UNIVERSITÀ
DEGLI STUDI
DI PADOVA

ISTITUTO ITALIANO
DI TECNOLOGIA

UNIVERSITA' DEGLI STUDI DI PADOVA

Department of Neuroscience

PhD Course in Neuroscience

XXXVI Cycle

Cortical Astrocytes Modulate Emotion Discrimination

Thesis written with the financial contribution of Italian Institute of Technology (IIT)

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Abbreviations

ASD	Autism Spectrum Disorder
AUC	Area Under the Curve
CB-R	Cannabinoid receptor
CB_1-R	Cannabinoid receptor-type 1
CB_2-R	Cannabinoid receptor-type 2
CNO	Clozapine-N-oxide
DI	Discrimination Index
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
eCBs	Endocannabinoids
ECS	Endocannabinoid System
EDT	Emotion Discrimination Test
ERTs	Emotion Recognition Test
GABA	Gamma-Aminobutyric acid
GCaMP	Genetically encoded calcium indicator
GFAP	Glial Fibrillary Acid Protein
IP3	Inositol 1,4,5-triphosphate
mPFC	Medial prefrontal cortex
PFC	Prefrontal cortex
PLC	Phospholipase C
PMCA	Plasma Membrane Calcium ATPase
PSTH	Peristimulus Time Histogram
PV+	Parvalbumin-positive neurons
SCZ	Schizophrenia
SOM+	Somatostatin-positive neurons
THC	Δ9-tetrahydrocannabinol
ToM	Theory of mind
VIP+	Vasoactive Intestinal Polypeptide-positive neurons

CHAPTER 1

General Introduction and Outlines of the Thesis

1.1 General Introduction

Emotion Recognition across species

Emotion recognition entails the ability to perceive, interpret, and identify emotional states in others (Adolphs, 2002a). It represents a pivotal component of the socio-cognitive repertoire enabling individuals to respond appropriately to emotional cues, empathize with others, and engage in the most suitable behavioral response (Brothers et al., 1990; Millan et al., 2012).
These processes encompass different cognitive processes. Understanding social cues from other individuals involves the ability to perceive and identify emotions through facial expressions, as well as comprehending acoustic properties in speech that reflect the speaker's emotional state. Consequently, the observation of such behaviors in others involves the activation of specific brain regions, mirroring the activation that would occur if the observer were performing the same actions, supporting the concept of *emotional sharing*. Building upon these observations, the ability to "mentally perceive" the intentions, thoughts, and emotions in others is achieved through the *mentalizing* process, allowing individuals to understand the inner world of others with their emotions. In the context of social interaction, individuals respond with their emotional reactions to the emotional cues they perceive in others. This process assumes a fundamental role in shaping interpersonal relationships and empathy (Green et al., 2015). It has a positive impact on functional outcomes, including social adjustment and mental health (Wells et al., 2021).

In human studies, the ability to recognize different emotions is typically evaluated through tests known as *Emotion Recognition Tasks* (ERTs) (Green et al., 2015; Henry et al., 2016). These tests evaluate one's ability to identify emotions portrayed in static or dynamic photographs that express various facial expressions. One of the most extensively validated used in the fields of psychology and behavioral research is "Ekman's Faces Test". It is a computerized task based on the identification of six basic innate human emotions (fear, anger, disgust, happiness, surprise, and sadness) which must be correctly recognized by the testing subject (Ekman, 1992). Furthermore, to assess the gravity of potential socio-cognitive deficits during the administration of the Ekman's Faces test, emotions can be presented with graded intensities scales and in a combination of different experimental paradigms (Blair et al., 2001; Dujardin et al., 2004; Henry et al., 2016; Kohler et al., 2003; Matsumoto et al., 2000).

Emotion recognition is not limited to humans. It represents an essential socio-cognitive ability for other species within social communities, including non-human primates, horses, dogs, and sheep (Ferretti & Papaleo, 2019). Notably, in non-human primates emotion recognition has been investigated as an empathy-related phenomenon with different methodologies, involving the presentation of subjects to drawings (Dittrich, 1990; Parr et al., 2007), static images, movies, and real-life interactions with fellow conspecific (Parr et al., 1998). Primates have shown a remarkable capacity to not only recognize conspecific's emotions (Parr, 2003), but also categorize their affective states and predict future actions (Morimoto & Fujita, 2012; Nakamura et al., 1992; Waller et al., 2016). Not by chance, electrophysiological studies of emotion recognition in monkeys revealed commonalities in the brain regions employed by both human and non-human primates for processing facial expressions in emotions. Specifically, amygdala cells process both negative and positive stimuli but with distinct activation patterns (Nakamura et al., 1992; Paton et al., 2006; Sanghera et al., 1979).

Traditionally, emotion recognition has been primarily associated with the most advanced species, like humans and non-human primates (van Hooff1, 1962). Nonetheless, understanding the emotions of others is fundamental for adapting to changing conditions within the social environment, such as recognizing the emotional state of potential predators (Puścian et al., 2022). Accordingly, emotion recognition abilities were also observed in other mammals, including horses, dogs, and sheep (Albuquerque et al., 2016; Tate et al., 2006; Wathan et al., 2016). Yet, the ability to convey emotions is highly species-dependent. Animals equipped with advanced visual systems, like horses, sheep, and dogs have demonstrated the ability to integrate a range of sensory signals to interpret and respond to emotional cues through facial expressions (Albuquerque et al., 2016; Tate et al., 2006; Wathan et al., 2016). Moreover, their responses include adaptive behavior, such as avoiding individuals displaying signs of stress compared to those with neutral expressions (Racca et al., 2012; Tate et al., 2006; Wathan et al., 2016). In dogs, the integration of sensory signals (e.g., visual and auditory inputs) culminates with the creation of an internal, multifaceted representation of emotions (Albuquerque et al., 2016), as was observed in both humans and monkeys for categorizing emotions into positive or negative affect.

Rodents, similar to many other mammals, are social animals. They engage in intricate social interactions and establish robust social hierarchies that are influenced by their

environmental conditions. In a recent study conducted by Dolensek et al., (2020) using machine video, it has been demonstrated that mice exhibit facial expressions in response to different sensory stimuli (e.g. tail shock, sweet sucrose, bitter quinine, and lithium chloride injection); each emotional event triggered distinct facial movements in mice that were detected and identified by unfamiliar observers.

Additionally, other works demonstrated that rodents are not only capable to perceive and react to different emotions displayed by their conspecifics (Atsak et al., 2011; Ben-Ami Bartal et al., 2011; Knapska et al., 2010; Meyza et al., 2017; Panksepp & Lahvis, 2011), but they also show empathic responses and pro-social behaviors driven by such shared experience (Ben-Ami Bartal et al., 2014; Burkett et al., 2016). Specifically, it has been shown that rodents exhibit a freezing response following the observation of another conspecific experiencing a distressing event. As a result, the negative emotional state of one individual appears to influence the emotional state of another one conspecific. This phenomenon is known as "vicarious freezing": it is considered a part of the so-called *emotional contagion*, a form of automatic empathic response characterized by the synchronization of emotional responses between individuals (Atsak et al., 2011; Meyza et al., 2017; Yusufishaq & Rosenkranz, 2013).

Furthermore, several studies have demonstrated that rodents respond to alterations in the emotional states of their conspecifics, even when they were not directly involved in emotional contagion by freezing response (Knapska et al., 2006, 2010). For instance, when an observer rat was exposed to a cage mate demonstrator that had undergone a fear-conditioning procedure just before the interaction, the observer exhibited an elevated social approach and allogrooming behavior towards the altered animal, indicating an affective response (Burkett et al., 2016; Knapska et al., 2010).

Together, these findings demonstrate that rodents consistently exhibit the capability to accurately perceive and interpret the emotional states of their fellow conspecifics, and their responses can be different. Nonetheless, investigating the ability to distinguish between distinct emotions requires the development of a specific behavioral paradigm that closely emulates the human emotion recognition ability. Given the lack of evidence indicating that mice, the most commonly used laboratory animals, possess the ability to differentiate between emotional states in others, developing a novel behavioral paradigm could serve as a powerful tool for investigating the neurobiological processes underpinning these crucial social functions.

The *Social Brain*: a neuronal network of emotion recognition

Over the past decades, the field of neuroscience has shown an increasing interest in elucidating the neurobiological mechanisms governing the recognition of emotions. Within the brain, the process of perceiving and understanding emotions relies on a network consisting of distinct brain areas, each playing a role in recognizing emotions.

This intricate network, originally defined as the "*Social Brain"* by Brothers in the 1980s, encompasses a variety of regions including those involved in processing visual information, limbic functions, prefrontal processes, subcortical functions, temporal and temporoparietal contributions, and cerebellar engagement. Together, these regions play key roles in identifying social cues, understanding the mental states of others, and driving motivated behaviors in social contexts (Adolphs, 2009; Fusar-Poli et al., 2009).

Particularly, the social cognitive processes of emotion recognition involve the interaction between limbic and frontal cortical regions (Gur & Gur, 2016); among these, the amygdala and the prefrontal cortex (PFC) have a crucial function (Maat et al., 2015).

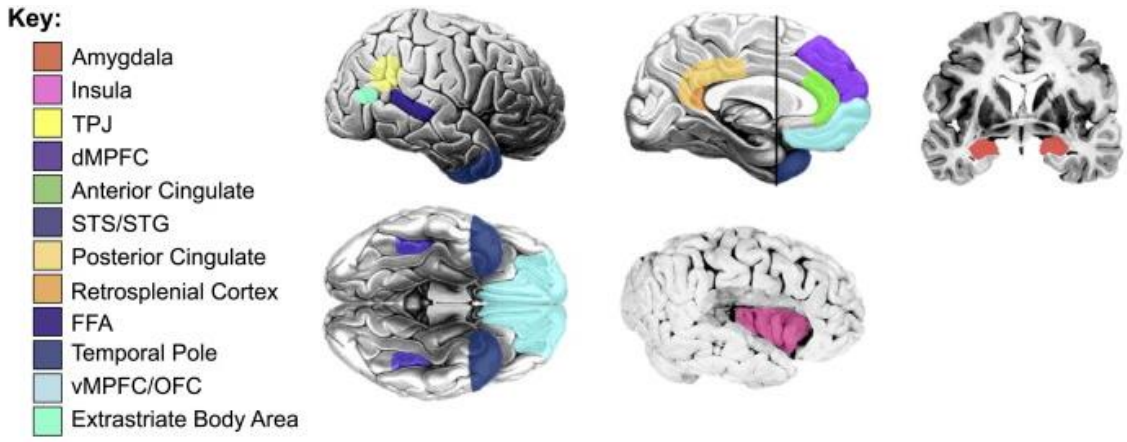

Figure 1. Figure adapted from Kennedy and Adolphs, 2012, *Neuron*. Graphical representation of the "*Social Brain"* illustrating all the brain structures engaged in socio-cognitive functions.

It is well-established that the amygdala, a core brain region within the limbic system, assumes a pivotal role in emotional processing. It serves as a key component in automatically integrating different input signals into cognitive processing, thereby eliciting an appropriate behavioral response (Adolphs R., 2002). Indeed, human lesion studies have proved the role of the amygdala in the social evaluation of emotions through other's facial expressions (Adolphs et al., 1994). Remarkably, impairment in emotion recognition following bilateral amygdala damage is most

pronounced for the identification of negative valance emotions, in particular, fear, anger, and sadness (Adolphs et al., 1999; Broks et al., 1998; Schmolck & Squire, 2001). However, functional neuroimaging studies have provided evidence supporting the amygdala's responsivity in the recognition of neutral and happy faces (Killgore & Yurgelun-Todd, 2004; Somerville et al., 2004). Within the brain, the amygdala is extensively connected with many other cortical structures including the prefrontal cortex (Felix-Ortiz et al., 2016). These interconnected regions communicate synergistically to carry out functions related to the recognition of emotions (Adolphs R., 2002). Remarkably, it is hypothesized that lesions in the amygdala in combination with reduced interconnectivity with the prefrontal cortex, may lead a diminished emotional expression and deficits in recognizing emotions (Aleman & Kahn, 2005).

Notably, the prefrontal cortex exerts the "top-down" executive modulatory control over socio-cognitive functions, and it is recognized as a fundamental hub for social cognition. (Adolphs, 2009; Amodio & Frith, 2006; Friedman & Robbins, 2022; Kietzman & Gourley, 2023). Anatomically, the prefrontal cortex is a complex brain structure composed of multiple sub-regions, collectively engaged in complex cognitive processes and goal-directed social behaviors (Haber et al., 2022; Ko, 2017). In particular, the medial regions of the PFC, such as the medial prefrontal cortex (mPFC) and the anterior cingulate cortex (ACC), are linked to social behaviors and are strongly associated with emotional self-awareness and the perceptions of similar individuals (Johnson et al., 2002; Lou et al., 2017; Mah et al., 2004; Mitchell et al., 2006). Specifically, medial regions of PFC receive direct afferents from the amygdala (Felix-Ortiz et al., 2016). Conversely, the lateral regions, including the dorsolateral prefrontal cortex (dlPFC) and ventrolateral prefrontal cortex (vlPFC), are active during social tasks processing information in the social domain but are considered "general fields" in their functions. (Nelson & Guyer, 2011; Weissman et al., 2008).

Overall, these findings suggest that socio-cognitive functions are primarily governed by the activity of the medial prefrontal cortex. Human lesion studies have consistently shown that individuals with damage to the mPFC exhibit difficulties in accurately interpreting emotions in others, leading to subsequent abnormal social behaviors and detrimental impact on their social life (Hornak et al., 2003; Mah et al., 2004; Monte et al., 2013).

Additionally, neuroimaging investigations involving individuals with autism spectrum disorders and schizophrenia have revealed altered activation patterns compared to healthy subjects within

the amygdala and prefrontal cortex during the recognition of emotions (Aleman & Kahn, 2005; Ibrahim et al., 2019; Taylor et al., 2012). Importantly, in autistic patients, there is evidence of disrupted functional connectivity between the amygdala and other brain regions during the observation of emotional faces. Similarly, individuals with schizophrenia exhibit altered activation in both the amygdala and PFC when exposed to emotional stimuli. Specifically, the amygdala appears to be impaired during the processing of facial expression, while the PFC is affected during tasks related to the theory of mind (Adolphs, 2009; Hill et al., 2004; Maat et al., 2016).

In addition, volumetric structural studies examining abnormalities in the morphology of the amygdala and PFC and their relationship with deficits in social cognition have revealed a link between reduced volume in the amygdala structure and difficulties in recognizing emotions (Exner et al., 2004; Namiki et al., 2007). Furthermore, diminished gray matter concentrations in the PFC have been associated with the theory of mind deficits in schizophrenia, especially in the medial prefrontal cortex (Bertrand et al., 2008; Yamada et al., 2007).

Nevertheless, the precise mechanisms governing emotion recognition functions at the circuit- and cell-specific levels in the mentioned brain regions remain elusive in humans. Indeed, it is plausible that within the same brain region, specific circuits or cell mechanisms regulate different facets of emotion recognition and processing. In this context, rodent studies could provide valuable insights into fundamental biological principles of social cognition, which may have broader implications for other mammal species.

Indeed, in the last years emotion-based behavioral investigations have proved the central role of specific brain regions, particularly the amygdala, insular cortex and anterior cingulate cortex (Allsop et al., 2018; Jeon et al., 2010; Rogers-Carter et al., 2018).

However, the precise neuronal mechanisms involved in processes regulating the recognition of emotions remain unclear. Therefore, it is essential to develop an appropriate behavioral paradigm in rodents that can mimic the human recognition task. This approach could have the potential to enhance our comprehension of social dysfunctions and find a way for more targeted and effective therapeutic interventions to improve the quality of life of patients with impaired ability to recognize emotion in others.

Emotion Recognition in brain disorders

Deficits in emotion discrimination have been documented across a spectrum of neurological conditions, encompassing disorders in which social cognitive deficits are an inherent characteristic of the diagnosis, such as autism spectrum disorder or following traumatic brain injury. Furthermore, in conditions like Parkinson's disease, Alzheimer's disease or post-traumatic stress disorder (PTSD), social challenges might be more subtle and challenging to clinically identify (Henry et al., 2016).

Traumatic Brain Injury (TBI) has been identified as a common feature of impaired processing of social cues conveyed through facial expressions (Adolphs et al., 1994; McDonald & Flanagan, 2004). In particular, neuroimaging studies shed light on the specific brain regions involved in regulating emotional responses (Fusar-Poli et al., 2009). The prefrontal cortex and amygdala have been implicated in tasks related to the identification of emotions (Gorno-Tempini et al., 2001; Habel et al., 2007; Hariri et al., 2000; Morris et al., 1996; Nakamura et al., 1999; Narumoto et al., 2000). Damage to the temporal (Adolphs R., 2002; Rosen et al., 2004) and frontal lobe (Hornak et al., 1996) (Marinkovic et al., 2000) (Heberlein et al., 2008) has been shown to impair the recognition of emotional expressions in faces and voices, leading to inappropriate behavioral responses.

Furthermore, impaired facial recognition and emotion processing have been largely documented in Parkison's disease, as evidenced by decreased accuracy in emotional categorization tasks (Borg et al., 2012; Heller et al., 2018; Jacobs et al., 1995; Mattavelli et al., 2021). In particular, individuals with Parkinson's disease exhibit greater difficulties in recognizing negative emotional states such as anger, disgust, and fear compared to positive emotions like happiness and surprise (Gray & Tickle-Degnen, 2010; Kan et al., 2002; Lawrence et al., 2007)

Dysfunction in social cognition is a common feature of neurodegenerative diseases such as Alzheimer's disease (AD), which affects between 30 and 35 million people worldwide (World Health Organization, 2021) and leads to dementia. In the early stages of Alzheimer's disease, when mild episodic memory impairment is often observed, deficits in emotion processing may be relatively subtle and challenging to clinically detect (Henry et al., 2016). However, as the disease progresses and memory deficits become more severe, impairments in other cognitive

domains become increasingly noticeable. Indeed, individuals with AD typically exhibit reduced emotion processing abilities, including difficulties in identifying and discriminating emotions, as assessed by validated tests such as the Ekman Face's test (Henry et al., 2016; Kumfor et al., 2014; Miller et al., 2012).

Post Traumatic Stress Disorder (PTSD), arising from exposure to traumatic or life-threatening events, is characterized by pronounced cognitive and affective dysregulation. Notably, individuals with PTSD frequently exhibit compromised cognitive abilities related to emotional information processing, resulting in aberrant emotional responses and diminished emotional expression (Ehring & Quack, 2010; Frewen et al., 2017; Miles et al., 2016). Specifically, individuals who experienced PTSD revealed deficiencies in the ability to accurately decipher emotions through facial stimuli when compared to healthy subjects (Bardeen et al., 2013; Castro-Vale et al., 2020). Moreover, evidence demonstrated that PTSD patients displayed difficulties in identifying specific emotions, particularly fear and sadness, in contrast to other emotional states, as indicated by the reduction of accuracy and sensitivity for perceiving these emotions (Poljac et al., 2011). Additionally, multiple neuroimaging studies conducted on individuals with post-traumatic stress disorder have unveiled a potential association between cognitive deficiency and substantial reductions in brain volume, notably affecting regions such as the hippocampus (Bremner et al., 1997; Gilbertson et al., 2002; Villarreal et al., 2002), amygdala (Morey et al., 2012), medial prefrontal cortex (Rauch et al., 2003), and anterior cingulate cortex (Kitayama et al., 2006; Woodward et al., 2006). Nevertheless, the precise origins of these structural alterations – whether they stem from pre-existing susceptibilities to PTSD or emerge because of the traumatic experience, remain undefined.

Deficits in the ability to recognize and convey facial emotions are a core feature in conditions associated with cognitive disturbances, such as neuropsychiatric disorders like Schizophrenia (SCZ) and Autism spectrum disorders (ASD). In the context of schizophrenia, several studies provided strong evidence that SCZ patients encounter significant difficulties in accurately identifying emotions in others through facial expressions (Gur et al., 2002; Kohler et al., 2010; Mandal et al., 1998). More specifically, individuals with SCZ exhibit alterations in their ability to represent and infer the mental states of others, indicating an impaired *theory of mind* (ToM) (Fett et al., 2011; Green et al., 2008; Pickup & Frith, 2001) which might explain a wide range of both positive and negative symptoms observed in SCZ individuals. Neuroimaging

studies on emotional processing have identified a connection between impaired emotional processing in SCZ patients and functional abnormal activity in specific neuronal circuits involving brain areas like the prefrontal cortex, amygdala, and visual areas (Dyck et al., 2014; Garcia-Leon et al., 2021; Spilka et al., 2015; Takahashi et al., 2004).

Autism spectrum disorder is a lifelong neurodevelopmental disorder characterized by dysfunctions in socio-cognitive domains which encompass difficulties in recognizing, distinguishing, and remembering different facial expressions (Lai et al., 2014). Thus, individuals with autism experience an atypical perception of faces (Adolphs et al., 2001; Davies et al., 1994), impaired emotional processing (Corbett et al., 2009), and altered mental state (Senju, 2012). Nevertheless, the state of research on emotion recognition abilities in individuals with autism spectrum disorders remains uncertain. While some studies indicate that ASD patients demonstrate an impaired ability to correctly match emotions, there is other evidence that demonstrates similar performance to healthy controls, often achieved through compensatory mechanisms like verbal meditation or feature-based learning (Harms et al., 2010). The heterogeneity of these results is given by a combination of different limited factors including demographic characteristics of study participants, diversity of task design, and different measurements assessed which underscore the complexity of understanding emotion recognition in the context of ASD (Fusar-Poli et al., 2009; Harms et al., 2010).

In summary, the skill of accurately interpreting other's emotions is a fundamental requirement for successful communication within social contexts. This skill plays a pivotal role in everyday social interactions and in establishing interpersonal relationships with conspecifics (Dunbar, 2009; Fett et al., 2011).

As a result, deficits in socio-cognitive abilities have a negative impact on the quality of social life of individuals (Penn et al., 1997), leading to a diminished quality of life, increased social isolation, and feelings of loneliness (Green et al., 2008; Penn et al., 1997). The clinical manifestations of these social deficiencies often include difficulties in understanding the theory of mind (ToM), which refers to the capability to perceive the mental states of others, recognizing that they may differ from our own. These alterations, in turn, result in disrupted social perception which prevents the accurate interpretation of facial expression, body language, and voices, with consequent altered behavioral responses (Henry et al., 2016).

Acknowledging the critical significance of these processes in social functioning, in the last two decades neuroscience has exhibited a great interest in unravelling the neurobiological mechanism underpinning emotion recognition abilities. Unfortunately, effective therapeutic strategies for such social behavioral deficits are still lacking, primarily due to the limited understanding of the biological substrates underlying them. Hence, it is fundamental to conduct investigations into the biology of emotion recognition at the cellular and circuit levels. This endeavor might potentially give new insights for a more precise and targeted therapeutic approach in the future.

Emotion Recognition in mice: a novel behavioral task

As previously discussed, the exploration of brain circuits involved in emotion recognition is an essential research area for comprehending the specific neuronal and non-neuronal mechanisms that drive these crucial functions. Considering the pivotal role of socio-cognitive abilities in daily social interactions, the absence of knowledge regarding the brain circuits governing the capability to perceive other's emotional states is a significant gap in our understanding. Thus, the investigation of the neurobiological processes underlying emotion recognition is indispensable for gaining a deeper understanding of the social brain functioning in healthy and disease conditions.

In line with increasing evidence suggesting rodents exhibit higher-order social-emotional processes (Meyza et al., 2017), a behavioral paradigm has recently been introduced to investigate the ability of laboratory mice to recognize emotions.

The *Emotion Discrimination Task* (EDT), used in this study, was developed in Papaleo's group (Ferretti et al., 2019; Scheggia et al., 2020), and was inspired by human emotion recognition tasks that rely on the ability to differentiate basic expressions of emotions in others. Human emotion recognition paradigms typically include the presentation of both positively and negatively valenced emotions. Accordingly, we designed a two-choice discriminative set-up focused on behaviors initiated by the observer mice. We incorporated manipulations aimed at inducing negative and positive emotional states in the demonstrator mice.

Our investigation aimed to determine whether a testing mouse, referred to as "observer", could discriminate unfamiliar conspecifics by detecting a positively valenced emotional state. Specifically, we exposed observer mice to two stimuli, or "demonstrators", differing in their affective state. One demonstrator remained in a neutral state, while the other received 1-h ad libitum access to water after 23 h of water deprivation (as depicted in Figures 2A and 2B). Water was chosen as a rewarding stimulus to eliminate olfaction-related cues that could differentiate the two demonstrators.

We hypothesized that the relief from the distressing water deprivation would result in a positively valenced emotional state, referred to as ''relief''. Notably, our findings demonstrated that the 1-h ad libitum access to water resulted in a conditional place preference in mice that experienced the 23 h water deprivation, but not in mice in ad libitum water condition (as shown

in Figures 2E and 2F). Additionally, 1-h ad libitum access to water after deprivation resulted in reduced corticosterone levels in relief mice (as seen in Figure 2G).

Observers of both sexes exhibited social exploration toward the relief demonstrator compared to the neutral, selectively in the initial 2 min of the task (Figures 2C and 2D). No changes in rearing and grooming patterns toward the demonstrators were observed throughout the task (Figures 2H and 2I). Moreover, observers showed a typical decrease in locomotor activity (Figure 2J) and did not show freezing behavior, escape attempts, or other stress-related behaviors during the entire test session.

These findings collectively indicate that mice possess the ability to detect and socially respond to unfamiliar conspecifics in a positively valenced emotional state.

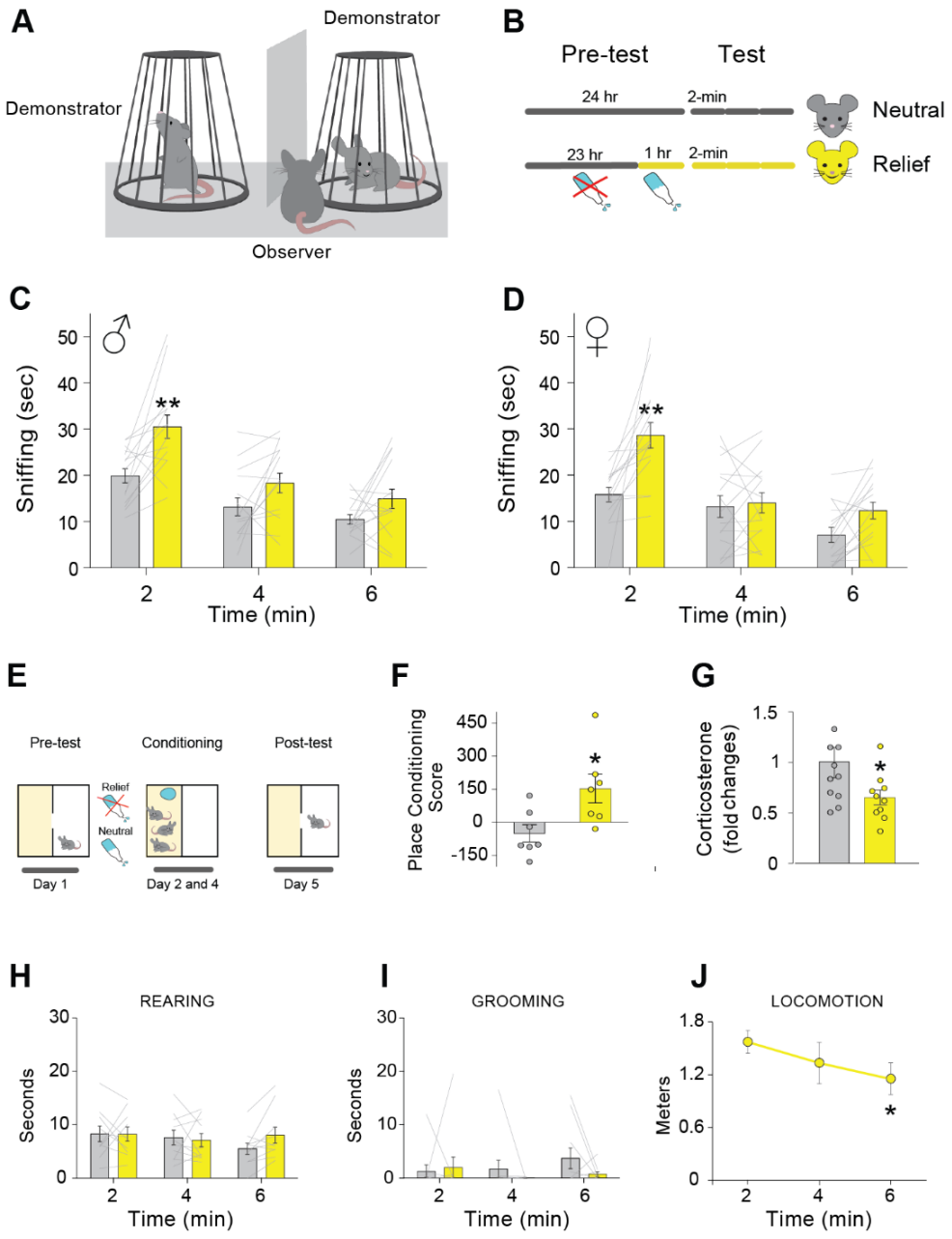

***Figure 2*. Mouse Emotion Discrimination for Relief.**
From Ferretti V., Maltese F., et al (2019) *Current Biology*
(**A**) Schematic drawing of the test setting. (**B**) Timeline of pre-test and test procedures to evoke in one of the two demonstrators a ‘‘relief’’ state during the testing phase. (**C-D**) Time (in seconds) spent sniffing demonstrators in neutral (gray bars) or water-induced relief (yellow bars) states displayed by (**C**) male and (**D**) female observer mice during the 6 min of the test, divided into three consecutive 2-min epochs (first 2-min RM ANOVA for males $F_{1,14} = 15.07$, $p = 0.001$ and females $F_{1,14} = 14.60$, $p = 0.001$; no significant differences for the 2- to 4-min and 4- to 6-min epochs). **$p < 0.005$ versus the exploration of the neutral demonstrator. n= 15 observers per group. (**E**) Place conditioning procedure used to assess whether the relief manipulation was associated with a negative-, neutral-, or positive-valence affective state. (**F**) Place conditioning scores (in seconds) displayed by mice conditioned during a neutral (gray bar) or relief (yellow bar) state. For each mouse, a place conditioning score was calculated as the post- minus

the pre-conditioning time spent in the conditioning-paired compartment of the apparatus. A positive score indicates place preference, a negative score a place aversion, and 0 no place conditioning (t test: df = 12; p = 0.02). *p < 0.05 versus the neutral control group. n = 7 per group. (**G**) Blood corticosterone levels displayed by demonstrator mice immediately after a period of 24-h water deprivation (gray bar) or after a period of 1 h ad libitum access to water following 23-h water deprivation (yellow bar; t test: df: 19; p = 0.05). *p = 0.05 versus water deprived mice. n = 11 per group. (**H-I**) Time (in seconds) spent in (**H**) rearing and (**I**) grooming in proximity of demonstrators in neutral (gray bars) or relief (yellow bars) state displayed by observer mice during the test (RM ANOVAs showed no significant differences). (J) Locomotor activity displayed by the same observer mice during the test (RM ANOVA F2,18 = 4.35; p = 0.04). *p < 0.05 versus minute 0–2. n = 10 observers.
Error bars represent standard error of the mean.

Additionally, we aimed to investigate whether mice had the ability to discriminate unfamiliar conspecifics in a negatively valenced emotional state. To test this hypothesis, we employed two different protocols.

In the ‘‘fear’’ paradigm, one of the two demonstrators had been fear conditioned to a tone cue at least one day before the test (Figure 3A 3B). The presentation of the tone during the test would then evoke a negatively valenced emotional state in the conditioned mouse (LeDoux, 1996). Specifically, the tone was delivered during the second 2-min epoch of the test (Figure 3B) to assess observers’ responses before, during, and after the induction of the altered emotional state in the demonstrators. Both male and female observers increased their sniffing toward the fear-conditioned demonstrator compared to the neutral one (Figures 3C, 3D, 3G–3I). This effect became evident after the 2-min tone presentation (Figures 3C, 3D). Although no discriminatory behavior was observed during the tone presentation, we found an inverse correlation between the time the fear demonstrator spent freezing and the time of observer sniffing, suggesting that freezing per se might influence observer discrimination (Figure 3E). However, we found no correlation between the demonstrator freezing and the observer sniffing after the tone presentation, suggesting that demonstrator’s freezing did not affect the discriminatory behavior we observed (Figure 3F).

In line with prior evidence (Jeon et al., 2010; Langford et al., 2006; Pisansky et al., 2017), we investigated signs of fear transfer from the emotionally -altered demonstrator to the observer by quantifying freezing behavior, escape attempts, changes in locomotor activity, and other stress-related behaviors (i.e., rearing and grooming). During the 6-min test, we detected no sign of emotion contagion (Figures 3G–3I). Moreover, corticosterone levels of observer mice exposed to the fear paradigm or to two neutral demonstrators did not differ (Figure 3J). These findings

collectively suggest that mice can detect and socially respond to unfamiliar conspecifics in a negative altered emotional state.

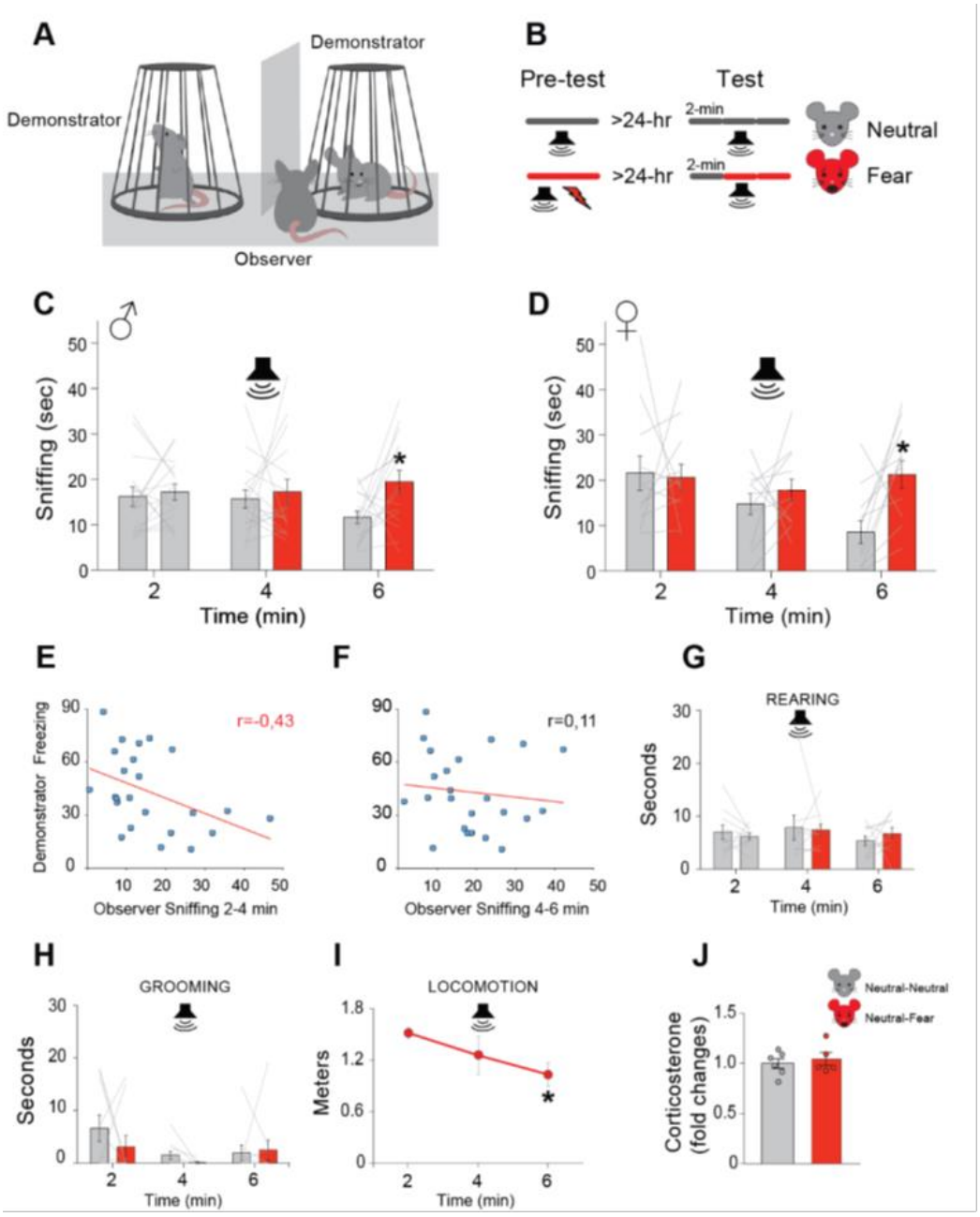

***Figure 3*. Mouse Emotion Discrimination for Fear.**
From Ferretti V., Maltese F., et al (2019) *Current Biology*
(**A**) Schematic drawing of the test setting. (**B**) Timeline of pre-test and test procedures to evoke in one of the demonstrators a ‘‘fear’’ state by delivering the conditioned tone in the 2- to 4-min epoch. (**C-D**) Time (in seconds) spent sniffing demonstrators in neutral (gray bars) or tone-induced fear (red bars) state displayed by (**C**) male and (**D**) female observer mice during the 6-min test, divided into three consecutive

2-min epochs (last 2-min repeated measurements [RM] ANOVA for males $F_{1,15} = 6.51$, $p = 0.022$, and females $F_{1,11} = 10.98$, $p = 0.006$; no significant differences in the 0- to 2-min and 2- to 4-min epochs). *$p < 0.05$ versus the exploration of the neutral demonstrator. n = 8/15 observers per group. (**E-F**) Correlation analyses between the time the fear-conditioned demonstrator spent freezing (in y axis) and the time the observer spent sniffing the fear conditioned demonstrator (in x axis; **E**) in the 2- to 4-min epoch or (**F**) in the 4- to 6-min epoch of the test ($r = -0.4310$ for 2–4 min; $r = -0.11$ for 4–6 min). n = 24 observers. (G and H) Time (in seconds) spent (**G**) rearing and (**H**) grooming in proximity of the demonstrators in neutral (gray bars) or fear states (red bars) displayed by the same observer mice during the test (RM ANOVAs showed no significant differences). (**I**) Locomotor activity displayed by the same observer mice during the test (RM ANOVA $F_{2,16} = 4.08$; $p = 0.03$). *$p < 0.05$ versus 0–2 min. n = 9 observers. (**J**) Blood corticosterone levels displayed by observer mice immediately after being exposed to two neutral demonstrators (gray bar) or one neutral and one fear demonstrator (red bar). Data are expressed as fold changes compared to observers exposed to two neutral demonstrators (t test: df: 9; $p = 0.58$). n = 5/6 observers per group.
Error bars represent standard error of the mean.

Next, we also developed a different negative affective state protocol (Scheggia et al., 2020). We used the same EDT setting, but the observers were presented with a demonstrator that underwent a mild stress protocol, consisting of 15 min of acute restraint before the beginning of the emotion discrimination, and a neutral demonstrator (Fig. 4a). We observed increased exploration towards the stressed demonstrator (Fig. 4b) and more time spent in the related zone of the apparatus (Fig. 4c). Additionally, mice first entered the zone related to the stressed demonstrator (Fig. 3d) and made longer visits to this zone during the first 2 min of the test (Fig. 4e). The total number of visits did not differ between the stressed and neutral demonstrators (Fig. 4f). Data from EDTs conducted in naive animals and in mice implanted with electrodes and under 'light off' conditions were pooled together and are shown as percent exploration towards the stress demonstrator (Fig. 4g). The affective state discrimination was a reliably observable behavior, with only 12% of tested mice not discriminating between a mouse in an altered or a neutral affective state (stress, 13 of 93; Fig. 4h). The scores of explorations towards the relieved and stressed demonstrators fit a normal distribution (D'Agostino and Pearson normality test, stressed: n=93, K2=1.54, P=0.46). These data showed that affective state discrimination is a stable trait in mice. No differences between male and female mice were evident in the discrimination of sex-matched stressed demonstrators (Fig. 4k).

Overall, these results showed that mice can similarly discriminate others based on both positive and negative affective states.

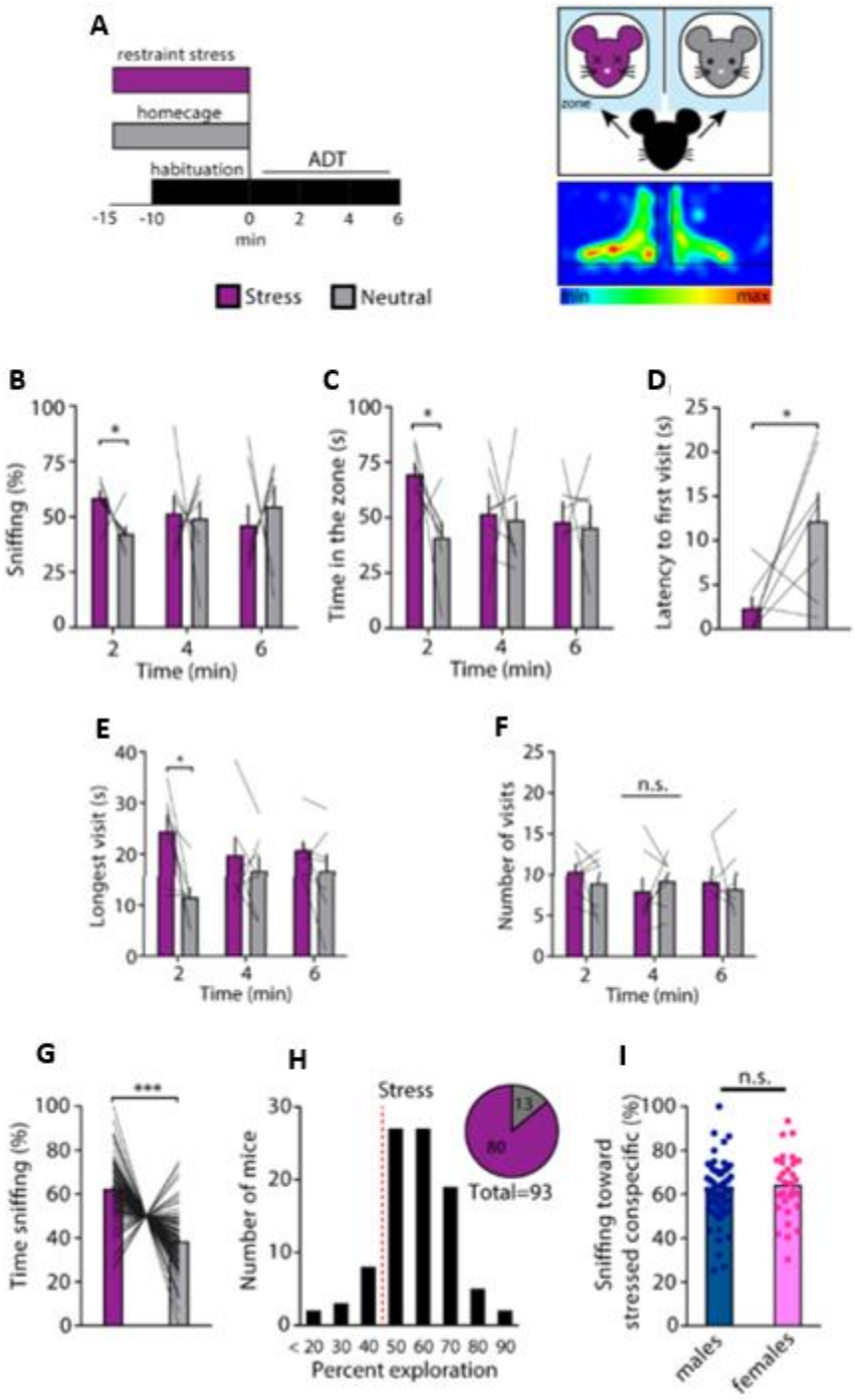

Figure 4. **Mouse Emotion Discrimination for Stress.**

Adapted from Scheggia D., Managò F., et al. (2020) *Nature Neuroscience*

(**A**) Timeline of pre-test and test procedures to evoke in one of the two demonstrators a ‘‘stress’’ manipulation during the testing phase. In the stress protocol, one demonstrator (stress, purple) was subjected to the restraint stress test for 15 min immediately before the beginning of the ADT. The other demonstrator (neutral, gray) waited undisturbed in the home cage. (**B**) Increased sniffing of the stressed

demonstrator (two-tailed multiple t-test, Bonferroni correction, 2 min: t = 3.22, d.f. = 12, P = 0.021) (**C**) and time spent in the zone with the stressed compared with the neutral demonstrator (two-tailed multiple t-test, Bonferroni correction, 2 min: t = 3.23, d.f. = 12, P = 0.021). (**D**) The latency to the first visit was significantly lower for the stressed demonstrator than for the neutral demonstrator (two-tailed paired t-test: t = 2.43, d.f. = 6, P = 0.050). (**E**), Average number of visits to each zone did not differ (two-tailed multiple t-test, Bonferroni correction, t=0.81, df=12, p>0.999). (**F**) Observers made longer visits in the zone related to the stressed demonstrators (two-tailed multiple t-test, Bonferroni correction, 2 min: t=3.46, df=12, p=0.017).

(**G**) More exploration of the stressed than of the neutral demonstrator in several replications of the ADT (two-tailed paired t-test: t = 8.22, d.f. = 92, P < 0.0001). (**H**) (**I**) Exploration of the stressed demonstrator was higher than chance in a large number of mice (80 of 93, one-sample t-test against chance, defined as 50%: t = 8.22, d.f. = 92, P < 0.0001) (**H**) and did not change depending on gender (63 male, 30 female; two-tailed unpaired t-test: t = 0.92, d.f. = 91, P = 0.357) (**I**).

Bar and line graphs show mean ± s.e.m. *P < 0.05. **P < 0.005. ***P < 0.0005. n.s., not significant.

Cortical neuronal brain mechanisms of emotion recognition

The orchestration of emotional processes within the human brain involves a sophisticated interplay among various brain regions, with a specific focus on the limbic and frontal areas (Adolphs, 2009; Fusar-Poli et al., 2009). While these brain regions play a general pivotal role in governing emotions, understanding their precise functioning is crucial. Whitin a singular brain region, the existence of intricate circuits and cell- specific heterogeneous mechanisms adds a layer of refinement to the modulation of distinct aspects of emotion recognition. Unsurprisingly, the investigation required to unravel these mechanisms remains beyond the reach of current human studies. Nevertheless, preclinical research employing rodents as model organisms provides a unique opportunity to delve into the biological microcircuits governing socio-cognitive aspects. Leveraging preclinical rodent models allows researchers to meticulously investigate and dissect intricate neuronal circuits and cellular mechanisms, employing innovative approaches such as optogenetics, chemogenetics, and calcium imaging. These techniques, facilitating the manipulation of specific cells or circuits combined with real-time recordings, offer specific insights. Unraveling the subtleties within brain regions associated with emotion recognition has significantly advanced our understanding of neuronal mechanism, paving the way for more precise interventions aimed at addressing social and emotional dysregulation in humans.

In-depth investigations into neuronal circuits have uncovered the critical role of maintaining a proper excitatory/inhibitory (E/I) balance for overall network stability. The orchestrated synaptic interplay between excitatory and inhibitory neurons, releasing glutamate and GABA, respectively, ensures a homeostatic equilibrium crucial for integrating inputs and generating appropriate responses (Shadlen & Newsome, 1994; Van Vreeswijk & Sompolinsky, 1996). Disruption in E/I balance within neuronal circuits leads to network instability, interfering with the brain's ability to adapt and respond to external stimuli (Yizhar et al., 2011). At the behavioral level, such alteration manifests as pronounced behavioral dysfunction, characteristic of different neurological conditions (Kobayashi et al., 2003; Zhou et al., 2009; Zhu & Roper, 2000). Notably, the negative impact of E/I perturbation has been demonstrated in cognitive deficits, prominently observed in neuropsychiatric diseases including schizophrenia and autism spectrum disorders (Akbarian,' et al., 1996; Cohen et al., 2015; Gogolla et al., 2009; Kehrer et al., 2008; Lee et al., 2017; Rubenstein, 2010; Rubenstein & Merzenich, 2003).

GABA-expressing interneurons are fundamental players in fine-tuning the E/I balance within neuronal circuits. Despite constituting a minority of the total cortical neuronal population, accounting for up to 20% (Meinecke & Peters, 1987) and displaying notable heterogeneity (Kawaguchi & Shindou, 1998; Markram et al., 2004), GABAergic interneurons sculpt network cortical dynamics by coordinating the activity of excitatory glutamatergic pyramidal cells (Isaacson & Scanziani, 2011). Due to their fundamental role in modulating neuronal circuitry, dysfunctions within GABAergic interneurons are the core of several neurological disorders (Ramamoorthi & Lin, 2011) (Sohal & Rubenstein, 2019). Alterations in the development, maturation, and integration of GABAergic interneurons contribute to the development of severe deficits commonly observed in conditions like schizophrenia, bipolar disorder, Rett syndrome, and epilepsy (Benes & Berretta, 2001; Chiapponi et al., 2016; Mossner et al., 2020; Righes Marafiga et al., 2021). Recognizing the significance of these dysfunctions, the term "interneurnopathies" was introduced in 2005 by Kato & Dobyns (2005) to specify disorders associated with GABAergic dysfunctions (Katsarou et al., 2017).

The primary classification of GABAergic interneurons is determined by the neuropeptides they express, giving rise to three distinct subpopulations, each playing a unique role in the regulation of neuronal function. These subtypes consist of *parvalbumin* (PV+)-expressing neurons, *somatostatin* (SOM+) expressing neurons and *vasoactive intestinal polypeptide* (VIP+) expressing neurons. These typological differences are associated with distinct functional roles, enabling them to exert unique control over excitatory input (Z. J. Huang, 2014).

Among cortical interneurons, both SOM+ and PV+ cells are well recognized for their contribution to social functions (Bicks et al., 2020; Cao et al., 2018; Kingsbury et al., 2020; Scheggia et al., 2020; Yizhar et al., 2011). Notably, a previously established reciprocal association between these interneurons and social behaviors underscores their relevant position. Photoinhibition of PV+ interneurons within the medial prefrontal cortex (mPFC) has been linked to impaired social interaction in mice, as evidenced by (Yizhar et al., 2011) and (Scheggia et al., 2020). In parallel, the general activity of SOM+ neurons in the mPFC increases during interaction with emotionally altered mice (Scheggia et al., 2020). Interestingly, reduced mPFC SOM+ activity has been associated with deficits in the recognition of affective states (Scheggia et al., 2020). This observation aligns with previous evidence highlighting a more specific role for cortical SOM inhibitory neurons in mediating socio-cognitive abilities (Keum et al., 2018).

These neurons appear to be less involved in basic sociability, social memory and social dominance processes (Scheggia et al., 2020; Zhang et al., 2022). Overall, this evidence suggests that the dual association between cortical interneurons and social functions warrants a more in-depth investigating into the microcircuits-level mechanisms governing the control of social behaviors.

However, it is crucial to note that the modulation of GABAergic interneuron transmission involves different mechanisms. Specifically, the interaction with neuromodulatory systems, such as the serotoninergic, dopaminergic or cholinergic system, plays a significant role in influencing GABAergic transmission through the release of neurotransmitters (Feng et al., 2001; Goral et al., 2022; Kusek et al., 2015; Lew & Tseng, 2014; Seamans et al., 2001). Furthermore, compelling studies have illustrated intricate interactions with neighboring cells, including astrocytes and microglia (Christian & Huguenard, 2013; Liu et al., 2022; Mariotti et al., 2018; Mederos et al., 2021; Shigetomi et al., 2011), which exert substantial influence on GABAergic interneurons and subsequently impact behavior.

Comprehending the intricate interplay between GABAergic neurons and different cell types is essential for understanding the regulation of inhibitory mechanisms in the brain. This will contribute to maintaining the delicate balance between excitatory and inhibitory signaling, thereby ensuring the proper functioning of neuronal circuits.

Astrocytes: pioneering players in shaping socio-cognitive processes

Astrocytes, historically considered supportive cells for neurons in the brain, have now gained recognition as active contributors in complex behavioral processes, primarily due to their essential role in neuronal network functions from early development to adulthood (Allen, 2013; Hodges et al., 2017; Kim & Chung, 2023).

Unlike neurons, responsible for generating action potentials and transmitting electrical signals, astrocytes do not partake in these functions. Nonetheless, their active involvement in synaptic activity is demonstrated by both spontaneous and stimulus-induced Ca^{2+} signals (Cornell-Bell et al., 1990; Nedergaard, 1994). The activation of G protein-coupled receptors (GPCRs) expressed within astrocytes by neuronal signals triggers intracellular Ca^{2+} activity, leading to the subsequent release of a repertoire of gliotransmitters, such as glutamate, GABA, ATP/adenosine, D-serine and lactate (Araque et al., 2014; Harada et al., 2016; Yin et al., 2020). Notably, the stimulation of metabotropic receptors triggers the activation of phospholipase C (PLC), which subsequently hydrolyses the membrane lipid phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and Inositol-3-Phosphate (IP3). Once released, IP3 binds specifically to the astrocytes-expressed isoform IP3R2 of the IP3 receptors (IP3R) (Sharp et al., 1999), leading to the release of calcium from intracellular stores within the endoplasmic reticulum (Agulhon et al., 2008; Kofuji & Araque, 2021).

This complex IP3R2-dependent process significantly contributes to the modulation of synaptic transmission, demonstrating the interplay between neurons and astrocytes in shaping brain functions (Halassa et al., 2007; Haydon & Carmignoto, 2006). Notably, astrocytes have been identified to functionally interact with GABAergic-inhibitory circuits. Accordingly, a short optogenetic stimulation of SOM + interneurons within the mouse somatosensory cortex induced a significant increase in astrocytic Ca^{2+} activity (Mariotti et al., 2018). Conversely, optogenetic stimulation of visual cortical astrocytes enhanced both excitatory and inhibitory transmission, with an increase of PV+ interneuron firing rate (Perea et al., 2014), suggesting the implicational role of astrocytes in the modulation of different processes controlled by cortical circuits. As a result, astrocytes are strongly implicated in modulating different behavioral processes (Kofuji & Araque, 2021; Lyon & Allen, 2022; Shigetomi & Koizumi, 2023). The initial evidence highlighting their role in behaviors emerged in 2009 by Halassa and colleagues, demonstrating

astrocytes' role in sleep regulation by inhibiting the gliotransmission process (Halassa et al., 2009).

Over the years, the fundamental role of astrocytes in behaviors has been extensively recognized. Their contribution to various behavioral outcomes including sleep (Halassa et al., 2009; Pelluru et al., 2016; Vaidyanathan et al., 2021; Yamashita et al., 2014), memory (Adamsky et al., 2018; Halassa et al., 2009; Licht-Murava et al., 2023; Mederos et al., 2021), sensory activity (Lines et al., 2020; Wang et al., 2006), fear and anxiety (Adamsky et al., 2018; Martin-Fernandez et al., 2017; Wiktorowska et al., 2021) and cognition (Corkrum et al., 2020; Dallérac & Rouach, 2016; Kang et al., 2020), has been established.

Recently, the neuroscience community has begun delving into the impact of astrocytes on social functions (Cheng et al., 2023; Noh et al., 2023; Wang et al., 2021). Innovative methodologies have emerged, enabling researchers to study the role of astrocytes in specific circuits and animal behaviors. These advanced techniques encompass the manipulation of astrocytic functions and molecules, combined with optical methods employed to monitor astrocytic activity and neurotransmitter release (Hirrlinger & Nimmerjahn, 2022; Yu et al., 2020). Together, these approaches promise to offer deeper insights into the impact of astrocytes on social processes.

The IP32 conditional knockout mice, displaying disruptions in intracellular calcium signals within astrocytes, but non-neurons, have demonstrated marked deficiencies in social interactions and repetitive behavior. In particular, the deletion of the IP3R2 gene significantly impairs the ability to approach social stimuli, while their preference for social novelty remains unaffected. These behavioral changes are accompanied by a remarkable decrease in ATP levels in the mPFC, suggesting a potential role of astrocyte-derived ATP as a molecular target in mice with IP3R2 deletion (Wang et al., 2021).

Furthermore, during a critical developmental period, adolescent mice exposed to diminished cortical astrocytes Ca^{2+} signaling have been documented to exhibit social deficits and depressive-like behaviors. Through the continuous extrusion of cytosolic Ca^{2+} ions from the plasma membrane, achieved by overexpressing the plasma membrane calcium transporting ATPase2 (PMCA2) in the bilateral cortex, cortical astrocyte activity is reduced. This novel manipulation strategy resulted in significant deficits in social interaction in mice. Interestingly, a partial rescue of synaptic and behavioral dysfunctions was found following the injection of hM3Dq DREADDs

in the cortex, suggesting that intact cortical astrocyte activity is important for social behaviors (Luo et al.,2023).

A recent study by Noh et al. demonstrated the fundamental role of astrocytes within the dorsomedial prefrontal cortex (dmPFC) in shaping the dominant behavior of mice. The social hierarchy among dominant and subordinate male mice relies on a distinct prefrontal synaptic excitatory/inhibitory (E/I) balance, significantly governed by the influence of astrocytes. Specifically, astrocytes modulate this balance by enhancing presynaptic-dependent excitatory transmission and down-regulating postsynaptic-independent inhibitory transmission through the release of glutamate and ATP, respectively (Noh et al., 2023).

Mice experiencing social isolation exhibit altered Ca2+ activity and global changes in gene expression within hippocampal astrocytes. Notably, following social deprivation, the Ca2+ channel TRPA1 shows increased expression in astrocytes. Deleting astrocytes-specific TRPA1 reverses the physiological and cognitive deficits linked to social isolation (Cheng et al., 2023).

Collectively, these recent findings underscore the active involvement of cortical astrocytes in the regulation of different aspects of social processes in mice. Yet, their role in socio-cognitive domain remains an unresolved question. Exploring the role of astrocytes as a dynamic contributor to higher-order brain functions has the potential to provide insights into the cellular mechanisms that underlie the ability to perceive and respond to emotional cues, ultimately influencing the quality of social interactions. From a translational perspective, delving into the influence of astrocytes on socio-cognitive functions not only enhances our comprehension of social cognition but also reveals promising avenues for identifying novel therapeutics targets. This could offer a novel approach for interventions and treatment strategies specifically tailored to conditions marked by socio-cognitive deficits.

As previously mentioned, deficits in understanding other's emotions are a hallmark of neurodevelopmental disorders such as autism spectrum disorders (ASD). Evidence in the literature demonstrated a potential connection between ASD and dysfunctional neuroglia, particularly astrocytes. Indeed, human studies involving post-mortem brain samples from individuals with ASD have revealed altered expression of glial-specific markers in the cortex of patients with autism (Edmonson et al., 2014; Vargas et al., 2005).

Additionally, the number of astrocytes within the II layers of the PFC was significantly reduced in ASD patients compared to healthy subjects. These observations highlight a possible

association between the dysregulation of astrocytes and behavioral alterations in autism (Falcone et al., 2021).

Preclinical studies involving animal models exposed to prenatal valproic acid (VA) have demonstrated the development of autistic-like behaviors, accompanied by alterations in the expression of neuroglia markers. Notably, these results identified substantial changes in astrocytic activity, particularly prevalent in the medial prefrontal cortex during adolescence and adulthood (Bronzuoli et al., 2018).

Overall, these findings underline the crucial role of astrocytes in shaping brain functioning, thereby influencing behavioral responses, particularly in pathological conditions.

While recent studies have associated astrocyte signaling at the synaptic or circuit level with cognitive processes in mice, their involvement in socio-cognitive abilities remains unknown. Given the multifaced nature of social cognition involving different brain areas and mechanisms, it is reasonable to consider the role of astrocytes in mediating these processes within neuronal circuits. This highlights an area of research which requires further exploration. Such insights could be fundamental in understanding how astrocytes influence social interaction and relationships within a social context, potentially leading to the discovery of novel therapeutic targets for enhancing social interaction and social communication skills, both in healthy and pathological conditions.

1.2 Aims and Outlines of the Thesis

The overarching goal of my doctoral research work was to investigate the potential involvement of astrocytes in the medial prefrontal cortex in the ability to recognize and respond to altered emotions in others. I specifically focused on the following topics:

1. **Exploring the Role of mPFC Astrocytes-Somatostatin Neurons Communication in Emotion Discrimination**

Chapter 2 was designed with the primary aim of investigating the role of mPFC astrocytes in socio-cognitive domain, particularly their ability in modulating emotional recognition processes. Despite the well-established fundamental role of astrocytes in the central nervous system, their active engagement in synaptic processing through bidirectional communication with neurons within the "tripartite synapse" (Araque et al., 1999) is increasingly recognized. As a consequence, it is unsurprising that astrocytes play pivotal roles in different behaviors (Kofuji & Araque, 2021; Lyon & Allen, 2022; Shigetomi & Koizumi, 2023). However, the specific involvement of astrocytes in modulating emotional recognition processing remains elusive. To address this gap, my initial focus in Chapter 2, was to investigate the potential involvement of astrocytes in socio-cognitive domain. Subsequently, the exploration extended to unraveling the interplay between astrocytes and somatostatin-positive (SOM+) inhibitory neurons in the context of emotion recognition. This exploration was motivated by their documented bidirectional communication with astrocytes, as highlighted by Mariotti et al., (2018). Furthermore, SOM+ neurons are recognized for their fundamental role in affective state discrimination (Scheggia et al., 2020). By investigating the dynamic communication between astrocytes and SOM+ inhibitory neurons, this research aims to expand the knowledge of the substrates underpinning socio-cognitive behaviors.

2. **Studying Astrocytic-Cannabinoid Signaling in Emotion Discrimination**

Given the increasing body of evidence highlighting the role of the endocannabinoid system (ECS) in social domains in humans (Bossong et al., 2013; Foltin et al., 1987; Hindocha et al., 2014, 2015) as well as in animal studies (Busquets-Garcia et al., 2017; Fyke et al., 2021; Jimenez-Blasco et al., 2020), **Chapter 3** was dedicated to investigate the impact of cannabinoids,

and the link with astrocytes, on the perceptions of emotions. Specifically, cannabinoid-type 1 receptors (CB_1R) found in astrocytes have been proposed to influence behavioral outcomes including sociability (Haller et al., 2004; Jimenez-Blasco et al., 2020) and learning and memory (Han et al., 2012; Robin et al., 2018) Nonetheless, their contribution to social cognitive functions is poorly addressed. In this study, we revealed a strong implication of astrocytic CB_1R in the recognition of emotions. Disruptions in CB_1R signaling within medial prefrontal cortex astrocytes emerged as a critical factor that contributes to social deficits, specifically impairing the ability to recognize emotions.

Furthermore, we extended our study by exploring the social effects induced by CB_1R activation through systemic administration of Δ9-Tetrahydrocannabinol (THC) on different aspects of social behaviors.

Collectively, these studies aim to enhance our understanding of the implications of cortical ECS activity within astrocytes on emotion recognition.

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CHAPTER 2

Cortical Astrocytes-Somatostatin Neurons Communication Modulates Emotion Discrimination

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In preparation

Abstract

The medial prefrontal cortex (mPFC) is a crucial hub for social cognition, encompassing the ability to decipher, interpret and respond to others' emotions. Within this domain, somatostatin-positive (SOM+) inhibitory neurons play a pivotal role in shaping the recognition and reaction to altered emotions in others. Despite the well-established functional connection between cortical inhibitory neurons and astrocytes, the specific contribution of astrocytes to emotion recognition and their potential involvement in connection with inhibitory neurons in social cognition remain elusive.

Here, we provided a quantitative analysis elucidating a distinct spatial connection between cortical astrocytes and SOM+ cells within the medial prefrontal cortex. Interestingly, similar to SOM neurons, mPFC astrocytes exhibited heightened calcium activity when an observer mouse interacts with an emotionally altered stimulus. In agreement, chemogenetic activation of mPFC astrocytes enhances the capacity to recognize both positive and negative emotions compared to neutral ones, without affecting basic sociability, social memory, social hierarchy, and social novelty abilities. Importantly, emotion recognition deficits resulting from somatostatin optogenetic inhibition were effectively compensated by the activation of astrocytes in the mPFC.

Overall, our findings provide novel insights into the significant role played by mPFC astrocytes-SOM neurons communication in the recognition of positive and negative emotional valences, contributing to a more profound understanding of the neurobiology processes underpinning emotion discrimination abilities.

Introduction

Emotion recognition is a crucial ability in shaping human interactions, requiring an understanding of social cues to perceive, identify, and respond appropriately to emotions expressed by others (Adolphs, 2002). This fundamental skill is essential for effective social communication within social domains (Green et al., 2015), significantly impacting mental health and social adjustment (Wells et al., 2021). Conversely, deficiencies in these socio-cognitive abilities, often indicative of different brain disorders, contribute to social isolation, consequently leading to a diminished quality of life for patients (Fett et al., 2011).

While the role of medial prefrontal cortex (mPFC) GABAergic interneuron cells in affective state discrimination has been extensively established (Scheggia et al., 2020), the contribution of astrocytes to this behavior remains largely unexplored. Considering their involvement in different brain functions including structural (Alvarez et al., 2013; Heithoff et al., 2021), metabolic (Barros & Weber, 2018; Suzuki et al., 2011), and homeostatic functions (Parpura & Verkhratsky, 2012), it is reasonable to hypothesize that astrocytes could play a role in social processes, given their influence in brain circuits. Recent findings strongly suggest the involvement of cortical astrocytes in regulating different aspects of social processes in mice (Cheng et al., 2023; Luo et al., 2023; Noh et al., 2023; Q. Wang et al., 2021). However, their specific role in the socio-cognitive domain remains elusive.

Inhibitory neurons in the mPFC have strongly implicated in different social processes (Bicks et al., 2020) (Cao et al., 2018) (Kingsbury et al., 2020) (Yizhar et al., 2011) (Scheggia et al., 2020). A discernible pattern is emerging, suggesting a dichotomous implication of SOM+ and PV+ cells in distinct social functions with PV+ neurons contributing to sociability (Scheggia et al., 2020; Yizhar et al., 2011), and SOM+ neurons being implicated in emotion recognition (Scheggia et al., 2020). Notably, photoinhibition of PV+ interneurons within the mPFC has been associated with impaired social interaction in mice (Scheggia et al., 2020; Yizhar et al., 2011), while reduced activity in mPFC SOM+ neurons has been linked to deficits in recognizing affective states (Scheggia et al., 2020). Despite these associations, the biological mechanisms governing such distinct implications of SOM+ and PV+ neurons in distinct social processes still need to be elucidated.

Intriguingly, ex vivo experiments identified distinct functional connection between PV and SOM cells with astrocytes (Mariotti et al., 2018). Accordingly, a short optogenetic

stimulation of SOM + interneurons within the mouse somatosensory cortex induced a significant increase in astrocytic Ca^{2+} activity (Mariotti et al., 2018). Conversely, optogenetic stimulation of visual cortical astrocytes enhanced both excitatory and inhibitory transmission, with an increase of PV+ interneuron firing rate (Perea et al., 2014), suggesting the implicational role of astrocytes in the modulation of different processes controlled by cortical circuits. Astrocytes play a dynamic role in modulating neuronal transmission by actively engaging in interaction with neurons in the so-called "tripartite synapse" (Araque et al., 1999). Within this framework, astrocytes sense synaptic activity through membrane receptors, culminating in an elevation of astrocytic Ca^{2+} signaling and consequent release of gliotransmitters (Araque et al., 2014).

Notably, in a mouse model lacking $GABA_B$ receptors in mPFC astrocytes, diminished GABAergic astrocytic signaling strongly correlates with cognitive impairments, disrupting prefrontal circuit dynamics and negatively impacting excitatory/inhibitory balance (Mederos et al., 2021). Intriguingly, optogenetic stimulation of astrocytes demonstrated their ability to fine-tune cortical circuit activity, ultimately leading to improved cognitive performance (Mederos et al., 2021). While the synaptic communication between GABAergic and astrocyte networks has been thoroughly characterized, their modulation in socio-cognitive processes remains elusive.

Given the fundamental role of SOM+ interneurons in affective state discrimination (Scheggia et al., 2020), we hypothesized that astrocytes-SOM+ neurons communication could be important for emotion recognition. To achieve this, we initially gained insight into the physical connection between astrocytes and different inhibitory neurons in the medial prefrontal cortex, employing a combination of viral vector transduction and brain clearing method. Our analysis revealed a notable spatial correlation between astrocytes and SOM+ inhibitory neurons. Functionally, in vivo fiber photometry recordings established increased mPFC astrocytes activity during interaction with emotionally-altered conspecific. In agreement, using the well-established chemogenetic approach to activate astrocytes, we demonstrated that these glial cells significantly enhance the ability of mice to recognize the positive or negative altered affective state expressed by another conspecific. Furthermore, deficits induced by SOM+ inhibition are rescued by astrocytes activation, which in turn influence SOM neurons activity.

Collectively, these findings support our hypothesis regarding the involvement of astrocytes-SOM neurons communication in emotion recognition, contributing to a deeper understanding of the circuits underlying this socio-cognitive function.

Results

Astrocytes envelop more mPFC SOM neurons compared to PV and VIP inhibitory neurons

The bidirectional synaptic communication between astrocytes and GABAergic interneurons has been extensively documented (Lia et al., 2019; Mariotti et al., 2018; Matos et al., 2018; Mederos et al., 2021). However, a comprehensive characterization of the spatial organization involving all inhibitory interneurons in proximity to astrocytes remains incomplete. While a structural and spatial study of hippocampal astrocytes has revealed their close proximity to somatostatin (SOM+) interneurons compared to parvalbumin (PV+) and VIP inhibitory neurons (Refaeli et al., 2021), a quantitative investigation in the medial prefrontal cortex is currently lacking.

To achieve this goal, we combined viral vectors injection and fluorescent tagging method in transgenic mice (Fig 1a). Specifically, a AAV8-GFAP-eGFP virus was injected into the medial prefrontal cortex in SOM::Cre x Ai14, PV::Cre x Ai14, and VIP::Cre x Ai14 mice (Fig.1b), in order to co-label GFAP cortical astrocytes (green) and tdTomato inhibitory neurons (red).

We then rendered thick brain slices (4-5mm) transparent using CLARITY and acquired high resolution 2D and 3D images (Fig.1b).

We first quantified the spatial proximity between astrocytes and inhibitory interneurons in the medial prefrontal cortex. We found that while the average distance between inhibitory neurons and their surrounding astrocytes in the medial prefrontal cortex does not differ between the three inhibitory cell types (Figure 1c), PV and VIP inhibitory neurons are unlikely to be engulfed by any astrocyte (25% and 34%, respectively), whereas most SOM neurons are engulfed by at least one (>60% of SOM neurons come in close proximity to nearby astrocyte) (Figure 1d).

In summary, our findings revealed that while the average spatial proximity between astrocytes and inhibitory interneurons in the medial prefrontal cortex remains consistent across parvalbumin, somatostatin and VIP- positive cells, the distinctive interaction patterns indicate that SOM neurons exhibited a higher degree of engulfment by astrocytes in comparison to PV and VIP, highlighting their specific higher connection with cortical astrocytes.

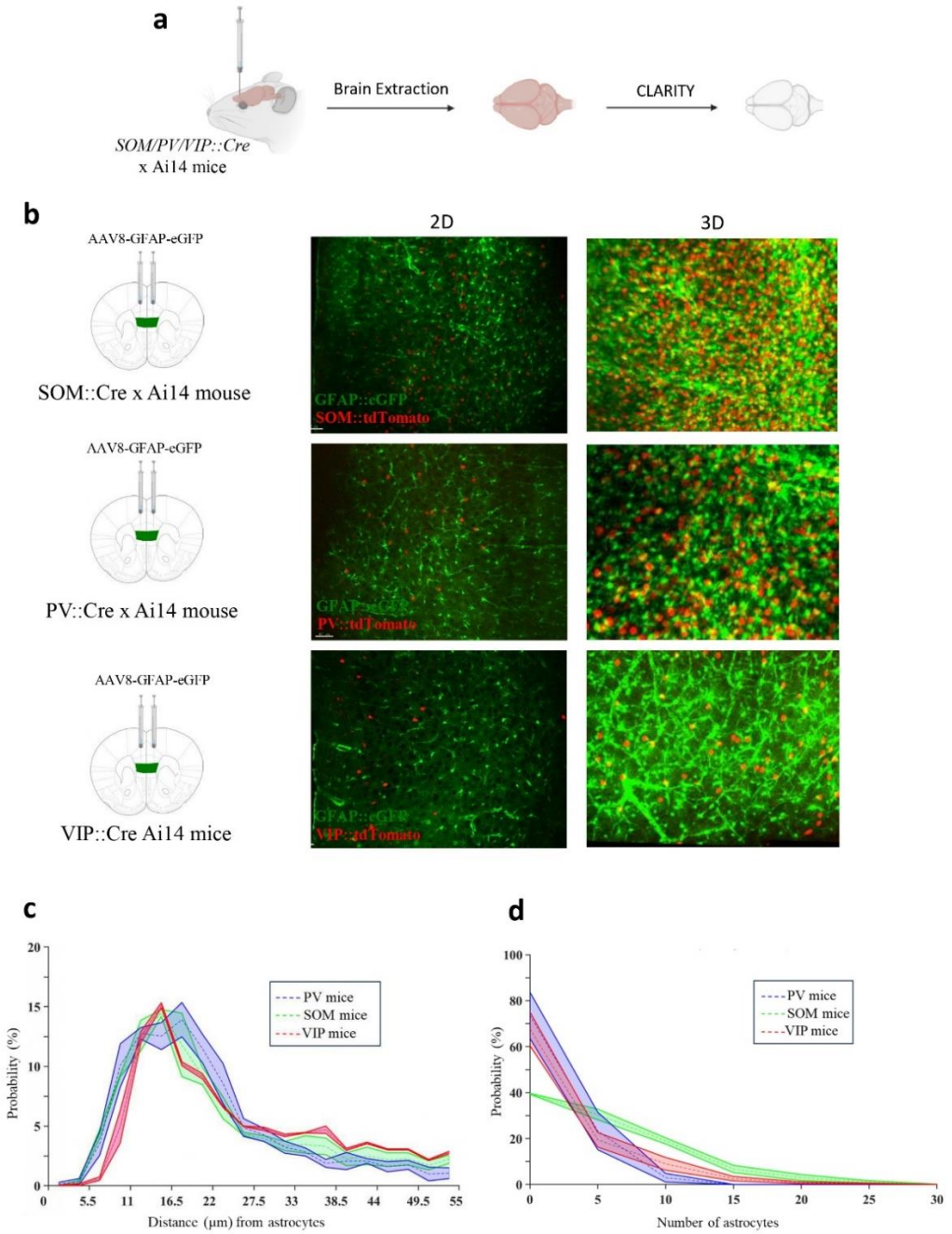


Figure 1 | Spatial distribution between astrocytes and inhibitory interneurons in the medial prefrontal cortex. **(a)** SOM-Cre, PV-Cre and VIP-Cre xAi14mice were injected in the mPFC with AAV8—GFAP-eGFP virus to induce the expression of fluorescent protein in astrocytes. Following >4 weeks, their brains were extracted, cut into thick cortical slices (4–5 mm) and were then made transparent by CLARITY after which they were imaged. **(b)** Co-expression of red fluorescent tdTomato (red) interneurons and GFP (green) astrocytes in the mPFC is shown in 2D and 3D images. **(c)** Astrocyte probability spatial distributions in SOM (green), VIP (red), and PV (blue) mice, as a function of distance. **(d)** Astrocytes probability to envelope SOM (green), VIP (red), and PV (blue) interneurons in the mPFC.

Increased astrocytes activity in mPFC with emotionally-altered mice

To investigate the physiological dynamics of mPFC astrocytes during emotion discrimination test (EDT), we performed fiber photometry recordings in the recognition of different affective states. In the medial prefrontal cortex of observer mice, we injected a fluorescent calcium indicator virus, AAV5-gfaABC1D-cyto-GCaMP6f, targeting astrocytes through the specific *gfaABC1D* astrocytic promoter (Fig.2a).

As discussed in the previous *chapter 1*, in the emotion discrimination test the observer mouse was exposed to both positive and negative emotional valence protocol, specifically relief and fear, respectively. To induce an altered affective state with positive valence, one unfamiliar demonstrator (‘*relief’*, yellow) received 1 hour of ad libitum access to water after 23 hours of water deprivation, while the other one was directly taken from its home cage (‘*neutral*’, grey) (Fig.2b).

Conversely, in the fear paradigm, the neutral demonstrator (‘*neutral*’, grey) was not subjected to any manipulation, while the fear one (‘*fear*’, red) experienced fear conditioning at least one day before being tested in the EDT, receiving three foot shocks (0.7 mA, 2s duration, 90s intershock interval) preceded by a 4000Hz beep tone. On the day of the EDT test, the presentation of the tone was specifically delivered in the second trial of the task for two minutes in order to study observers’ response before, during, and after tone presentation (Fig.2e).

We analyzed fiber photometry data by correlating astrocytes' activity with behavior, recorded using Anymaze tracking software. Specifically, we aligned calcium activity with the mice’s entrance into different zones of the EDT apparatus, categorically divided into the *altered-zone* (= associated with emotionally altered demonstrator quarter circle, yellow or red), the *neutral zone* (= associated with neutral demonstrator quarter circle, grey) and *no-stimuli zone* (= with no demonstrator’s present) (Fig.2b, Fig.2e).

Our data demonstrated an increase in astrocytic calcium activity when the observer mouse entered the zone associated with the relief demonstrator (AUC; p=0.075 compared to neutral), particularly during the initial 0-2-minute trial of the task (Fig.2c, Fig.2d), when the most significant discrimination is observed (Ferretti et al., 2019).

Similar results were observed in the fear paradigm. Following tone presentation in the 4-6 minute trial, observer mice exhibited an increased tendency astrocyte activity upon entering the fear zone compared to the neutral (AUC; 0.087) (Fig.2f , Fig.2g)

Overall, these findings suggest the involvement of mPFC astrocytes in the recognition of altered affective states, as indicated by increased activity when interacting with emotionally altered stimuli.

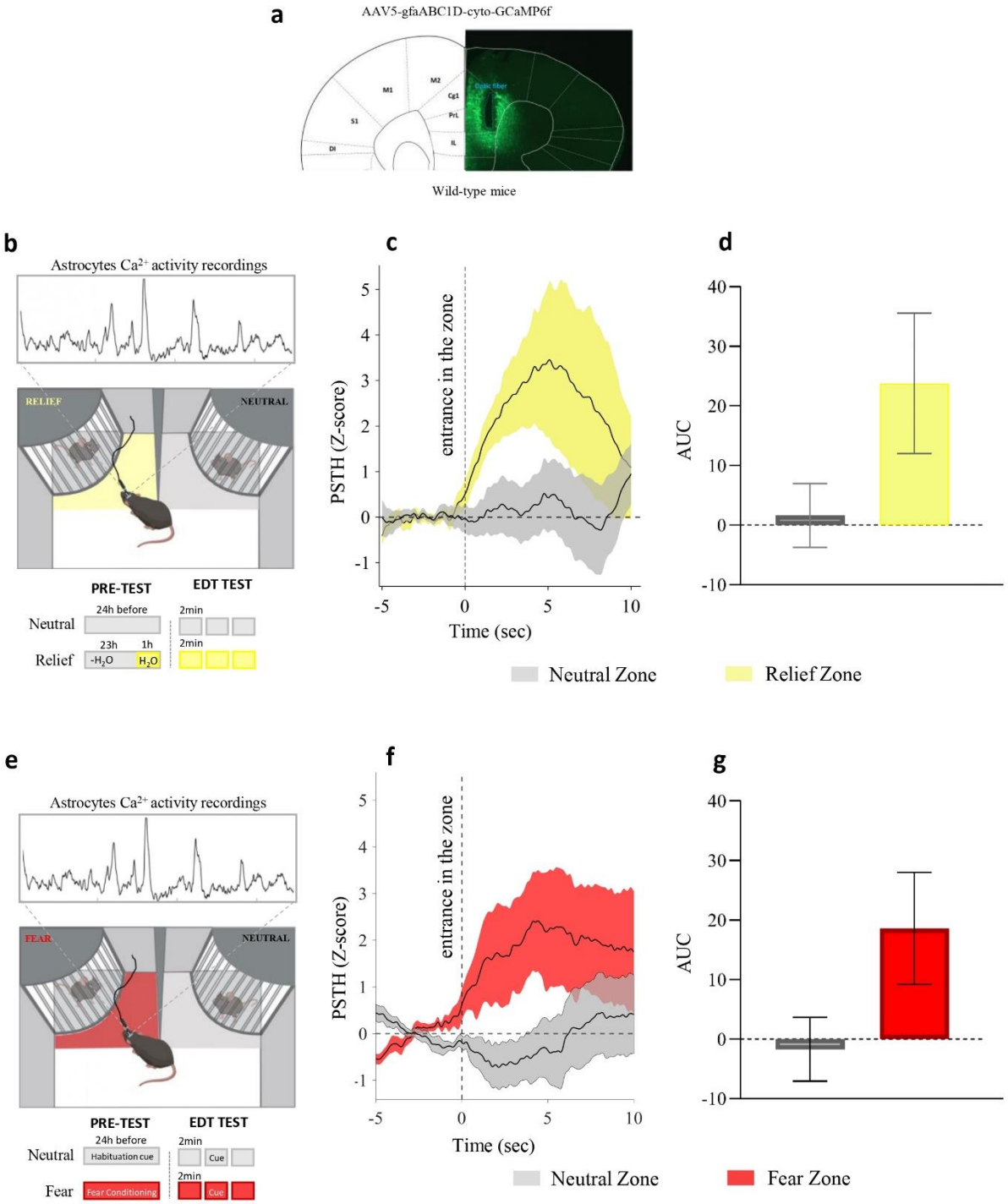


Figure 2 | mPFC astrocytic activity correlates with the altered-emotional state interaction **(a)** Representative image of coronal mPFC section. Wild-type mice were injected with AAV5-gfaABC1D-cyto-GCaMP6f and implanted unilaterally with optic fiber in mPFC. **(b)** Schematic illustration of the testing arena strategically divided into relief (yellow) and neutral (gray) demonstrator's zone. During the relief test, one demonstrator received 1-h ad libitum access to water after 23h of water deprivation (relief, yellow) while the other demonstrator (neutral, gray) waited undisturbed in its home cage before being tested. The astrocytic cortical activity of observers' mice was concurrently recorded as they interacted with neutral and relief demonstrators. **(c)** GCaMP6f fluorescence changes in the mPFC were observed in response to the observer's entry into the relief (yellow) or neutral (grey)- associated zone of the EDT apparatus during the initial trial. The signal was aligned to behavioral outcomes, considering a baseline preceding the events (T=5 seconds) and a post-baseline following the events (T=10 seconds). The *x-axis* indicates time, with 0 denoting the exact moment of entry into the zone. The *y-axis* represents the peristimulus analysis computed in correlation with the corresponding behavior. **(d)** Area under the curve of GCaMP PSTH according to the entrance in the relief (yellow) and neutral (gray) zone. **(e)** Schematic illustration of the testing arena strategically divided into fear (red) and neutral (gray) demonstrator's zone. In the fear protocol, one demonstrator (fear, red) was fear-conditioned at least one day before. The other demonstrator (neutral, gray) was not manipulated.The astrocytic cortical activity of observers' mice was concurrently recorded as they interacted with neutral and fear demonstrators. **(f)** GCaMP6f fluorescence changes in the mPFC were observed in response to the observer's entry into the fear (red) or neutral (grey)- associated zone of the EDT apparatus during the last trial. The signal was aligned to behavioral outcomes, considering a baseline preceding the events (T=5 seconds) and a post-baseline following the events (T=10 seconds). The *x-axis* indicates time, with 0 denoting the exact moment of entry into the zone. The *y-axis* represents the peristimulus analysis computed in correlation with the corresponding behavior. **(g)** Area under the curve of GCaMP PSTH according to the entrance in the fear (red) and neutral (gray) zone.

Chemogenetic mPFC astrocytes activation enhances emotion discrimination

To further investigate the potential involvement of astrocytes in the ability to discriminate and approach their conspecifics based on different altered affective states, we employed chemogenetics to selectively activate GPCR signaling in astrocytes during emotion discrimination task (EDT). Our aim was to determine whether astrocytes contribute to the recognition of both positive and negative emotional states. To achieve this goal, observer mice underwent bilateral injection of AAV5-GFAP-hM3D(Gq)-mCherry into the mPFC area (Fig. 3a and 3b) and emotion discrimination tests were conducted 30 minutes after administering CNO or a control treatment. The observer was exposed to both positive and negative emotional valenced protocols including relief as well as fear and stress as a positive and negative protocol, respectively (Fig. 3c, 3d, 3e). As a control, we included a separate group of mice expressing AAV8-GFAP104-mCherry in the mPFC (Supplementary Fig.1a), which received control or CNO injections, as well.

As previously shown (Ferretti et al., 2019) (Scheggia et al., 2020), in the emotion discrimination task (EDT) the observer mouse is placed in front of two unfamiliar conspecifics (demonstrators) expressing different emotional states for a 6-minute session.

To induce an altered affective state with positive valence, one unfamiliar conspecific demonstrator (‘*relief’*, yellow bars) received 1 hour of ad libitum access to water following 23 hours of water deprivation, while the other one remained in a neutral state (‘*neutral*’, grey bars) (Fig.2c). Consistent with prior findings (Ferretti et al., 2019), observer mice treated with saline (“control”) spent more time sniffing towards relieved-altered demonstrators than toward neutrals, selectively in the first trial (0 - 2 minutes) of the task (Fig.3f). 2wayANOVA RM revealed a significant trial x affective state interaction effect ($F_{(2,56)}$=4.983, p=0.0102) and multiple comparison analysis highlighted a significant difference between groups selectively in the 0-2 minutes trial (p= 0.0396). Surprisingly, we observed a significant increase in the observer’s social approach toward relief stimulus in the first 2 minutes of the task when CNO was injected. This effect was also prolonged in the second trial (2 - 4 minutes), as suggested by an increase in sniffing towards the relief demonstrators compared to the control group (Fig.3g). A significant trial x affective state interaction effect was demonstrated by 2wayANOVA RM ($F_{(2,64)}$= 5.428 p=0.0066). Multiple comparison analysis revealed a significant difference between neutral and relief in the first trial (p<0.0001) and second trial (p= 0.0105). Indeed, these observations were

substantiated also by the discrimination index (DI), revealing a significant treatment effect ($F_{(1,30)}$=8.037, p=0.0081) in the discrimination of different emotional stimuli (Fig.3h) No significant effects (p=0.2124) were observed when analyzing the total sniffing time over the entire 6-minute duration of the emotion discrimination task between CNO-treated and control mice (Fig.3i).

Furthermore, our additional analysis revealed that CNO-treated mice spent a greater amount of time in the zone of EDT apparatus associated with relief stimuli than neutral (Fig.4e) (2wayANOVA treatment x zone $F_{(1,61)}$= 0.0154, multiple comparisons control vs CNO p<0.0001), highlighting their preference for the altered demonstrators. Notably, no ambulatory effects, such as total distance travelled (Fig.4f) and average speed (Fig.4g), were observed between CNO and the control-treated group during EDT, indicating the absence of alterations in general locomotor behavior induced by astrocyte activation (Mann-Whitney test: locomotion p=0.4114, speed p=0.3952).

In addition, CNO *di per se* did not elicit differences in emotion discrimination for relief (Supplementary Fig.1c and 1d), as evidenced by no significant effect in the discrimination index (Supplementary Fig.1e) when analyzed with 2wayANOVA RM (trials x treatment $F_{(2,16)}$=1.381, p=0.2797). Moreover, no effects were observed in the total sniffing time over the entire 6 minutes of EDT (Supplementary Fig.1g) when compared to the control group (p=0.5476). These findings are in line with previous findings reported in Ferretti et al (2019). Moreover, additional analysis revealed no differences in the zone preference (Supplementary Fig.1f) (trials x zone $F_{(1,8)}$=0.2778, p=0.6125) and locomotor parameters (Supplementary Fig.4h and 4i) (p=0.6905, speed p=0.6508).

Overall, these data suggest that astrocytic activation by hM3D(Gq) can lead to a pronounced social preference for the relief demonstrator without affecting sociability, revealing the first evidence for the involvement of astrocytes in the recognition of positive-emotional stimuli.

We next explored the implication of astrocytic modulation in the recognition of negative emotional valence with a fear stimulus (Fig.3d). In this paradigm, the neutral demonstrator (grey bars) was not subjected to any manipulation, while the fear one (red bars) was subjected to fear conditioning experience at least one day before being tested in the EDT. The conditioned stimulus received three foot shocks (0.7 mA, 2s duration, 90s intershock interval), each preceded by a beep tone released for consecutive 30s. On the day of the EDT test, the presentation of the

tone was specifically delivered in the second trial of the task for two minutes. This approach was designed to ekove a negative-valenced emotional state in the fear demonstrator and consequently examine observers' response before, during, and after tone presentation (Ferretti et al., 2019b).

In agreement with our previous work, the observer of the control group showed an increase sniffing behavior toward the fear-conditioned demonstrator compared to the neutral one in the third trial (Fig.3j), after the 2-minute duration of the beep tone release (second trial), when the fear mouse display the classical freezing response due to the presentation of the conditioned stimulus. Indeed, a main interaction trial x affective state effect was detected by 2way ANOVA RM and multiple comparison analysis revealed a significant difference between neutral and fear sniffing in the last trial ($p=0.0087$).

In line with the previous experiment, the CNO-treated group exhibited a pronounced discriminatory behavior throughout the last trial of the test (4 -6 minutes) (Fig.3k). Notably, the activation of mPFC astrocytes following CNO injection anticipated emotional discrimination in the second trial during tone presentation (second trial $p=0.0069$, third trial $p=<0.0001$). This effect was evidenced by an increase in sniffing behavior towards the fear stimulus compared to neutral, suggesting a potential involvement of astrocytes in the recognition of negative states that may override conditioned freezing-inhibitory behavior. These findings were further corroborated by the discrimination index analysis, revealing a significant difference for treatment ($F_{(1,30)}=4.878$, $p=0.0350$) between CNO and control group (Fig.3l). However, no significant differences were observed in terms of the total sniffing time over the entire 6-minute duration of the emotion discrimination task (Fig.3m) ($p=0.1890$).

Furthermore, we observed a heightened preference for the altered demonstrators, as indicated by the analysis of the total amount of time spent in the EDT apparatus (Fig.4i). A significant interaction of treatments x zone effect was observed ($F_{(1,60)}=12.94$, $p = 0.0007$). Specifically. observers' mice treated with CNO, but not with the control treatment ($p=0.9912$), spent more time positioned in the zone related to the fear-conditioned demonstrators than the neutral ones ($p=0<0.0001$). Importantly, no locomotor alteration was evident, as suggested by the absence of significant effects in total distance travelled (Fig.4j) ($p=0.1425$) and average speed (Fig.4k) ($p=0.2795$).

Notably, the control group injected with the control virus AAV8-GFAP104-mCherry did not exhibit significant effects following CNO injection in the recognition of fear stimulus

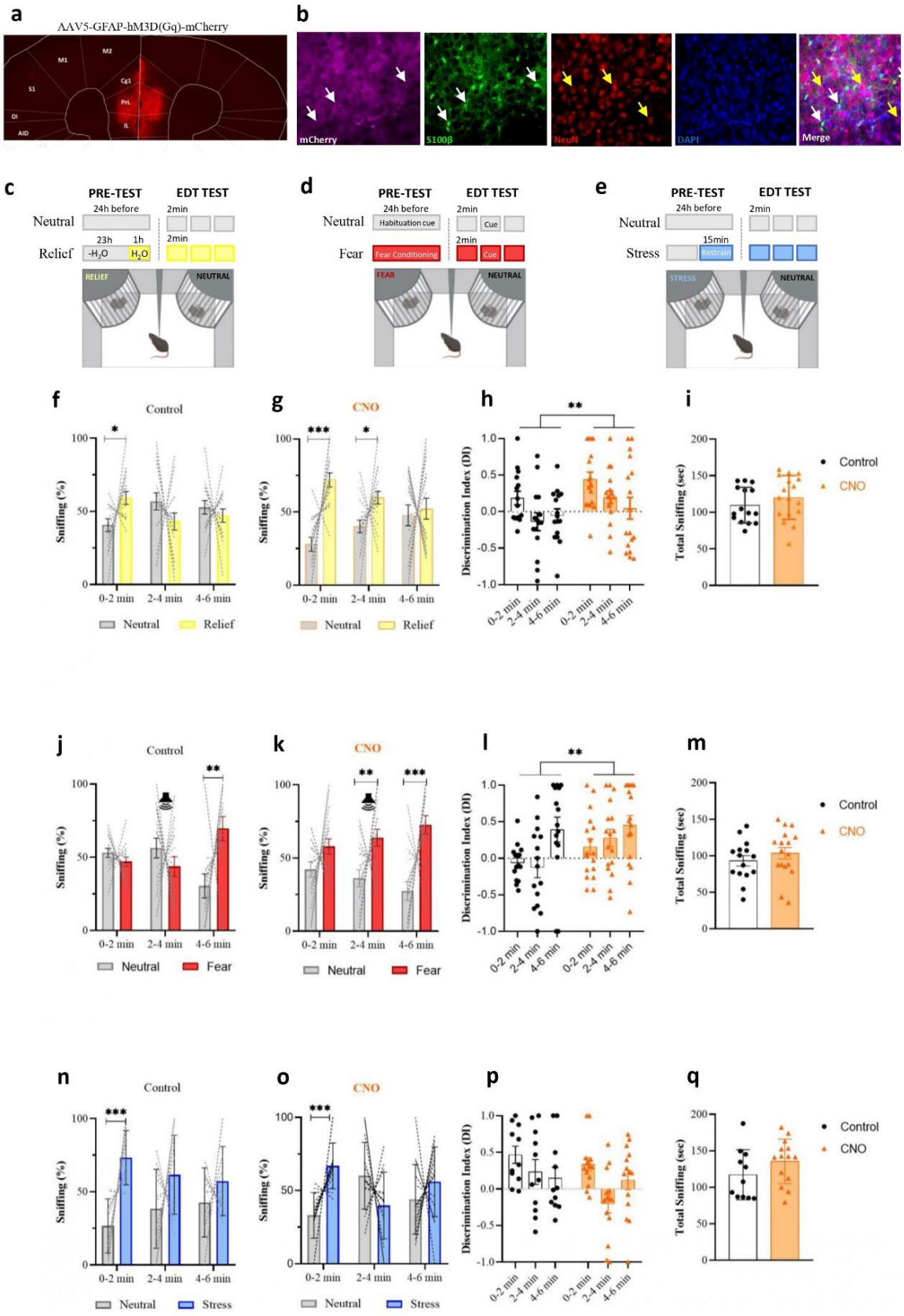
(Supplementary Fig. 1k, 1l and 1m), as evidence by no statistical significant effect in 2wayANOVA analysis (treatment x trials: $F_{(2,16)}$=0.8651, p=0.8651), and in the sociability behavior (Supplementary Fig.1o) (p=0.0952). No differences were observed in zone preference (Supplementary Fig.1n) ($F_{(1,8)}$=3.438, p=0.1008) and ambulatory parameters (Supplementary Fig. 1p and 1q) (locomotor p=0.4206, average p=0.4444).

These comprehensive results strongly support the role of astrocytes in influencing social responses to negative emotional stimuli in the context of fear conditioning.

We further explored the involvement of astrocytes in recognizing negative-emotional models by applying stressful manipulation. In this paradigm, the altered demonstrator ("*stress*", blue bars) underwent a mild stress protocol, involving 15 minutes of acute restrain in a 50mL falcon tube immediately before EDT. Instead, the neutral demonstrator ("*neutral*", grey bars) remained unmanipulated and was directly taken from its home cage (Fig.3e), following a similar approach as in the other emotional protocols described above.

Similarly to the control group, observer mice treated with CNO demonstrated a preference for the stress demonstrator compared to the neutral one, selectively in the 0–2-minute epochs of the test (Fig.3n and 3o). In both treatment conditions, statistical analysis with 2wayANOVA RM revealed a significant trials x affective state interaction effect (control: $F_{(2,40)}$=4.125, p=0.0235; CNO: $F_{(2,52)}$=12.82, p<0.0001), followed by a selective sniffing difference in the first trial (control, p<0.0001; CNO, p<0.0001). Accordingly, the discrimination index analysis between the control and CNO did not reveal significant differences (Fig.3p) ($F_{(2,46)}$=1.807, p=0.1755), suggesting that they spent more time sniffing towards the altered stimulus without any significant difference between the two experimental groups in term of sociability (Fig.2q) (p=0.1492). In accordance, further analysis did not reveal significant differences in term of time in the zone (Fig.4m), as evidenced by no statistical significant effect in treatment x zone interaction (2wayANOVA RM, $F_{(1,46)}$=2.633, P=0.1115). No significant ambulatory differences, such as distance travelled (Fig.4n) (p=0.8508) and average velocity (Fig.4o) (p=0.8196), were observed between groups. This indicates that astrocytic modulation did not produce a discernible impact on the preference for the stress-related emotional state, suggesting a possible involvement of different biological processes that require further investigation.

Overall, these data provide support for the hypothesis that mPFC astrocytes may play a key role in mediating emotion discrimination processes in response to altered affective states.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M1
M2
S1
Cg1
PrL
IL
DI
AID
b
mCherry
S100β
NeuN
DAPI
Merge
c
PRE-TEST
EDT TEST
Neutral
24h before
2min
Relief
23h
1h
-H2O
H2O
2min
RELIEF
NEUTRAL
d
PRE-TEST
EDT TEST
Neutral
24h before
Habituation cue
2min
Cue
Fear
Fear Conditioning
2min
Cue
FEAR
NEUTRAL
e
PRE-TEST
EDT TEST
Neutral
24h before
2min
Stress
15min
Restrain
STRESS
NEUTRAL
f
Control
Sniffing (%)
0-2 min
2-4 min
4-6 min
Neutral
Relief
g
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Sniffing (%)
0-2 min
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Neutral
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Discrimination Index (DI)
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CNO
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Control
Sniffing (%)
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Sniffing (%)
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Sniffing (%)
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Neutral
Stress
p
Discrimination Index (DI)
0-2 min
2-4 min
4-6 min
q
Total Sniffing (sec)
Control
CNO

Figure *3* | Chemogenetic mPFC astrocytes activation enhances emotion discrimination
(a) Representative image of coronal mPFC section. Wild-type mice were bilaterally injected in the mPFC with AAV5-GFAP-hM3D(Gq)-mCherry virus. 30 minutes before starting emotion discrimination tests (EDT), mice received an intraperitoneal injection of Clozapine-N-oxide (CNO) or control (saline) treatment. **(b)** Immunohistochemistry confirming AAV5-GFAP- hM3D(Gq)-mCherry virus specificity in mPFC GFAP+ cells. White arrows indicate infected cells express mCherry and are co-labeled with S100β+ cells. Yellow arrows indicate that NeuN + cells (red) are not co-labeled with either S100β or mCherry. **(c) (d) (e)** Experimental design of EDT for relief (yellow), fear (red) and stress (blue) paradigm. In the relief (c), one demonstrator received 1-h ad libitum access to water after 23h of water deprivation (relief, yellow) while the other demonstrator (neutral, gray) waited undisturbed in its home cage before being tested. In the fear paradigm (d), one demonstrator (fear, red) was fear-conditioned at least one day before. The other demonstrator (neutral, gray) was not manipulated. (e) In the stress protocol, one demonstrator (stressed, blue) was subjected to the tube restraint stress for 15 min immediately before the beginning of the EDT. The other demonstrator (neutral, gray) waited undisturbed in the home cage. (**f) (g)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. control (f) and CNO (g) treatment. N= 15 control observers and N= 17 CNO observers (**h**) Mice in control (black dots) and CNO (orange triangles) condition significantly differ based on discrimination index ([sniffing towards relief– sniffing towards neutral] / [sniffing towards relief + sniffing towards neutral]) (**i**) No difference between control and CNO group was found in the total sniffing time during relief test. **(j) (k)** Percentage of time spent sniffing demonstrators towards fear (red bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. control (f) and CNO (g) treatment i.p. control (j) and CNO (k) treatment. N= 15 control observers and N= 17 CNO observers (**l**) Mice in control (black dots) and CNO (orange triangles) condition significantly differ based on discrimination index ([sniffing towards fear– sniffing towards neutral] / [sniffing towards fear + sniffing towards neutral]).(**m**) No difference between control and CNO group was found in the total sniffing time during fear test. **(n) (o)** Percentage of time spent sniffing demonstrators towards stress (blue bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. control (f) and CNO (g) treatment i.p. control (n) and CNO (o) treatment. N= 11 control observers and N= 14 CNO observers (**p**) Mice in control (black dots) and CNO (orange triangles) condition did not differ based on discrimination index ([sniffing towards stress– sniffing towards neutral] / [sniffing towards stress + sniffing towards neutral]). (**q**) No difference between control and CNO group was found in the total sniffing time during stress test.
Bar and line graphs show mean ± s.e.m. *P < 0.05. **P < 0.005. ***P < 0.0005.

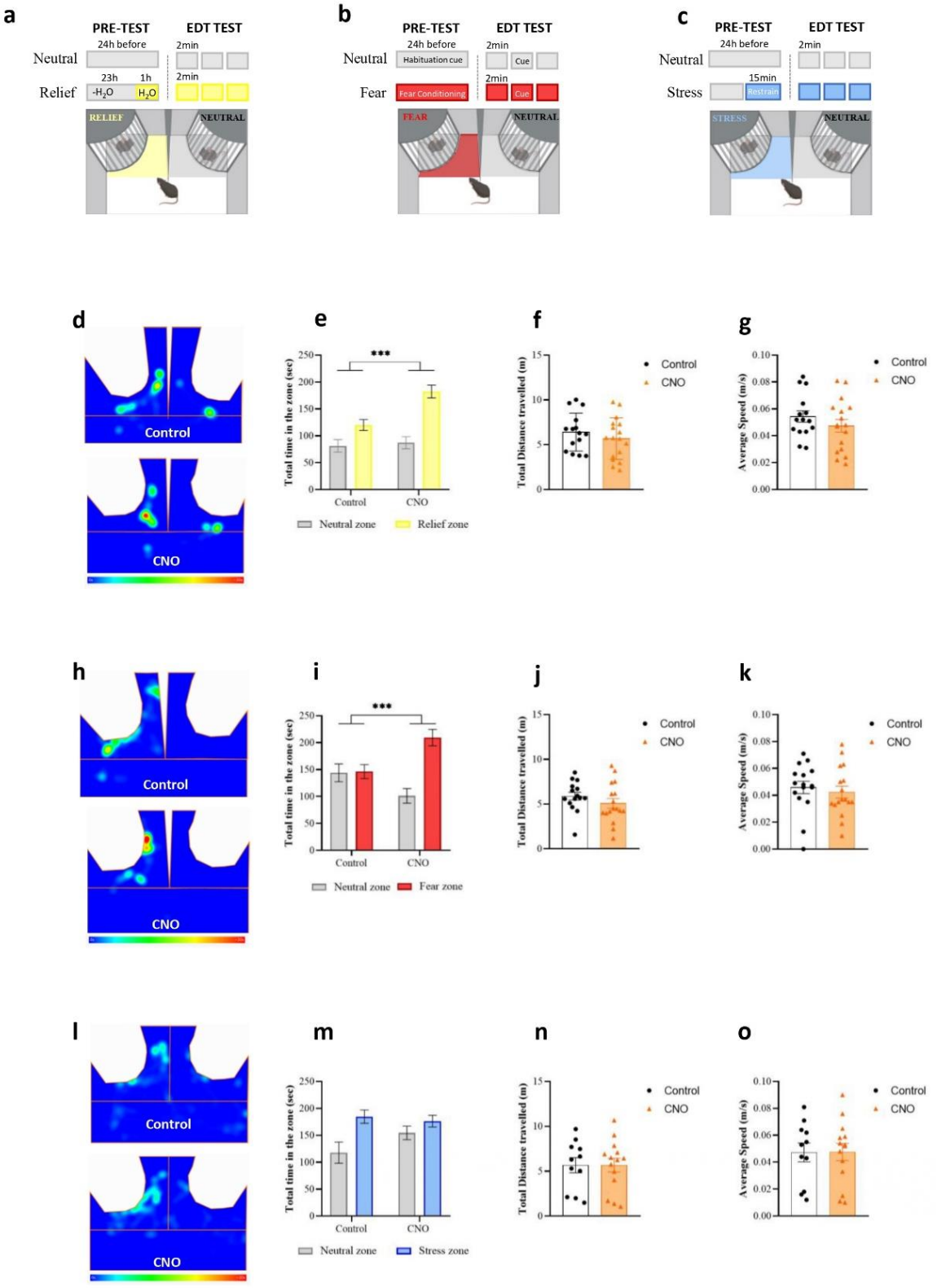

a
PRE-TEST
EDT TEST
24h before
2min
Neutral
23h
1h
2min
Relief
-H2O
H2O
RELIEF
NEUTRAL
b
PRE-TEST
EDT TEST
24h before
2min
Neutral
Habituation cue
Cue
2min
Fear
Fear Conditioning
Cue
FEAR
NEUTRAL
c
PRE-TEST
EDT TEST
24h before
2min
Neutral
15min
Stress
Restrain
STRESS
NEUTRAL
d
Control
CNO
e
Total time in the zone (sec)
***
Control
CNO
Neutral zone
Relief zone
f
Total Distance travelled (m)
Control
CNO
g
Average Speed (m/s)
Control
CNO
h
Control
CNO
i
Total time in the zone (sec)
***
Control
CNO
Neutral zone
Fear zone
j
Total Distance travelled (m)
Control
CNO
k
Average Speed (m/s)
Control
CNO
l
Control
CNO
m
Total time in the zone (sec)
Control
CNO
Neutral zone
Stress zone
n
Total Distance travelled (m)
Control
CNO
o
Average Speed (m/s)
Control
CNO

Figure 4 / Enhanced zone preference for the altered emotional stimuli induced by mPFC astrocytes chemogenetic activation

(a)(b)(c) Experimental design and set up characteristics of EDT for relief (yellow), fear (red) and stress (blue) protocols. The EDT apparatus was strategically divided into distinct zones to evaluate the behavioral responses of observer mice following i.p. control (f) and CNO (g) treatment in response to the different emotional stimuli presented during the tests. For the relief (a), the EDT testing cage was divided into two zones. The first zone, marked by a yellow square, was specifically linked to the altered demonstrator (relief, yellow zone), while the second zone, denoted by a gray square, was associated with the neutral demonstrator (neutral, gray zone). For the fear (b), the testing cage was divided into a red square associated with the fear demonstrator (fear, red zone) and a gray square associated with the neutral demonstrator (neutral, gray zone). For the stress (c), the testing cage was divided into a blue square associated with the stress demonstrator (stress, blue zone) and a gray square associated with the neutral demonstrator (neutral, gray zone). (**d**) Representative heat maps during EDT relief following control or CNO treatment. **(e)** Increased time spent in the zone with the relief demonstrator (yellow bars) compared to neutral (gray bars) following CNO administration during the relief EDT test. N= 15 control observers and N= 17 CNO observers **(f) (g)** No differences between control and CNO-treated groups were observed in total distance travelled (f) and average speed velocity (g) during the relief EDT test. (**h**) Representative heat maps during EDT fear following control or CNO treatment. **(i)** Increased time spent in the zone with the fear demonstrator (red bars) compared to neutral (gray bars) following CNO administration during the fear EDT test. N= 15 control observers and N= 17 CNO observers **(j) (k)** No differences between control and CNO-treated groups were observed in total distance travelled (j) and average speed velocity (k) during the fear EDT test. (**l**) Representative heat maps during EDT stress following control or CNO treatment. **(m)** No differences were observed in terms of time in the zone associated with the stress demonstrator (blue bars) compared to neutral (gray bars) following CNO administration during the stress EDT test. N= 11 control observers and N= 14 CNO observers **(n) (o)** No differences between control and CNO-treated groups were observed in total distance travelled (n) and average speed velocity (o) during the stress EDT test.

Bars show mean ± s.e.m. ***P < 0.0005.

Chemogenetic activation modulate mPFC astrocytes Ca^{2+} activity

In our study, we first elucidate the potential involvement of cortical astrocytes in emotion discrimination. Using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), we selectively modulated G protein-coupled receptor (GPCR) signaling (Roth et al., 2016) in mPFC astrocytes. To validate the effectiveness of the chemogenetic approach, we co-injected an adeno-associated virus (AAV) carrying a hM3D(Gq) fused with mCherry fluorescent protein under the control of the astrocytic glial fibrillary acidic protein (GFAP) promoter (AAV5-GFAP-hM3D(Gq)-mCherry) and the genetically encoded calcium indicator GCaMP6f (AAV5-pZac2.1 gfaABC1D-cyto-GCaMP6f) within the medial prefrontal cortex (Fig 5a). Then we implanted an optic fiber terminating dorsally to this area and we performed fiber photometry in freely moving mice (Fig5b). The astrocytic activity was recorded during a baseline period of 10 minutes prior to intraperitoneal (i.p.) administration of either saline (control) or Clozapine-N-oxide (CNO, 3mg/kg). After 20 minutes from injection, calcium dynamics were monitored for 10 minutes during the post-treatment period to identify changes in calcium fluctuations (Fig.5c). A main treatment x time interaction effect was detected by 2wayANOVA RM ($F_{(1,16)}$=7.866 p=0.0127) suggesting that CNO injection triggered a significant increase in peak frequency (peaks/min) across time. Specifically, multiple comparison analysis revealed a statistical significance in peak frequency variation between pre and post CNO injection (p=0.0179) and between CNO and control treatment in the post injection phase (p=0.0070). In contrast, the control did not elicit significant effects driven by the treatment injection (p =0.9357) (Fig.5d). Interestingly, during the post-CNO injection phase, we observed a significant reduction in peak intensity (ΔF/F), indicating a decrease in the amplitude of astrocytic peaks. 2wayANOVA RM revealed a significant treatment x interaction time effect ($F_{(1,16)}$=10.19,p=0.0057), followed by multiple comparison analysis that showed statistical significant differences between pre and post CNO injection (p=0.0218) and post control and CNO treatment (p=0.0218) (Fig.5e).

Consistent with previous findings (Adamsky et al., 2018; Codeluppi et al., 2023; Durkee et al., 2019), our results confirm that hM3D(Gq)-DREADDs induce the cellular activation of astrocytes, resulting in an increase in the frequency, but not intensity, of Ca^{2+} events following CNO injection.

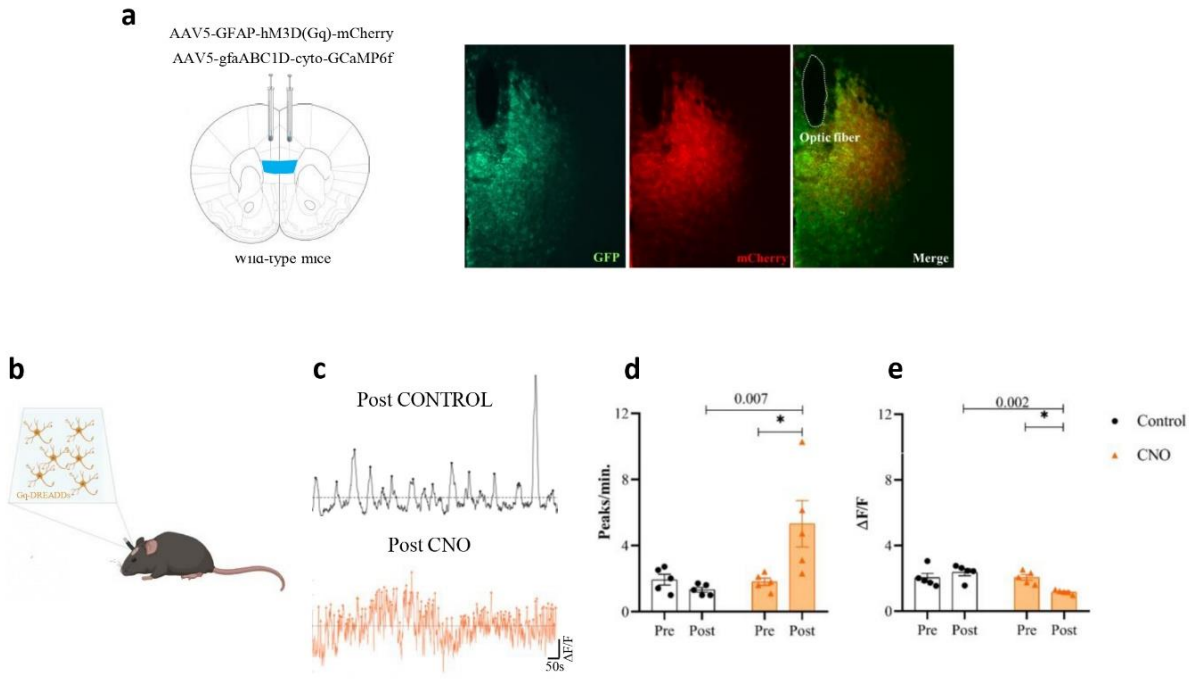


Figure 5 | Chemogenetic activation modulate mPFC astrocytes Ca^{2+} activity
(a) Left: Wild-type mice were injected in the mPFC with AAV5-GFAP-hM3D(Gq)-mCherry and AAV5-gfaABC1D-cyto-GCaMP6f and implanted bilaterally with optic fibers terminating dorsally to the injection area. Right: representative image of a coronal mPFC section with GFP and mCherry expression **(b)** Astrocytic activity was recorded with fiber photometry in freely moving mice before and after clozapine-n-oxide (CNO) or control treatment injection **(c)** Representative traces of calcium transients post control and CNO administration. Dotted black and orange line: Z-score = 0 respectively for the control and CNO group. Black dots and orange triangles indicate local maxima peaks respectively for the control and CNO group after injection. **(d)** Peak frequency and **(e)** peak intensity of calcium transients recorded for 10 mins before and after acute administration of control or CNO. N=5 mice.
Treatments were counterbalanced.
Bars show mean ± s.e.m. *P < 0.05.

mPFC astrocyte activation does not affect sociability, social memory and social hierarchy

To validate our hypothesis concerning the involvement of cortical astrocytes in selectively modulating emotion recognition abilities, we conducted additional social behavioral tasks in mice expressing GFAP-hM3D(Gq) in mPFC (Fig.6a) to explore their contribution to various aspects of social behaviors.

CNO administration had no significant effect on sociability and social memory processes, as assessed through the Social Habituation Dishabituation Task (Huang et al., 2014) (Fig.6b). Both control and CNO-treated mice exhibited equal progressive reduction in the social interaction with their conspecifics from Trial 1 to Trial 4, as evidenced by a main effect of trial within groups (2wayANOVA RM $F_{(2.237,53.69)}$=26.09, p<0.0001). No interaction effect of trial x treatment was found ($F_{(4,96)}$=0.02563, p=0.9987), neither any significant difference between groups as effect of treatment ($F_{(1,24)}$=0.01522, p=0.9028). Notably, they demonstrated increased social exploration when a novel conspecific stimulus was introduced in the testing cage in Trial 5 (Fig.6c). No significant effects were observed in the total social exploration time (Fig.6d) (p=0.9197) and in the social novelty score (Fig.6e) (p>0.999). Similarly, no significant effects were found in the GFAP104-mCherry control group following CNO intraperitoneal injection when we analyzed social interaction across trials (Supplementary Fig.1s) (trials x treatment interaction F(4,32)=0.5360, p=0.7103). Additionally, no statistical differences were observed in sociability (Supplementary Fig.1t) (p= 0.4762) and social novelty (Supplementary Fig. 1u) (p=0.9143).

Social dominance plays a pivotal role in rodent behavior, influencing both social structures and motivation (Komori et al., 2019; Kunkel et al., 2018). To examine the involvement of mPFC astrocytes in this context, we employed the tube dominance test (Fig.6f), a reliable paradigm for determining social rank (Fan et al., 2019). Following the assessment of social rank of male cage mate mice in baseline condition, the test was subsequently repeated at intervals of 30 minutes, 2 hours, 6 hours, and 24 hours post vehicle (Fig. 6g) and CNO (Fig. 6h) injection to monitor potential changes in social dominance associated with astrocytic activation. However, no significant changes in hierarchical rank among cage mates were observed across different time intervals.

These collective findings suggest that mPFC astrocytes may not play a substantial role in modulating sociability, social memory, and social dominance processes.

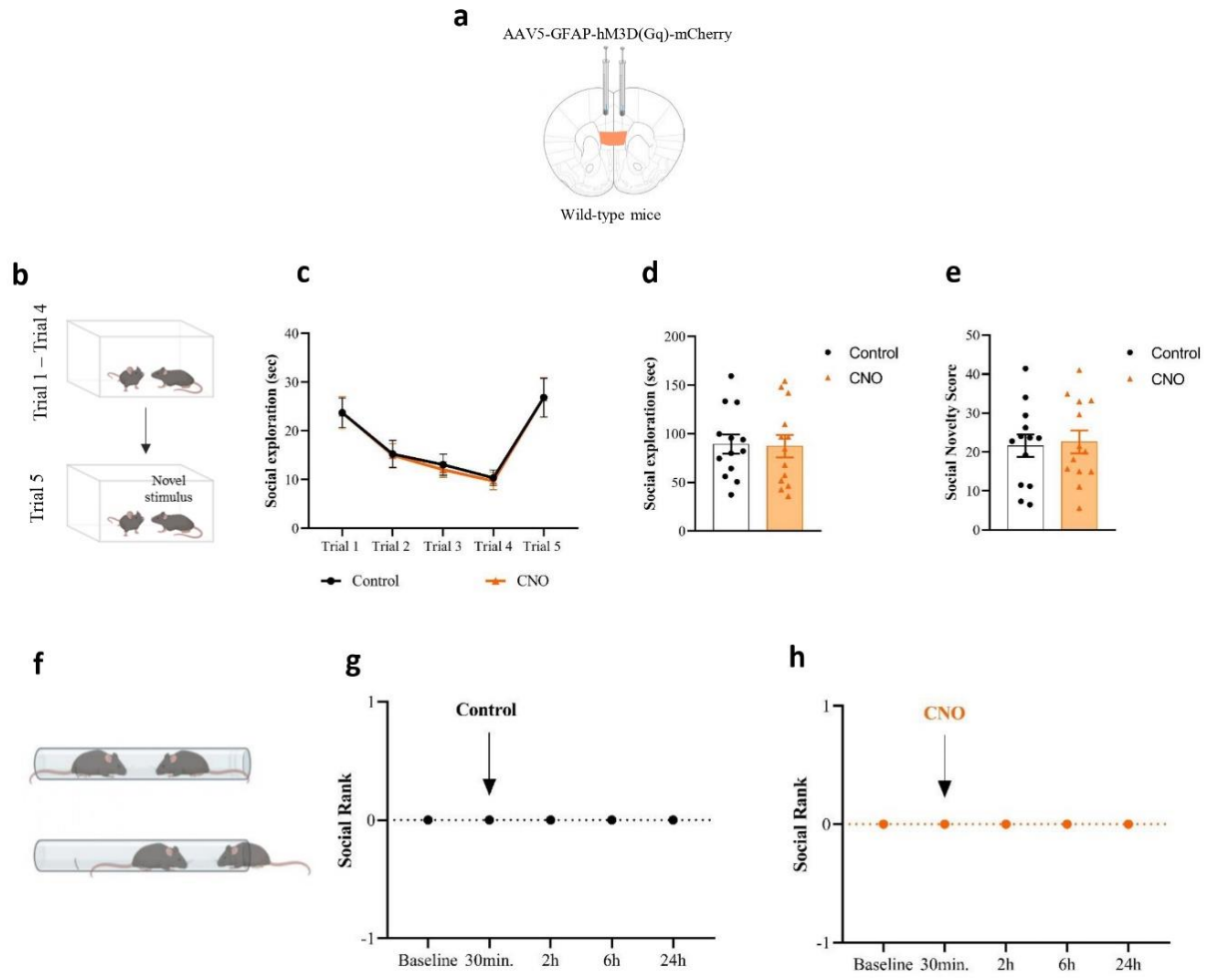


Figure 6 / mPFC astrocyte activation does not affect sociability, social memory and social hierarchy

(a) Wild-type mice were bilaterally injected in the mPFC with AAV5-GFAP-hM3D(Gq)-mCherry virus. 30 minutes before starting social tests, testing mice received an intraperitoneal injection of Clozapine-N-oxide (CNO) or control (saline) treatment. **(b)** Schematic illustration of the habituation dishabituation social interaction test. An unfamiliar stimulus mouse was introduced into the testing cage, engaging in a 1-minute interaction with the testing subject. At the end of the 1-minutes trial, the stimulus animal was removed and placed in an individual holding cage. This sequence was repeated for four trials, separately by 3-minutes inter trial interval, reintroducing the same stimulus mouse. In the fifth 'dishabituation' trial, a novel unfamiliar stimulus mouse was introduced in the testing cage to assess social memory. **(c)** No differences were observed analyzing the total amount of time in seconds allocated to investigation of the unfamiliar stimulus from trial1 to trial5 between control (black) and CNO (orange)-treated mice. N= 13 control testing mice and N= 13 CNO testing mice. **(d)** Total exploration time was calculated summing the social exploration times from trial 1 to trial 5. No difference was found between control (black dots) and CNO (orange triangles) groups during the entire social task. **(e)** Social novelty score was calculated using the following formula: ((T1-T4)+(T5-T4))/2 where "T" are the social interaction times sessions. No difference was found between control (black dots) and CNO (orange triangles) groups during the entire social task. **(f)** Schematic illustration of the tube test paradigm for evaluating social hierarchy among cage mates. Adult male mice, housed in pairs, were introduced to the test by being placed at opposite ends of

the plastic tube. They subsequently met in the middle of the tube, and their interaction was observed until one of the mice retreated or was completely pushed out of the tube by the other. The mouse successfully forced its counterpart out of the tube was considered the dominant one (=social rank 1), while the retreating mouse was considered subordinate (=social rank -1). Following the determination of their innate social dominant status, mice underwent either control or CNO treatment. Tests were conducted at various time points post injection, including 30 minutes, 2 hours, 6 hours and 24 hours. **(g) (h)** Social dominant status did not change across different time points following injection for both control (g) and CNO (h) groups**.** N= 10 cages for control and N= 10 cages for CNO. Treatments were counterbalanced.
Bar and line graphs show mean ± s.e.m.

Chemogenetic astrocytes activation restores emotion recognition impairments following SOM+ photoinhibition in the medial prefrontal cortex

SOM+ interneurons in the mPFC play a fundamental role in affective states discrimination. A prior study definitively demonstrated that optogenetic inhibition of somatostatin led to socio-cognitive deficits (Scheggia et al., 2020). To investigate whether selective activation of mPFC astrocytes-expressing hM3D(Gq) could mitigate the discrimination deficiencies induced by selective optogenetic SOM+ photoinhibition, we employed a combination of optogenetics and chemogenetics. For this purpose, we used transgenic SOM-Cre mice expressing a Cre recombinase in somatostatin expressing neurons. We bilaterally induced a co-expression of a Cre-dependent halorhodopsin vector (AAV5-Ef1a-DIO eNpHR 3.0-EYFP) and a hM3Dq virus under GFAP promotor (AAV5-GFAP-hM3Dq-mCherry) in the medial prefrontal cortex and subsequently, mice were implanted with optic fibers terminating dorsally to this brain area (Fig.7a). We performed the emotion discrimination test (EDT) with both *relief* and *fear* paradigm, wherein we observed significant effects primarily driven by astrocytes activation (Fig.3h; Fig3l). During EDT, astrocytes activity was modulated via intraperitoneal injection of clozapine-N-oxide (CNO, 3mg/kg) 30 minutes prior behavioral test. Simultaneously, somatostatin neurons activity was silenced during the initial 2 minutes of the test for the relief (Fig,7b) and in the last 2 minutes for fear (fig.7i), by delivering a continuous green light (532nm) stimulation, following the established protocol applied in our previous study (Scheggia et al., 2020). This approach strategically targeted the time windows when observers typically increase social exploration towards demonstrators in altered affective states. Mice underwent testing for consecutive weeks, with the order of treatments (vehicle and CNO) and laser stimulation (light off and light on) counterbalanced.

As previously documented, observer mice exposed to light stimulation (*Light ON*) during the initial two minutes of the *relief* test and treated with control treatment (saline) exhibited deficits in discriminating altered demonstrators, spending an equivalent amount of time sniffing both relief and neutral conspecifics. Notably, this negative behavioral effect was transient and reversible, as, following the laser interruption, mice demonstrated an increased social exploration towards emotionally altered stimulus compared to neutral (Fig.7c). 2wayNOVA RM revealed a significant trial x affective state interaction effect ($F_{(2,36)}$=3.615, p=0.0371), followed by post-hoc

multiple comparative analysis highlighting a difference by trial (I trial: p=0.8933; II trial: p=0.0121). Surprisingly, our findings revealed that astrocytes' chemogenetic activation upon CNO injection successfully restored socio-cognitive impairments induced by SOM+ inhibition in the first session of EDT as evidenced by an increased social exploration toward altered demonstrator (Fig.7d) (affective state: $F_{(1,22)}$=8.183, p=0.0091). Importantly, this manipulation did not alter basic sociability, as suggested by the absence of effects on total exploration time (Fig.7e) (p=0.5387).

In *Light OFF* control condition, when mice were tested without light stimulation, observer mice showed emotion discrimination for the relieved demonstrator independently to the treatment (Fig.7f and Fig.7g), with no differences in social exploration time between control and CNO groups (Fig.7h) (p=0.6505).

Next, we applied the same methodology to evaluate the impact of our approach using a different affective protocol with a negative valence. In the fear paradigm, the continuous green light stimulation was delivered in the last two minutes of the test, after beep tone presentation. Consistent with the positive protocol, the optogenetic inhibition of SOM+ interneurons under control treatment condition led to deficits in recognizing the fear demonstrator, as indicated by the lack of a social preference for either conspecific (Fig.7j). No trials x affective state interaction effect was observed with 2wayANOVA RM ($F_{(2,36)}$=1.021, p=0.3703). Remarkably, CNO injection for the selective astrocyte activation rescued recognition deficits with an enhancement of social preference for the conditioned stimulus compared to neutral (Fig.7k) (trials x affective state $F_{(2,48)}$=3.978, p=0.0252). However, no significant effects in sociability were observed between control and CNO-treated groups under light stimulation (Fig.7l) (p=0.2839).

As expected, in *Light OFF* condition, both experimental groups spent more time sniffing the fear-conditioned demonstrator compared to neutral ones in the last 2 minutes of the task, indicating recognition of the altered affective state following fear recall by tone (Fig.7m and Fig. 7n). Compared to the control group (trials x affective state $F_{(2,36)}$=2.692, p=0.0813), astrocyte activation in mPFC following CNO treatment significantly enhanced the discrimination of the fear conspecific (Fig. 7n) (trials x affective state $F_{(2,44)}$=18.41, p<0.0001), as previously shown in *chapter 2.*

Overall, these data indicate the fundamental role of mPFC astrocytes in restoring and compensating emotion recognition deficits induced by optogenetic inhibition of SOM+ neurons.

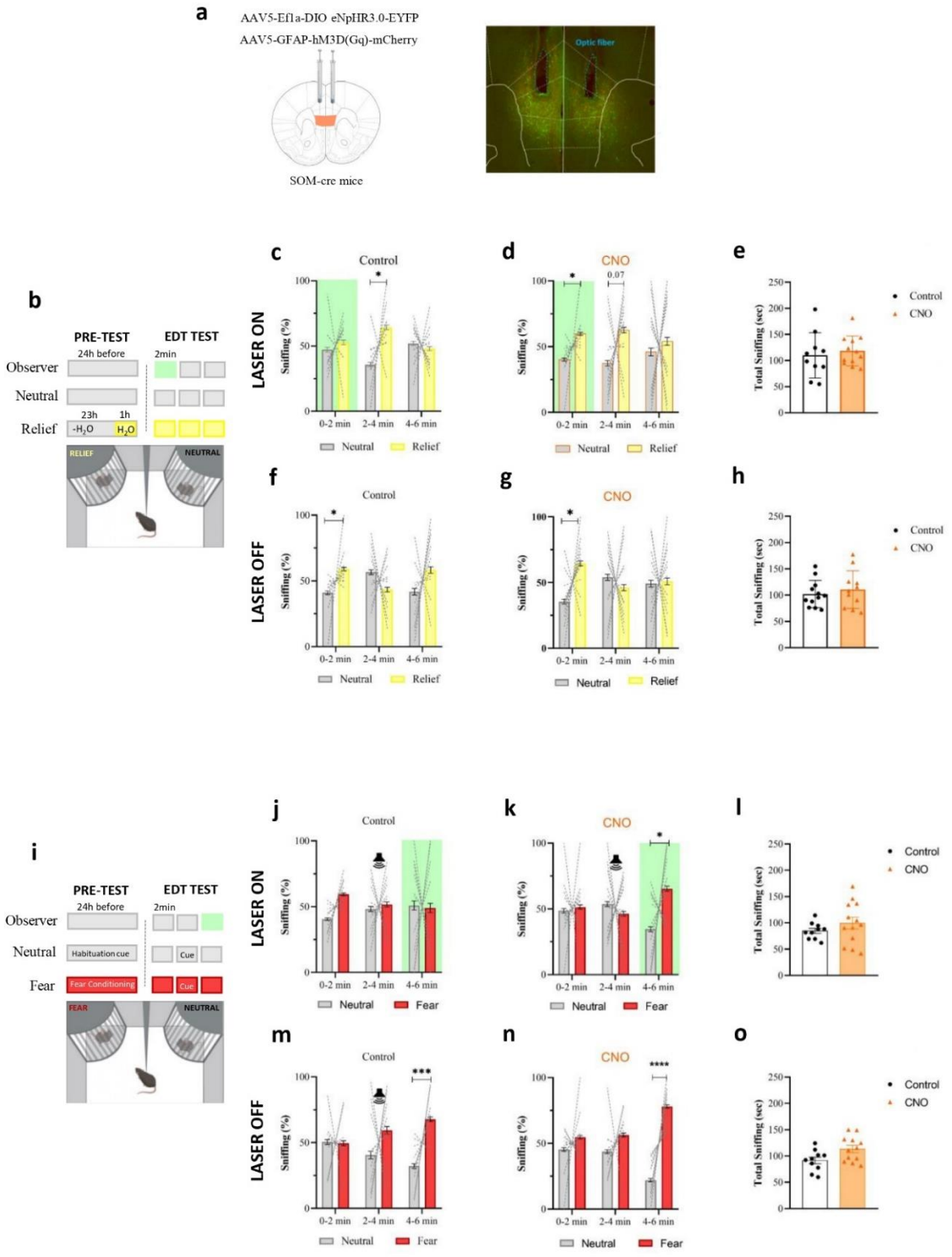
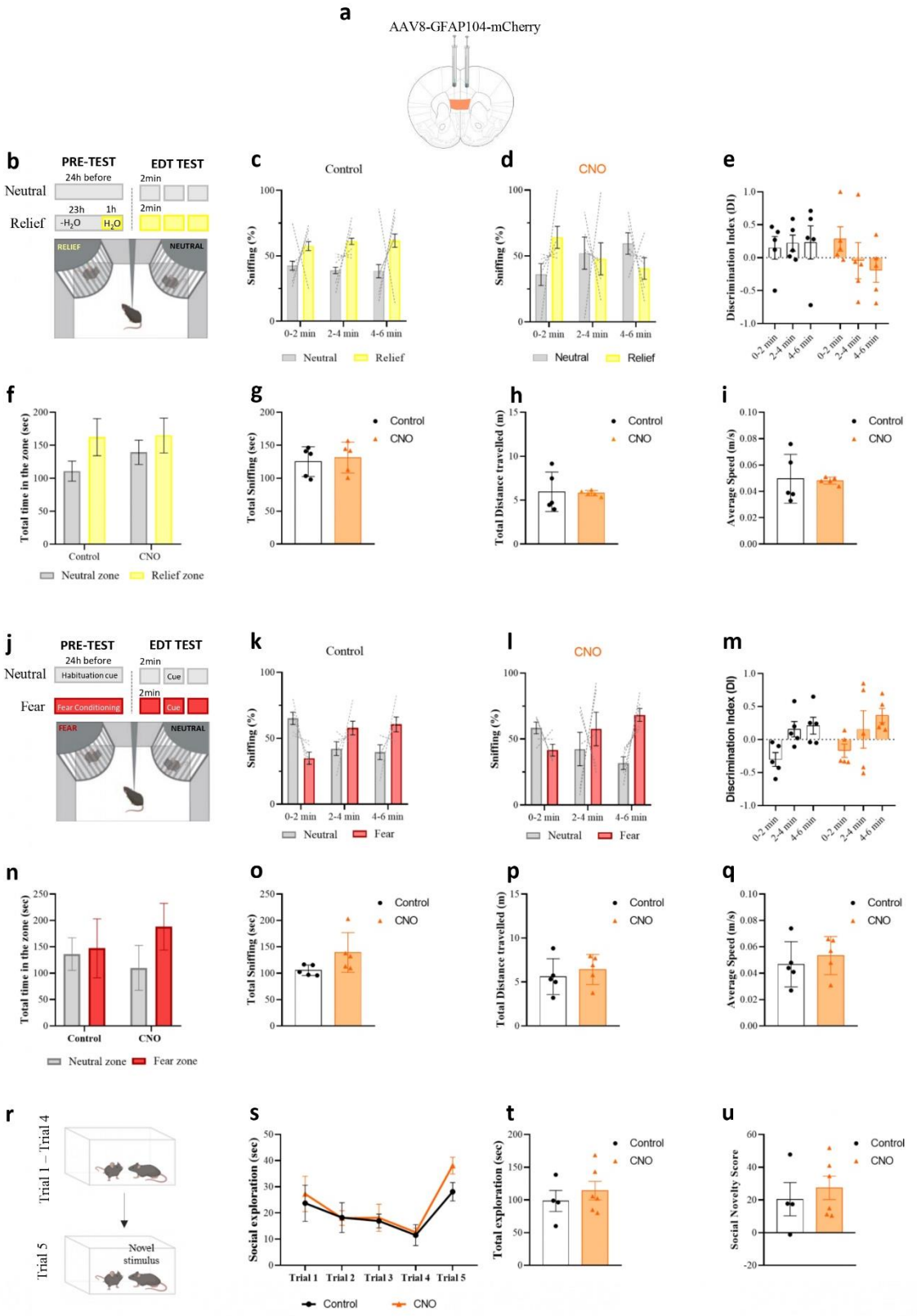


Figure 7 | Chemogenetic astrocytes activation restores emotion recognition impairments following SOM+ photoinhibition in the medial prefrontal cortex

(a) Representative image of coronal mPFC section. SOM+cre mice were bilaterally injected with AAV5-Ef1a-DIO eNpHR 3.0-EYFP and AAV5-GFAP-hM3Dq-mCherry in the mPFC and subsequently implanted with optic fibers terminating dorsally to this brain area. **(b)** Experimental design of the EDT with optogenetic stimulation with one neutral (gray) and one relief (yellow) demonstrator in naïve condition. Observers did not undergo any manipulation, but they were subjected to optogenetic stimulation. Photoinhibition (λ = 532 nm) was delivered during the first 2min of test, when discrimination occurs. **(c)(d)** Percentage of time spent sniffing two neutral demonstrators in neutral (gray bars) or relief (yellow bars) during the 6-min test displayed by naïve observer mice tested in laser-ON condition that received control (c) or CNO (d). treatment. N= 10 control observers and N= 12 CNO observers **(e)** Under the laser-ON condition, both control and CNO-treated groups did not exhibit any significant differences in terms of total sniffing time throughout the entire EDT session. **(f)(g)** Percentage of time spent sniffing two neutral demonstrators in neutral (gray bars) or relief (yellow bars) during the 6-min test displayed by naïve observer mice tested in laser-OFF condition that received control (f) or CNO (g) treatment. N= 11 control observers and N= 11 CNO observers **(h)** Under the laser-OFF condition, both control and CNO-treated groups did not exhibit any significant differences in terms of total sniffing time throughout the entire EDT session. **(i)** Experimental design of the EDT with optogenetic stimulation with one neutral (gray) and one fear (red) demonstrator in naïve condition. Observers did not undergo any manipulation, but they were subjected to optogenetic manipulation. Photoinhibition (λ = 532 nm) was delivered during the third trial of test. **(j)(k)** Percentage of time spent sniffing two neutral demonstrators in neutral (gray bars) or fear (red bars) during the 6-min test displayed by naïve observer mice tested in laser-ON condition that received control (j) or CNO (k) treatment. N= 10 control observers and N= 13 CNO observers **(l)** Under the laser-ON condition, both control and CNO-treated groups did not exhibit any significant differences in terms of total sniffing time throughout the entire EDT session. **(m)(n)** Percentage of time spent sniffing two neutral demonstrators in neutral (gray bars) or fear (red bars) during the 6-min test displayed by naïve observer mice tested in laser-OFF condition that received control (m) or CNO (n) treatment. N= 10 control observers and N= 12 CNO observers **(o)** Under the laser-ON condition, both control and CNO-treated groups did not exhibit any significant differences in terms of total sniffing time throughout the entire EDT session.

Bar and line graphs show mean ± s.e.m. *P < 0.05. **P < 0.005. ***P < 0.0005.

Supplementary



Supplementary Figure 1 | CNO *di per se* did not induce any behavioral changes
(a) Wild-type mice were bilaterally injected in the mPFC with the control virus AAV8-GFAP104-mCherry expressing the fluorescent protein. 30 minutes before starting emotion discrimination tests (EDT), observers mice received an intraperitoneal injection of Clozapine-N-oxide (CNO) or control (saline) treatment. **(b)** Experimental design of EDT relief. Observers did not undergo any manipulation. One demonstrator (relief, yellow) received 1-h ad libitum access to water after 23h of water deprivation. The other demonstrator (neutral, gray) waited undisturbed in the home cage**.(c)(d)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after i.p. control (c) and CNO (d) treatment. N= 5 control observers and N= 5 CNO observers. **(e)** Mice in control (black dots) and CNO (orange triangles) condition did not differ based on discrimination index ([sniffing towards relief– sniffing towards neutral] / [sniffing towards relief + sniffing towards neutral]). **(f)** No differences were observed in terms of time in the zone associated with the relief demonstrator (yellow bars) compared to neutral (gray bars) following CNO administration during the relief EDT test. **(g)** No significant effect in sociability was observed under both control and CNO condition **(h) (i)** No differences between control and CNO-treated groups were observed in total distance travelled (n) and average speed velocity (o) during the relief EDT test. **(j)** Experimental design of EDT fear. Observers did not undergo any manipulation. One demonstrator (fear, red) was fear-conditioned at least one day before. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(k)(l)** Percentage of time spent sniffing demonstrators towards fear (red bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after i.p. control (k) and CNO (l) treatment. N= 5 control observers and N= 5 CNO observers. **(m)** Mice in control (black dots) and CNO (orange triangles) condition did not differ based on discrimination index ([sniffing towards fear– sniffing towards neutral] / [sniffing towards fear + sniffing towards neutral]). **(n)** No differences were observed in terms of time in the zone associated with the fear demonstrator (red bars) compared to neutral (gray bars) following CNO administration during the fear EDT test. **(o)**No significant effect in total sniffing time was observed under both control and CNO condition **(p) (q)** No differences between control and CNO-treated groups were observed in total distance travelled (p) and average speed velocity (q) during the fear EDT test. **(r)** Schematic illustration of the habituation dishabituation social interaction test. An unfamiliar stimulus mouse was introduced into the testing cage, engaging in a 1-minute interaction with the testing subject. At the end of the 1-minutes trial, the stimulus animal was removed and placed in an individual holding cage. This sequence was repeated for four trials, separately by 3-minutes inter trial interval, reintroducing the same stimulus mouse. In the fifth 'dishabituation' trial, a novel unfamiliar stimulus mouse was introduced in the testing cage to assess social memory. **(s)** No differences were observed analyzing the total amount of time in seconds allocated to investigation of the unfamiliar stimulus from trial1 to trial5 between control (black) and CNO (orange)-treated mice. N= 5 control testing mice and N= 5 CNO testing mice. **(t)** Total exploration time was calculated summing the social exploration times from trial 1 to trial 5. No difference was found between control (black dots) and CNO (orange triangles) groups during the entire social task. **(u)** Social novelty score was calculated using the following formula: ((T1-T4)+(T5-T4))/2 where "T" are the social interaction times sessions. No difference was found between control (black dots) and CNO (orange triangles) groups during the entire social task.

Discussion

In our study, we have unveiled novel insights into the neurobiological underpinnings of emotion recognition processes, with a specific focus on the interplay between astrocytes-somatostatin interneurons within medial prefrontal cortex. Building upon existing observations of the involvement of somatostatin interneurons in socio-cognitive processes (Scheggia et al., 2020), our investigation contributes new findings regarding their functionality in social behaviors.

The analysis of spatial distribution unveiled a stringent communication pattern between astrocytes and somatostatin interneurons within medial prefrontal cortex, aligning with numerous studies that have demonstrated a functional connection between these cells (Henriques et al., 2022; Mariotti et al., 2018; Matos et al., 2018; Mederos et al., 2021). Consequently, we deeply investigated the potential interaction of cortical SOM+ neurons and astrocytes in modulating emotion discrimination processes. Consistent with prior research on SOM+ activity in the recognition of different emotions (Scheggia et al., 2020), our fiber photometry data demonstrated an elevation in astrocytes calcium activity when an observer mouse is exposed to emotionally altered stimulus. Significantly, astrocytes manipulation with chemogenetic Gq-DREADDs signaling resulted in an augmented recognition of emotionally altered stimuli, shedding light on the pivotal role of astrocytes within cortical microcircuits in shaping emotion discrimination ability.

Among cortical areas, the medial prefrontal cortex serves as a key regulator of higher-order functions (Frith and Dolan, 1996; Haber et al., 2022; Ko, 2017), including the control of emotions (Hiser and Koenigs, 2018). Crucial to this regulation is the maintenance of excitatory/inhibitory (E/I) balance, essential for sustaining optimal cortical excitability. This synaptic balance is intricately orchestrated by the interplay between excitatory pyramidal neurons and inhibitory interneurons, releasing glutamate and GABA, respectively. Such synchronization is fundamental for the integration of information and for generating appropriate behavioral responses (Sohal and Rubenstein, 2019). Disturbances in cortical E/I balance are well documented in postmortem studies of autistic and schizophrenic patients, where cognitive impairments arise from cortical excitatory/inhibitory imbalance (Chattopadhyaya and Cristo, 2012; Glausier and Lewis, 2013; Hutsler and Zhang, 2010). In this regard, a previous work demonstrated that somatostatin activity inhibition within mPFC impaired the ability to recognize

emotions (Scheggia et al., 2020), as a consequence of cortical GABAergic network alteration. Surprisingly, our results demonstrated that astrocyte activation could potentially restore these socio cognitive deficits, mitigating the impact of somatostatin interneurons silencing in socio cognitive function. The well documented reciprocal interactions between astrocytes and GABAergic interneuron represent a fundamental phenomenon ensuring circuit stability for healthy brain function (Lia et al., 2019; Mederos et al., 2019). Astrocytes actively participate in the synaptic modulation of E/I balance by releasing different chemical gliotransmitters into the extracellular space, including glutamate, GABA, ATP/adenosine, D-serine and lactate (Araque et al., 2014; Harada et al., 2016; Liu et al., 2022; Yin et al., 2021), which collectively influence synaptic activity and subsequently impact behavioral responses. However, the specific intracellular signaling mechanism through which the activation of astrocytes modulates socio-cognitive functions and compensates for social impairments is still not fully characterized and will require further investigations. Nevertheless, the behavioral effect induced by Gq-DREADDs astrocyte activation through chemogenetic could be mediated by the release of neurotransmitters, subsequently influencing neuronal circuits and restoring synaptic functionality. This interpretation aligns with previous studies demonstrating that Gq-DREADDs activation results in the release of glutamate from hippocampal and nucleus accumbens astrocytes (Bull et al., 2014; Durkee et al., 2018), D-serine from hippocampal CA1 astrocytes, and ATP from striatal astrocytes (Kang et al., 2020). In the scenario of GABAergic-somatostatin interneurons inhibition, astrocytes may directly impact overall synaptic activity by adjusting the balance of neurotransmitters, particularly releasing GABA. To rigorously test our hypothesis, further investigations will be necessary, combining in vivo calcium imaging and cellular manipulating approaches within the context of our social task.

Notably, somatostatin interneurons within the cortex are recognized for co-releasing both GABA and somatostatin neuropeptide (van den Pol, 2012), exerting modulation over synaptic activity. Recently, Mariotti et al. demonstrated an enhanced response of astrocytes following the release of somatostatin neuropeptide by cortical interneurons, through somatostatin receptors type 4 (SSTR4) located in astrocytic processes. Given the existence of substantial evidence reporting lower level of somatostatin peptide in the cortical areas of schizophrenic patients (Dienel et al., 2023; Joshi et al., 2015; Morris et al., 2008) characterized by socio cognitive deficits, we will further investigate the potential involvement of somatostatin in astrocytes-SOM

interneurons interaction related to emotion discrimination. The development of specific neuropeptide biosensors (Wang et al., 2023) will facilitate our exploration into whether somatostatin directly influences emotion discrimination and whether astrocytes release this neuropeptide compensating the effect related to SOM+ photoinhibition.

Traditionally considered supportive cells for neurons, accumulating evidence now demonstrates the influential role of astrocytes in behavioral processes (Kofuji and Araque, 2021; Lyon and Allen, 2022; Shigetomi and Koizumi, 2023). Specifically, alteration in cortical astrocyte activity during a critical developmental phase has been shown to result in synaptic dysregulation and pronounced social-behavioral deficits (Luo et al., 2023). In our study, aimed at a comprehensive understanding of the role of astrocytes in brain circuits related to social cognition, we plan to enhance our investigation using a plasma membrane Calcium-ATPase (PMCA) machinery designed for the selective manipulation of astrocytic calcium homeostasis. This approach will be fundamental to investigate the socio-cognitive response in mice following a reduction in cortical astrocytic activity.

While we observed a significant impact in emotion discrimination, our findings indicate that the activation of mPFC may not influence social hierarchy. This contrasts with recent results reported by Noh et al., who demonstrated astrocytic influence in the dmPFC potentiating dominance behavior. However, accumulating evidence underscores the heterogeneity of the astrocyte population in terms of morphological, physiological and functional properties (Ben Haim and Rowitch, 2017; Khakh and Sofroniew, 2015; Lanjakornsiripan et al., 2018; Zhang and Barres, 2010). Notably, distinct subpopulations of astrocytes may contribute to different behavioral responses. Investigating the specific roles of different astrocyte subtypes in shaping complex functions will be crucial to unraveling their contribution to behaviors.

In conclusion, our findings underscore the potential involvement of cortical astrocytes-SOM+ interneurons communication in modulating the ability to discriminate the expression of affective states in others, offering a novel potential microcircuit player for future mechanistic research concerning socio-cognitive deficits.

Materials and Methods

Mice

All procedures were approved by the Italian Ministry of Health (permit n. 749/2017-PR 176AA.34, 639/2020-PR 1766AA.82) and by the Hebrew University Animal Care and Use Committee. Routine veterinary care and animals' maintenance was provided by dedicated and trained personnel.

For Clarity experiments, Pv-IRES-Cre (B6.129P2-Pvalbtm1(cre)Arbr/J—stocknumber 017320; Hippenmeyer et al., 2005), Sst-IRES-Cre (Ssttm2.1[cre]Zjh/J—stock number 013044; Taniguchi et al., 2011), and VIP-IRES-Cre (Viptm1[cre]Zjh/J—stock number 010908; Taniguchi et al., 2011), were used. All mice included in this study were male, hence it does not account for potential gender differences. Using the Allen Brain Atlas Mouse Connectivity datasets, we found that the different transgenic mouse lines show reasonable penetrance (>94%, >75%, and >92% for SOM, PV, and VIP, respectively) and high specificity (>87%, 95%, and >97% for SOM, PV, and VIP, respectively) when crossed with Ai14 mice, as indicated by fluorescence in situ hybridization (FISH) co-staining.

For behavioral experiments, adult males and females C57BL/6J mice and Somatostatin cre-recombinase mice (SOM-cre, Jackson: 013044) on a C57BL/6J background were used. SOM-Cre mice were heterozygote, and their genotype was determined by Polymerase chain reaction (PCR) of ear snip tissue.

Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with ad libitum access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). Experiments were run during the light phase (between 10am-5pm). All mice were handled on alternate days during the week preceding the first behavioral testing. Experimenters were blind to mouse treatments during testing.

Female mice were visually checked for estrus cycle immediately after the test and no correlation was found between estrus status and performance in the test.

Viral Injections and fiber implantation

Naïve mice were prepared for stereotaxic surgeries at 3 months old. All of them were anaesthetized with a mix of isoflurane (2%) in Oxygen (O_2, 1%) by inhalation before being mounted onto a stereotaxic frame (Kopf) linked to a digital reader. Brain coordinates of viral injection in the mPFC were chosen in accordance with the mouse brain atlas: anterior–posterior (AP), +1.9 mm; medial–lateral (ML): ± 0.25 mm; and dorsal–ventral (DV): −2.7; - 2.4; - 2.1 mm.

We infused AAVs virus through a borosilicate micropipette connected to a 10µl Hamilton syringe filled with mineral oil. The injection rate was set to last between 5 and 10 minutes and the pipette was maintained in position for at least 10 min to allow proper diffusion and then slowly withdrawn. The following viral vectors were used: AAV8-GFAP-eGFP (UNC vector gene, AV5105E), AAV5-Ef1a-DIO eNpHR 3.0-EYFP (Addgene AV4806H), AAV5-GFAP-hM3Dq-mCherry (Addgene 50478) AAV5-gfaABC1D-cyto-GCaMP6f (Addgene 52925), AAV5-GFAP-hM3Dq-mCherry (Addgene 50478), AAV8-GFAP104-mCherry (Addgene 58909). The injected volumes were adjusted based on the type of experiment.

Fiber photometry implantation. After viral injection, mice used for fiber photometry experiments were given between 2 and 3 weeks to recover before undergoing optic fiber cannulae implantation (200 µm, 0.50 numerical aperture, fiber distance 3.5 mm; RWD). The skull was exposed, and the two previous holes were used to target the mPFC. Fiber optic cannulae were secured to the skull with VetBond glue and dental cement. After optic fiber implantation, mice were allowed to recover for 7–10 d depending on the general health.

Optic fibers implantation. After viral injection, mice underwent stereotaxic surgery for fiberoptic implantation. The skull was exposed and the two previous holes were used to target the mPFC. Dual fiberoptic cannulae (200µm, 0.37 numerical aperture, fiber distance 0.7mm; Doric Lenses) were lowered 2mm from the surface of the skull at roughly 400µm dorsal to the virus injection site, and implants were secured to the skull with MetaBond and dental cement. After optic fiber implantation, mice were allowed to recover for 7–10 d depending on the general health.

Clarity

Mice were transcardially perfused with ice cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA), brains were removed and kept in 4% PFA overnight at 4°C. Brains were then transferred to a hydrogel solution (PBS with: 2% acrylamide, bio-rad #161-0140; 0.1% Bisacrylamide, bio-rad #161-0142; 0.25% VA-044 initiator, Wako, 011-19365; 4% PFA) for 2 days. The samples were then degassed with N_2 for 45 min and polymerized in 37°C for 3.5 hr. The samples were then washed overnight in 200 mM NaOH-Boric buffer (sigma, #B7901) containing 8% sodium dodecyl sulfate (SDS) (sigma, #L3771), to remove PFA residuals. Samples were then stirred in a clearing solution (100 mM Tris-Boric buffer, bio-lab, #002009239100 with 8% SDS) at 37°C for 3–4 weeks. After the samples became transparent, they were washed with PBST (PBS with 0.5% tritonX100; ChemCruz, #sc-29112A) for 24 hr at 37°C with mild shaking and for another 24 hr with fresh PBST 0.5% at RT. Finally, the samples were incubated in the refractive index matched solution Rapiclear (RI = 1.47; SunJin lab, #RC147002) over night at 37°C and 2 days at room temperature before imaging.

Drugs

For hM3D astrocytes activation, we used i.p. administration of clozapine-N-oxide dihydrochloride (CNO, 4936 Tocris) dissolved in physiological saline (0.9% NaCl) at a dose of 3 mg kg^{-1} in a volume of 10 ml kg^{-1}, 30 min before the behavioral experiments. Saline injection in a volume of 10 ml kg^{-1} was used as a control treatment.

Behavioral assays

Emotion discrimination test

Habituation of the mice to the testing setting occurred on three consecutive days before the first experiment; each habituation session lasted 10 minutes. Test observer mice were habituated inside a 3D printed square apparatus (34 x25x19 cm) containing a separator (150 cm) and a two-quarter circle (4cm ray) on two opposite sides. Each quarter-circle is formed with metal rods (0.8cm) that allow sufficient space for social contact between the observers (freely moving in the apparatus) and the demonstrators (freely moving in the quarter-circle). A 3D-printed separator is thus inserted between both quarter-circles to block the reciprocal view of the stimuli animals while leaving the observer mice free to move between the two sides of the cage. For scoring, a

virtual square (11 x 11 cm) is placed between each quarter-circle and the separator to define a zone associated with each stimulus. The experimental cages were replaced after each subject with clean copies to avoid scent carryover. Similarly, the rest of the apparatus was wiped down with water and dried with paper towels for each new subject.

Demonstrator mice – matched by age and sex to the observers – were habituated inside the same 3D printed cage (34 x25x19 cm), into quarter-circle space three consecutive times, ten minutes each. During both habituation and behavioral testing, the cages were placed inside soundproof cubicles (TSE Multi Conditioning Systems) homogeneously and dimly lit (6 ± 1 lux) to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference. Digital cameras (imaging Source DMK 22AUC03 monochrome, Ugo Basile) were placed on top of the cage to record the three consecutive two-minute epochs, using the Anymaze program (Stoelting, Ireland). Behavioral scoring was performed a posteriori from videos by trained experimenters, blind to the manipulations of both the observers and demonstrators. Three independent people scored the same data with an inter-rater reliability r score of 0.954. A sniffing event was considered when the observer touched with the nose the demonstrators' wire cup or when the observer's nose directly touched the demonstrator.

Observers

Before the test, mice were habituated to the experimental setting as reported above. Only in the fear paradigm, on the third day of habituation, mice were also habituated to the tone cue (4 kHz, 80 dB sound pressure level, three times for 30 s each with an intertrial interval of 90 s) without any conditioning. One hour prior to behavioral testing, mice were placed in the testing cage, in an experimental setting (i.e., separator and two wire cups), in a room adjacent to the testing room. Five minutes before the experiment, the testing cages containing the observer mice were gently moved into the testing cubicles. The 6-minute experiment began after placing one emotionally 'neutral' and one 'emotionally altered' demonstrator in the EDT apparatus. The order of insertion of the neutral or emotionally-altered demonstrator was randomly assigned.

Neutral demonstrators

In the days before the test, all neutral mice were habituated to the experimental setting as reported above. For the relief condition, neutral demonstrators underwent no manipulation the day before the test. For the fear condition, the day before the test, neutral demonstrators were habituated to the tone cue inside the cups as for the experimental setting and as done for the

observer mice. On the testing day, neutral demonstrators were brought inside their home cages in the experimental room one hour before the experiment began. Demonstrators were test-naive and used only once. In some cases, we re-used the same demonstrator for maximum two/ three times, with always at least one week between each consecutive test. No differences were observed in the performance of the observer mice depending on the demonstrators' previous experience.

Relief demonstrators

In the days before the test, mice were habituated to the experimental setting as reported above. Relief demonstrators were then water deprived 23 hours before the experiment. One hour before the test, ad libitum access to water was reestablished, and mice were brought inside the experimental room in their home cages. Food was available ad libitum all the time and some extra pellets were put inside the home cage during the 1-hour water reinsertion.

Fear demonstrators

In the days before the test, mice were habituated to the experimental setting as reported above. Fear demonstrators were fear conditioned (from one day to one week before the test) using the parameters and context previously described (Scheggia et al., 2018), and using the same tone delivered to the observers and neutral demonstrators during their habituation process. In particular, the conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30 s) and the unconditioned stimulus was three scrambled shocks (0.7 mA, 2 s duration, 90 s intershock interval) delivered through the grid floor that terminated simultaneously with the tone (2 s). On the day of the test these mice were habituated, inside their home cages, in a room adjacent to the testing room for one hour prior to the test; they were consequently brought inside the experimental room one by one, before placing them under their designated wire cup. Fear mice were conditioned only once, in a separate room and using a distinct apparatus (Ugo Basile SRL, Italy) from the one where the emotion recognition task would be performed. Fear demonstrators were generally used only once. In the case of a second exposure to the test, these demonstrators were just re-exposed to the same conditioned tone, at least one week apart from the previous exposure and maximum of 1 month from the initial conditioning.

Stress demonstrators

Mice were subjected to a mild stress consisting of the restraint test for 15 min before the beginning of the EDT. These mice were then immediately moved to the testing arena.

Digital cameras (Imaging Source, DMK 22AUC03 monochrome) were placed facing the long side of the cage and on top of the cage to record the test from different angles using a behavioral tracking system (Anymaze 6.0, Stoelting). These videos were used offline by experimenters blind to the manipulations of both the observers and demonstrators for a posteriori scoring of sniffing.

Social Habituation/Dishabituation

The test consisted of five repeated social interaction sessions between the experimental subject and an unfamiliar stimulus mouse followed by the exposure of the experimental subject to a new unfamiliar mouse. Briefly, mice were tested in 2150E Tecniplast cages (35.5 x 23.5 x 19 cm) lightly illuminated (5±1 lux) and video-recorded using a UniBrain Fire-i™ Digital Camera. The video camera was mounted facing the front of the cage to record the session for subsequent scoring of social investigation parameters. All the equipment was kept in a sound-attenuating chamber (TSE Multi Conditioning Systems). Adult mice were individually placed in the testing cage and left to habituate for 1 hour. Single housing manipulation was not carried out to avoid any instauration of home-cage territory and aggressive behaviors. Testing began 5 minutes after the habituation in the apparatus when a stimulus mouse of the same sex and age was introduced into the testing cage for a 1-min interaction. At the end of the 1-min inter-trial, we removed the stimulus animal and placed it in an individual holding cage. We repeated this sequence for four trials with 3-min inter-trial intervals. In a fifth trial, we introduced a new unfamiliar stimulus mouse in the testing cage. Videos of behaviors were recorded and subsequently scored offline (ANY-maze, Stoelting Co.). We measured the duration of the following behavioral responses performed by the tested mice, considering as social interaction anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), head sniffing (sniffing or snout contact with the head/neck/mouth area) and following (time spent in following the stimulus mouse). The “social exploration” score was obtained by summing all time spent in social interaction during the 5 minutes interaction sessions. The “social novelty score” was obtained by this formula ((T1-T4)+(T5-T4))/2 where “T” are the social interaction sessions, to measure the ability of each mouse to distinguish between a familiar and an unfamiliar mouse.

Tube Test

Social hierarchy of male mice was assessed as previously described (Fan et al., 2019). Briefly, this test consisted of two phases: training and testing. Training phase lasted two consecutive days during which mice were trained to walk through a Plexiglas tube ten times, five times from each side. In the testing phase, cage mate mice were tested in a pair-wise fashion in the tube, and mouse rank was assessed by the number of times each mouse wins or loose. The rank was considered stable when all mice maintained the same ranking for four consecutive days. After each trial, the mice were put back into the home cage and left for 2 min before starting the next trial, to reduce the potential immediate impact of recent winning or losing.

In vivo fiber photometry recordings and analysis

To analyze calcium fluctuations of mPFC astrocytes, the fluorescence signal emitted by GCaMP6f-expressing astrocytes was recorded using fiber photometry system. A signal processor (RZP5, Tucker Davis Technologies) was used to control two light sources (465-nm LED, CLED_465; 405-nm LED, CLED_405, Doric Lenses), which were modulated at 211 and 539 Hz, respectively. The two wavelengths were combined by a fluorescence minicube (Doric Lenses) and transmitted through an optical patch cable (Doric Lenses) to the mouse head implant. Emitted fluorescence was collected by the same patch cable, delivered back to the same minicube through a 525-nm filter and sent to a photoreceiver (Femtowatt Silicon Photoreceiver, DC-750 Hz; Newport). Real-time signals were acquired, lowpass filtered (3 Hz) and demodulated with Synapse Essentials software (Tucker Davis Technologies). Anymaze tracking system program (Stoelting, Ireland) was used to generate TTLs to time-stamp specific events during EDT task (e.g., entrance in the zone). Data were extracted from TDT files and analyzed using custom MATLAB scripts.

Before starting the analysis, bleaching was corrected by fitting a double exponential decay to the fluorescent signal, capturing the exponential decay of the isosbestic. Subsequently, the raw signal was divided by the fitted decay, and Z-score normalization was performed to standardize the signal by expressing it in terms of units of standard deviation.

For the PSTH analysis, the signal was aligned to behavioral outcomes (entrance in the zone), considering a baseline preceding the events (T=5 seconds) and a post-baseline following the events (T=10 seconds). The PSTH quantities were then obtained by subtracting the mean of the

baseline activity from the restricted signal and then dividing by the standard deviation of the same. Average plots of PSTH were represented as mean and standard error of the mean (S.E.M.), combining all the behavioral outcomes across all the mice. Areas under the curve (AUC) of the peristimulus curves have been computed over an interval of 10 seconds after the entrance in the zone (at time T=0), and subdivided into altered and neutral cases, if they corresponded to the entrance in the altered or neutral zone.

Identification of calcium signal events was performed via custom MATLAB scripts, using the findpeaks function to find the local maxima of calcium signals. Such maxima were considered calcium events if they overcame thresholds on the minimum event height (MinHeight, set as the average of the signal), minimum event prominence (MinProm, set as the standard deviation of the signal), minimum distance between consecutive events (MinDist = 1 second). The event frequency in a given time window was computed by dividing the number of events identified in such window by its length. Peak intensity was calculated as the $\Delta F/F$ value of the event.

In vivo optogenetic behavior

During behavioral testing, fiber optic cannulae were connected to patch cords (Doric Lenses), which were in turn connected to green light (532 nm) or blue light (473 nm) lasers (CNI Laser) using a 1 × 2 intensity division fiberoptic rotary joint (Doric Lenses) located above the cubicle containing the testing arena. Laser power was adjusted such that the light exiting the fiberoptic cable was approximately 4.5 mW. For photoinhibition experiments we used continuous green light.

Histology

At the end of the behavioral procedures, we checked viral expression and the position of the optic fibers. Mice were deeply anesthetized (urethane 20%) and transcardially perfused with 4% paraformaldehyde in PBS at pH 7.4. Brains were dissected, fixed overnight, and cryoprotected in 30% sucrose in PBS. Coronal sections (40 μm) were cut using a HM450 microtome (Thermo Fisher Scientific). For immunohistochemical studies free-floating sections of selected areas were washed in PBS three times for 10 min, permeabilized in PBS plus 0.4% Triton X-100 for 30 min, blocked by incubation in PBS plus 4% normal goat serum (NGS) and 0.2% Triton X-100 for 30 min (all at room temperature, 20–23 °C), and subsequently incubated in PBS plus 2% NGS and

0.1% Triton X-100 overnight at 4 °C with the following primary antibodies: GFP polyclonal antibody (1:1000, Abcam, AB13970), dsRed polyclonal antibody (1:1000, Takara, 632496), s100β monoclonal antibody (1:700, Synaptic system, 287 108) and . Neun monoclonal antibody (1:1000, Sigma, MAB377). Incubated slices were washed three times in PBS plus 1% NGS for 10 min at room temperature, incubated for 2 h at room temperature the following secondary antibodies: Alexa Flour 488 goat anti-chicken IgG (1:1000, Molecular Probes, A11039), Alexa Flour 568 goat anti-rabbit IgG (1:1000, Molecular Probes, A11036) and Alexa Flour 647 goat mouse IgG (1:1000, Molecular Probes, A21236)in PBS plus 2% NGS and 0.1% Triton X-100. Subsequently, slices were washed three times in PBS for 10 min at room temperature. The sections were mounted on slides and covered with cover slips.

Statistics

Results are expressed as mean ± s.e.m. throughout the manuscript. For the analyses of emotion discrimination, each observer behavior towards the two different demonstrator mice was calculated as a percentage of sniffing time to allow direct comparison between different experimental conditions. 2wayANOVA repeated measure within-groups was used to analyze these data, followed by Sidak's post-hoc test with multiple comparison corrections for making comparisons within groups when the overall ANOVA showed statistically significant differences. The same approach was used for the peak analysis, discrimination index and social habitation dishabituation analysis when different treatments were involved.

Mann-Whitney nonparametric test was used to analyzed different conditions including total sniffing, social novelty score, locomotor activity, average speed, and areas under the curve corresponding to altered and neutral zone. The accepted value for significance was $P < 0.05$. Statistical analyses were performed using GraphPad Prism 8. Numbers of mice are reported in the figure legends. The experiments reported in this work were repeated independently two to four times, using mice from at least three different generations. Mice were excluded post-hoc when optic fiber placement or viral expression patterns were not appropriate (outside the target region or week viral expression). For all behavioral tests, littermates were randomly assigned to the different groups. Specific randomization in the organization of the experimental conditions is described in the results and figure legends. Experimenters were not blinded during data acquisition, but all analyses were performed with blinding of the experimental conditions.

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CHAPTER 3

Astrocytic-Cannabinoid Signaling in Emotion Discrimination

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In preparation

Abstract

Astrocytes play a crucial role in many brain functions. Their activity is regulated by different mechanisms, often mediated by receptors of neurochemicals that integrate synaptic and networks information. Among such regulatory machineries, the endocannabinoid system is considered an important intracellular signaling pathway known for its modulation of diverse physiological responses. Notably, cannabinoid-type 1 receptors (CB_1-R) are expressed in both neurons and astrocytes, exerting distinct functions in a cell-type-dependent manner.

Despite extensive research has elucidated the impact of the cannabinoid system on social cognition, the interplay between astrocytes and cannabinoid signaling in socio- cognitive functions remains not well-characterized. This study aims to address this gap by exploring their interaction in social cognition, specifically focusing on CB_1 receptors within astrocytes.

Our findings show that the selective ablation of CB_1-R within astrocytes in the medial prefrontal cortex results in a reduction of overall astrocytic activity, accompanied by behavioral impairments in the recognition of selective emphatic states. Importantly, we also observed that chemogenetic astrocytes activation is sufficient to rescue socio-cognitive deficits induced by both acute and chronic delta-9-tetrahydrocannabinol (THC) administration. Overall, our results contribute to a deeper understanding of the strict interaction between astrocytes and cannabinoid system in the regulation of emotional processes.

Introduction

In the central nervous system, astrocytes constitute approximately 10-20% of the entire cellular population(Verkhratsky et al., 2017) Distinguished from other glial cells, their significant role in the brain is well recognized, given the involvement in different functions, such as structural (Alvarez et al., 2013) (Heithoff et al., 2021), metabolic (Barros & Weber, 2018) (Suzuki et al., 2011), and homeostatic functions (Parpura & Verkhratsky, 2012). Furthermore, their bidirectional communication with neurons (Araque et al., 1999) underscores their crucial role in governing synaptic activities. Indeed, astrocytic calcium activity, triggered by the release of neurotransmitters and subsequent binding to astrocytic receptors (Araque et al., 2014), is a pivotal aspect of their function. In response, astrocytes release gliotransmitters influencing neuronal activity and impacting behaviors (Kofuji & Araque, 2021) (Lyon & Allen, 2022)(Shigetomi & Koizumi, 2023). Therefore, the manipulation of astrocytic function has an impact on neuronal network activity through different mechanisms, subsequently influencing behavioral outcomes (Park & Lee, 2020) Several molecules and systems emerged as promising candidates for perturbating astrocytic activity.

The endocannabinoid system (ECS) serves as a complex neuromodulatory system intricately involved in controlling a plethora of physiological processes (Fride, 2005). The ECS operates through G-protein-coupled cannabinoid receptors (CB-R), endogenous lipid ligands (endocannabinoids, eCBs), and enzymes responsible for eCBs synthesis and degradation (De Petrocellis & Di Marzo, 2009) (Piomelli, 2003), which works synergistically to regulate different processes. The molecular target of cannabinoid compounds is represented by cannabinoid type 1 and type 2 receptors (CB_1-R and CB_2-R), each with distinct localizations. While CB_2-R are predominantly distributed in the peripheral areas (Mackie, 2008), CB_1-R are notably abundant in the brain, specifically in forebrain regions (including cortex, hippocampus, amygdala, striatum, and substantia nigra) and hindbrain regions, such as cerebellum and brainstem (Mackie, 2005).
CB1Rs are primarily located on the presynaptic terminals of neurons (Piomelli, 2003) (Castillo et al., 2012), and particularly abundant in cortical GABAergic interneurons (Marsicano & Lutz, 1999). Here, CB_1-R activation by endogenous (e.g. anandamide or 2-arachydonoylglycerol) or exogenous (e.g. THC) cannabinoids - modulates synaptic transmission leading to a general decrease in neuronal excitability, influencing many physiological processes.

Upon ligand binding and subsequent receptor activation, they promote specific molecular pathways that are cellular and subcellular-type dependent (Covelo et al., 2021). In neurons, they are predominantly coupled with $G_{\alpha i/o}$ proteins. At the synaptic level, CB_1-R activation inhibits neurotransmitter release through the inhibition of adenyl cyclase (AC) and subsequent downregulation of cAMP/PKA pathway, and the inhibition of presynaptic calcium influx mediated by voltage-gated Ca^{2+} channels (Kano et al., 2009) (Castillo et al., 2012). However, cases of functional selectivity were also reported. In specific brain areas characterized by substantial colocalization of both CB_1-R and dopamine D_2R, a functional interplay between these receptors was identified. The interconnection involved a functional and/or physical interaction between CB_1-R and D_2R, capable of shifting CB_1 receptor signaling from $G_{\alpha i/o}$ to $G_{\alpha s}$, resulting in a subsequent increase in cAMP. (Glass & Felder, 1997) (Kearn et al., 2005).

Other studies have also shown that some clusters of CB_1-R are expressed in postsynaptic compartments (Bacci et al., 2004) (Maroso et al., 2016), but further investigations will be needed to address their physiological role.

Importantly, the expression of CB_1-R is not limited to neurons. Notably, these receptors are also detectable in astrocytes (Navarrete & Araque, 2008) (Covelo et al., 2021), where their relatively modest expression is not indicative of a lack of functional relevance (Metna-Laurent & Marsicano, 2015) (Busquets-Garcia et al., 2018). Indeed, the initial debates about the expression of CB_1-R within astrocytes were primarily attributed to their comparatively low expression levels compared to neurons, posing a challenge for detection using conventional techniques like immunohistochemistry and *in situ* hybridization. Thanks to the use of mutant mouse lines, astrocytic expression of CB1R could be observed in brain areas like hippocampus (Han et al., 2012) (Robin et al., 2018) (Gutiérrez-Rodríguez et al., 2018) and cerebral cortex (Bosier et al., 2013).

Interestingly, and in contrast to neurons, astrocytic CB1-R were suggested to couple with $G_{\alpha q/11}$ proteins instead of $G_{\alpha i/o}$ or $G_{\alpha s}$. Upon activation, these receptors induce phospholipase C (PLC) activation, leading to the release of inositol 1,4,5-triphosphate (IP3). Consequently, these events trigger the release of calcium from internal stores, elevating cytosolic calcium levels in both soma and processes of astrocytes (Navarrete & Araque, 2008). Notably, the application of a PLC inhibitor, but not $G_{\alpha i/o}$ pertussis toxin blocker, definitively stops CB_1-R evoked calcium signal, suggesting that at least in astrocytes CB_1-R might couple to Gq proteins instead (Navarrete &

Araque, 2008), and therefore underscoring the different signaling pathways promoted by different cellular expression of CB_1-R.

The versatility of their signaling and regulatory functions within astrocytes is also dependent on their subcellular distribution (Covelo et al., 2021). Like many other GPCRs, CB_1-R are prominently expressed on the plasma membrane, where they govern synaptic transmission and cellular communication. However, recent evidence has demonstrated the presence of CB1 receptors also on astroglial mitochondria (mtCB1R), suggesting potential involvement in cellular processes related to energy metabolism (Gutiérrez-Rodríguez et al., 2018) (Jimenez-Blasco et al., 2020). Similarly to CB_1-R expressed in neurons, these mitochondrial receptors were shown to be couple with $G_{\alpha i/o}$ proteins. When endogenous or exogenous cannabinoids actives mtCB1R, the inhibition of mitochondrial soluble adenyl cyclase (sAC) promotes the decrease of mitochondrial respiration (Bénard et al., 2012) (Fernández-Moncada et al., 2023), and consequent impact on social behaviors (Jimenez-Blasco et al., 2020), learning and memory (Hebert-Chatelain et al., 2016) and food intake (Koch et al., 2015).

The diverse distribution of CB_1-R across different cellular compartments underscores the pivotal role of the cannabinoid system in shaping a spectrum of behavioral response. While the effects of cannabinoids on social cognition are extensively documented, the specific mechanism underpinning these behaviors remains not well understood.

Given their distribution in brain areas associated with emotional processes (e.g. cortex and amygdala), the cannabinoid system significantly influences socio-cognitive functions, affecting the ability to decipher emotional stimuli and respond appropriately to non-verbal cues. Initial clinical studies have reported alteration in social interaction (Galanter et al., 1974) (Salzman et al., 1976) and empathic behavior (Janowsky et al., 1979) following marijuana smoking. These findings were corroborated by further studies demonstrating impaired recognition of human emotional faces after both acute (Clopton et al., 1979) (Bossong et al., 2013b) and chronic (Platt et al., 2010) (Hindocha et al., 2014b) (Zimmermann et al., 2017) cannabis administration, particularly for stimuli with negative emotional valence rather than positive emotional content (Bossong et al., 2013b) (Ballard et al., 2012).

Neuroimaging analysis coupled with validated cognitive task assessing facial affect recognition (*ERTs*; see Chapter 1) in cannabis users have consistently demonstrated a global reduction in brain activity when processing stimuli with negative valence (Bossong et al., 2013b). Notably,

alterations in the activity of cortical and limbic brain regions responsible for recognizing emotional stimuli are well-established. Heavy marijuana smokers have exhibited significant reductions in the cingulate cortex and amygdala activity in response to both negative and positive stimuli (Gruber et al., 2009). Furthermore, oral THC administration in cannabis users' dose-dependently decreases blood oxygen level-dependent (BOLD) signal in the amygdala in response to threat-related stimuli (Phan et al., 2008). Hypoactivity of the medial prefrontal cortex (mPFC) during an emotional evaluation task, involving the presentation of 100 photographic stimuli, was observed in heavy cannabis users who were not acutely intoxicated before the test (Wesley et al., 2016). Notably, functional connectivity analysis revealed decreased amygdala-dorsolateral prefrontal cortex activity during reappraisal of negative stimuli in regular marijuana users (Zimmermann et al., 2017), highlighting an intriguing association between behavioral deficits and altered frontal-limbic activity. While these clinical studies collectively provide a consistent narrative on the detrimental impact of marijuana on facial emotion identification, their overall effectiveness is somewhat constrained. This limitation arises from various factors contributing to increased study variability, including diverse methodological approaches employed, variation in the intensity of the emotional stimuli presentation; and challenges associated with the participants' prior substance experiences.

Therefore, conducting more precise studies with controlled experimental conditions could offer valuable insights into the neurobiological mechanism underlying social cognition following cannabinoid intoxication.

Results

Astrocytic CB_1-R ablation within mPFC alters astrocytic activity

The neurobiological effects orchestrated by the endocannabinoid system are intricately regulated by the activity of CB_1 receptors (Di Marzo et al., 2004), primarily distributed throughout the brain in both neurons and astrocytes (Navarrete & Araque, 2008) (Covelo et al., 2021). To unravel the specific physiological role of astrocytic CB_1-R within the medial prefrontal cortex (mPFC) and its implication in mediating socio-cognitive behavior, we employed a targeted approach to selectively silence CB_1-R expression within astrocytes in CB_1-flox mice.

First, we investigated the potential impact of astrocytic CB_1 deletion on astrocyte signaling by recording their Ca^{2+}basal activity for 1 hour. To achieve this goal, we performed a co-injection of a Cre-expressing viral vector (AAV5-GFAP-mCherry-Cre) and a GCaMP (AAV5-pZac2.1 gfaABC1D-cyto-GCaMP6f) under an astrocytic promoter in the mPFC of CB_1-flox mice (Fig. 1a). 2-3 weeks after virus injection, we implanted an optical fiber upon the targeted brain area, and recorded mPFC astrocytes activity using fiber photometry (Fig.1b and Fig.1c).

Compared to wild-type mice, the calcium activity of astrocytes in mice lacking CB_1-R (called *CB_1-GFAPcre mice*) was decreased. We observed a significant decrease of peak frequency (Fig.1d) (p=0.0165) and peak intensity (Fig.1e) (p=0.0006) in the CB_1-GFAPcre mice, suggesting the potential role of CB_1-R in shaping astrocytic calcium activity.

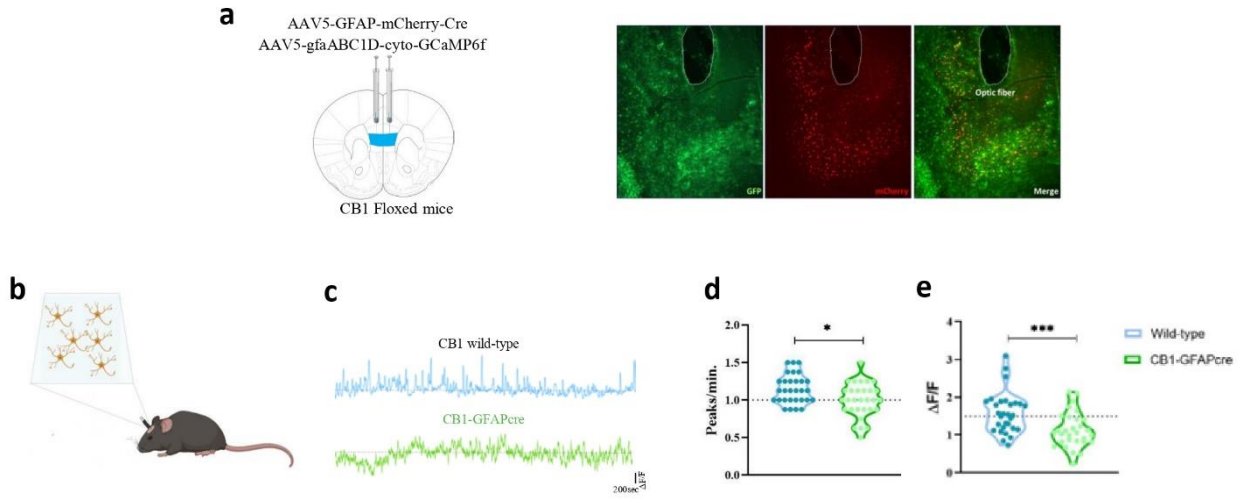


Figure 1 | Astrocytic CB_1-R ablation within mPFC alters astrocytic activity

(a) Left: CB_1-flox mice were injected in the mPFC with AAV5-GFAP-mCherry-Cre and AAV5-gfaABC1D-cyto-GCaMP6f and implanted bilaterally with optic fibers terminating dorsally to the injection area. Right: representative image of a coronal mPFC section with GFP and mCherry expression **(b)** Cortical astrocytic activity was recorded with fiber photometry in freely moving mice **(c)** Representative traces of calcium transients in CB1 wild-type (blue) and CB1-GFAP cre (green) mice. Dotted black line: Z-score = 0 **(d)** Peak frequency and **(e)** peak intensity of astrocytic calcium transients recorded for 1 hour of basal activity.
Dots represent the average peak of a 300-second transient event. *P < 0.05. **P < 0.005

Astrocytic CB_1-R ablation within mPFC alters emotion discrimination abilities

Next, we investigated the influence of mPFC-specific $CB1_R$-knock-downon socio-cognitive behaviors. In the mPFC, CB_1-flox mice were injected with AAV5-GFAP-mCherry-Cre (or AAV-GFAP-eGFP as a control) virus (Fig.2a) and then tested in emotion discrimination test (EDT), adopting both positive (Fig.2b *relief*, yellow) and negative (Fig.2g *fear*, red) (Fig.2l *stress*, blue) protocols.

In the relief paradigm, the altered state of one of the two demonstrators (*relief*, yellow bars) was induced by providing 1h of *ad libitum* access to water following 23h of water deprivation. Notably, while the control group (CB_1 wild-type) exhibited an increased sniffing time towards the relief demonstrator compared to the neutral one during the initial 0-2 min epochs of the task (Fig. 2c) (trial x affective state $F_{(2,60)} = 5.557$, p=0.0061; 0-2 minutes epochs p=0.0181), the CB_1-GFAPcre group showed a deficit in recognizing the altered emotional stimulus (Fig.2d) (trial x affective state $F_{(2,36)} = 1.048$, p=0.3610; 0-2 minutes epochs p=0.9176). Consistently, also the discrimination index analysis (Fig. 2e) (p=0.0460), revealed a significant reduction in the CB_1-GFAPcre group compared to control selectively in the first session of the task where we observed the emotion discrimination for relief. However, no discernible effects were observed when analyzing the total sniffing time over the entire 6-minute duration of the task (Fig.2f) (p=0.5049).

Then, we extended our investigation to assess the impact of mPFC astrocytic CB_1-R ablation on the recognition of negative emotional states. In the fear protocol, the fear conditioning paradigm was employed to induce the altered affective state in the fear demonstrator (*fear*, red bars), though the release of a three-foot shock series preceded by a beep tone, at least one day prior EDT. As expected, observers of the control group (CB_1 wild-type) spent more time sniffing the emotional altered stimulus in the last epoch (4-6 minutes of the test; Fig. 2h). Indeed, 2wayANOVA RM analysis revealed a main trial x affective state interaction effect ($F_{(2,66)} = 10.53$, p=0.0001), and post hoc multiple comparison analysis showed a significant effect in the 4-6 minutes epochs of the test (p<0.0001), coinciding with the induction of classical fearful behavior of the altered stimulus in response to the tone released during the middle trial of the task. Relevantly, CB_1-GFAPcre mice exhibited impairments in the recognition of fear stimulus compared to neutral (Fig.2i) (trial x affective state: $F_{(2,36)} = 1.750$, p=0.1883), as indicated by the discrimination index data related to the last EDT trial (Fig.2k) (p=0.0074). No significant

differences in total sniffing were observed between wild-type and CB_1-GFAPcre mice (Fig.2k) (p=0.1687).

In the stress paradigm, the altered demonstrator (*stress*, blue bars) underwent tube-restraint for a consecutive 15-minute period preceding the test. During the initial 0-2 min epoch, our data indicated that mice lacking the CB_1-R within astrocytes spent more time exploring the stress stimulus compared to the neutral one (trial x affective state $F_{(2,40)} = 4.856$, p=0.0129) (Fig. 2m), similar to the control group ($F_{(2,60)} = 4.618$, p=0.0136) (Fig. 2n). Furthermore, the discrimination index (Fig. 2o) (p=0.9934) and overall social interaction (Fig. 2p) (p=0.9751) did not manifest any statistically significant differences between groups. These results suggest that both groups perceive the altered stressed condition of the conspecific, even in absence of the receptor, highlighting the importance to deeply investigate compensatory pathways that might be involved in the recognition and processing of stress-related stimuli in the absence of astrocytic CB_1-R.

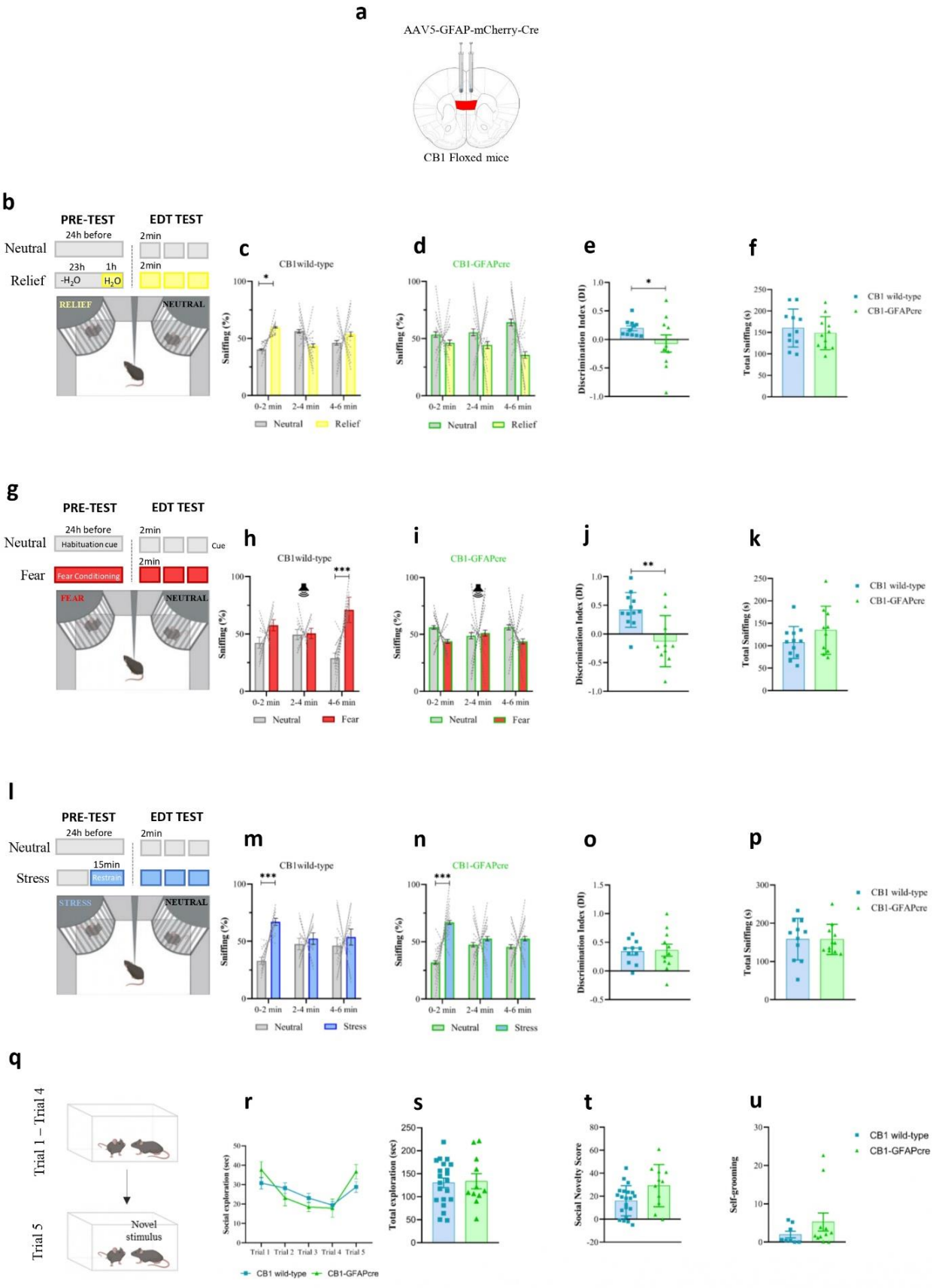
Our findings demonstrate that selective deletion of CB_1-R within mPFC astrocytes negatively influences overall astrocytes activity and impairs the recognition of both positive and negative emotional states, indicating the importance of these receptors in mediating socio-cognitive processes.

Astrocytic CB_1-R ablation within mPFC did not alter basic sociability and social memory

To further investigate the potential impact of astrocytic-cannabinoid manipulation within mPFC in mediating various aspects of social behaviors, we conducted additional behavioral analysis to assess the influence of astrocytic CB_1-R ablation in the Social Habituation Dishabituation Task, as described by Huang et al., 2014. In brief, a test mouse is exposed to the same novel stimulus for four trials of 1 min, separated by 3 min of non-social interaction. Over these four trials, the social interaction of the test mouse decreases due to habituation to the stimulus. Subsequently, in the fifth trial, the test mouse is exposed to a novel social stimulus, leading to an increase of social interaction (Fig.2q).

As illustrated in Fig.2r, the deletion of CB1-R did not interfere with basic sociability and social recognition memory. Remarkably, both CB_1 wild-type and CB_1-GFAPcre mice exhibited similar social interaction patterns with unfamiliar conspecific from Trial 1 to Trial 4, with an increase in social exploration observed only in Trial 5 when a novel conspecific stimulus was introduced into the testing arena. 2wayANOVA repeated measure did not revealed any significant effect of manipulation ($F_{(1,30)}$=0.03797, p=0.8468) such as multiple comparison analysis relative to the social exploration in trial 5 between wild-type and CB_1-GFAPcre mice (p=0.4048). Additionally, no significant changes were observed in the total social exploration time (Fig.2s) (p=0.9014), social novelty score (Fig.2t) (p=0.0594) and self-grooming (Fig.2u) (p=0.3229).

Overall, these data suggest that astrocytic CB_1-R deletion in mPFC does not influence basic sociability and the memory ability to recognize a novel stimulus.


a
AAV5-GFAP-mCherry-Cre
CB1 Floxed mice
b
PRE-TEST
EDT TEST
Neutral
Relief
24h before
2min
23h
1h
-H2O
H2O
RELIEF
NEUTRAL
c
CB1wild-type
Sniffing (%)
0-2 min
2-4 min
4-6 min
Neutral
Relief
d
CB1-GFAPcre
e
Discrimination Index (DI)
f
Total Sniffing (s)
CB1 wild-type
CB1-GFAPcre
g
Fear
Habituation cue
Cue
Fear Conditioning
FEAR
h
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k
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Stress
15min
Restrain
STRESS
m
n
o
p
q
Trial 1 – Trial 4
Trial 5
Novel stimulus
r
Social exploration (sec)
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
s
Total exploration (sec)
t
Social Novelty Score
u
Self-grooming

Figure 2 | Astrocytic CB_1-R ablation within mPFC alters emotion discrimination abilities but not sociability and social memory

(a) CB_1-flox mice were bilaterally injected in the mPFC with AAV5-GFAP-mCherry-Cre. **(b)** Experimental design of EDT relief. Observers did not undergo any manipulation. One demonstrator (relief, yellow) received 1-h ad libitum access to water after 23h of water deprivation. The other demonstrator (neutral, gray) waited undisturbed in the home cage**.(c)(d)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs in CB1 wild-type (c) and CB1-GFAPcre (d) mice. N= 11 CB1 wild-type observers and N= 10 CB1-GFAPcre observers. **(e)** Wild-type (blue square) and CB1-GFAPcre (green triangles) mice significantly differ based on discrimination index ([sniffing towards relief– sniffing towards neutral] / [sniffing towards relief + sniffing towards neutral]). **(f)** No differences between CB1 wild-type and CB1-GFAPcre mice were found in the total sniffing time during the relief test. **(g)** Experimental design of EDT fear. Observers did not undergo any manipulation. One demonstrator (fear, red) was fear-conditioned at least one day before. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(h)(i)** Percentage of time spent sniffing demonstrators towards fear (red bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs in CB1 wild-type (h) and CB1-GFAPcre (i) mice. N= 12 CB1 wild-type observers and N= 10 CB1-GFAPcre observers. **(j)** Wild-type (blue square) and CB1-GFAPcre (green triangles) mice significantly differ based on discrimination index ([sniffing towards fear– sniffing towards neutral] / [sniffing towards fear + sniffing towards neutral]). **(k)** No differences between CB1 wild-type and CB1-GFAPcre mice were found in the total sniffing time during the fear test. **(l)** Experimental design of EDT stress. Observers did not undergo any manipulation. One demonstrator (stressed, blue) was subjected to the tube restraint stress for 15 min immediately before the beginning of the EDT. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(m)(n)** Percentage of time spent sniffing demonstrators towards stress (blue bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs in CB1 wild-type (m) and CB1-GFAPcre (n) mice. N= 11 CB1 wild-type observers and N= 11 CB1-GFAPcre observers. **(o)** Wild-type (blue square) and CB1-GFAPcre (green triangles) mice did not differ based on discrimination index ([sniffing towards stress– sniffing towards neutral] / [sniffing towards stress + sniffing towards neutral]). **(p)** No differences between CB1 wild-type and CB1-GFAPcre mice were found in the total sniffing time during the stress test. **(q)** Schematic illustration of the habituation dishabituation social interaction test. An unfamiliar stimulus mouse was introduced into the testing cage, engaging in a 1-minute interaction with the testing subject. At the end of the 1-minutes trial, the stimulus animal was removed and placed in an individual holding cage. This sequence was repeated for four trials, separately by 3-minutes inter trial interval, reintroducing the same stimulus mouse. In the fifth 'dishabituation' trial, a novel unfamiliar stimulus mouse was introduced in the testing cage to assess social memory. **(r)** No differences were observed analyzing the total amount of time in seconds allocated to investigation of the unfamiliar stimulus from trial1 to trial5 between CB1 wild-type and CB1-GFAPcre mice. N= 22 CB1 wild-type testing mice and N= 11 CB1-GFAPcre testing mice. **(s)** Total exploration time was calculated summing the social exploration times from trial 1 to trial 5. No difference was found between wild-type (blue square) and CB1-GFAPcre (green triangles) mice during the entire social task. **(t)** Social novelty score was calculated using the following formula: ((T1-T4)+(T5-T4))/2 where "T" are the social interaction times sessions. No difference was found between wild-type (blue square) and CB1-GFAPcre (green triangles) mice during the entire social task.**(u)** No difference was found in the self-grooming analysis between CB1 wild-type (blue square) and CB1-GFAPcre (green triangles) mice.
Bar and line graphs show mean ± s.e.m. *P < 0.05. **P < 0.005. ***P < 0.0005.

The acute and chronic THC treatment impair emotion discrimination

Recognizing the inherent limitations of clinical studies (see *Introduction*), preclinical mouse models of social behaviors offer a powerful instrument for more controlled exploration into the systemic effects of cannabinoids (Amhed et al., 2022), providing the opportunity to manipulate variables within a controlled experimental environment and granting potential translational approaches.

Thus, we started to investigate the systemic effects of both acute and chronic delta-9-tetrahydrocannabinol (THC) administration in social behaviors. As shown in the experimental timetable (Fig. 3a), on DAY-1 mice received a single intraperitoneally injection of THC (0.25mg/kg, 10mL/Kg^{-1} of volume) or vehicle (= control) and, 30 minutes later, underwent the initial EDT test (relief or fear). Doses were selected according to a previous study (Busquets-Garcia et al., 2017). Subsequently, daily THC chronic treatments started and continued until DAY-8, culminating in a reevaluation of social behaviors through a repeated series of EDT experiments (relief or fear).

On Day 1, our observations revealed that acute THC administration impaired the ability to discriminate between conspecifics when neutral and a relieved demonstrators were presented in the testing cage (Fig.3b). In contrast to the vehicle-treated group, which exhibited a distinct preference by spending more time sniffing the altered demonstrator than the neutral one in the 0-2 epochs of EDT (Fig. 3c), the THC-treated group did not show any discernible preference (fig. 3d). While in the control, 2wayANOVA RM revealed a significant affective state effect ($F_{(1,16)}$=5.132, p=0.0377), in the THC group no effect was observed ($F_{(1,16)}$=0.1245, p=0.7288). However, no significant difference between the groups were observed in the total sniffing time (Fig.3e) (p=0.8633), suggesting that THC treatment did not influence overall sociability.

Similar patterns were observed in the fear paradigm (Fig. 3f). When presenting a fear-conditioned demonstrator, the control group successfully recognized the altered state, particularly following the tone presentation, indicative of fear memory recall (Fig.3g) (trial x affective state: $F_{(2,28)}$=11.33, p=0.0002). In contrast, the THC-treated group did not exhibit any discernible preference for the fear stimulus (Fig. 3h) (trial x affective state: $F_{(2,32)}$=1.142, p=0.3320). Notably, no significant effect was observed in sociability, as evidenced by similar results in total sniffing time (fig.3i) (p=0.6058).

On Day 8, after daily chronic THC treatments, emotion recognition abilities were further analyzed.

Notably, our data revealed that chronic THC treatments impaired the recognition of positive (relief) emotional state (trial x affective state: $F_{(2,24)}$=4.464, p=0.0225) compared to vehicle-treated group (trial x affective state: $F_{(2,24)}$=1.311, p=0.2882). Differently from the control (Fig. 3k), the chronic THC treatment negatively influenced the social preference for the altered demonstrators over neutral ones, as evidenced by the analysis of sniffing time (Fig.3l). No effects were observed in the total sniffing time (Fig. 3m) (p=0.8048).

To exclude potential confounding effects arising from THC administration for incorrect social behavior evaluation and biases in data interpretation, we also checked locomotor activity. Building upon previous findings (Busquets-Garcia et al., 2017) indicating an influence of a comparable THC dose on locomotor, we implemented the same experimental timetable described above (Fig.4a) to track locomotor activity following acute (Fig.4b) and chronic THC treatments (Fig. 4g). The open field test was used to monitor 1h of spontaneous locomotion post-vehicle or THC administration. Our data indicated an absence of significant treatment effects between groups after both acute ($F_{(1,10)}$= 0.01912, p=0.8928) and chronic ($F_{(1,10)}$=0.5848, p=0.4621) conditions. As expected, both vehicle and THC groups exhibited a decline in the distance traveled in the arena over time (Fig.4c and Fig 4h). 2wayANOVA revealed a main effect of time following acute (F(4.039,40.39)=3.554, p=0.0139) and chronic ($F_{(3.936,39.36)}$=14.03, p<0.0001) treatment. However, the mean total distance covered across time did not significantly differ after both acute (Fig.4d) (p=0.9372) and chronic (Fig.4i) (p=0.5887). Additionally, the absence of statistically significant differences in term of average speed (Fig.4e; Fig.4j) and time in the center arena (Fig.4f; Fig.4k) excluded any ambulatory effects or anxiety-like behaviors induced by both acute and chronic THC treatment.

Overall, these findings demonstrate that both acute and chronic THC treatment impair emotion discrimination for positive and negative emotions, while no discernible effects were observed in locomotion, supporting the strong implication of the endocannabinoid system in socio-cognitive processes.

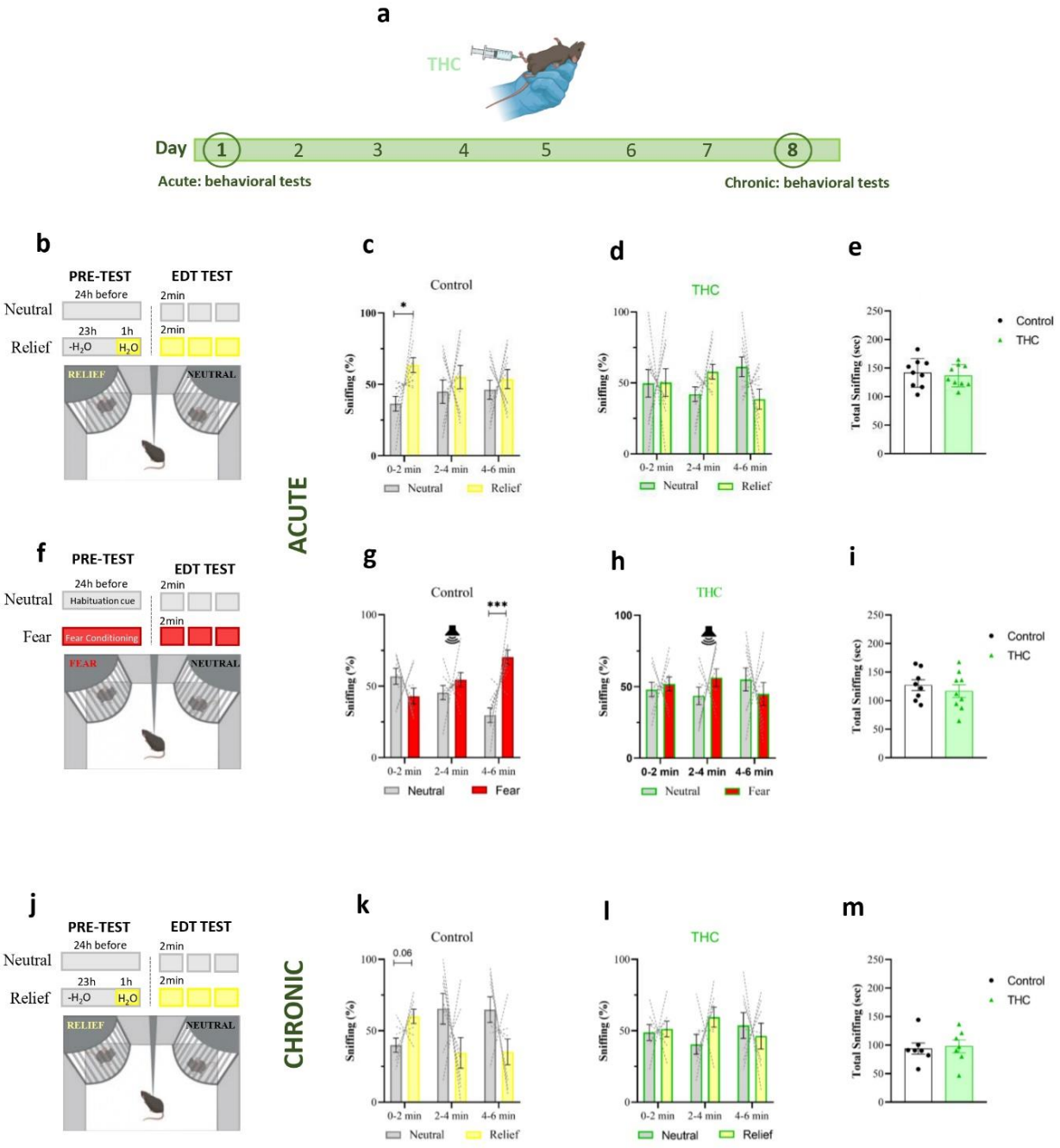


Figure 3 | The acute and chronic THC treatment impair emotion discrimination
(a) Experimental timetable of THC treatment. On day 1, mice received the initial THC injection, followed by social behavioral tasks 30 minutes later. Subsequently, after seven consecutive days of daily chronic treatments, behavioral assessments were repeated to evaluate outcomes following prolonged exposure to THC. **(b)** Experimental design of EDT relief. Observers did not undergo any manipulation. One demonstrator (relief, yellow) received 1-h ad libitum access to water after 23h of water deprivation. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(c)(d)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. acute control (c) and THC treatment

(d). N= 9 control observers and N= 9 THC observers **(e)** No differences were observed analyzing the the total sniffing time between observer mice that received i.p. acute control and THC treatment in the relief test. **(f)** Experimental design of EDT fear. Observers did not undergo any manipulation. One demonstrator (fear, red) was fear-conditioned at least one day before. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(g)(h)** Percentage of time spent sniffing demonstrators towards fear (red bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. acute control (g) and THC treatment (h). N= 8 control observers and N= 9 THC observers **(i)** No differences were observed analyzing the the total sniffing time between observer mice that received i.p. acute control and THC treatment in the fear test. **(j)** Experimental design of EDT relief. Observers did not undergo any manipulation. One demonstrator (relief, yellow) received 1-h ad libitum access to water after 23h of water deprivation. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(k)(l)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. chronic control (k) and THC treatment (l). N= 7 control observers and N= 7 THC observers **(m)** No differences were observed analyzing the the total sniffing time between observer mice that received i.p. chronic control and THC treatment in the relief test.
Bar and line graphs show mean ± s.e.m. *P < 0.05. **P < 0.005. ***P < 0.0005.

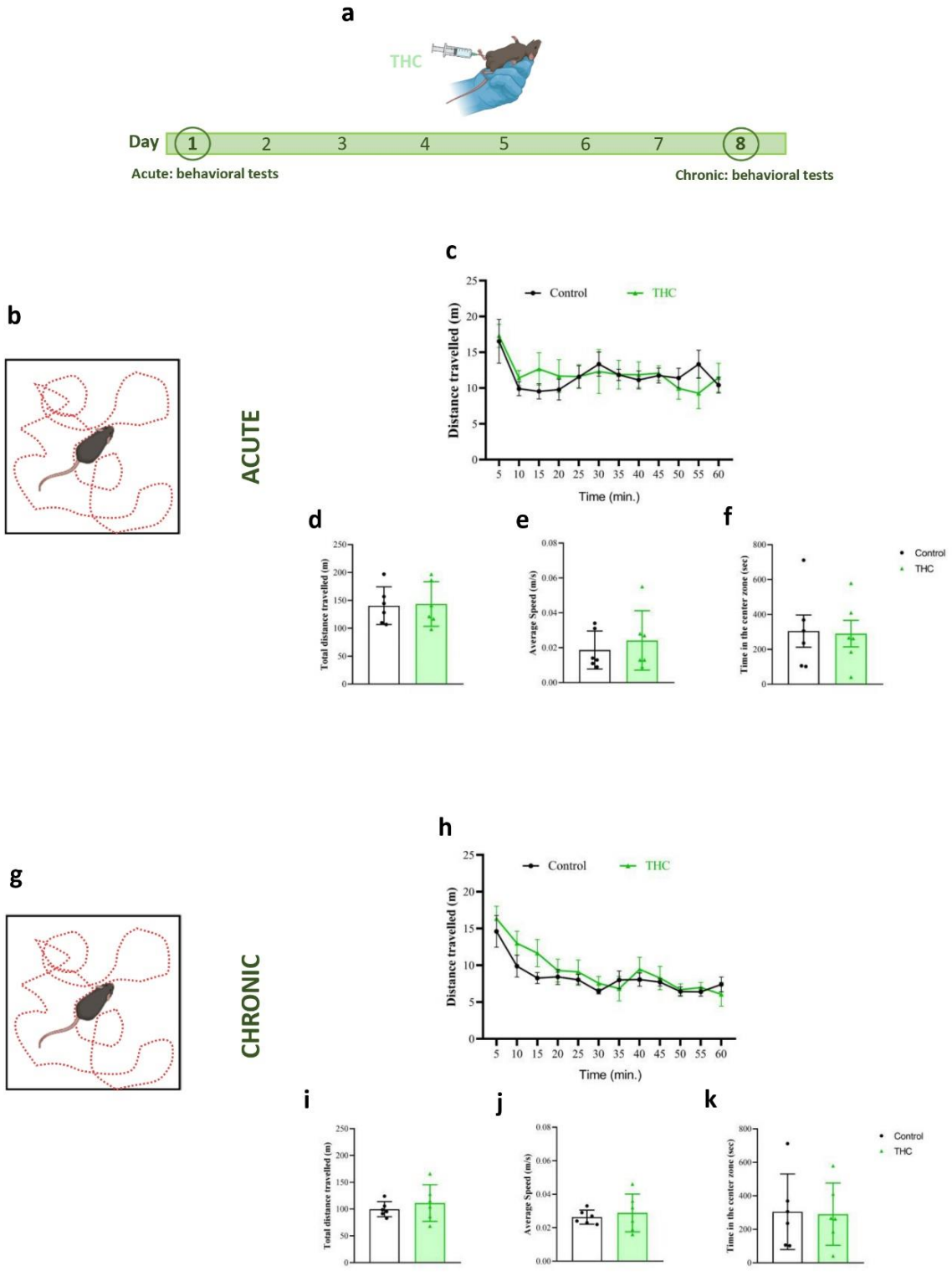


Figure 4 | The acute and chronic THC treatment did not affect locomotion

(a) Experimental timetable of THC treatment. On day 1, mice received the initial THC injection, followed by open field test 30 minutes later. Subsequently, after seven consecutive days of daily chronic treatments, behavioral assessment in open field was repeated to evaluate locomotion following prolonged

exposure to THC. **(b) (c)** The locomotor activity was assessed over a 1-hour period using the classical open field apparatus (b) following acute treatment. (c) The distance travelled was measured at 5-minute intervals after acute control (black line) and THC (green line) treatment. N= 6 control observers and N= 6 THC observers **(d) (e) (f)** No differences were observed in total distance travelled (d), average speed (e), and time in the center (f). between control (black dots) and THC (green triangles) groups.**(g) (h)** The locomotor activity was assessed over a 1-hour period using the classical open field apparatus (g) following chronic treatment. (h) The distance travelled was measured at 5-minute intervals after chronic control (black line) and THC (green line) treatment. N= 6 control observers and N= 6 THC observers **(i) (j) (k)** No differences were observed in total distance travelled (i), average speed (j), and time in the center (k) between control (black dots) and THC (green triangles) groups.
Bar and line graphs show mean ± s.e.m.

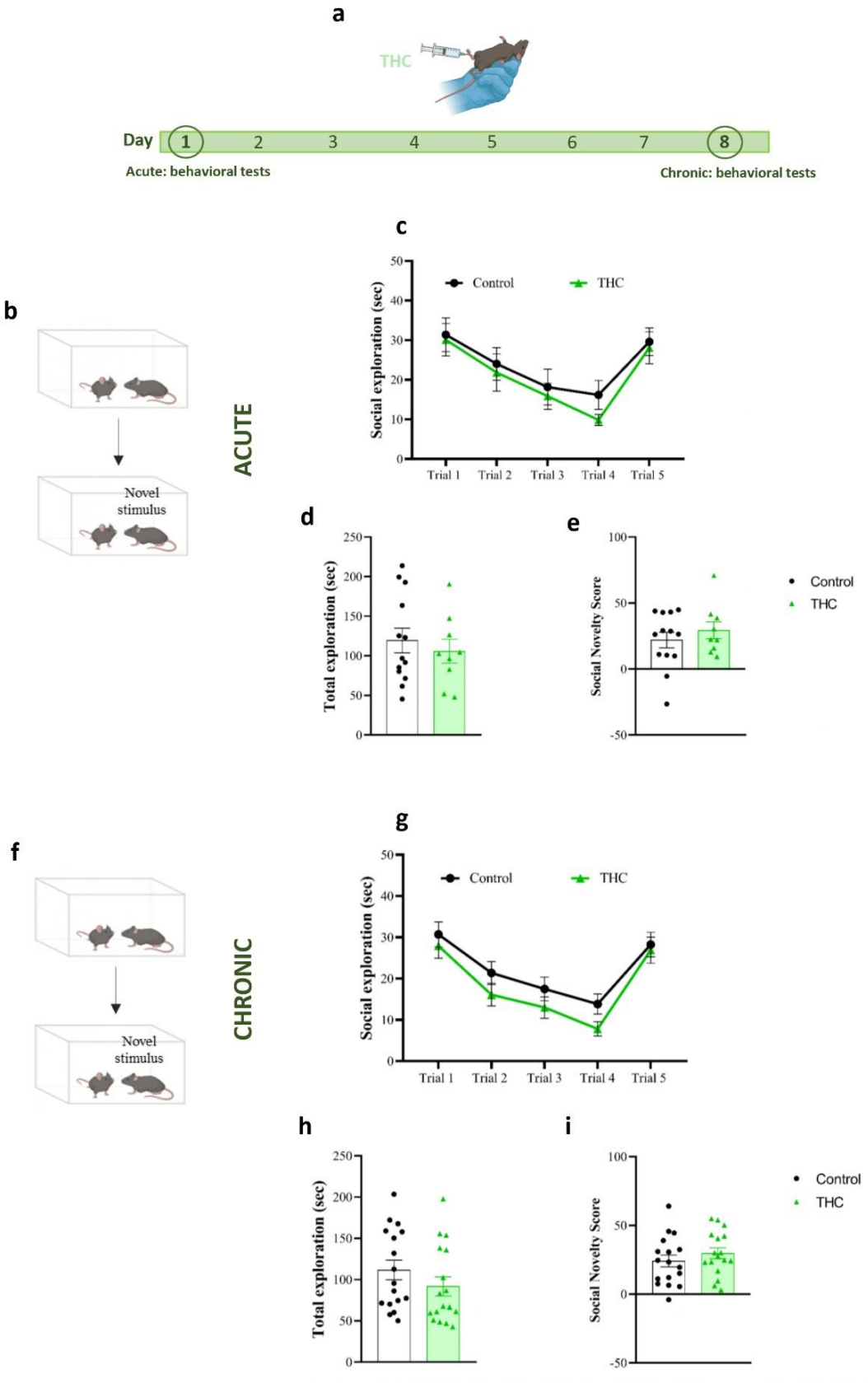
The acute and chronic THC treatment does not influence sociability and social memory

Applying the same experimental timetable previously described (Fig.5a), we expanded our investigations to assess the impact of both acute and chronic THC treatment on sociability and social memory processes. To achieve this, we employed the Social Habituation Dishabituation paradigm to study social behaviors following acute (Day 1) and chronic (Day 8) THC treatment.

We found that acute administration of THC did not alter sociability or social memory performance 30 minutes after the intraperitoneal injection of the drug, as compared to the control group (Fig.5c). As expected, a significant main effect of trial was observed ($F_{(3.029, 60.58)}$ =14.49, $p<0.0001$), reflecting a progressive reduction in social exploration toward the unfamiliar conspecific stimulus across trials. However, no significant differences were observed between the experimental groups following treatments ($F_{(1,20)}$=0.3637, p=0.5533). Importantly, when a novel stimulus was introduced in the testing cage, both experimental groups showed an equal increase of social exploration (Fig.5c). Additionally, neither the total social interaction analysis (Fig.5d) (p=0.7938) nor the social novelty score (Fig.5e) (p=0.8446) revealed significant differences between the groups.

Similar results were observed after daily THC chronic treatments on Day 8, where a main effect of trial was assessed (Fig.5g) ($F_{(2.876, 92.02)}$ =45.47, $p<0.0001$). However, no significant effect of treatment (2wayANOVA-RM: $F_{(1,32)}$ =1.472, p=0.2339) on social recognition memory performance and basic sociability(Fig.5h) (p=0.1401) was observed. Also, the social novelty score did not differ between the control and THC-treated group (p=0.3940).

Overall, our data revealed that THC administration did not influence social interaction and social recognition memory, under both acute and chronic conditions.


a
THC
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2
3
4
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6
7
8
Acute: behavioral tests
Chronic: behavioral tests
b
Novel stimulus
ACUTE
c
Control
THC
Social exploration (sec)
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
d
Total exploration (sec)
e
Social Novelty Score
Control
THC
f
Novel stimulus
CHRONIC
g
Control
THC
Social exploration (sec)
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
h
Total exploration (sec)
i
Social Novelty Score
Control
THC

Figure 5 | The acute and chronic THC treatment did not influence sociability and social memory

(a) Experimental timetable of THC treatment. On day 1, mice received the initial THC injection, followed by social habituation dishabituation test 30 minutes later. Subsequently, after seven consecutive days of daily chronic treatments, social behavioral assessments were repeated to evaluate sociability and social memory following prolonged exposure to THC. **(b)** Schematic illustration of the habituation dishabituation social interaction test. **(c)** No differences were observed analyzing the total amount of time in seconds allocated to investigation of the unfamiliar stimulus from trial1 to trial5 between control (black line) and THC (green line)-treated mice under acute condition. N= 13 control testing mice and N= 9 THC testing mice. **(d)** Total exploration time was calculated summing the social exploration times from trial 1 to trial 5. No difference was found between control (black dots) and THC (green triangles)-treated mice under acute condition mice during the entire social task. **(e)** Social novelty score was calculated using the following formula: ((T1-T4) + (T5-T4))/2 where "T" are the social interaction times sessions. No difference was found between control (black dots) and THC (green triangles)-treated mice under acute condition mice during the entire social task. **(f)** Schematic illustration of the habituation dishabituation social interaction test. **(g)** No differences were observed analyzing the total amount of time in seconds allocated to investigation of the unfamiliar stimulus from trial1 to trial5 between control (black line) and THC (green line)-treated mice under chronic condition. N= 17 control testing mice and N= 17 THC testing mice. **(h)** Total exploration time was calculated summing the social exploration times from trial 1 to trial 5. No difference was found between control (black dots) and THC (green triangles)-treated mice under chronic condition mice during the entire social task. **(i)** Social novelty score was calculated using the following formula: ((T1-T4) + (T5-T4))/2 where "T" are the social interaction times sessions. No difference was found between control (black dots) and THC (green triangles)-treated mice under chronic condition mice during the entire social task.

Bar and line graphs show mean ± s.e.m.

mPFC astrocytes chemogenetic activation restore emotion discrimination deficits given by both acute and chronic THC-treatment

Considering the decreased astrocytic calcium activity resulting from the mPFC-specific deletion of CB1-R, we proposed that chemogentic activation of mPFC astrocytes independent of CB1-R signaling could potentially restore socio-cognitive deficits induced by THC treatment.

To address this, we employed chemogenetics. Specifically, we bilaterally injected AAV5-GFAP-hM3D(Gq)-mCherry virus into the medial prefrontal cortex to selectively manipulate G protein-coupled receptor (GPCR) signaling in astrocytes. The DREADDs selective agonist Clozapine- N-Oxide (CNO, 3mg/kg) or control (saline) treatment was intraperitoneally injected 30 minutes prior to the EDT test, following a second i.p. injection of THC (0.25mg/kg) treatment (Fig.6a).

As previously reported, impairment in the recognition of positive emotional states (*relief*) compared to neutral was confirmed by the acute administration of THC in combination with control treatment (Fig.6c). Surprisingly, CNO i.p. injection re-established the social preference for the relief demonstrator in the first 0-2 epochs ($p=0.0141$), although statistical significance could not be reached for the discrimination index ($p=0.1225$). Notably, no significant effects were observed in the total sniffing time for the entire 6 minutes of the test. (Fig.6f). ($p=0.0976$). Similarly, emotional discrimination deficit for negative state (*fear*) induced by acute THC (Fig. 6h) was completely rescued upon CNO injection, as evidenced by the increase of social approach towards the fear stimulus compared to neutral in the last trial of the task (Fig.6i). This effect is also confirmed by the discrimination index (Fig.6j) ($p=0.0499$), while no significant effects were observed in total social interaction (Fig.6k) ($p=0.7984$).

Remarkably, we noted comparable effects even under chronic conditions. Specifically, a significant increase in social exploration toward the relief demonstrator was observed in the group with activated astrocytes following CNO injection (Fig. 6n), effectively overcoming the avoiding effect for the altered stimulus induced by chronic THC treatment (Fig.6m). This effect was corroborated by the discrimination index, even if not statistically significant (Fig.6o) ($p=0.0546$). However, no differences were observed in social interaction (Fig.6p) ($p=0.5941$).

Overall, these findings demonstrated the potential role of astrocytes activation in restoring emotion discrimination deficits produced by both acute and chronic systemic THC

treatment, highlighting the fundamental role of cortical astrocytes in ameliorating socio-cognitive impairments.

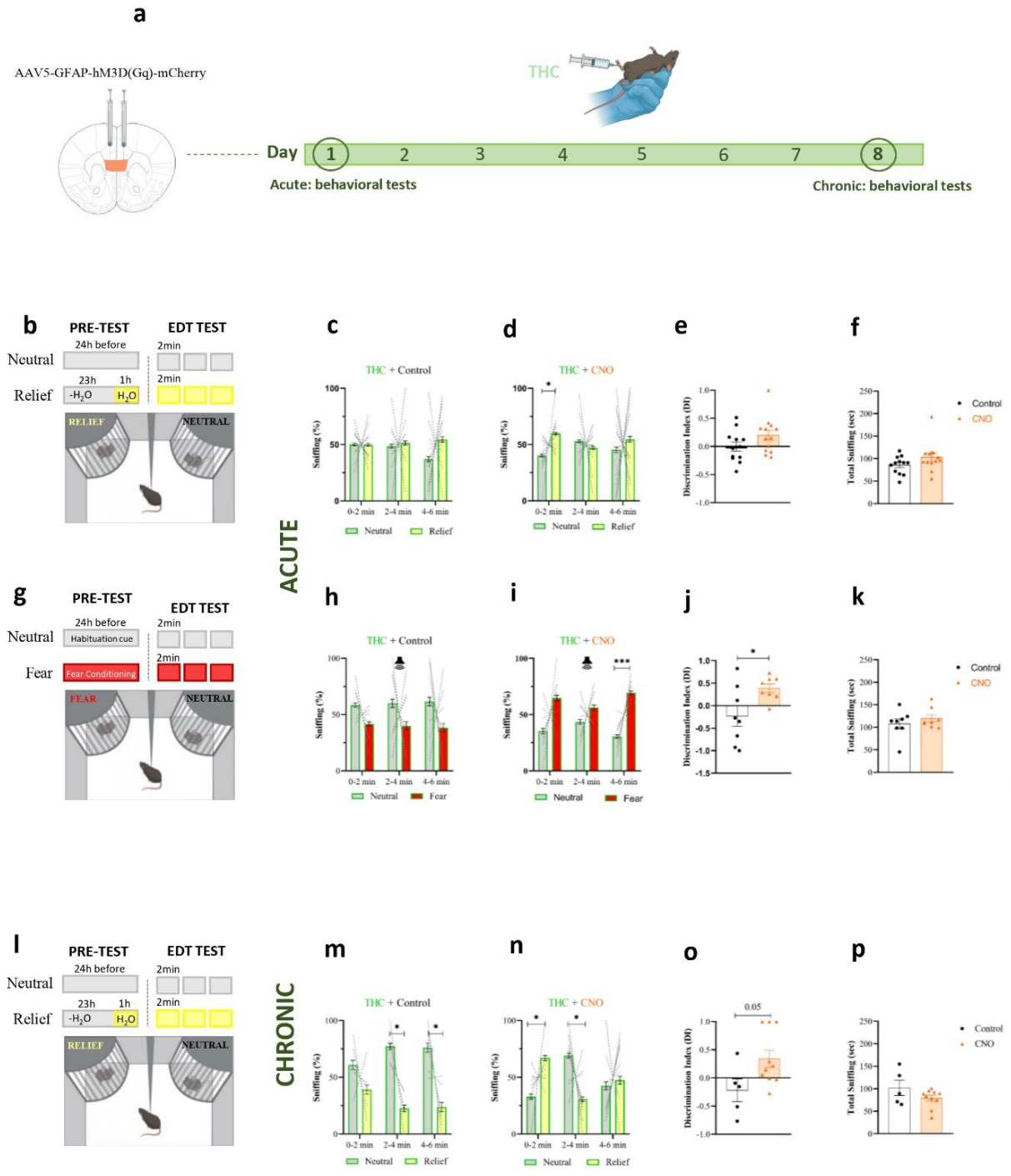

Figure 6 | mPFC astrocytes chemogenetic activation restore emotion discrimination deficits given by both acute and chronic THC-treatment

(a) Experimental design for chemogenetic astrocytes activation under THC treatment condition. Mice were bilaterally injected in mPFC with AAV5-GFAP-hM3D(Gq)-mCherry. After 4 weeks from virus injection, experiments were performed under both acute (day1) and chronic (day 8) THC condition. On day 1, 30min.before the test, mice received both THC and CNO (or vehicle) treatment. Subsequently, following seven consecutive days of daily chronic THC treatments, social behavioral assessments were repeated to evaluate emotion discrimination abilities following astrocytes activation upon CNO injection in prolonged exposure to THC. **(b)** Experimental design of EDT relief. Observers did not undergo any manipulation but 30min.before test they received both THC (or control) and CNO (or vehicle) treatment. One demonstrator (relief, yellow) received 1-h ad libitum access to water after 23h of water deprivation. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(c)(d)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. control (c) and CNO (d) injection in combination of acute THC treatment. N= 12 THC+control observers and N= 13 THC+CNO observers **(e)** The discrimination index between THC+control (black dots) and THC+CNO (orange triangles) groups was calculated using the following formula: ([sniffing towards relief– sniffing towards neutral] / [sniffing towards relief + sniffing towards neutral]). **(d)**No differences were observed analyzing the total sniffing time between THC+control and THC+CNO observer mice under acute THC conditions during relief EDT test**. (g)** Experimental design of EDT fear. Observers did not undergo any manipulation but 30min.before test they received both THC (or control) and CNO (or vehicle) treatment. One demonstrator (fear, red) was fear-conditioned at least one day before. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(h)(i)** Percentage of time spent sniffing demonstrators towards fear (red bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. control (h) and CNO (i) injection in combination of acute THC treatment. N= 8 THC+control observers and N= 8 THC+CNO observers **(j)** The discrimination index between THC+control (black dots) and THC+CNO (orange triangles) groups was calculated using the following formula: ([sniffing towards fear– sniffing towards neutral] / [sniffing towards fear + sniffing towards neutral]). **(d)**No differences were observed analyzing the total sniffing time between THC+control and THC+CNO observer mice under acute THC conditions during fear EDT test.
(l) Experimental design of EDT relief. Observers did not undergo any manipulation but 30min.before test they received both THC (or control) and CNO (or vehicle) treatment. One demonstrator (relief, yellow) received 1-h ad libitum access to water after 23h of water deprivation. The other demonstrator (neutral, gray) waited undisturbed in the home cage. **(m)(n)** Percentage of time spent sniffing demonstrators towards relief (yellow bars) or neutral (gray bars) during the 6-min test, divided into three consecutive 2-min epochs after observer mice received i.p. control (c) and CNO (d) injection in combination of chronic THC treatment. N= 5 THC+control observers and N= 11 THC+CNO observers **(o)** The discrimination index between THC+control (black dots) and THC+CNO (orange triangles) groups was calculated using the following formula: ([sniffing towards relief– sniffing towards neutral] / [sniffing towards relief + sniffing towards neutral]). **(p)**No differences were observed analyzing the total sniffing time between THC+control and THC+CNO observer mice under chronic THC conditions during relief EDT test

Discussion

The main finding of this study is the established influence of astrocytic-cannabinoid interaction in modulating socio-cognitive abilities. Employing a comprehensive approach that combines pharmacological and genetic manipulation with emotion discrimination test, we studied mice's ability to distinguish between different emotions following cannabinoid modulation at the level of mPFC astrocytes.

Our compelling results underscore the strong involvement of CB_1-R within astrocytes in recognizing altered emotional states. Specifically, the targeted deletion of these receptors from mPFC astrocytes led to a noteworthy decrease in overall activity of astrocytes, highlighting their relevant role in intracellular astrocytic signaling. Consistent with existing data (Navarrete & Araque, 2008), as these receptors are coupled to $G_{\alpha q/11}$ proteins, their removal resulted in a reduction of calcium levels. Consequently, this effect may lead to a decline in gliotransmitter release, ultimately modulating signaling in the cortex.

Concerning behavioral outcomes, these modulating effects translated into impairments in emotion recognition abilities for both positive and negative emotions. Surprisingly, social interaction and social recognition memory were not impacted indicating that astrocytic CB_1-R modulation can selectively modulate emotion recognition in mice. This aligns with certain studies reporting no significant difference in the overall sociability of mice lacking CB_1-R either totally (Litvin et al., 2013) or partially (Gould et al., 2012). However, evidence on social recognition memory is somewhat conflicting, with Litvin et al. reporting an increased discrimination index for social novelty in CB_1-R knockout mice, while others evidence observed no changes (Jacob et al., 2012). Notably, our study is the first to specifically investigate such social cognitive features following selective astrocytic CB_1-R deletion in the medial prefrontal cortex. Taken together, these findings suggest that astrocytic CB_1 receptors may play a crucial role in the accurate recognition of emotions, though the underlying mechanism remains unknown.

Nevertheless, several questions remain still unresolved. The behavioral effects induced by the deletion of astrocytic CB_1 receptors parallel findings in pharmacological data. Surprisingly, we found that THC treatment, activating the primary target CB_1 receptor, impairs the recognition of different emotions, as evidenced by clinical studies (Clopton et al., 1979) (Bossong et al., 2013) (Platt et al., 2010) (Hindocha et al., 2014b) (Zimmermann et al., 2017) (Ballard et al., 2012). It is

noteworthy, however, that systemic THC administration could trigger a cascade of events not necessarily linked to astrocytes signaling. The abundant presence of CB_1-R in GABAergic interneurons (Marsicano & Lutz, 1999) suggests that their activation, induced by systemic THC administration, could potentially disrupt the cortical circuitries responsible for emotion discrimination, leading to behavioral deficits. Indeed, it has been demonstrated that prolonged exposure to THC during adolescence promotes alterations in the GABAergic system within prefrontal cortex, marked by a reduction in cortical GAD67 expression with consequent psychotic-like behaviors (Zamberletti et al., 2014). Considering the pivotal role of somatostatin interneurons in mediating emotion recognition abilities (Scheggia et al., 2020), it is plausible that THC negatively regulates the activity of these cells, thereby contributing to behavioral deficits. Nevertheless, conducting future studies is imperative to investigate the modulation of somatostatin activity following THC administration, providing a more comprehensive understanding of the impact of THC on emotional processing.

Additionally, the heterogeneous distribution of CB_1-R suggests that their activation, whether by endogenous or exogenous cannabinoids, can trigger diverse biological processes across cellular and subcellular compartments. In line with this perspective, a recent study by Jimenez-Blasco et al. (2020) demonstrated that persistent activation of mtCB1 receptors in astrocytes induced by THC administration leads to impairments in mice's social behaviors. This activation causes the inhibition of glycolysis through HIF1 activity, resulting in alteration in lactate production and consequent neuronal metabolic stress. These observations, which cannot be excluded in our approach, underscore the intricate complexity of CB1receptor pathways.

We also observed that acute as well as chronic THC treatment might not exert an impact on sociability and social memory, in contrast to the findings reported by Busquets-Garcia et al. (2017) and Long et al. (2010). It is crucial to note that drugs acting on the cannabinoid system can display divergent effects on social functions depending on the dosage administered. In the present study, we deliberately utilized a low dose (0.25mg/kg) to mitigate potential biases in data interpretation. This decision was based on previous findings indicating that higher THC doses could influence both social interactions and locomotor activity, factors that might adversely impact subject's social behaviors in our EDT test. However, our analysis excluded any discernible effects on locomotion and sociability.

Crucially, our observations suggest that emotion discrimination deficits induced by systemic THC treatment could potentially be reversed through the activation of astrocytes within mPFC. The chemogenetic manipulation of astrocytes within the mPFC enhances intracellular calcium levels, which, in turn, contributes to the modulation of neuronal circuits of social cognition through the release of gliotransmitters. However, the exact mechanism by which astrocytes' activation compensates for behavioral deficits induced by cannabinoids has yet to be fully elucidated.

In conclusion, our findings build upon previous reports regarding the role of the eCB system in social cognition, offering new insights into the potential pathophysiological implications of the astrocytic CB_1-R in the medial prefrontal cortex in the context of emotional discrimination ability.

Materials and Methods

Mice

All procedures were approved by the Italian Ministry of Health (permit n. 749/2017-PR 176AA.34, 639/2020-PR 1766AA.82) and strictly adhere to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Routine veterinary care and animals' maintenance was provided by dedicated and trained personnel.

Adult males and females C57BL/6J mice and, homozygous CB_1-flox (breeders were kindly gifted by Giovanni Marsicano) were used. Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with ad libitum access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). Experiments were run during the light phase (between 10am-5pm). All mice were handled on alternate days during the week preceding the first behavioral testing. Experimenters were blind to mouse treatments during testing. Female mice were visually checked for estrus cycle immediately after the test and no correlation was found between estrus status and performance in the test.

Viral Injections and fiber implantation

Naïve mice were prepared for stereotaxic surgeries at 3 months old. All of them were anaesthetized with a mix of isoflurane (2%) in Oxygen (O_2, 1%) by inhalation before being mounted onto a stereotaxic frame (Kopf) linked to a digital reader. Brain coordinates of viral injection in the mPFC were chosen in accordance with the mouse brain atlas: anterior–posterior (AP), +1.9 mm; medial–lateral (ML): ± 0.25 mm; and dorsal–ventral (DV): −2.7; - 2.4; - 2.1 mm.

We infused AAVs virus through a borosilicate micropipette connected to a 10µl Hamilton syringe filled with mineral oil. The injection rate was set to last between 5 and 10 minutes and the pipette was maintained in position for at least 10 min to allow proper diffusion and then slowly withdrawn. The following viral vectors were used: AAV5-gfaABC1D-cyto-GCaMP6f (Addgene 52925, 450nL), AAV5-GFAP-hM3Dq-mCherry (Addgene 50478, 300nL), AAV8-GFAP104-eGFP (UNC Vector AV5105E), and AAV8-GFAP-mCherry-CRE (UNC Vector AV5056C). The injected volumes were adjusted based on the type of experiment.

Fiber implantation. After viral injection, mice used for fiber photometry experiments were given between 2 and 3 weeks to recover before undergoing optic fiber cannulae implantation (200 µm, 0.50 numerical aperture, fiber distance 3.5 mm; RWD). The skull was exposed, and the two previous holes were used to target the mPFC. Fiber optic cannulae were secured to the skull with VetBond glue and dental cement. After optic fiber implantation, mice were allowed to recover for 7–10 d depending on the general health.

Drugs

THC ((-)-trans-Δ-9-tetrahydrocannabinol, 0.25mg/kg) in absolute ethanol solution was purchased from National Institute on Drug Abus (Bethesda, USA). To avoid ethanol-related bias on behavior, the alcoholic component was evaporated using a continuous nitrogen flow to obtain THC resin, that was dissolved in 1%DMSO, 1% Tween80 and 98% saline. As a control treatment, we injected a vehicle solution of 1%DMSO, 1% Tween80 and 98% saline. For both acute and chronic treatments the drug solution (or vehicle) was administered through intraperitoneal injections of a 10 ml kg^{-1} volume, 30 minutes before the test.

For hM3D astrocytes activation, we used i.p. administration of clozapine-N-oxide dihydrochloride (CNO, 4936 Tocris) dissolved in physiological saline (0.9% NaCl) at a dose of 3 mg kg^{-1} in a volume of 10 ml kg^{-1}, 30 min before the behavioral experiments. Saline injection in a volume of 10 ml kg^{-1} was used as a control treatment.

Behavioral assays

Emotion discrimination test

Habituation of the mice to the testing setting occurred on three consecutive days before the first experiment; each habituation session lasted 10 minutes. Test observer mice were habituated inside a 3D printed square apparatus (34 x25x19 cm) containing a separator (150 cm) and a two-quarter circle (4cm ray) on two opposite sides. Each quarter-circle is formed with metal rods (0.8cm) that allow sufficient space for social contact between the observers (freely moving in the apparatus) and the demonstrators (freely moving in the quarter-circle). A 3D-printed separator is thus inserted between both quarter-circles to block the reciprocal view of the stimuli animals while leaving the observer mice free to move between the two sides of the cage. For scoring, a virtual square (11 x 11 cm) is placed between each quarter-circle and the separator to define a

zone associated with each stimulus. The experimental cages were replaced after each subject with clean copies to avoid scent carryover. Similarly, the rest of the apparatus was wiped down with water and dried with paper towels for each new subject.

Demonstrator mice – matched by age and sex to the observers – were habituated inside the same 3D printed cage (34 x25x19 cm), into quarter-circle space three consecutive times, ten minutes each. During both habituation and behavioral testing, the cages were placed inside soundproof cubicles (TSE Multi Conditioning Systems) homogeneously and dimly lit (6 ± 1 lux) to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference. Digital cameras (imaging Source DMK 22AUC03 monochrome, Ugo Basile) were placed on top of the cage to record the three consecutive two-minute epochs, using the Anymaze program (Stoelting, Ireland). Behavioral scoring was performed a posteriori from videos by trained experimenters, blind to the manipulations of both the observers and demonstrators. Three independent people scored the same data with an inter-rater reliability r score of 0.954. A sniffing event was considered when the observer touched with the nose the demonstrators' wire cup or when the observer's nose directly touched the demonstrator.

Observers

Before the test, mice were habituated to the experimental setting as reported above. Only in the fear paradigm, on the third day of habituation, mice were also habituated to the tone cue (4 kHz, 80 dB sound pressure level, three times for 30 s each with an intertrial interval of 90 s) without any conditioning. One hour prior to behavioral testing, mice were placed in the testing cage, in an experimental setting (i.e., separator and two wire cups), in a room adjacent to the testing room. Five minutes before the experiment, the testing cages containing the observer mice were gently moved into the testing cubicles. The 6-minute experiment began after placing one emotionally 'neutral' and one 'emotionally altered' demonstrator in the EDT apparatus. The order of insertion of the neutral or emotionally-altered demonstrator was randomly assigned.

Neutral demonstrators

In the days before the test, all neutral mice were habituated to the experimental setting as reported above. For the relief condition, neutral demonstrators underwent no manipulation the day before the test. For the fear condition, the day before the test, neutral demonstrators were habituated to the tone cue inside the cups as for the experimental setting and as done for the observer mice. On the testing day, neutral demonstrators were brought inside their home cages in

the experimental room one hour before the experiment began. Demonstrators were test-naive and used only once. In some cases, we re-used the same demonstrator for maximum two/ three times, with always at least one week between each consecutive test. No differences were observed in the performance of the observer mice depending on the demonstrators' previous experience.

Relief demonstrators

In the days before the test, mice were habituated to the experimental setting as reported above. Relief demonstrators were then water deprived 23 hours before the experiment. One hour before the test, ad libitum access to water was reestablished, and mice were brought inside the experimental room in their home cages. Food was available ad libitum all the time and some extra pellets were put inside the home cage during the 1-hour water reinsertion.

Fear demonstrators

In the days before the test, mice were habituated to the experimental setting as reported above. Fear demonstrators were fear conditioned (from one day to one week before the test) using the parameters and context previously described (Scheggia et al., 2018), and using the same tone delivered to the observers and neutral demonstrators during their habituation process. In particular, the conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30 s) and the unconditioned stimulus was three scrambled shocks (0.7 mA, 2 s duration, 90 s intershock interval) delivered through the grid floor that terminated simultaneously with the tone (2 s). On the day of the test these mice were habituated, inside their home cages, in a room adjacent to the testing room for one hour prior to the test; they were consequently brought inside the experimental room one by one, before placing them under their designated wire cup. Fear mice were conditioned only once, in a separate room and using a distinct apparatus (Ugo Basile SRL, Italy) from the one where the emotion recognition task would be performed. Fear demonstrators were generally used only once. In the case of a second exposure to the test, these demonstrators were just re-exposed to the same conditioned tone, at least one week apart from the previous exposure and maximum of 1 month from the initial conditioning.

Social Habituation/Dishabituation

The test consisted of five repeated social interaction sessions between the experimental subject and an unfamiliar stimulus mouse followed by the exposure of the experimental subject to a new unfamiliar mouse. Briefly, mice were tested in 2150E Tecniplast cages (35.5 x 23.5 x 19 cm)

lightly illuminated (5±1 lux) and video-recorded using a UniBrain Fire-i™ Digital Camera. The video camera was mounted facing the front of the cage to record the session for subsequent scoring of social investigation parameters. All the equipment was kept in a sound-attenuating chamber (TSE Multi Conditioning Systems). Adult mice were individually placed in the testing cage and left to habituate for 1 hour. Single housing manipulation was not carried out to avoid any instauration of home-cage territory and aggressive behaviors. Testing began 5 minutes after the habituation in the apparatus when a stimulus mouse of the same sex and age was introduced into the testing cage for a 1-min interaction. At the end of the 1-min inter-trial, we removed the stimulus animal and placed it in an individual holding cage. We repeated this sequence for four trials with 3-min inter-trial intervals. In a fifth trial, we introduced a new unfamiliar stimulus mouse in the testing cage. Videos of behaviors were recorded and subsequently scored offline (ANY-maze, Stoelting Co.). We measured the duration of the following behavioral responses performed by the tested mice, considering as social interaction anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), head sniffing (sniffing or snout contact with the head/neck/mouth area) and following (time spent in following the stimulus mouse). The "social exploration" score was obtained by summing all time spent in social interaction during the 5 minutes interaction sessions. The "social novelty score" was obtained by this formula ((T1-T4)+(T5-T4))/2 where "T" are the social interaction sessions, to measure the ability of each mouse to distinguish between a familiar and an unfamiliar mouse.

In vivo fiber photometry recordings and analysis

To analyze calcium fluctuations of mPFC astrocytes, the fluorescence signal emitted by GCaMP6f-expressing astrocytes was recorded using fiber photometry system. A signal processor (RZP5, Tucker Davis Technologies) was used to control two light sources (465-nm LED, CLED_465; 405-nm LED, CLED_405, Doric Lenses), which were modulated at 211 and 539 Hz, respectively. The two wavelengths were combined by a fluorescence minicube (Doric Lenses) and transmitted through an optical patch cable (Doric Lenses) to the mouse head implant. Emitted fluorescence was collected by the same patch cable, delivered back to the same minicube through a 525-nm filter and sent to a photoreceiver (Femtowatt Silicon Photoreceiver, DC-750 Hz; Newport). Real-time signals were acquired, lowpass filtered (3 Hz) and

demodulated with Synapse Essentials software (Tucker Davis Technologies). Anymaze tracking system program (Stoelting, Ireland) was used. Data were extracted from TDT files and analyzed using custom MATLAB scripts.

Before starting the analysis, bleaching was corrected by fitting a double exponential decay to the fluorescent signal, capturing the exponential decay of the isosbestic. Subsequently, the raw signal was divided by the fitted decay, and Z-score normalization was performed to standardize the signal by expressing it in terms of units of standard deviation.

Identification of calcium signal events was performed via custom MATLAB scripts, using the findpeaks function to find the local maxima of calcium signals. Such maxima were considered calcium events if they overcame thresholds on the minimum event height (MinHeight, set as the average of the signal), minimum event prominence (MinProm, set as the standard deviation of the signal), minimum distance between consecutive events (MinDist = 1 second). The event frequency in a given time window was computed by dividing the number of events identified in such window by its length. Peak intensity was calculated as the ΔF/F value of the event.

Histology

At the end of the behavioral procedures, we checked viral expression and the position of the optic fibers. Mice were deeply anesthetized (urethane 20%) and transcardially perfused with 4% paraformaldehyde in PBS at pH 7.4. Brains were dissected, fixed overnight, and cryoprotected in 30% sucrose in PBS. Coronal sections (40 μm) were cut using a HM450 microtome (Thermo Fisher Scientific). For immunohistochemical studies free-floating sections of selected areas were washed in PBS three times for 10 min, permeabilized in PBS plus 0.4% Triton X-100 for 30 min, blocked by incubation in PBS plus 4% normal goat serum (NGS) and 0.2% Triton X-100 for 30 min (all at room temperature, 20–23 °C), and subsequently incubated in PBS plus 2% NGS and 0.1% Triton X-100 overnight at 4 °C with the following primary antibodies: GFP polyclonal antibody (1:1000, Abcam, AB13970) and dsRed polyclonal antibody (1:1000, Takara, 632496). Incubated slices were washed three times in PBS plus 1% NGS for 10 min at room temperature, incubated for 2 h at room temperature with the following secondary antibodies: Alexa Flour 488 goat anti-chicken IgG (1:1000, Molecular Probes, A11039) and Alexa Flour 568 goat anti-rabbit IgG (1:1000, Molecular Probes, A11036) in PBS plus 2% NGS and 0.1% Triton X-100.

Subsequently, slices were washed three times in PBS for 10 min at room temperature. The sections were mounted on slides and covered with cover slips.

Statistics

Results are expressed as mean ± s.e.m. throughout the manuscript. For the analyses of emotion discrimination, each observer behavior towards the two different demonstrator mice was calculated as a percentage of sniffing time to allow direct comparison between different experimental conditions. 2wayANOVA repeated measure within-groups was used to analyze these data, followed by Sidak's post-hoc test with multiple comparison corrections for making comparisons within groups when the overall ANOVA showed statistically significant differences. The same approach was used for the peak analysis, discrimination index and social habitation dishabituation analysis when different treatments were involved.

Mann-Whitney nonparametric test was used to analyzed different conditions including total sniffing, social novelty score, locomotor activity, average speed, and areas under the curve corresponding to altered and neutral zone. The accepted value for significance was $P < 0.05$. Statistical analyses were performed using GraphPad Prism 8. Numbers of mice are reported in the figure legends. The experiments reported in this work were repeated independently two to four times, using mice from at least three different generations. Mice were excluded post-hoc when optic fiber placement or viral expression patterns were not appropriate (outside the target region or week viral expression). For all behavioral tests, littermates were randomly assigned to the different groups. Specific randomization in the organization of the experimental conditions is described in the results and figure legends. Experimenters were not blinded during data acquisition, but all analyses were performed with blinding of the experimental conditions.

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CHAPTER 4

General Conclusions and Future Perspectives

Synthesis of Key Findings

In the present study, we have provided novel insights into the complex neurobiological mechanisms underpinning emotionally-linked social cognitive processes.

Specifically, throughout *Chapter 2*, we demonstrated the fundamental role of astrocytes in the recognition of different emotional states. This goal was achieved through the implementation of a rodent behavioral task developed to study in mice the ability to discriminate different emotions. Combined manipulating approach, the task resulted a powerful instrument in unraveling the interplay of both neuronal and non-neuronal substrate contributing to emotion discrimination.

While the role of SOM interneurons in the ability of recognizing emotions has been previously characterized, our exploration moved to study the specific interaction between astrocytes and these cortical inhibitory neurons within the medial prefrontal cortex. Although a large body of research has demonstrated their interaction, the specific involvement of astrocytes-SOM interneurons in socio-cognitive processes remains not fully elucidated.

We first explore the spatial communication between astrocytes and GABAergic neurons within the medial prefrontal cortex, demonstrating a notable interaction particularly with SOM+ neurons. This observation prompted us to delve into the prospect of this interaction influencing behavioral processes.

Subsequently, we extended our investigation into the physiological contribution of mPFC astrocytes to emotion discrimination, taking advantage of fiber photometry technique. Surprisingly, heightened astrocytic calcium activity was observed when an observer mouse interacted with an emotionally altered conspecific stimulus. Similar results were found with somatostatin activity, as evidenced in a previous study of the lab.

These compelling results motivated an in-depth exploration of the behavioral role of astrocytes in emotion discrimination. Employing chemogenetic, we demonstrated that mPFC astrocytes activation potentiate the ability to recognize different emotions without affect other aspects related to social behaviors (e.g. sociability, social memory and social dominance). Importantly, our research found that emotion discrimination deficits induced by optogenetic inhibition of mPFC SOM+ interneurons can be ameliorated through astrocytes activation.

The specific mechanism by which astrocytes mediated the communication with somatostatin interneurons to modulate emotional processes remains a gap in our understanding, needing future investigations.

Moving forward to *Chapter 3*, we started to examine the behavioral outcomes resulting from the manipulation of cortical astrocytes through the cannabinoid system, a known modulator system of emotional processes. The complexity of astrocytic signaling arises from their peculiar structure and intricate communication with neurons. Astrocytes express a variety of receptors that, in turn, modulate synaptic processes. Notably, the expression of cannabinoid CB_1 receptors within astrocytes has been extensively documented. However, their functional role in socio-cognitive processes remains an unexplored domain.

We first elucidated the correlation between these receptors and socio cognitive behavior. Importantly, we demonstrated that the targeted removal of CB_1-R within mPFC astrocytes not only drastically affects overall astrocytic signaling but also exerted a detrimental impact on emotion recognition. This impairment manifested as a diminished ability to discern altered emotional states, highlighting the pivotal role of astrocytic CB_1-R signaling within the neurobiological processes of emotion discrimination.

Parallel behavioral deficits were observed when CB_1-R were activated through the administration of an exogenous cannabinoid compound. THC, whose primary target is represented by CB_1-R, has been acknowledge for its negative influence on emotion recognition in cannabis users. In our study, both acute and chronic exposure to THC were found to lead an impaired ability to accurately perform socio-cognitive tests, emphasizing the influence of CB_1-R signaling on social abilities. It is important to note that we cannot exclude potential effects at the level of inhibitory interneurons, given their abundant expression in these neurons.

Nevertheless, we observed that THC-induced emotion discrimination deficits could be reversed by chemogenetic activation of astrocytes, underscoring the pivotal role of these glial cells in compensating for alteration in cortical circuits related to emotion discrimination. Further studies will be essential to elucidate the potential mechanism driving this effect.

In summary, our research contributes novel insights into the pathophysiological implications of cortical circuits in social cognition, offering valuable avenues for future neurobiological targets.

Future research perspectives

In our study titled “Cortical Astrocytes Modulate Emotion Discrimination” we have delved into the intricate cortical mechanisms governing emotion discrimination, aiming to make a significant contribution to the identification of novel substrates that could prove valuable for future therapeutics targets against social impairments in neuropsychiatric disorders. While our findings have provided valuable insights, several questions still require clarification. To enhance the depth of our comprehension of cortical circuits in emotion discrimination, we will augment our study with additional investigations.

In *Chapter 2*, we have clarified the role of astrocytes in modulating socio-cognitive behaviors. Nevertheless, future studies could be necessary to better understand their functional signaling relevance within medial prefrontal cortex circuits in modulating emotion discrimination abilities. However, the complexity of astrocytes biology makes challenging the technical development of specific molecular tools that can selectively manipulate in vivo these cells without affecting other cell types or functions. Notably, specific tools widely used to manipulate neurons may exhibit divergent effects in astrocytes, exemplified by optogenetic approach (Octeau et al., 2019) (Poskanzer & Yuste, 2016). Despite these challenges, there has been a growing interest in astrocytes research, and efforts are being made to develop more sophisticated molecular tools for manipulating astrocytes in vivo.

In our study, we plan to investigate a novel approach developed by Yu et al. (2018) aimed at reducing astrocytes Ca^{2+} signaling. This approach involves the expression of a plasma membrane Ca^{2+} pump (PMCA). In contrast to the classical method of attenuating calcium signaling through IP_3 receptor deletion in mice, the PMCA approach offers greater selectivity. Specifically, through local microinjections of adeno-associated virus with astrocytes promoter, PMCA can be selectively expressed in specific brain regions. This targeted manipulation will provide valuable insights into the role of astrocytes in emotion discrimination, enhancing the depth of information in our study.

Moreover, we will investigate neurotransmission patterns following astrocyte modulation, utilizing state-of-the-art biosensors designed to monitor real-time release of neurotransmitters or neuropeptides (H. Wang et al., 2023) (Marvin et al., 2019). As discussed in the conclusion of Chapter 2, we firmly believe in the implication of GABA or somatostatin peptide in the modulation of behavioral responses. This exploration will provide novel information about the

dynamic neurochemical changes that underlie the observed behavioral alterations, shedding light on the specific contributions of GABA and somatostatin in astrocyte-mediated modulation.

In addition, we aim to enhance our investigation of the communication between astrocytes and somatostatin interneurons within the context of emotion discrimination. This will be achieved by combining chemogenetic and/or PMCA approaches with microendoscopic calcium imaging. By employing this approach, we can simultaneously capture somatostatin neuronal activity in freely behaving mice during emotion discrimination test, following astrocytes manipulation. Using one-photon miniature miniscope technology, we will further refine our investigation enabling a more in-depth analysis of somatostatin activity with single cell resolution. This will facilitate the analysis of neuronal clusters following astrocytes activation and/or deactivation, providing a comprehensive understanding of their interplay in the intricate processes of emotion discrimination.

In *Chapter 3*, we have uncovered a noteworthy correlation between the cannabinoid system and astrocytic activity in the modulation of emotion discrimination abilities. While the established impact of CB_1-R deletion on impairing emotion discrimination behaviors is well confirmed, the data concerning the social effect of THC systemic administration show promising results. However, to fortify our scientific idea, we intend to replicate these experiments, increasing the number of mice tested. Specifically, we aim to extend our investigation under chronic conditions, where the effects of chronic THC treatment in fear paradigm are still unexplored.

Furthermore, we plan to conduct a more in-depth investigation into how cannabinoid manipulation, involving both CB_1-R deletion and THC treatment, could affect SOM+ interneurons activity. To achieve this goal, we will take advantage of fiber photometry technique to record overall somatostatin activity following manipulation. This approach will better define the impact of the cannabinoid system on the cortical circuits involved in emotion discrimination.

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Acknowledgments

I would like to express my sincere gratitude to my supervisor, Dr. Francesco Papaleo, for the support provided throughout my academic journey. His enthusiasm for our work and encouragement of my ideas have been fundamental in shaping the trajectory of my research.

A special acknowledgment goes to my “mentor” Francesca Managò. She significantly contributed to my scientific growth. Her teachings were essential, as well as the scientific and non-scientific support provided during these years at IIT.
I am immensely grateful to have been her student (of course, her favorite one).

I would like to extend my gratitude to all the people who constantly contribute to technically support our research “behind the curtain”, particularly the precious assistance from the animal facility team for taking care of animals.

Lastly, I am grateful for the opportunity to have interacted and learned from each single person I encountered during the PhD, enriching my scientific and personal experience.
Thanks to: Federica Maltese, Gabriella Trigilio, Giulia Chiacchierini, Cinzia Molent, Anna Monai, Arianna Benedetti, Federica Antonelli, Fabrizio Bernardi, Zisis Bimpisidis, Alessia Polito, Alessandra Cucinelli, Roman Walle, Christine Stubbendorf, Zichen Zang and Ilaria Carta.

Finally, a special thank to Marco Niello for supporting (and bearing with) me from the beginning.