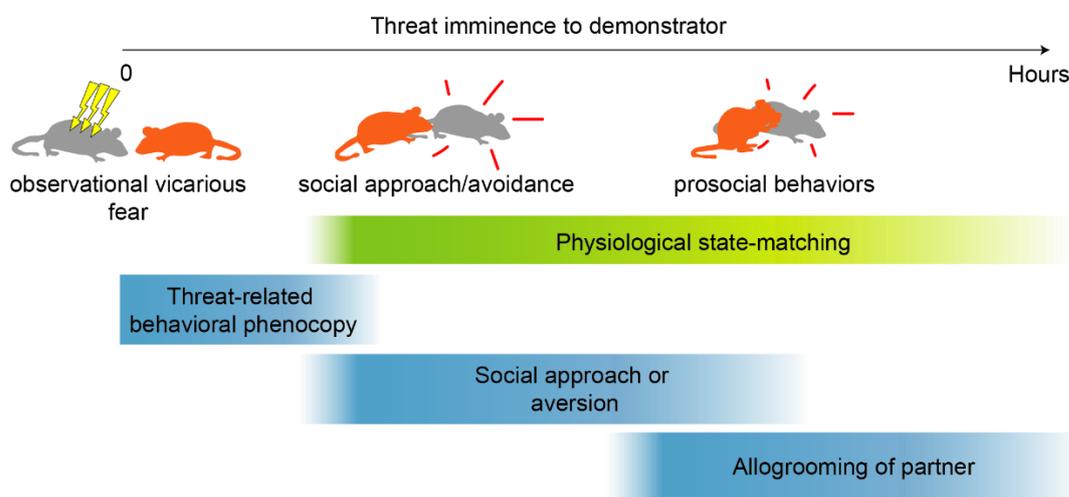


Figure 11., Threat imminence to demonstrator continuum. Adapted from the theory of Fanselow (Fanselow and Lester, 2020), I propose that social observers adapt their behavioral strategies to demonstrators depending on the time in which the demonstrator experienced the threat or stressor. When an observer witnesses a demonstrator receive a threat (time 0), the behavioral consequence of the socialization will be a phenocopy of the demonstrator's behavior that is guided by visual cues. If the socialization occurs with

a stressed demonstrator but in the absence of a threat, the responses of the observers will change depending on the delay between the threat-exposure to the demonstrator and the social interaction. In early stages of the social interaction, they might exhibit approach/avoidance behaviors and later exhibit prosocial behaviors, like allogrooming of the partner.

In support of this hypothesis, whereas social transmission of stress did not increase allogrooming during the 5-min test of our tests, Sterley and colleagues (2018) observed significant allogrooming during their 30-minute observation period. Figure 11 schematically summarizes the idea that the behavioral outcome of a social interaction with a stressed partner depends on the time elapsed from the stress experience. In other words, whereas observers tend to mimic threat responses of demonstrators when the interaction occurs close in time to the stressful experience, these responses tend to evolve with time into approach/aversion and eventually in prosocial allogrooming. This implies that the communication between demonstrator and observer is not fixed, but it changes over time and conditions. For instance, one study addressed the impact of stress related olfactory social communication on the modulation of the fear response freezing (**Figure 12**)(Finkelstein et al., 2022). Fear-conditioned mice were exposed to a familiar previously shocked conspecific in different manners, and then tested for fear recall. Animals were paired with the shocked familiar with a 1-way mirror division (in which the stressed partner could see them but not the opposite, but with access to auditory and olfactory signals), with an opaque wall or allowing full interaction. Only in the case of the 1-way mirror, fear-conditioned mice displayed increased freezing, suggesting that stressed partners were emitting specific olfactory or auditory signals. Following this idea, we could say that mice are able to produce different non-visual cues depending on their social context that could convey information to conspecifics concerning the features of the experienced stress.

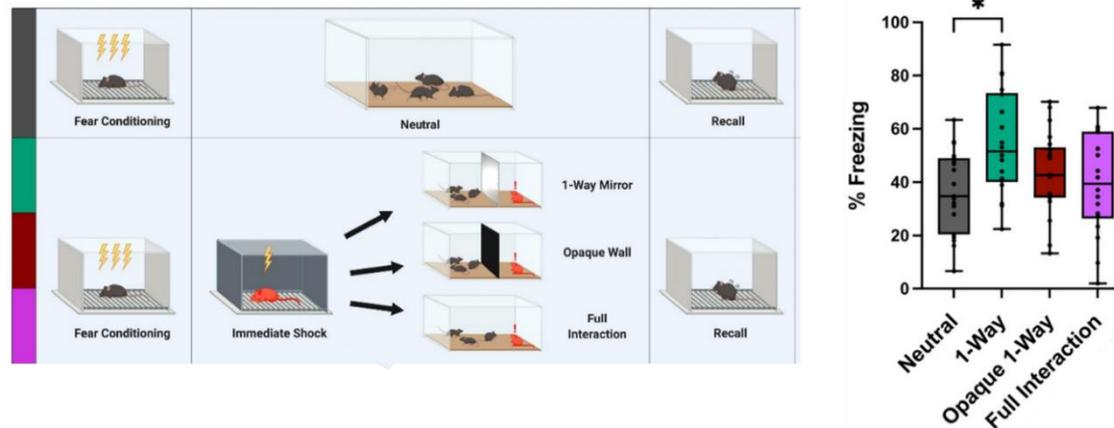


Figure 12: Adapted from (Finkelstein et al., 2022). (A) Schematic representation of the behavioral schedule. (B) Freezing levels during a 5-min fear recall test for male mice.

Social transmission of stress has relatively recently started to be investigated, and the data available do not allow concluding general rules concerning its modes and constraints. However, it is already clear that this is a complex and modular phenomenon that can be regulated and adjusted to different conditions, thereby displaying strong flexibility and high adaptive value.

2. CB1 receptors in the context of social functioning.

Social behaviors shape the way we interact with our environment (Karlson et al., 2016). The endocannabinoid system is involved in social behaviors (see Chapter 5), although it is still unclear how. The use of global genetic deletion of CB1 receptors in social tasks provide variable results (pro- or anti-social phenotypes) that might depend on specific experimental conditions (Häring et al., 2011; Jacob et al., 2009; Terzian et al., 2014). Conversely, the modulation of endogenous cannabinoid levels have provided more consistent results, with increased levels very often associated to enhanced social interactions (Servadio et al., 2016; Wei et al., 2017). Furthermore, other functions that impact social behavior, such as anxiety, motivation, reward, or learning and memory, are also affected by CB1-mediated regulation (Karlson et al., 2016).

Our results suggest that astrocytic and mitochondrial CB1 receptors play specific functions in social communication of stress, as body exploration was not changed while exploring a stressed partner, and neither did any social parameters during interaction with a neutral one. However, we observed other

phenotypes in mice lacking glutamatergic CB1 receptors that could also be linked with social functioning. Glu-CB1-KO mice had a deficit in anogenital investigation, body exploration and allogrooming. These mice have been reported to be impaired in several social tests, like direct social interaction with a stranger (Jacob et al., 2009c) or the 3-chamber test (Häring et al., 2011). Furthermore, CB1-positive glutamatergic projections from insula to NAc control social approach towards stressed juveniles in rats (Rogers-Carter et al., 2019). These published studies could explain the results we observed in STS using Glu-CB1-KO mice. However, glutamatergic CB1 receptors control novelty seeking (Lafenêtre et al., 2007) and behavioral responses to stressors like shock or force swimming (Kamprath et al., 2006; Steiner et al., 2008). In this context, glutamatergic CB1 receptors could impact social transmission of stress by altering related processes, such as anxiety or stress-reactivity. Experiments manipulating glutamatergic CB1 receptor subpopulations in STS and other stress-related behavioral paradigms would determine the specificity of the phenotypes presented here. Nevertheless, a synergistic role between CB1 in astrocytes and glutamatergic neurons in different brain regions could be involved in different aspects of social transmission of stress, namely detection and process, or behavioral expression.

Impairments of social functions, such as deficits in social emotional reciprocity or in nonverbal communication, are in the core of sociopathologies like autism spectrum disorder (DSM-5; Karhson et al., 2016). Endocannabinoids activating CB1 receptors can rescue some autistic-like phenotypes seen in mouse models of autism (Hosie et al., 2018b; Wei et al., 2016). The ASD-like BTBR mice have specific deficits in social interaction with stressed partners, in presence of otherwise normal social behaviors (Meyza et al., 2015). These mice therefore represent a specific model of the most common symptom in ASD, namely social emotional reciprocity deficits. We observed that social emotional reciprocity in mice is controlled by astrocytic CB1 receptors, that modulate anogenital exploration and subsequent cognitive adaptations. Interestingly, I performed some experiments showing that SHANK3-KO mice (Song et al., 2019) mode spend less time investigating the anogenital region of stressed partners (data not shown). Given the relationship between SHANK3 and the

endocannabinoid system (Folkes et al., 2020; Wang et al., 2017), we could hypothesize that modulation of astrocytic CB1 receptors might impact the capacity of SHANK3 to react appropriately to stressed congeners. This preliminary hypothesis opens the door to further studies of CB1 receptors in the context of empathic-like behaviors altered in pathology.

3. Circuitry mediating social transmission of stress and its cognitive adaptations.

Social transmission of stress involves OB processing of nonvolatile (or low-volatile) stress odorants (present results), activation of hypothalamic PVN^{CRH} neurons (Sterley et al., 2018) and of specific insular connections (Rogers-Carter et al., 2018a), and plasticity in CA1 hippocampal synapses (Lee et al., 2021). Moreover, the results of this Thesis show that STS leads to impairment of NOR, a mostly hippocampal-dependent, non-aversive and non-olfactory cognitive test (Antunes and Biala, 2012; Robin et al., 2018). How is it possible that the processing of a specific odor can lead to the engagement of brain regions that are so distant? Sterley et al observed that a separation with a Plexiglass with holes inhibited STS expression of synaptic plasticity, proposing the idea of this chemosignal to not be volatile (Sterley et al., 2018). Usually, nonvolatile odors are processed by the VNO (although not exclusively as observed with MHC peptides), which projects to the AOB sending in turn projection to emotion-related regions like the medial or posterior cortical amygdala or the BNST (Hussain, 2011). Surprisingly, however, STS and its cognitive consequences are altered by viral manipulations targeting the MOB, indicating that the stress odor is processed by this system. Interestingly, mutants lacking astrocytic CB1 receptors in the OB did not react to 2-SBT, a putative alarm chemosignal processed by the MOB that induces innate aversive responses upon presentation, and were immune to the effects of this odor in NOR. This supports the hypothesis that specific MOB circuits controlled by astrocytic CB1 are able to mediate the effects on cognitive performance of social alarm odors. However, the profile of MOB astrocyte mitochondrial calcium dynamics induced by exposure to 2-SBT appears to be different (more persistent in time) than the one induced by anogenital secretions of stressed partners (compare **Fig. Chapter 1 -3c** and **Chapter 2 – 4c,d**). This suggests that, if 2-SBT is indeed one component of stress odors from

demonstrators, other chemicals are likely present and able to modulate cellular responses in the MOB.

After being processed in the MOB, the information relayed by the stress odor(s) is transferred towards other brain regions, of which PVN and hippocampus are the only ones surely identified so far (Lee et al., 2021; Sterley et al., 2018). This implies the existence of a putative network involving the MOB, the PVN and the hippocampus. It is currently impossible to identify all the stations linking this network. However, three main brain regions might act as hubs of this network, because they are involved in emotion processing and are simultaneously connected to MOB, PVN (particularly CRH-positive neurons) and hippocampal CA1 region.

The medial amygdala is located in the intersection between main and accessory olfactory systems, and receives inputs from key brain regions like the BLA, hypothalamus, hippocampus, VTA or PFC (Dwyer et al., 2022; Sotoudeh et al., 2022). The neurons in this region display specific responses tuned to positive and aversive social stimuli, thereby eliciting consequent behaviors (Chen et al., 2019; Demir et al., 2020; Hong et al., 2014; Nordman et al., 2020; Shemesh et al., 2016). This suggests that this region is not only a relay station for social olfactory information but could act an integration hub for sensory information and valence-encoding processes to decide an appropriate response and relay it to the specific downstream areas. In this context, valence-encoding projections from mPFC or hippocampus to BLA (Huang et al., 2020; Redondo et al., 2014) could assign appetitive or aversive values to social information in the MeA based on the animal past experience (familiarity, own emotional state). Furthermore, MeA sends direct projections to the entorhinal cortex and the ventral hippocampal CA1 (Pardo-Bellver et al., 2012), suggesting that a bidirectional modulation between MeA-hippocampus activity could be beyond the hippocampal-dependent cognitive adaptations in response to social transmission of stress.

The lateral entorhinal cortex is a direct output region of the MOB and projects to the dorsal hippocampus (Li et al., 2017b; Lopez-Rojas et al., 2022; Salimi et al., 2022). As mentioned, social transmission of stress was associated with meta plasticity at dorsal CA1 synapses (Lee et al., 2021). Interestingly,

projections from the lateral entorhinal cortex to dorsal CA1 mediate olfactory associative learning (Li et al., 2017b). Furthermore, the connectivity between the OB-entorhinal cortex-hippocampus is synchronized in the form of oscillations that are seen during olfactory-related tasks like odor go/no-go tests (Gourévitch et al., 2010; Martin et al., 2007). This data suggests that olfactory information can influence not-odor-related cognitive functions by sending information to the entorhinal cortex which in turn might modify CA1 hippocampal activity.

Retrograde trace labeling of PVN^{CRH} revealed that these neurons receive inputs from the cortical amygdala, and in particular from its posterolateral portion (PLCo) and the piriform transition area (AmPir)(Kondoh et al., 2016). The PLCo receives projections from the MOB and bidirectionally modulates the innate behavioral responses to predator chemosignals and attractive nonsocial odors (Root et al., 2014). On the other hand, the AmPir, located between the piriform cortex and the anterior cortical nucleus of the amygdala (Pardo-Bellver et al., 2012), is also activated by several types of predator odors, and its inactivation blocks the raise of CORT levels, but not the freezing, induced by these odors (Kondoh et al., 2016). While the PLCo is known to project to CA1 directly (Kemppainen et al., 2002), connections between AmPir and hippocampus have not yet been reported. This anatomical data could suggest a possible parallel but distinct circuit from the MOB to the PLCo and AmPir independently modulating the behavioral and endocrine responses to the stress odor of conspecifics, explaining why a CORT increase is not always predictive of the behavioral outcome associated with social transmission of stress (Sterley et al., 2018).

Instead of a parallel circuit mediating social transmission of stress effects on PVN and hippocampus activity independently, it is also plausible that the activation of CRH neurons affect hippocampal activity directly. The hippocampus has both CRH1 receptors (Bagosi et al., 2023) and CRH-positive neurons, which represent a subset of GABAergic interneurons that have been involved in the modulation of object recognition memory (Hooper et al., 2018). Interestingly, CRH1 receptors modulate hippocampal changes centrally in response to chronic stress, independently of CORT levels (Ivy et al., 2010). CORT injections modulate NOR recognition retrieval through CA1 GABAergic interneurons, which could be recapitulated by a foot-shock (Skupio et al., 2023), supporting the idea

that transmission of stress could directly impact hippocampal CA1 functions through local CRH signaling.

Other regions might play important roles in different aspects of STS. The results presented in this Thesis showed that olfactory chemosignals impair NOR independently of the temporal length of exposure, as either 2 seconds or 20 seconds led to the same impairment in WT mice. This suggests that the stress odor conveys information in an all-or-none type of way. Interestingly, presentations of different concentrations of a fear odor to humans always led to the same responses (de Groot et al., 2021b). This might have an adaptive value, as rapidly understanding the danger when exposed to a stressed partner can favor fast responses. However, we observed that the anogenital exploration of a stressed conspecific is sustained in time, with a higher frequency at the beginning of the interaction and other sparse contacts throughout. This suggests that (i) the stress odor is not aversive *per se*, and (ii) that anogenital contacts are likely reinforced, as the animals continue doing them after already having received the stress-related information. In this context, the nucleus accumbens, which modulates motivated approach social responses (Gunaydin et al., 2014; Le Merrer et al., 2023), might be behind the STS-associated sustained social interaction. Interestingly, attractive odorants induce approach responses via MOB-olfactory tubercle projections that are mediated by dopaminergic signaling (Midroit et al., 2021). The olfactory tubercle interacts with the reward system by sending projections to both the nucleus accumbens and the VTA (Wesson and Wilson, 2011). Furthermore, insula to nucleus accumbens projections mediate social approach to stressed juveniles in rats (Rogers-Carter et al., 2019). This is thought to be mediated by a connection of the BLA to posterior insula (Djerdjaj et al., 2022)d. This anatomical data suggests the existence of both a bottom-up (from the MOB, perhaps involving astrocytic mtCB1 receptors) and top-down (from Insula) control of the NAc-dependent motivational aspects of interacting with a stressed conspecific. This would paradoxically suggest that stress odors can be rewarding.

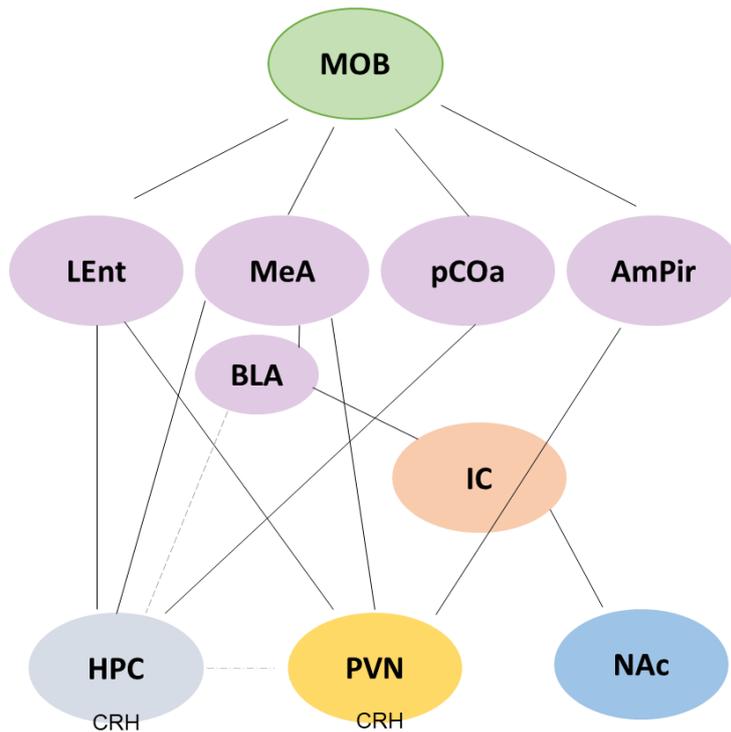


Figure 11: Brain regions possibly involved in social transmission of stress. Lines are direct characterized connections (grey lines putative ones). MOB, main olfactory bulb; LEnt, lateral entorhinal cortex; MeA, medial amygdala; pCOa, posterior cortical amygdala; AmPir, amygdaloid piriform transition area; BLA, basolateral amygdala; IC, insular cortex; HPC, hippocampus; PVN, paraventricular nucleus of the hypothalamus; NAc, nucleus accumbens; CRH, corticotropin-releasing-hormone positive neurons.

Astrocytic mitochondrial calcium regulation of olfactory processing: meaning and mechanisms

Astrocytic-CB1 mediated mitochondrial calcium responses are necessary for the correct processing of stress odors and its subsequent cognitive effects. Astrocytic mitochondrial calcium signals recorded by fiber photometry in the MOB are higher in response to stress odor than to saline. Indeed, increasing cytosolic calcium in astrocytes impairs odor discrimination, which suggests that these cells are involved in different responses depending on the nature of the odor. Furthermore, preliminary data shows that other odors associated with salient stimuli, like female urine, modulate specific mitochondrial calcium responses, while the effects of other odors do not differ from saline. This suggests that mitochondrial calcium signals in the astrocytes of the bulb are not involved in the encoding of the odor identity, but could be encoding some of its characteristics, like salience of affective value. In line with this idea, electrophysiological experiments suggested that the affective value of an odor is associated with specific M/T cell responses (Doucette et al., 2011). Moreover, manipulations of astrocytic calcium affects performance of a rewarded odor discrimination Go/No-Go task (Ung et al., 2020). This supports a role for the early encoding of complex

odorant characteristics in the MOB, which could be reflected in astrocytic activity, that directly modulate M/T. Technical limitations are to be taken into account while interpreting fiber photometry data, particularly related to the lack of single cell resolution. An increase in the mitochondrial calcium signal could mean two things: (i) a recruitment of a bigger number of astrocytic mitochondria or (ii) a higher calcium activation in each mitochondrion. As this might have an impact on understanding the cellular processes associated with STS (e.g. modulation of the same circuits or recruitment of additional ones), future experiments will use single cell imaging tools to investigate the details of these processes.

In our hands, the exposure to a stress odor induces a higher response in astrocytic mitochondrial calcium than a saline swab. However, the preliminary analysis of cytosolic calcium signals in the astrocytes showed the contrary effect: saline swab exposure induced a higher increase of calcium in comparison to the stress odor exposure. As discussed above, this opposite regulation of cytosolic vs. mitochondrial calcium levels cannot so far be ascribed to any specific cellular process (e.g. are the same astrocytes involved or not?). However, it could reflect a simple buffering function of mitochondria in the MOB. In other words, where mitochondrial calcium increases, cytosolic one decreases and viceversa. However, the only data available concerning the role of mtCB1 receptors indicate that the relationship between the effects of cannabinoid signaling on the two compartments are more complex.

Astrocytic mitochondrial calcium uptake is coupled to IP₃-induced calcium release, shaping cytosolic calcium dynamics, which determine the astrocyte “excitability” (Eraso-Pichot et al., 2017; Li et al., 2014). However, we know that our mitochondrial calcium increase is mediated by mtCB1 in astrocytes. In a previous publication of the team, Serrat and colleagues showed that mtCB1 receptors in astrocytes control mitochondrial calcium increases but they do not affect cytosolic signals. Moreover, blocking MCU transport did not affect the amplitude of CB1 receptor-dependent control of cytosolic calcium, but it decreased the spatial and temporal dynamics of the signals. The effect of mtCB1 receptors is mediated by IP₃ receptors, likely at mitochondrial-ER contact sites (MERCs). Interestingly, a similar effect was observed in the OSNs of the MOE: while the amplitude of the cytosolic signals did not change with MCU inhibition,

the signals became longer in time (Fluegge et al., 2012). Moreover, mitochondrial calcium in OSNs is necessary for the increasing firing pattern of these cells upon the exposure to increasing odor concentrations. When mitochondrial functions are impaired, OSNs work as simple stimulus detectors rather than as intensity encoders. Although the mechanisms that regulate neuronal responses can hardly be extrapolated to astrocytes due to the different biophysical principles that control them (Semyanov et al., 2020), together this data implies a role of mtCB1 modulation in astrocytes that could be regulating calcium in microdomains, and the propagation of these signals in active synaptic sites.

In this context, one type of plasticity is dependent on mitochondrial calcium, the astrocytic-mediated lateral synaptic potentiation (Serrat et al., 2021). In this type of plasticity, neuronal depolarization induces eCBs which leads to neuronal DSE in that synapse but also binds to astrocytic CB1 receptors, that modulate calcium signal increases and glutamate gliotransmission to distant synapses, inducing excitatory short term potentiation (Covelo and Araque, 2016). This phenomenon did not occur at hippocampal synapses in DN22-CB1 mice not expressing mtCB1 but also after blocking MCU function. The lateral regulation by of both inhibition and excitation of synapses that are close by would represent a nice example on how odor discrimination could be modulated in the mitral cell layer. In this layer, granule cells control lateral dendrodendritic inhibition of mitral cells (Yokoi et al., 1995). Astrocytes could represent a link between mitral and granule cells: mitral cell activity due to specific salient odors would induce eCBs release, which would lead to DSE in the cells associated with that odor, but would mediate potentiation of granule cell inhibition to other non-relevant mitral cells. Another scenario would involve astrocytes mediating cortical activity upon granule and mitral cells: eCBs mediated DSE from centrifugal projections onto granule interneurons (Pouille and Schoppa, 2018), so astrocytic mtCB1 activation could lead to a refinement of other close-by synapses, which could be short deep axon cells further inhibiting granule cells (Burton and Urban, 2015), or the potentiation of other granule cells modulating not relevant mitral cell noise.

In conclusion, although the exact mechanisms are far from being understood, mitochondrial calcium dynamics likely participate in the coding properties of astrocytes in the MOB, particularly in circuits determining the

saliency of specific stress odors. To further investigate this, experiments blocking the MCU when recording cytosolic and mitochondrial calcium signals could offer additional information of the contribution of mitochondrial buffering in these responses.

Future perspectives

The presented data of this Thesis shows that astrocytic processing in the MOB of specific odors related to the emotional state of the individual can affect cognitive functioning. However, the mechanism by which this happens remains to be completely elucidated.

First, the contribution of astrocytic mtCB1 receptors in the olfactory to M/T cell output activity should be addressed. This can be studied *ex vivo* and *in vivo*. In slices from the olfactory bulb, lateral synaptic potentiation of mitral cell activity could be assessed in WT and mutants lacking astrocytic mtCB1 receptors (OB-GFAP-mtCB1-KO) in the granule cell layer (Serrat et al., 2021). Moreover, using either fiber photometry or electrophysiological recordings, we could record mitral cell activity in both WT and KO mice. Interestingly, mitral cell activity calcium events and spikes can decode odor identity (Xu et al., 2021), so the results will tell us whether astrocytic mtCB1 can affect the encoding of odorant representations.

Secondly, the mechanisms by which mitochondrial calcium dynamics affect olfactory bulb astrocytic function remain underexplored. Using *in vivo* recordings in head-fixed mice, we could analyze calcium activation profiles of both astrocytes and neurons in the olfactory bulb through a cranial window, in response to the presentation of odors (Ung et al., 2020). Genetic manipulation of the MCU will determine whether blocking mitochondrial calcium transfer affects single-astrocyte responses to odors, and how this affects neuronal activity. Other types of genetic manipulations, such as the impairment of astrocytic mtCB1-mediated mitochondrial respiration control (using a non-phosphorylatable form of NDFUS4, a key subunit phosphorylated due to mtCB1 activation; Jimenez-Blasco et al., 2020), would explore the role of the other characterized function of astrocytic mtCB1 receptors, the regulation of bioenergetics, to odorant processing.

Lastly, the interaction between the hypothalamus, hippocampus and olfactory bulb in the processing of transmitted stress odors is not clear. Using multi-site fiber placements, we could image neuronal activity in these brain regions in response to the stress odor of a conspecific in WT and astrocytic mtCB1 KO mice. In the hippocampus, the targeting of genetic calcium indicators to GABAergic interneurons (Hoshino et al., 2021) would contribute to understand whether the mechanisms of direct stress mediated NOR impairment are similar to those mediating transmitted stress (Skupio et al., 2023).

These experiments would contribute to a better understanding on the role of olfactory bulb astrocytes to social transmission of stress, which is the main aim of the Thesis work.

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