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Central amygdala - ventral tegmental area - cortical circuits mediate initiation and maintenance of social interaction

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Achievements

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Abstract

Successful social interaction involves reciprocal contact with other individuals and requires well-orchestrated responses from interaction partners. In social species, specialized brain areas and neural networks ('social brain') mediate social interaction and allow individuals to survive and thrive. Dysfunctions of these brain networks result in decreased motivation to initiate social interaction and/or incapacity to communicate and understand social information, which causes problems with maintaining social interaction. Different mental disorders affect various aspects of social interactions. However, the neuronal circuits underlying the initiation and maintenance of social contact have yet to be discovered. As one of the primary hypotheses explaining social dysfunctions is a deficiency in reward processing, one of the promising targets to treat social impairments are neuronal circuits known to process rewards.

Here, I investigated the neuronal circuit comprising the anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), central amygdala (CeA), and ventral tegmental area (VTA) to verify their role in the initiation and maintenance of social interaction. Further, I tested the specificity of these circuits in social interaction by comparing their role in social interaction and food motivation. I found that the CeA cells activated by social interaction or food rewards receive projections from the ACC and OFC. Next, I discovered that chemogenetic inhibition of the ACC-CeA projection modulates the maintenance of social interaction but not the initiation of social interaction. On the other hand, inhibition of the OFC-CeA projection diminished both the social approach and the maintenance of social contact. Inhibition of either projections decreases food motivation. Further, using a *c-fos*-dependent construct containing opsins that targets behaviorally activated neurons, I labeled the CeA cells involved in social and food reward. Optogenetic manipulations revealed that the functional overlap between these circuits is limited. To identify the CeA outputs that can be specific to social interaction, I used chemogenetic manipulations. I found that the CeA-VTA projection and the dopaminergic VTA-ACC and VTA-OFC pathways are involved in social interaction but not food motivation. Moreover, I identified the CeA-VTA and VTA-ACC projections as critical for initiating social contact and the CeA-VTA for

maintaining it. Unlike most published studies, I define the circuits regulating appetitive social behaviors by their functional connectivity with other structures rather than the markers they express. Such defined neuronal circuits could serve as therapeutic targets for rescuing social deficits.

Streszczenie

Udane interakcje społeczne wymagają wzajemnego kontaktu pomiędzy osobnikami, podczas którego partnerzy muszą w dobrze zsynchronizowany sposób przekazywać i odbierać informacje społeczne. U gatunków społecznych, obszary mózgu, jak i połączenia między nimi, zaangażowane w kontakty z innymi (tzw. „mózg społeczny") pełnią istotną rolę zarówno w rozwoju, jak i przeżyciu osobnika. Dysfunkcje tych sieci mózgowych skutkują obniżoną motywacją do inicjowania interakcji społecznych i/lub niemożnością komunikowania się i rozumienia informacji społecznych, co powoduje problemy z utrzymaniem interakcji społecznych i budowaniem więzi między osobnikami. W różnych zaburzeniach neuropsychicznych trudności z nawiązywaniem i utrzymywaniem interakcji społecznej mogą występować w różnym nasileniu. Jednak obwody neuronalne leżące u podstaw inicjowania i utrzymywania kontaktów społecznych nie zostały jeszcze dobrze poznane. Zgodnie z jedną z wiodących hipotez tłumaczących dysfunkcje interakcji społecznych, deficyty w podejmowaniu interakcji z innymi spowodowane są problemami z przetwarzaniem informacji o nagrodzie. Z tego względu jednym z obiecujących celów badań są obwody neuronalne tworzące tzw. układ nagrody.

W swojej pracy zbadałam obwód neuronalny obejmujący przednią korę zakrętu obręczy (ang. *anterior cingulate cortex,* ACC), korę oczodołowo-czołową (ang. *orbitofrontal cortex,* OFC), jądro środkowe ciała migdałowatego (ang. *central amygdala,* CeA) i brzuszny obszar nakrywki (ang. *ventral tegmental area,* VTA), aby zweryfikować ich rolę w inicjowaniu i utrzymywaniu interakcji społecznych. Dodatkowo, rozróżniłam udział tych obwodów w koordynowaniu odpowiedzi na nagrodę społeczną i pokarmową. Poprzez wyciszenie projekcji ACC-CeA za pomocą technik chemogenetycznych odkryłam, że pełni ona istotną rolę w utrzymywaniu interakcji społecznej, ale nie zmienia motywacji do ich rozpoczynania. Natomiast projekcja OFC-CeA jest istotna dla obu etapów, zarówno rozpoczęcia, jak i utrzymania interakcji. Następnie, wykorzystując techniki optogenetyczne wykazałam, że populacje komórek w CeA zaangażowane w pozytywne interakcje społeczne i nagrodę pokarmową są różne. Zbadanie połączeń pomiędzy CeA-VTA oraz dopaminergicznych połączeń między VTA-

ACC oraz VTA-OFC wykazało ich istotną rolę w interakcjach społecznych, ale nie w reakcjach na nagrodę pokarmową. Ponadto wykazałam, że projekcje CeA-VTA oraz VTA-ACC są istotne dla nawiązywania interakcji społecznych, a projekcja CeA-VTA jest niezbędna dla ich utrzymania. W przeciwieństwie do większości opublikowanych badań definiuję obwody regulujące apetytywne zachowania społeczne na podstawie ich funkcjonalnych połączeń z innymi strukturami, a nie markerów, które wyrażają. Tak zdefiniowane obwody neuronalne mogą w przyszłości służyć jako cele terapeutyczne w terapiach deficytów zachowań społecznych.

Abbreviations

ACC	anterior cingulate cortex
AP-1	activator protein 1
ASD	autism spectrum disorder
BLA	basolateral amygdala
c21	compound 21
cAMP	cyclic adenosine monophosphate
CAV	canine adenovirus
CeA	central nucleus of the amygdala
ChR2	channelorhodopsin-2
CNO	clozapine-N-oxide
CREB	response element binding protein
DA	dopamine
DIO	reading frame constructs
DREADDs	designer receptors exclusively activated by designer drugs
DV	dorsoventral
GPCRs	G protein-coupled receptors
GABA	gamma-aminobutyric acid
GAD1	glutamate decarboxylase 1

Glu	glutamic acid
Hipp	hippocampus
HPLC	High Performance Liquid Chromatography
IEGs	immediate early genes
MDD	unipolar major depressive disorder
mOFC	medial orbitofrontal cortex
NA	noradrenalina
NAc	nucleus accumbens
NpHR	halorhodopsin
OFC	orbitofrontal cortex
pgACC	pregenual anterior cingulate cortex
PFC	prefrontal cortex
PHA-L	Phytohemagglutinin-L
PKCδ	protein kinase Cδ
PPC	posterior cingulate cortex
PR	Progressive Ratio test
sgACC	subgenual anterior cingulate cortex
SNc	substantia nigra pars compacta

STS	superior temporal sulcus
TH	tyrosine hydroxylase
TOM	theory of mind
USVs	ultrasonic vocalizations
VGluT2	vesicular glutamate transporter 2
VMAT2	vesicular monoamine transporter 2
vmPFC	ventromedial prefrontal cortex
vOFC	ventral orbitofrontal cortex
VTA	ventral tegmental area
5-HT	serotonin

1. Introduction

1.1. Social interaction

Social interaction is a process of reciprocal contact with other individuals. This process is based on integration of internal information, such as own physical and emotional state, with external information, such as cues emitted by conspecifics or coming from the environment. Integrated internal and external information motivates individuals to initiate contacts with others and adapt their behavior in a way allowing to maintain social interaction (Fisher et al., 2021; Han et al., 2021). The capacity to engage in and maintain social interactions includes affective components such as social motivation and pleasure from social contact, as well social cognition, which encompasses social perception, emotional recognition, memory, and judgement. Additionally, successful social interaction requires social skills including coordinated behaviors aimed at gaining social goals (Morrison et al., 2020). During healthy development, social involvement promotes social skills learning; however, it is also feasible to train individuals with a diminished social motivation to improve their social skills (Chevallier et al., 2012).

Initiation and maintaining social contact activates the reward system, which leads to rewarding experiences that drive social interaction (Solié et al., 2022). Notably, the quality of social interactions contributes significantly to overall life satisfaction. Understanding how the brain processes the initiation and maintenance of social interaction is fundamental to defining the causes of social interaction deficits that significantly affect life quality. The present study aimed to investigate whether there is a shared neural substrate underlying motivation to initiate social contact and abilities required to maintain social interaction, such as social skills and cognition.

1.2. Functions of social interaction

For social species, including humans and many other animals, social contacts are crucial for survival, and individuals express complex and specialized social behaviors. Such behaviors are acquired and mastered during development. Successful social interaction provides information about emotional states of others but also about the environment, enabling an individual efficiently navigate social and physical worlds (Silk, 2007; Tamir and Hughes, 2018). In this section, I will summarize how social interactions support socio-cognitive development, facilitate collaboration and competition within social groups, and provide crucial information about the environment.

1.2.1. Socio-cognitive development

Close contact with a caregiver is critical for the survival of newborns in many species. The need for social attachment may be stronger than the need for food, as demonstrated by the early Harlow's studies. After birth, he separated infant rhesus monkeys from their mothers and placed them with two dolls intended to mimic motherly contact. The first doll was covered by a soft fabric but did not deliver any food, whereas the second was made of wire but provided nourishment from an attached baby bottle. Harlow noticed that the baby monkeys spent substantially more time with the soft doll, which gave soothing and comfort, than with the wire doll, which provided food. He also demonstrated that the presence of the cloth doll (an artificial mother) led to greater sense of security observed as higher room exploration. In contrast, removing the artificial mother from the room led to decreased exploration, increased screaming, and freezing (Harlow et al., 1965).

Social interaction requires a series of complex and well-timed behaviors tailored to the partner's responses. Successful interaction needs recognition of complex social stimuli, such as face expression and body language, and a close cooperation with the interaction partner, which promotes development of cognitive abilities (Hari et al., 2015). Observation of faces and gaze direction is a powerful

stimulus for developing shared attention, i.e., the process in which one person purposefully coordinates their focus of attention with that of another person (Carpendale and Lewis, 2004). Additionally, the eyes and surrounding regions communicate complex emotional information (Federico, 2020; Fisher et al., 2021), which, if perceived correctly, facilitate children's social understanding and, consequently, their ability to function within a social group (Carpendale and Lewis, 2004; Jenkins et al., 2003). Sharing emotional states with others activates the brain's empathy-related network, leading to a better understanding of the past and future social conduct of others, such as in tasks requiring knowledge of others' internal states (Alcalá-López et al., 2019; Keysers and Gazzola, 2007). Thus, social contact with parents/caregivers is also critical to developing the ability to build mental models of others' actions. Children who are better at sharing attention and building mental models of other people's actions at 6 months have higher cognitive outcomes at 18 months. Apparently, better social understanding boosts one's ability to use existing learning opportunities (Marciszko et al., 2020).

The importance of efficient social communication for cognitive development is illustrated by a delay in mental processes development (as measured by the false-belief test) in deaf children having hearing parents. In contrast to deaf children of deaf parents, deaf children of hearing parents initially experience communication problems. Importantly, only the former show a delay in cognitive development (Peterson and Siegal, 2000). Apparently, parents who are native sign language users are able to efficiently explain the actions and emotions of others to their children. Conversing about the mental environment and exchanging experiences supports the development of social cognition.

Early social engagement also fosters development of executive function, i.e., the capacity to facilitate appropriate activity at the same time inhibiting other activities that could prevent achieving certain goals (Dempster, 1992). The early child-adult relationship provides the child with alternative viewpoints on how to address different situations, which can be utilized in later development (Moriguchi, 2014).

Numerous animal studies have investigated the effects of social deprivation at various stages of development. Long-term solitude throughout adolescence

disrupts cognitive processes, including learning and attention (Amitai et al., 2014; Schrijver and Würbel, 2001). Also, increased stress impacts the quality of social interactions in early life and reduces spatial working memory in preadolescence (Perry et al., 2019). On the other hand, social support is associated with reduced stress and depression symptoms (Sherman et al., 2016). Therefore, children with high stress levels and inadequate social contact in their early years have greater difficulty controlling negative emotions (Burkholder et al., 2016).

Collectively, social interaction promotes acquisition of the skills necessary for social understanding and serves as a scaffold for building relationships with other individuals living in the social group.

1.2.2. Social skills: Competition and cooperation

Effective navigation through the social environment requires knowledge of one's own and others' abilities as well as social skills that facilitate the free flow of information (Zink et al., 2008). This knowledge establishes fundamental social interaction modes, such as competition or cooperation (Wang et al., 2011).

Animals learn how to compete throughout development, e.g., through play fighting. Play fighting allows mammals and birds to learn how to obtain an advantage over an opponent, such as biting a partner without being bitten. Play fighting can imitate competitive interactions, such as social grooming, or non-social rivalry, like predation (Pellis and Pellis, 2017). Moreover, competition among young individuals increases their motivation, physical effort, and attention (Burguillo, 2010; DiMenichi and Tricomi, 2015), and facilitates healthy maturation of social behavior (Pellis and Pellis, 2017; Trezza et al., 2011). Social hierarchies facilitate access to high-profitable food or reproductive possibilities for those with the most desired characteristics (Drews, 1993; Sapolsky, 2005; Wang et al., 2011). Once established, the group's hierarchy is relatively stable and can reduce the group's members' aggressiveness (Drews, 1993).

There is a correlation between the frequency of social engagement and social hierarchy. Compared to lower-ranking-male monkeys, higher-ranking males

devote more time to affiliative behaviors and spend less time feeding themselves (Guo et al., 2020). Additionally, understanding relationships between other individuals affects social interactions and requires specific brain regions activity. For instance, the combination of social network analysis and functional brain imaging in humans demonstrated that the position of an individual within a social network and knowledge about the placements of others within the social network influence brain activity (Parkinson et al., 2017)

Cooperation is essential to the survival of numerous species (de Waal, 2000; Kern, 2021; Wright et al., 2010). The influence of non-social elements, such as learning, on cooperation seems to be comparatively lower than that of social factors, such as rank, affiliation, tolerance, coordination, and prosociality. (Dale et al., 2020). As a result, social engagement within a social group is critical to a successful collaboration (Capraro and Cococcioni, 2015; Dale et al., 2020; Kern, 2021; Łopuch and Popik, 2011; Molesti and Majolo, 2016). However, efficient collaboration requires numerous skills, including prosocial behaviors, spatial and temporal coordination of actions, and adverse reactions to an inequitable outcome rather than an instinctual impulse. Social animals learn how to collaborate throughout development (Kendal et al., 2015; Shorland et al., 2022). Thus, individuals engaging in social interactions are more likely to learn how to cooperate and develop social relationships. Prosocial acts, such as assisting, comforting, sharing, and supporting the feelings of others, are crucial for group bonding, cohesion, and the development of trust. They strengthen social links, positively impact communities, and contribute to an individual's well-being (de Waal, 2008; de Waal and Preston, 2017).

1.2.3. Social interaction as a source of information

Social learning, defined as learning from others or during social interaction, has been observed in many species. It provides information about food and other desirable resources, predators, and appropriate social behavior, which is essential for an animal's survival (Fiorito and Scotto, 1992; Heyes, 2012). Numerous species can detect social cues and use them to adapt to their environment. For instance, the

alarm chemical chondroitin released from the injured fish's skin induces alarm behavior in conspecifics, i.e., increases swimming speed and promotes escape behavior (Mathuru et al., 2012; Pijanowska et al., 2020). The animals also learn by observation. For example, in an experiment that displayed two different colored items, octopuses were more likely to attack the object that a conspecific previously attacked (Fiorito and Scotto, 1992). Further, observation of demonstrators selecting meal types in bonobos increases the preference for the food chosen by the demonstrators in observing animals (Shorland et al., 2019). Similarly, hearing the calls of a demonstrator with specific food preferences that they previously displayed triggered an investigation of the region where the food preferred by the demonstrator was accessible (Shorland et al., 2022). Also, copying the prevailing behaviors that characterize behavioral traditions is observed across various species. Such copying strengthens social ties (Kendal et al., 2015; Whiten et al., 1999). In humans, observing the actions of others is sufficient to develop a preference based on the prior positive experiences of others (Denrell, 2008; Denrell and Le Mens, 2007; Wood, 2000).

To summarize, interaction with others promotes healthy growth, allows learning how to compete and collaborate efficiently, and provides essential information about the environment.

1.3. Interactions with others recruit the reward system

The neuronal basis of social interaction has attracted increasing attention in recent years. Motivation for a reward, including food, drugs, and social engagement, is mediated by dopaminergic mesocorticolimbic circuits (Berridge, 2018; Schultz et al., 1992; Tsai et al., 2009; Waelti et al., 2001). The structures responsible for processing social reward within these circuits include the ventral tegmental area (VTA), the nucleus accumbens (NAac), the hippocampus (Hipp), the orbitofrontal cortex (OFC), and the anterior cingulate cortex (ACC, (Apps et al., 2016; Bariselli et al., 2018, 2016; Dölen et al., 2013; Gangopadhyay et al., 2021; Gunaydin et al., 2014; Guo et al., 2020; Hung et al., 2017; Jennings et al., 2019; Mague et al., 2022; Nieh et al., 2016; Olsson et al., 2020).

Reward comprises several psychological components. Incentive salience, also called “wanting”, refers to motivation for rewards driven by both physiological state and previously learned associations about a reward cue and produced primarily by the mesolimbic dopamine system. In contrast, pleasure is caused by reward intake, often called “liking”, and does not depend on dopaminergic transmission (Berridge et al., 2009; Berridge and Robinson, 2016). The “wanting” is triggered by Pavlovian reward cues (conditioned stimuli) and unexpected contact with rewards (unconditioned stimuli). The cue producing “wanting” induces motivation to attain or approach; thus, the cue itself can be sought after even without a reward. The strength of “wanting” can be altered by relevant physiological states such as hunger, thirst, or stress and is controlled by the mesocorticolimbic circuitry (Warlow and Berridge, 2021). Social interaction is rewarding, which drives animals to initiate and maintain social contacts and allows for establishing and maintaining stable relationships (Kawamichi et al., 2016). Most likely, the ‘wanting’ circuit’s inability to activate motivated behaviors, especially in social contexts, accounts for the absence of social-seeking tendencies in individuals with autism spectrum disorder (ASD). It has been found that babies with ASD diagnoses who are genetically predisposed to the condition show lessened social motivation and interest. This deficit fundamentally modifies how individuals with ASD attend to and interact with the world, depriving them of vital opportunities to acquire social perceptual and social cognitive skills (Chevallier et al., 2012; Kohls et al., 2012; Zwaigenbaum et al., 2005).

Also, perception of positivity and pleasure, commonly referred to as "liking", significantly impacts social interactions. For example, people with higher levels of social anxiety are liked less by their interaction partners, which may contribute to even higher levels of social anxiety (Tissera et al., 2021) putting them in the vicious circle. Also, social anxiety often results in the negative interpretations of positive social events, resulting in low positive affect experienced during social interaction (Alden et al., 2008; Kawamichi et al., 2016).

Collectively, the initiation and maintenance of social interaction encompasses two dimensions, including the motivation for social interaction (referred to as “wanting”) and the experience of gratification derived from social interaction (referred to as “liking”).

1.4. The social brain: neuroanatomical components

The essential components of the "social brain" include the anterior cingulate cortex (ACC), the orbitofrontal cortex (OFC), the amygdala, the temporal cortex (mainly the superior temporal sulcus - STS), the medial prefrontal cortex (mPFC), the ventral tegmental area (VTA), and the hypothalamus. Recent theories about how the social brain works say that it is made up of a dynamic, hierarchical network of automated circuits that detect socially relevant stimuli and partially overlap with networks reflecting one's own or other mental states (Baron-Cohen et al., 2000; Porcelli et al., 2019). One of the concepts delineated five brain networks comprising the social brain. Three, partially distinct, networks are anchored in the amygdala and involved in perception of social stimuli, and processing information about affiliation and aversion. The other two social assemblies include the mirror network and the mentalizing network (Bickart et al., 2014). These networks are probably inter-dependent, and their relationships have yet to be fully understood.

1.4.1. Anterior cingulate cortex

The ACC is a subregion of the prefrontal cortex. In humans, it was originally divided by Brodmann into the precingulate [areas 24, 25, 32, and 33 comprising the anterior cingulate cortex (ACC)] and the postcingulate [areas 23, 29, 30, and 31 comprising the posterior cingulate cortex (PPC), (Stevens, 2011)]. Currently, the ACC can be categorized based on anatomical criteria such as receptor mapping and connectivity. This classification results in two subdivisions, namely the subgenual (sgACC) and pregenual (pgACC). In the dorsal ACC the division also includes the middle cingulate cortex (MCC, Caruana et al., 2018; Sakata et al., 2019; van Heukelum et al., 2020). The ACC plays an important role in control of positively valanced emotions, and action-outcome learning maximizing reward and minimizing punishment (Rolls, 2019). The ACC is closely linked to other structures that process social information, including the amygdala, the orbitofrontal cortex, the hippocampus, and the hypothalamus (van Heukelum et al., 2020), and is involved in fundamental cognitive processes such as cost-benefit analysis (Apps et

al., 2016). The ACC is believed to regulate autonomic responses, emotional processing, and motivational components of behavior, while the middle cingulate cortex (MCC) is implicated in attentional processes and decision-making (van Heukelum et al., 2020). The ACC is also involved in observational learning by transmitting information about the unpleasant value of the cue received from observation to the BLA. Inhibition of the ACC-BLA projection leads to a reduction of observational fear conditioning (Allsop et al., 2018).

In humans, the ACC is involved in empathy. Understanding the feelings of others and sharing affective states is important for successful social interactions. Empathy helps to recognize the feelings of other individuals, increase social bonds and maintain social relationships. Neuroimaging shows that the ACC is active when someone feels pain or sees someone else in pain (Lamm et al., 2011). This activation is reduced in people with a profound lack of empathy (Meffert et al., 2013). Also, animal research has confirmed the role of the ACC in processing social emotions. The ACC is activated when rats experience aversive shocks and witness other rats receiving shocks (Carrillo et al., 2019; Jeon et al., 2010). In addition, the elevated expression of c-Fos in oxytocin receptor-containing neurons in the ACC and the amygdala during helping behaviors indicates that the ACC-amygdala circuit is implicated in these empathy-motivated behaviors (Yamagishi et al., 2020). In line, blocking oxytocin receptors in the ACC disrupted helping behavior learning (Yamagishi et al., 2020). Furthermore, rats display harm aversion by reducing usage of the lever that guarantees a reward but simultaneously induces pain in their conspecifics, and the pharmacological deactivation of the ACC reduces the harm aversion (Hernandez-Lallement et al., 2020).

The ACC is ascribed the role of an "alarm system" or ‘conflict monitor’, as it is instrumental for recognizing when an instinctive reaction is improper or contradicts present aims. The ACC is activated in response to pain, is the first indicator that "something goes wrong" (Sawamoto et al., 2000). Increase in the ACC activity during social exclusion suggests that social pain involves similar structures as physical pain (Eisenberger et al., 2003), and underlies a critical role of social relationships for survival.

In sum, the ACC has a unique place in the brain since it is linked to both the "emotional" limbic system and the "cognitive" prefrontal cortex. Therefore, the ACC plays a significant role in integration of neural circuits regulating different emotional states, including the responses to emotional states of others.

1.4.2. Orbitofrontal cortex

The orbitofrontal cortex (OFC), a ventral subregion of the PFC, engages in sensory and emotions processing, and cost-benefit decision-making (Hong et al., 2019; Izquierdo, 2017; Rudebeck et al., 2008, 2006). In particular, the OFC has been implicated in regulating emotional states by modifying and adapting emotional responses on the moment-by-moment basis (Reekie et al., 2008).

The rats' OFC is anatomically divided into medial, ventral, lateral, dorsolateral, and agranular areas (Ray and Price, 1992). It has been shown that the subregions of the OFC (medial and ventral parts) specialize functionally and display distinct cortical and subcortical connectivity patterns. The mOFC (medial orbitofrontal cortex) projects more widely to the limbic structures than the vOFC (ventral orbitofrontal cortex). The target structures of the mOFC include the amygdala, the VTA, and the substantia nigra. On the other hand, the vOFC has stronger connections with the ACC, the sensorimotor and the temporal cortices. The connectivity pattern suggests that the mOFC could be more engaged in goal-directed behavior, whereas the vOFC in directed-attention decisions (Hoover and Vertes, 2011). Recently published data also suggest functional diversity depending the anterior-posterior axis of the OFC that stems from different connectivity (Barreiros et al., 2021). However, the OFC is usually not divided into parts in the studies investigating its function.

The OFC has been implicated in reward value prediction (Hong et al., 2019) and encoding information about satiety. Neuronal recordings from the monkey OFC showed that reward-specific satiety during ongoing consumption, is followed by reduced signals from the OFC (Pastor-Bernier et al., 2021). Notably, the OFC contains distinct populations that selectively respond to either food rewards or

social stimuli, and activation of the OFC social populations inhibits feeding behavior (Jennings et al., 2019).

Emotions provide an appraisal of external events. However, emotional reactions are not always beneficial, and, therefore, the ability to adapt and rapidly modulate emotional responses during unpredictable situations like interaction with others is required. The OFC has been identified as a region contributing to emotional regulation and processing hedonic properties of rewards (Kringelbach et al., 2008; Reekie et al., 2008).

The OFC controls advanced decision-making motivated by rewards (Rudebeck et al., 2008; Wallis, 2007). The lesions of the OFC have no effect on simpler processes, such as formation of stimulus-reward associations, but they impair the capacity to flexibly reassign value to previously unrewarded stimuli. Monkeys with the OFC lesions have difficulties with two types of response selection: based on the value of the reward (as measured by the reinforcer devaluation task) and based on when the reward would come (as measured by the object reversal task, Izquierdo, 2017). The OFC is also involved in regulation of motivation elicited by fear-related cues. Many studies of the OFC's function have relied on macaques' inherent fear of snakes and perception of unfamiliar persons as dangerous. The macaques' fear of snakes is tested by putting a valued food item near a toy snake. The macaques' delay of the food retrieval reflects their fear of the snake. The control macaques dread snakes and retrieve food only after relatively long time. However, macaques with the OFC damage are less afraid of snakes and get the piece of food almost instantly. Also, compared to unoperated control animals, the OFC-damaged macaques are more hostile to strangers (Izquierdo et al., 2005). Consistently, the OFC lesions in rats (Rudebeck et al., 2008) and macaques (Izquierdo et al., 2005) resulted in increased aggressive behavior, which may be the consequence of impaired reward processing or decreased fear (Rudebeck et al., 2008). In humans the role of the OFC in processing social stimuli has not been extensively studied. However, it has been regularly reported that the OFC lesions lead to decreased emotion identification and impairments of social judgement (Beer et al., 2006; Willis et al., 2010), which may be the effect of disconnecting the OFC and the BLA (Li et al., 2021). In summary, the OFC is

involved in processing stimuli-response actions, which can either enhance or diminish the tendency to approach rewards.

1.4.3. Amygdala

The amygdala comprises multiple interconnected nuclei nested deep in the temporal lobe. The amygdala nuclei are anatomically and functionally distinct. These include the central nucleus of the amygdala (CeA, made up of lateral and medial subdivisions) and the lateral and basal nuclei (together referred to as the basolateral amygdala, BLA; (Knapska et al., 2007). The BLA consists of about 80% of principal glutamatergic neurons and 20% of inhibitory interneurons, whereas the CeA neurons are primarily GABAergic (Ehrlich et al., 2009). The CeA receives an excitatory or feedforward inhibitory projection from the BLA, as well as cortical and subcortical structures, including the insular cortex, VTA, and paraventricular thalamus (Jolkkonen and Pitkänen, 1998; Kim et al., 2017; Pitkänen et al., 2006; Warlow and Berridge, 2021). The CeA neurons express serotonin, dopamine type-1, dopamine type-2, orexin, and oxytocin receptors. Also, the CeA contains a large population of neurons expressing corticotropin-releasing hormone, protein kinaseC δ, somatostatin, or neurotensin (Cai et al., 2014; Hu et al., 2020; Kim et al., 2017; McCullough et al., 2018).

The amygdala is a hub for processing both negative and positive emotions. The first studies on the amygdala's role focused on negative emotions, such as fear (LeDoux et al., 1988; Phelps and LeDoux, 2005; Rodrigues et al., 2004). They revealed distinct function of the CeA and BLA in emotional responses to threat (Killcross et al., 1997). They also showed essential role of the amygdala in modulation of attention, perception, and memory. The subsequent studies revealed the role of the amygdala in processing positive stimuli, including food and drug rewards (Bermudez and Schultz, 2014; Douglass et al., 2017; Everitt et al., 2003; Fadok et al., 2018; Knapska et al., 2013, 2007, 2006; Phelps and LeDoux, 2005; Robinson et al., 2014). In particular, they showed that the CeA neuronal circuits regulate food consumption (Douglass et al., 2017). Optogenetic or chemogenetic stimulation of the CeA circuitry increases motivation to pursue and consume food reward or addictive drug or even create maladaptive 'wanting what hurt' behavior

by activating the mesocorticolimbic system (Hardaway et al., 2019; Robinson et al., 2014; Warlow et al., 2020, 2017). Further, pairing a reward with optogenetic activation of the CeA cells increases preference to choose this reward, compared to an alternative reward that is available (Robinson et al., 2014), pointing out to the role of the CeA in incentive motivation. Moreover, optogenetic activation of the CeA paired with touching a rod providing shocks in rats leads to repeated approaches and touching the rod (Warlow et al., 2020), showing that even harmful stimuli can acquire rewarding properties through association with the CeA stimulation. Stimulation of the neuronal circuits in the CeA mostly increases incentive motivation ('wanting') but not hedonic response 'liking' (Warlow and Berridge, 2021). Targeted activation of genetically-defined CeA neurons types has shown that photoactivation of the CeA neurons expressing protein kinase Cδ (PKCδ +) suppresses food intake. In contrast, activation of the CeA PKCδ (-) neurons promotes food intake in the presence of lithium chloride (an anorexigenic agent). This suggests that populations within the CeA have specific roles in food-directed motivation (Cai et al., 2014). Also, optogenetic stimulation of the projections from the CeA to the parabrachial nucleus (brain region involved in controlling feeding behaviors) increases ethanol consumption and intake of sucrose solutions (Torruella-Suárez et al., 2020).

The amygdala has also long been thought to be a component of the "social brain" (Baron-Cohen et al., 2000). When humans decode social cues such as gaze and recognize emotions of others (particularly fearful expressions) the amygdala is activated (Kawamichi et al., 2016; Morris et al., 1996). Baron-Cohen suggested that abnormalities in the amygdala structure and function, such as reduced volume and decreased activity during a mentalizing task, may contribute to the autistic condition (Baron-Cohen et al., 2000).

Most of the studies on the role of the amygdala in social interaction focused on the BLA. The electrophysiology recordings showed changes in the BLA neuronal activity during social interaction (Katayama et al., 2009). The activation of mouse BLA inputs to the ventral hippocampus through optogenetics has been found to have a negative impact on social behaviors, while inhibition of these projection improved social contacts (Felix-Ortiz and Tye, 2014). Much less is known about the involvement of the CeA in social interaction. It has been shown

that oxytocin signaling in the CeA modulates emotion discrimination in mice during social contact (Ferretti et al., 2019). Further, the data show that the CeA contains distinct populations orchestrating responses to emotional states of conspecifics signaling imminent and remote threats (Andraka et al., 2021).

Taken together, the amygdala is critical for emotional appraisal of both positive and negative environmental stimuli, and this process involves both the basolateral and central parts of the amygdala. The amygdala is also involved in decision making and choosing an appropriate response to the external conditions. Prior studies have indicated that the CeA plays a role in social engagement. However, the extent to which the CeA drives social interactions has yet to be thoroughly investigated. Specifically, whether social and non-social reward populations are identical has yet to be resolved.

1.4.4. Ventral tegmental area

The Ventral Tegmental Area (VTA) is a brain structure pivotal for regulating motivation. The VTA comprises dopaminergic (DA), glutamatergic, and GABAergic neurons that collectively mediate reward signaling and motivate individuals to pursue rewards (Volkow et al., 2017, 2011). The VTA has been implicated in responses to both, positive and negative reinforcements, through distinct neuronal populations (Volkow et al., 2017; Zweifel et al., 2011). The DA neurons in the VTA, together with and the DA neurons in the substantia nigra pars compacta (SNc) are involved in detection of salient stimuli. When such stimuli are detected their phasic activity increases facilitating attention reorientation and alerting to potentially significant sensory cues (Berridge, 2007). The VTA consist of approximately 60% dopaminergic neurons, 35% GABAergic neurons, and 5% glutamate-releasing neurons (Cai and Tong, 2022; Nair-Roberts et al., 2008; Yamaguchi et al., 2015). Dopamine-releasing neurons produce dopamine by converting L-tyrosine via the enzyme tyrosine hydroxylase (TH) to L-DOPA (3,4-dihydroxyphenylalanine). Then, L-DOPA is converted into dopamine by the enzyme aromatic-l-amino-acid decarboxylase and accumulated into vesicles by vesicular monoamine transporter 2 (VMAT2). Glutamate-releasing neurons contain the enzyme glutaminase, which converts glutamine to glutamate and vesicular

glutamate transporter 2 (VGluT2). GABA is synthesized from glutamate by glutamate decarboxylase 1 (GAD1, known as GAD67) and packed into vesicles by the vesicular GABA transporter (Morales and Margolis, 2017). Additionally, some VTA neurons exhibit combinatorial neurotransmitter characteristics, e.g., part of the TH-neurons also co-express vesicular glutamate transporter 2 -VgluT2 (Yamaguchi et al., 2015). DA neurons are activated by reward and reward-predictive signals (Cohen et al., 2012; Haber and Knutson, 2010; Waelti et al., 2001; Zweifel et al., 2011). Light stimulation of the VTA DA neurons expressing ChR2 causes macaques to self-stimulate those neurons, confirming that the stimulation of the VTA DA neurons is indeed reinforcing (Stauffer et al., 2016). In line, activation of the VTA DA neurons through optogenetic manipulation enhances social engagement (Gunaydin et al., 2014). The activity of the VTA DA neurons is controlled by inputs from various brain structures and local GABA-releasing or glutamate-releasing neurons (Morales and Margolis, 2017). For example, inhibitory projection from the lateral hypothalamus to the VTA increases DA release in the nucleus accumbens (Nac) via inhibition of the local VTA GABAergic neurons, leading to increased social contact and seeking a sugar reward (Nieh et al., 2016, 2015). Lack of sociability has been connected to problems with excitatory transmission development in the VTA DA neurons. In SHANK3-deficient mice, a mouse model of autism, optogenetic stimulation of the DA VTA neurons consistently increases social interaction (Bariselli et al., 2016). Deficits in social novelty exploration, reported in ASD, e.g., diminished attention to social stimuli (Pierce 2011), may be relieved via the VTA DA neurons stimulation (Bariselli et al., 2018).

Taken together, the VTA is instrumental for responses to food, drug and social rewards. In particular, the VTA plays a crucial role in regulating social behavior, including social affiliation and recognition, and creating and maintaining social bonds. Notably, social deficits in various psychiatric disorders, such as ASD, have been linked to the VTA dysfunctions.

1.4.5. Social vs. non-social reward

The rewarding properties of social engagement are essential to forming social bonds. Here, I compare the brain processing of social rewards with non-social rewards, focusing on the various elements that distinguish them. Multiple dimensions can be used to define rewards, and each can be associated with distinct brain correlates and psychological processes. To assist in comparison and improve classification, I distinguished these two types of rewards according to dimensions such as primacy, temporal closeness, familiarity and size (Matyjek et al., 2020).

Primacy is a dimension that classifies rewards into innate or biologically pre-programmed reinforcers (like hunger being satisfied by food or an infant's need for touch being satisfied by being close to its mother) and learned or acquired rewards (by associations with primary reinforcers; like money as a way to get food or a Facebook thumbs-up as a way to have social attention, (Delgado et al., 2006)). Thus, primary and secondary incentives exist in the social (touch, thumbs-up) and non-social (food, money) domains. The ventromedial prefrontal cortex (vmPFC) is involved in processing both primary (biologically reinforced) and secondary (learned/acquired) rewards. In contrast, it has been shown that the hypothalamus is activated specifically to primary rewards, and posterior cingulate cortex (PCC) to secondary rewards (Levy and Glimcher, 2011). Thus, when comparing social and non-social incentives, choosing rewards of the same primacy is crucial.

Temporal closeness links motivated behavior to reward. The midbrain, frontal cortex, and amygdala are sensitive to reward timing, which suggests that rewards can be processed differently depending on their proximity. Both timing and expectation are crucial for the brain to guide behaviors and adapt to the environment effectively. It was shown that rewards delivered earlier than expected resulted in a greater activation in VTA than when given at the learned time (Hollerman and Schultz, 1998). Temporal discounting may also motivate people to choose immediate smaller rewards over delayed greater ones (Bermudez and Schultz, 2014). Typically, social rewards are supplied promptly after a trial through a smile or social feedback. However, with non-social rewards, there is often a delay between trial reward (e.g., a picture of money) and real reward after effort (i.e.,

receiving the physical money). Thus, comparing social and nonsocial incentives, their temporal closeness has to be taken into account (Matyjek et al., 2020).

Familiarity distinguishes between novel and familiar stimuli. It is signaled by the striatum and midbrain. Human neuroimaging research shows novelty benefits non-social stimuli, while familiar faces are more rewarding than strangers' faces (Guitart-Masip et al., 2010; Pankert et al., 2014). Overall, familiarity may influence social and non-social incentives differentially, which should be considered when comparing rewards along the social-non-social dimension.

Individuals evaluate reward size, and both objective and subjective values are essential for the evaluation. The level of activation of the ventral striatum correlates with the objective magnitude of rewards, while activation of the vmPFC correlates with the subjective magnitude of both social and non-social rewards (Haber and Knutson, 2010; Lin et al., 2012; Spreckelmeyer et al., 2009). Incentives valued higher are likely to elicit stronger responses than less valuable rewards. Therefore, when comparing social and non-social rewards, the difference in the magnitude of their value should be avoided (Matyjek et al., 2020).

Since perceiving social contact as rewarding is essential to initiate and maintain it, in my studies I focused on the reward system. In particular, I asked a question whether social and non-social motivation utilizes the same neural networks. It has been shown that positive non-social motivation, i.e., motivation to drink, eat, or take drugs, is mediated by the frontal-striatal-amygdala circuit. The well-characterized part of this circuit is the dopaminergic projections from the VTA to the medial prefrontal cortex, BLA, hippocampus, and nucleus accumbens, the set of structures together forming the mesocorticolimbic system, which is critically involved in generating motivated behaviors (Castro et al., 2015; Kelley et al., 2005; Wise, 2006). Much less is known about the neuronal circuits involved in social motivation. Importantly, however, the recent analysis of a functional connectome of the prelimbic cortex, amygdala, and VTA showed that this set of structures is capable of encoding a social-appetitive brain state in mice (Mague et al., 2022).

The currently accepted hypothesis proposes a general motivational system that is independent of incentive type (i.e., social vs monetary). The human neuroimaging data suggest that the same set of brain structures, including the

ventral striatum, VTA, ACC, OFC and amygdala, is engaged in anticipation and receipt of social and non-social rewards (Gu et al., 2019; Martins et al., 2021). However, the results of studies on processing appetitive stimuli in autism patients suggest specific deficits in social but not non-social reward processing (Chevallier et al., 2012; Cox et al., 2015; Delmonte et al., 2012). If so, the social and non-social rewards may be processed by, at least partially, different brain circuits.

1.5. Dysfunctions of social interaction

In the previous sections, I briefly summarized how social interaction helps individuals to develop their socio-cognitive abilities and function in their environments. Also, I described the involvement of brain regions, including the ACC, OFC, CeA, and VTA, in reward processing, positive motivation, and decision making. Now I would like to focus on social impairments, in which the abilities facilitating social interaction, like social skills and social cognition are impaired (Morrison et al., 2020).

Social motivation and pleasure, social cognition, and social skills are instrumental in initiating and maintaining social interaction. Social motivation is the need to interact with others (Berridge et al., 2009; Chevallier et al., 2012). Social cognition refers to the perception and evaluation of social information and is necessary to understand the emotional states and intents of an individual and others (Baron-Cohen, 1991). Social skills are a broad category of actions employed in various social circumstances to meet social expectations and achieve social goals (Nangle et al., 2010).

Social impairments are caused by social cognition, motivation and/or skills dysfunctions. Social impairments are hallmarks of many mental disorders, including autism spectrum disorder (ASD), schizophrenia, and unipolar major depressive disorder-MDD, (Dichter et al., 2012).

Social deficits are the most prominent symptom of ASD, a complex, heterogeneous neurodevelopmental and neuropsychiatric condition with early onset (Estes et al., 2015; Plavén-Sigray et al., 2017; Wang et al., 2023). Individuals with ASD have difficulties recognizing social signals and diminished social motivation.

The main theories accounting for cognitive and social impairments in individuals with ASD include the theory of mind (TOM), the weak central coherence theory, and social motivation deficits (SMD). According to the TOM theory of ASD, deficits in the ability to attribute mental states (beliefs, desires, intentions) to oneself and others, are the primary cause of difficulties in social interaction in ASD. The difficulties in social understanding lead to impaired socio-cognitive and social skills development, which results in diminished social motivation and decreased number of social interactions (Morrison et al., 2020). The weak central coherence theory posits that individuals with ASD cannot effectively integrate information from diverse sources, which results in a limited ability to process and comprehend intricate information, including social cues and emotions (Happé and Frith, 2006). On the other hand, the SMD theory postulates that the difficulty with social interaction, including establishing and maintaining relationships, reciprocating social contact, and communicating with others results from decreased motivation to interact with other individuals, minimizing the opportunity to engage in social interactions and develop cognitive and practical social skills (Chevallier et al., 2012). The theories discussed above suggest impairments of distinct processes required for successful social interaction (social cognition, social skills or social motivation). Notably, it is not clear, whether the same or different neuronal mechanisms underly these processes. Such knowledge could help us to decide which theory better identifies the origin of deficits observed in ASD.

ASD is characterized by decreased motivation to engage in social interaction and difficulty in responding to social cues. Typically developing individuals tend to find social interactions rewarding. Children diagnosed with ASD exhibit decreased activation of the VTA during the anticipation of social and non-social rewards comparing to typically developing children (Stavropoulos and Carver, 2014). Notably, individuals with ASD, exhibit heightened activation of the reward system when presented with personalized non-social rewards, such as, e.g., video games, as compared to social rewards like social approval. Furthermore, abnormal responses of the reward system, particularly of the caudate nucleus, are associated with social dysfunctions (Kohls et al., 2018). Due to impairments in social cognition and communication, ASD patients develop poorer social skills. Meta-analysis showed differences in brain activation between ASD and control

individuals in the amygdala, insula, middle temporal gyrus, and cingulate cortex (Patriquin et al., 2016). These structures are implicated in executive functions, specifically social decision-making and social cognition, encompassing the processing of social cues and the capacity to adopt alternative perspectives.

Schizophrenia is a debilitating and persistent psychiatric condition that impacts roughly 1% of the worldwide population. Social deficits are a prominent symptom of schizophrenia. They can profoundly affect an individual's capacity to establish and maintain social relationships, navigate social environments, and pursue a satisfying existence. Diminished sensitivity for social rewards leads to a low initiative in approaching others and paying less attention to socially rewarding clues, which diminishes the number of positive social interactions and memory formation. Consequently, the expectation of failure and increased concentration on signs of rejection appear (Fulford et al., 2018). Schizophrenic patients display social anhedonia, i.e., a deficit of pleasure in interpersonal relationships. The perception of the pleasantness of social interactions seems distinct from the motivation to initiate social contacts. For instance, people with schizophrenia express less enjoyment about participating in social interactions than healthy controls, even if they clearly express the need for social contact (Engel et al., 2016; Trémeau et al., 2013). In schizophrenia, some degree of social withdrawal begins in adolescence or even childhood, well before the disease's full manifestation, and is a major component of the disease (Howes and Murray, 2014). It has been proposed that this early social retreat may precipitate the onset of psychosis (the so-called social deafferentation hypothesis, Hoffman, 2007). It has been demonstrated that individuals diagnosed with schizophrenia exhibit impairments in diverse aspects of social cognition, such as the ability to recognize emotions, understanding others' perspective, and perceiving social cues. Individuals with this condition have difficulties in understanding social cues and nonverbal communication, which could result in the misinterpretation of social circumstances (Green et al., 2015; Porcelli et al., 2019). Social cognition deficits in schizophrenia, such as emotion recognition, are associated with reduced activation of the areas engaged by emotional stimuli, including the amygdala, ACC, PFC, dorsolateral frontal cortex, and medial frontal cortex, as well as visual processing areas (Bickart et al., 2014; Taylor et al., 2012). At the neurotransmitter level, schizophrenia affects the

dopaminergic (DA) system, resulting in an increase in presynaptic DA production and release (Howes and Murray, 2014). The abnormal DA function may interfere with the specific mechanisms underlying social reward/pain, hence interfering with forming bonds with a caregiver which are necessary for social growth (Porcelli et al., 2019).

One of the key symptoms of major depressive disorder (MDD) is anhedonia, i.e., diminished affective and motivational responses to appetitive stimuli, including social stimuli (Davidson, 1998; Russo and Nestler, 2013). Social dysfunction includes decreased motivation to establish and maintain social connections, decreased cooperativeness, competition avoidance, and changes in the socio-cognitive domain, including social perception and decision-making (Kupferberg et al., 2016). The reduced emotion perception in MDD patients and a negative emotional bias lead to communication problems (Dalili et al., 2015; Kupferberg et al., 2016). MDD patients are also more sensitive to peer rejection, which causes more powerful and lasting unpleasant emotions (i.e., social pain) than in healthy subjects (Ehnvall et al., 2014; Hsu et al., 2015). The rejection sensitivity typically leads to maladaptive behaviors, such as social withdrawal and aggression (Kupferberg et al., 2016). Changes the in ACC activity have been linked to negative affective interpretations of social rejection in MDD (Kupferberg et al., 2016). Further, the enhanced connection between the ACC and the amygdala was suggested to underlie the increased perception of a social threat observed in MDD patients (Stuhrmann et al., 2011). The increased ACC activity has also been linked to aberrant social cognition and negative emotional bias (Stuhrmann et al., 2011).

In sum, different social processes, such as social cognition, motivation, pleasure, and social skills, are prone to dysfunctions observed in neuropsychiatric illnesses, including ASD, schizophrenia, and MDD. Difficulties in initiating and maintaining social interaction result in social exclusion and problems in interpersonal relationships.

1.6. Methods used in the studies

In the following sections, I will briefly overview three approaches I employed in my research on the neuronal circuits underlying the initiation and maintenance of social interaction in rats, namely c-Fos expression mapping, chemogenetics, and optogenetics. I focus on each technique's underlying mechanisms, common uses, and main limitations.

1.6.1. c-Fos as a marker of neuronal activation

The *c-fos* gene belongs to a group of early response genes (i.e., Immediate Early Genes, IEGs) that are activated upon some form of cell stimulation and whose expression cannot be prevented by protein synthesis inhibitors. Activation of the CREB/CRE complex, cAMP and Ca^{2+} drives *c-fos* expression in neurons. The *c-fos* gene encodes the c-Fos protein, which dimerizes with transcription factors of the Jun family to form the transcription factor AP-1 (Activator Protein-1). IEGs encode transcription factors that influence neuronal physiology by regulating the expression of downstream target genes, often referred to as late-response genes (Gallo et al., 2018). The AP-1 is a transcription factor which, through regulation of late-response genes expression, regulates many biological processes, including increasing the number and strength of synaptic connections (Chiu et al., 1988; Sanyal et al., 2002; Sheng and Greenberg, 1990). Since the finding that neuronal activity regulates c-Fos expression in the brain, c-Fos has been extensively used as a marker in the studies mapping brain activity during behavioral tasks. The baseline c-Fos expression in the brain is low. It increases when an animals is subjected to novel stimuli (Kaczmarek et al., 1988; Nikolaev et al., 1992). After the stimulation, *c-fos* mRNA level is already increased after 15 minutes, and it reaches its maximum level between 30 and 60 minutes after the stimulation. The maximal protein level is achieved 1.5 to 2 hours after the stimulation, and it returns to its baseline after around 6 hours (Kaczmarek, 2002; Kaczmarek et al., 1988; Nikolaev et al., 1991).

c-Fos expression is linked to learning and memory, as its expression is significantly increased during behavioral tests in which animals learn new responses. Memory consolidation is a process that is thought to take place at the

same synapses that were involved in the encoding of the information. It involves the activation of transcription factors, the production of proteins, and the post-translational modification of those proteins, all of which lead to plastic changes that stabilize the memory trace (Alberini, 2009; Dudai, 2002). Learning and memory tests are frequently used as readouts of plastic alterations facilitating long-term memory development and maintenance of a memory trace in specific neuronal populations (Gallo et al., 2018). The largest changes in the c-Fos expression were found during the first sessions of multiple-session training procedures, which suggests its role in an adaptive response of neurons (Kaczmarek, 2002; Nikolaev et al., 1992). Forming a memory trace requires synapses remodeling and formation of new synaptic connections (Lamprecht and LeDoux, 2004). Expression of effector genes involved in synaptic remodeling associated with memory persistence depends on late *c-fos*-dependent transcription (Katche et al., 2010). For instance, fear memory formation requires c-Fos expression, and inhibition of c-Fos expression by infusion of *c-fos* antisense oligonucleotide into the dorsal CA1 region of the hippocampus or retrosplenial cortex impairs consolidation and durability of fear memory (Katche et al., 2010; Katche and Medina, 2017).

Memory reactivation during retrieval might result in two distinct processes: extinction or reconsolidation. In the process known as reconsolidation, the reactivated memory trace becomes labile and sensitive to modification or disruption. If the original trace is reinforced during retrieval, the protein synthesis-dependent reconsolidation mechanism re-stabilizes the original memory trace (Lee, 2008). Notably, c-Fos level in some brain areas is elevated during the memory recall, even if the animal was trained 30 days earlier, suggesting c-Fos involvement in reconsolidation or extinction (Frankland et al., 2004).

c-Fos is a very useful neuronal activation marker, as it can be reliably detected with immunohistochemistry, has low baseline levels, and is situated in cell nuclei, which gives single-cell resolution (Krukoff, 1998). There are, however, also some limitations of using c-Fos for mapping neuronal activation. First, since c-Fos is easily induced by new stimuli, it is crucial that laboratory animals are well-acclimatized to non-specific elements of experimental situation like human handling or experimental rooms, otherwise the high expression evoked by these non-specific elements may obscure the results. Second, not all activated neurons

express c-Fos. Thus, positive findings are less ambiguous than negative ones, the presence of c-Fos is a valid indication of neuronal activation but the absence of c-Fos cannot be used to reliably infer that the cell was not activated (Cruz et al., 2015; Peter et al., 2013).

1.6.2. Chemogenetics

The expression of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in neurons is the most frequently used chemogenetic approach. With this method the target neurons can be activated through systemic delivery of exogenous chemicals that are otherwise physiologically inert, e.g., clozapine-*N*-oxide (CNO), a metabolite of a neuropsychiatric drug clozapine (Armbruster et al., 2007; Sternson and Roth, 2014). Because of the potential side effects caused by back-metabolism of CNO to clozapine, in my studies I used compound 21 (c21), which activates DREADDs but is not metabolized to clozapine or any related compound and thus represents a safe alternative to CNO. The c21 is a suitable DREADD agonist, effective at latest 15 min after intraperitoneal application and showing a superior penetration and long-lasting presence in the brain (60 minutes, Chen et al., 2015; Jendryka et al., 2019).

One of the most popular DREADDs are synthetic variants of muscarinic acetylcholine receptors coupled to Gi (e.g., mutant muscarinic receptors 4; hM4Di which allows for reduction of neuronal activity) or Gq (e.g., mutant muscarinic receptors 3; hM3Dq which allows to stimulate neuronal activity, Armbruster et al., 2007; Roth, 2016). Different DREADDs enable us to limit the changes they cause to certain features of neuronal activity or plasticity. For instance, it has been shown that hM4Di inhibits the release of synaptic vesicles from axon terminals, while only mildly depolarizing soma (Armbruster et al., 2007; Stachniak et al., 2014).

The DREADDs include several chemogenetically engineered protein classes, including kinases, non-kinases, G protein-coupled receptors (GPCRs), and ligand-gated ion channels (Roth, 2016), which address different experimental needs. Developing FLEX switch vectors, i.e,, double-floxed inverse open reading frame constructs (DIO), allows for expressing DREADDSs in genetically specified

cells (Lee and Saito, 1998). Using the canine adenovirus (CAV) expressing Cre-recombinase (CAV-Cre) and AAV-DIO-DREADD makes the projection-specific expression of DREADDs possible. CAV-Cre is a reliable retrogradely transported genetic vector with minimal immunogenic and cytotoxic reactivity. The retrograde transduction of CAV-CRE in diverse projection neurons initiates the expression of DIO-DREADD previously injected to the structure sending the projection. Combined using CAV-Cre and FLEX-DREADD constructs has been called 'retro-DREADD approach' (Kakava-Georgiadou et al., 2019; Li et al., 2018).

DREADDs are very potent tools, enabling effective manipulation of neuronal activity. However, they also have some limitations. One of the most significant drawbacks of DREADDs is their low temporal resolution. In contrast to optogenetics, discussed below, it often takes several minutes to half an hour for DREADDs to become active and the effects they exert are relatively long-lasting, spanning from 2 to 4 hours. Consequently, the temporal control over DREADDs activation is very limited. Notably, however, DREADDs, unlike optogenetics, do not require local activation. The light fibers are not necessary, making the surgeries less invasive and allowing animals to move freely without tethering, which can be crucial during, e.g., in social interaction experiments.

1.6.3. Optogenetics

Optogenetics is an experimental method that allows for regulating neural activity using light (Fenno et al., 2011). Photoreceptors, which are light-sensitive proteins, modify cellular activity when light stimuli are applied. The method utilizes microbial rhodopsins, also known as type I rhodopsins, which are proteins found in archaea, bacteria, algae, and fungi. In these organisms, type I rhodopsins either generate membrane ion gradients for energy production or have photosensory functions such as mediating phototactic and photophobic responses. These heptahelical proteins are distinguished by light-induced conformational changes within the opsin, which result in protein activation (Nagel et al., 2002; Watanabe et al., 2021).

Channelrhodopsins (ChRs), light-gated ion channels that passively transport ions along electrochemical membrane gradients, are the optogenetic analogues of microbial ion pumps. Channelorhodopsin-2 (ChR2) was identified in the green alga Chlamydomonas reinhardtii, where it facilitates phototaxis (Boyden et al., 2005; Foster et al., 1984; Nagel et al., 2002). ChR2 is an ion channel that is activated by blue light, facilitating the passage of cations such as H^+, Na^+, Ca^{2+}, and Mg^{2+} (Schneider et al., 2015). This feature may be exploited to regulate neuronal activity; when ChR2 is expressed in neurons and activated with light, membrane depolarization and action potentials are induced (Rost et al., 2017; Yizhar et al., 2011). On the other hand, suppression of neuronal activity is accomplished, for instance, by halorhodopsin (NpHR), an orange-light-driven proton pump (Yizhar et al., 2011). Activation of NpHR introduces additional chloride ions into the cell, hyperpolarizing it and preventing spiking (Rost et al., 2017; Yizhar et al., 2011).

Over the past decade, optogenetics thrived in neuroscience for several reasons. First, optogenetics permits an experimental intervention to be confined to specified time windows, as well as it enables repeated studies with stimulation intensity set over lengthy timeframes. The light may be turned on and off almost instantly, which, under some circumstances, enables regulating neural activity with millisecond-resolution (Boyden et al., 2005; Watanabe et al., 2021). Second, genetically specified expression of optogenetic opsins allows to manipulate and monitor neural circuits of scattered well-defined cell populations in real time (Rost et al., 2017). Finally, the methodology may be utilized both in vitro and in vivo in conjunction with other techniques (e.g., electrophysiology, imaging, pharmacology), which significantly expands the spectrum of potential applications (Gradinaru et al., 2007).

Despite many advantages, optogenetics has also some limitations. The primary drawbacks stem from its propensity to cause unintended side effects. Some opsins, for instance, need a high intensity of light, which might lead to tissue heating and interfere with neuronal function in a way that is not specific (Owen et al., 2019). Moreover, the physiological movement of ions is very unpredictable, and its disruption might have unexpected consequences (Allen et al., 2015). Many opsins operate for far longer times when activated by light than the ion channels do in response to electrical stimulation because of the channels' inherent kinetics.

Optogenetic driving may thus cause slow depolarizations, which might activate endogenous ion channels, likely leading to calcium entry via voltage-gated calcium channels (Allen et al., 2015; Zhang and Oertner, 2007). Additionally, prolonged channelrhodopsin-mediated depolarization may lead to a depolarization block, a condition in which light pulses of sufficient length prevent further action potential firing by inactivating sodium channels (Herman et al., 2014). Finally, optogenetics usually requires optic fibers mounted in the head, which, if not wireless, may affect animals' behavior.

2. Research aims

Most of what we know about the neural mechanisms underlying social interactions comes from non-invasive human brain imaging studies. We know little about the causal role of the specific neuronal circuits of the reward system in control of social interaction. In particular, we do not know **how social interaction initiation is controlled** at the neuronal level, and **which neuronal circuits are necessary to coordinate the complex behavior required for a successful social contact**. It is also unclear **whether the neuronal circuits of the reward system that are involved in food motivation are utilized to control social interaction**. Thus, through the experiments presented in this thesis I aimed at verifying the following hypotheses:

1. **Initiation and maintenance of social interaction engage different neuronal circuits.**

2. **The neuronal populations involved in positive social interaction and incentive drive to pursue food rewards are, at least partially, different. Thus, manipulation of some social interaction-related circuits does not alter motivation for food reward.**

In my studies I used rats, social animals, which readily initiate and maintain social interactions. Their advanced social abilities allow to study the underlying neuronal circuits. Rats initiate social contact by approaching a partner, and are able to maintain social contact by a coordinated response to partner's socially oriented behaviors. In my studies, I focused on the brain structures forming the reward system, including the CeA, VTA, OFC and ACC, and their connectivity.

3. Methods

3.1. Animals

For my experiments, I used male, naïve Wistar rats, supplied by the Center of Experimental Medicine in Bialystok, Poland (for experiments with optogenetic and chemoinhibition manipulations, total n=100); c-fos-PSD95Venus-Arc rats bred at the Nencki Institute Animal House and the Animal House at the Faculty of Biology, University of Warsaw, Poland (for experiments with axonal tracing, n=4), and Sprague Dawley tyrosine hydroxylase (TH) IRES-Cre +/- transgenic rats supplied by the Institute of Zoology and Biomedical Research, Jagiellonian University, Krakow, Poland (for experiments with chemoactivation of dopaminergic pathways, n=38). I used only the male animals with a weight of 130–150 g at the beginning of the experiments (Table 1).

In each experiment, animals were randomly paired and housed together in standard home cages (43.0 × 25.0 × 18.5 cm) under a 12-hour light/dark cycle (lights on at 7 am) at 22°C and controlled humidity (around 55%). During social separation, all animals were singly housed (in standard cages, 26.5 cm x 42.5 cm x 18.5 cm) for 24h (baseline tests) or 21 days (final test). I conducted all experiments during the light phase of the cycle. Mild food restriction, achieved by providing 15 g of food per day (standard laboratory chow), resulted in rat weight becoming approximately 85% of their free-feeding weight. The food restriction began seven days before the training for operant conditioning for food and was continued until the end of the experiments. Water was freely available. All experiments I carried out following the Polish Act on Animal Welfare after obtaining specific permission from the First Warsaw Ethical Committee on Animal Research (permission LKE 954/2019).

3.2.Behavioral testing

3.2.1. Habituation

I habituated the rats to the experimenter's hand for 14 days preceding the experiment. This was followed by habituation to the transport, experimental rooms,

and equipment (Skinner box and box for social interaction) three days before the first training in pressing a lever.

3.2.2. Operant conditioning for food reward

All experiments assessing food motivation had the same general structure, involving food reward training. The behavioral training and testing were conducted using Skinner boxes (Med Associates, St Albans, Vermont, USA-30cm x 25 cm x 25 cm). Each box contained a light, a speaker, a food dispenser, and a lever on the right side of the feeder (Fig. 3). Three days of acclimatization to the Skinner box preceded general training. I placed rats in the apparatus for 30 minutes, during which the food dispenser delivered one sucrose pellet every two minutes (each weighing 45 mg, Bio-Serv). The pellet delivery was accompanied by a sound (5s, 2000 Hz at 75 dB). In addition, the lever was present, and its activation yielded an additional reward.

The training consisted of two distinct phases. During the initial 60 seconds, the lever was concealed. The lever then appeared and remained visible cyclically for 20 seconds, with 30-second intervals between trials. Each delivery of a reward (one pellet per lever press) was accompanied by a sound (5s, 2000 Hz at 75 dB). Each session lasted thirty minutes (35 presentations of the lever). Rats were trained to press the lever to receive sucrose pellets as the initial training step. Each lever press resulted in the presentation of a single pellet. Before moving on to the second phase of training, the animals completed the first phase until their performance was stable: at least 70 presses of the lever were maintained over three consecutive training sessions. In the second phase of training, rats were required to press the lever five times in order to receive the same food reward (1 pellet). Rats needed at least 250 lever presses over three consecutive training sessions to pass the second portion of the training. The rats that passed the second phase of training continued their daily training sessions as described previously. The animals were trained in 10 to 12 daily training sessions.

3.2.3. Social interaction test

I conducted the social interaction test after 24 hours (baseline) or 21 days after social separation (Hamed and Kursa, 2020). The examination was performed in the social interaction chamber (1m x 0.5 m, Fig. 1). Before the test, I marked the animals with red or green dye on their backs and introduced them to the chamber at roughly the same time. The behavior of rats was recorded for 10 minutes using a video camera (ImagingSource, Germany) positioned above the apparatus (70 cm).

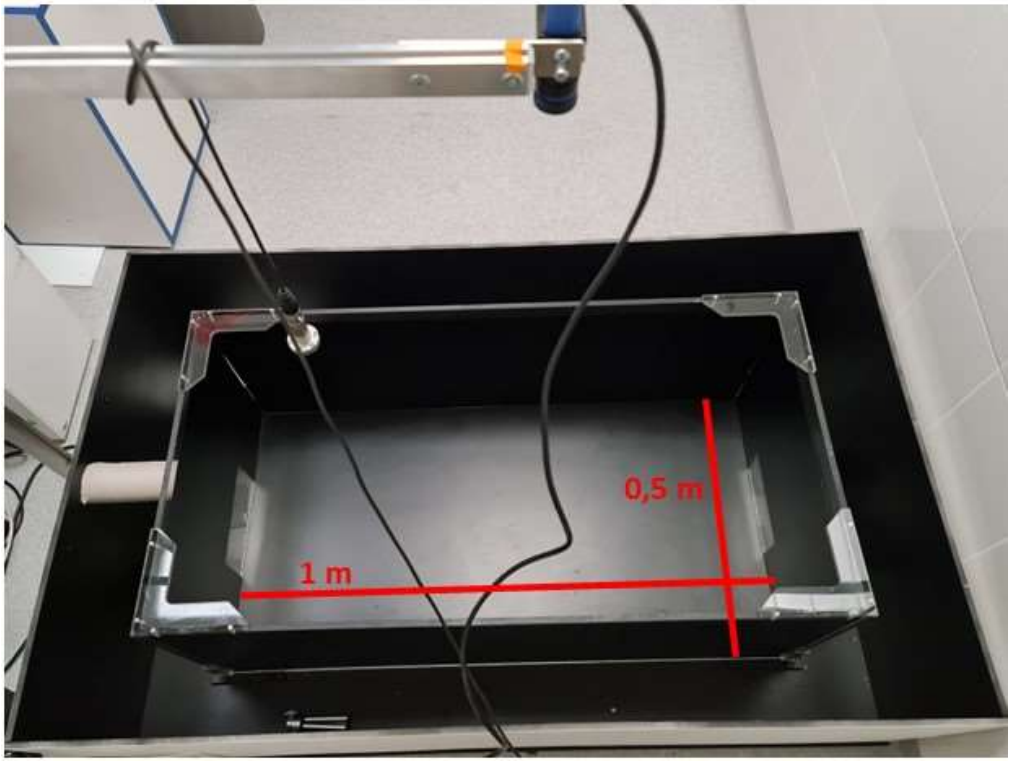

Fig. 1. Social interaction chamber.

3.2.4. Measuring social interaction during chemogenetic manipulation

For the analysis of social interaction, I divided behaviors into their component stages. The first category was initiation, which demonstrates the capacity to initiate social contact. The second category was maintenance of social interaction, which demonstrates the ability to react appropriately when a partner

initiates social contact. The third category was social interaction avoidance, which demonstrates anxiety and avoidance of social interaction.

The initiation was scored whenever an animal approached a partner and displayed affiliative behaviors, such as nape contact, nose-to-body contact, genital investigation, following, pouncing, allogrooming, wrestling, or crawling/climbing under the partner's body. The new approach to partner was scored if rats walked away from the partner (a distance greater than one body length) and then returned (with their heads facing the partner's body) to the partner (Fig. 2).

The maintenance of social interaction was evaluated based on the proportion of positive reactions and the number of crossings. The positive reaction ratio was calculated by dividing the number of positive responses to the partner's approach by the number of approaches the partner made multiplied by 100. The reaction was considered positive whenever the animal: directed his head to the partner's body (nose to the body, genital investigation), allowed for genital examination or allogrooming, positively responded to pouncing (e.g., rolled onto its back or engaged in pinning) and/or wrestling (the on-top and on-bottom positions alternate during social play), or permitted a partner to crawl over/under. Positive responses to the approach of the partner did not include pushing or running away. Crossings were scored whenever two rats passed at a close distance, such as skin-to-skin contact, maximal length of one half of their bodies between rats, or crawling over/under the other rat during an interaction. Given that crossing requires the participation of both rats, the total number of "crossings" for one rat is divided by two (Fig. 2).

The blocking of social interaction signified a lack of desire for interaction with others. Avoidances were recorded when rats fled when their partner approached. The push-back was accounted for when animals pushed back the partners when partners tried to initiate social interaction (Fig. 2).

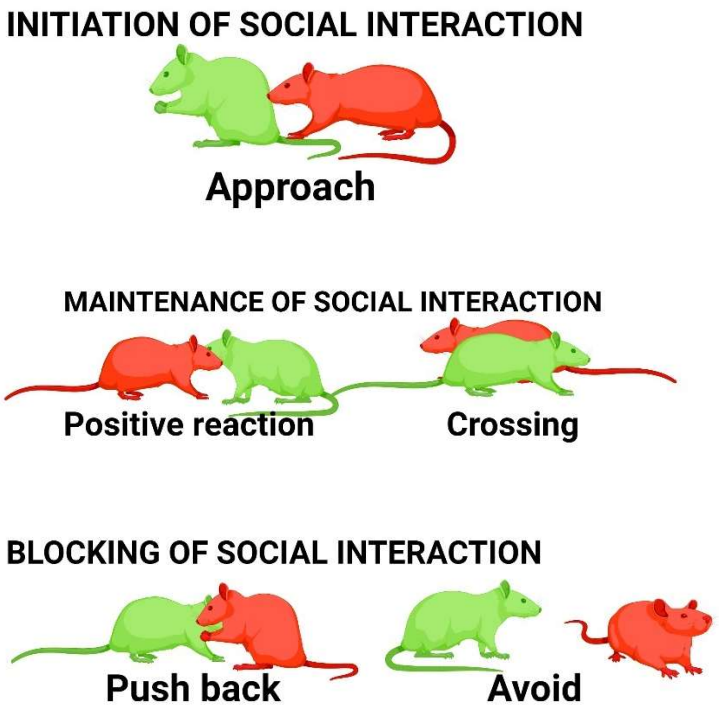

Fig. 2. Behaviors included in an assessment of social interaction.

3.2.5. Measuring social interaction during photomanipulation

In experiments involving photomanipulation of neuronal cells in the CeA that were activated by social interaction or by lever pressing for food, the social interactions took place in a social interaction chamber. To measure positive social interaction, I recorded rats' active approaches to the partner (initiation of social interaction) and a positive reaction to the partner's approach (continued interaction after being approached by the partner). The rats underwent 24 hours of social separation and 10 minutes of social interaction to determine the baseline level of positive social interaction after Skinner box training (as described above). I compared the behavioral measures and appetitive USVs between baseline social interaction and social interaction after three weeks of social isolation.

3.2.6. Measuring food motivation – Progressive Ratio schedule of reinforcement

Upon completion of the initial training, but before 21 days of social separation, I subjected rats to three food motivation tests. The latest test served as the baseline.

At the start of the Progressive Ratio (PR) test, a lever was extended into the operant chamber. The lever press was reinforced with a sucrose reward, and the number of responses required for each successive reward increased progressively. The reward delivery was accompanied by a tone (5s, 2000 Hz, 75 dB) and then the lever was concealed for 5 seconds. In subsequent steps, the number of required lever presses to receive a reward was determined in accordance with Robert and Bennett's implementation (Roberts and Bennett, 1993). The precise values were as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, and so on. Tests lasted thirty minutes.

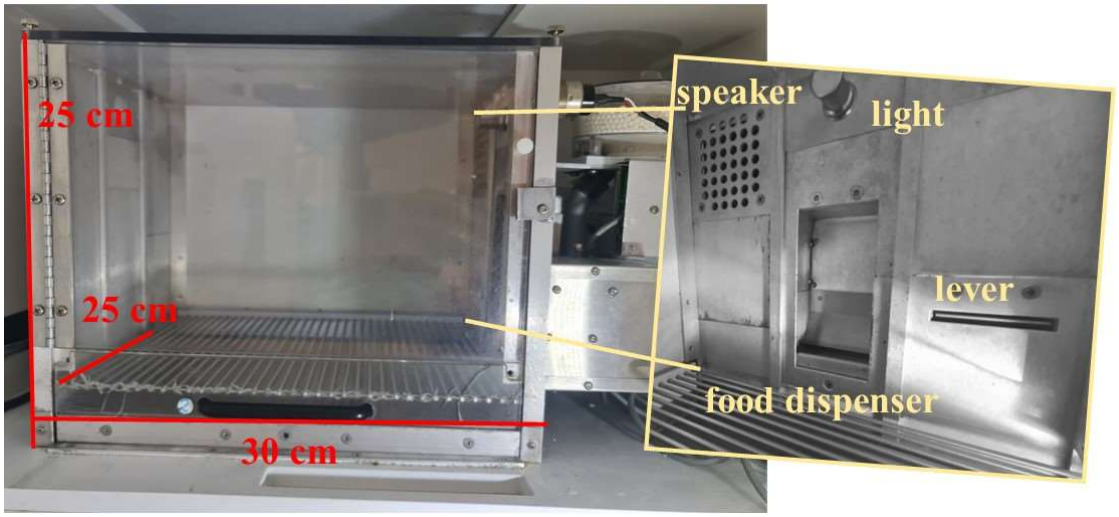

Fig. 3 Behavioral apparatus used for food reinforcement operant conditioning.

3.3. Experimental design

3.3.1. Experimental design for tracing experiments

After completing training for sucrose reward, I subjected all rats from this experimental group to intracranial injection of anterograde tracer PHA-L. After

surgery, I placed the animals back in the animal facility in single cages for three weeks. Then half of the animals I reunited with the original partners in home cages, and the other half I subjected to 30 minutes testing session in .the Skinner's box as described above (Fig. 4).

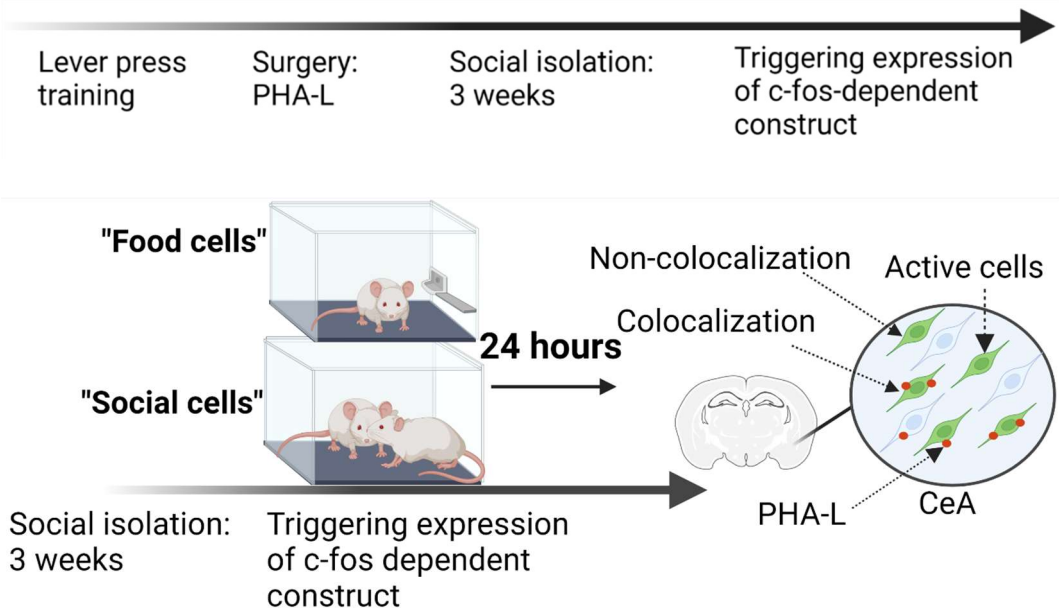

Fig. 4. The experimental scheme and timeline of the functional tracing experiment.

3.3.2. Experimental design for chemogenetic experiments

In the middle of food reward training (described above), the rats had their first surgery with intracranial delivery of DREADDs. Next, rats returned to the animal facility to the pair-together home cage, and after one week of recovery, they returned to training in the Skinner Box. After completion of training, I subjected the rats to 24 hours of social isolation and 10 min social interaction to establish the baseline of social interaction. Before the second surgery (infusion CAV-Cre or CAV-flxflp done three weeks after the first surgery), rats had established a baseline motivation for a food reward. Then I separated all animals from their cage mates for 21 days. During social isolation, the rats were additionally exposed, three times per week, to 30 minutes sessions in a Skinner box (where five presses of the lever guaranteed one reward). After 21 days, I subjected animals to social interaction test. From every pair, one rat was injected with c21 diluted in NaCl (3 mg/kg,

1mg/100ul, intraperitoneally [i.p.]), and the second rat was injected with NaCl 30 min before the testing session. After 10 minutes of social interaction, the rats returned to the animal facility and were placed in home cages with their partners. In the case of groups with activation of dopaminergic pathways, animals were back to a single cage without a partner.

Further isolation was necessary because activated rats displayed a high level of social interest, disregarding social information from partners. This resulted in an increased level of fear in partners (they performed freezing behaviors). Twenty-four hours later, I tested the rats for sucrose reward motivation. The animals injected previously with NaCl (before the social interaction test) prior to the Progressive Ratio test were given with c21 diluted in NaCl (3 mg/kg, 1mg/100ul, intraperitoneally [i.p.]). The rest of the animals were injected with NaCl (Fig 5).

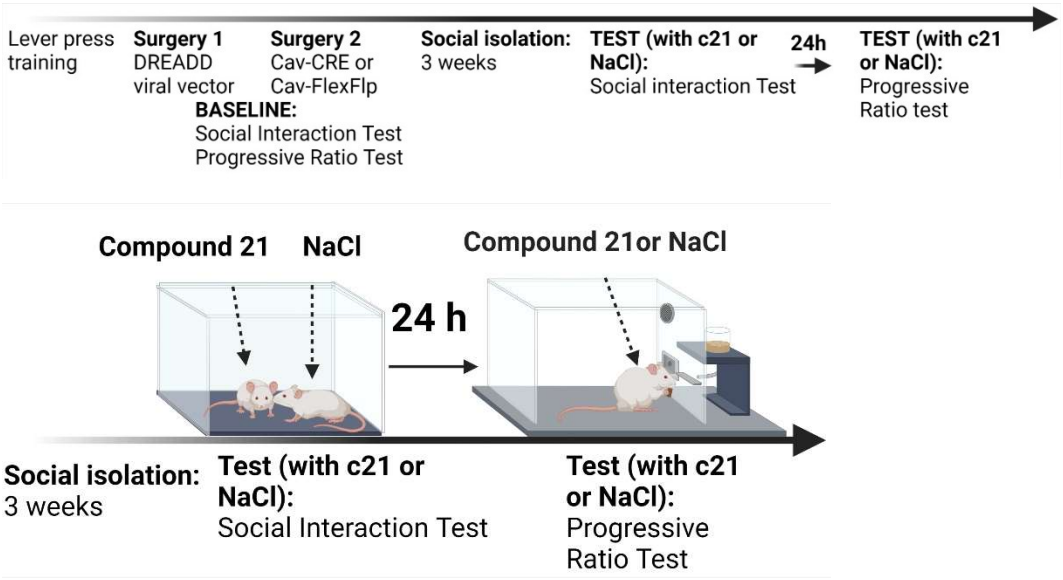

Fig. 5. The experimental scheme and timeline of the chemogenetic manipulation experiment.

3.3.3. Experimental design for photomanipulation during lever pressing for food

After the food reward training (described above), rats underwent 24 hours of social separation and 10 minutes of social interaction to determine the baseline level of positive social interaction after completing Skinner box training for the

reward (as described above). In addition, using the PR test (described above), I determined the baseline level of lever pressing for food. Then, I subsequently subjected rats to surgery (opsin delivery plus cannula implantation). The animals were returned to the animal facility in single cages for three weeks following surgery. During that time period, no behavioral procedures were conducted. Then, I exposed the animals to either 10 minutes of social interaction or 30 minutes of lever pressing for food in the Skinner box.

Following social interaction, the animals returned to the animal facility, with their partners, to the home cage. After the Skinner box session, rats returned to the animal facility in single cages. Approximately 24 hours later, I tested all rats for motivation for a food reward, while the activity of their CeA was manipulated optogenetically (Fig 6).

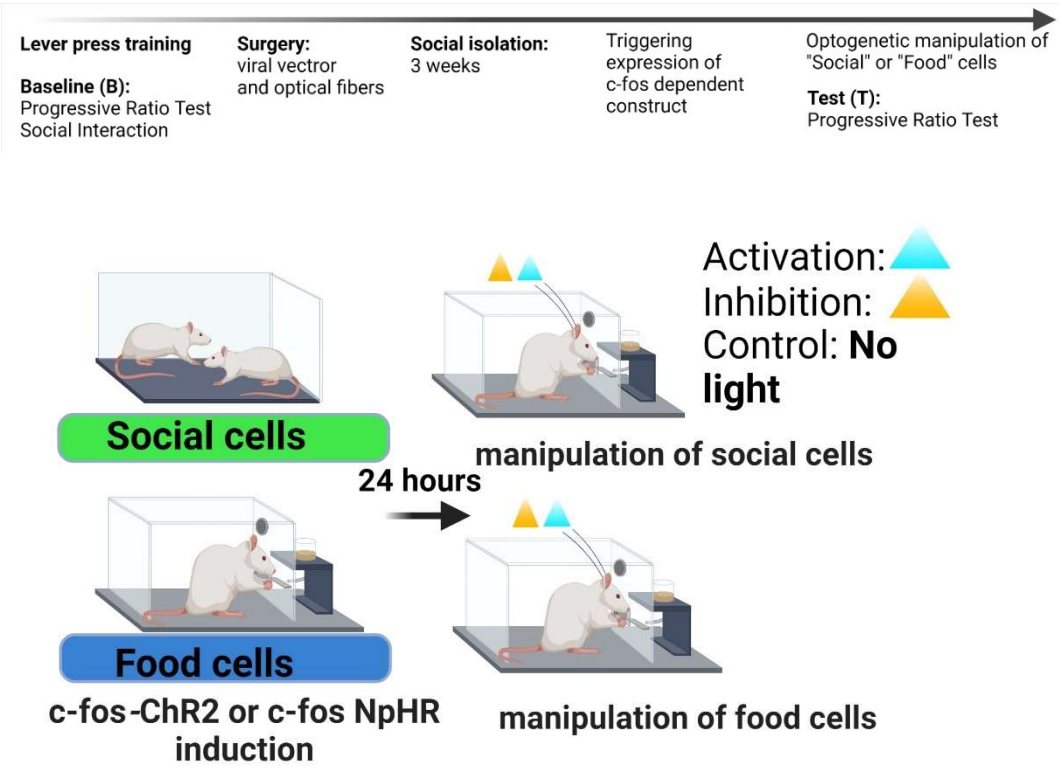

Fig. 6. The experimental schedule for photomanipulation during lever pressing for food.

Light delivery: custom-made optic cannulas (200 um fiber, 0.39NA, Thorlabs) were prepared before the surgeries and glued into M3 metal joints. Blue (472 nm) or yellow (589 nm) laser light was provided by fiber-coupled lasers (CNI), split by an optical rotary joint (Doric Lenses, 0.22NA), and delivered through armored patch

cords (Doric Lenses). Power was adjusted to obtain 10 mW at the cannula tips. The laser was triggered by an ArduinoUno microcontroller to provide 1-min long LIGHT ON with 2-min OFF periods (blue: 5ms pulses, 30 Hz; yellow: continuous stimulation, Fig. 7).

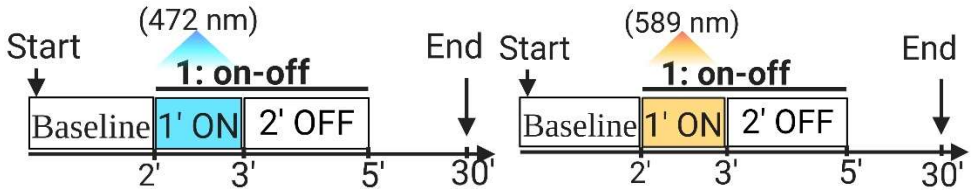

Fig. 7. The protocol for optogenetic activation (left) and optogenetic inhibition (right).

3.4. Surgical procedures

3.4.1. Surgical procedures for the anterograde tracing experiments

I performed surgeries 21 days before the behavioral experiment. I injected the rats with anterograde axonal transport tracer PHA-L Alexa Fluor 647 conjugate (2.5% wt/vol, dissolved in 0.1 M sodium PBS, pH 7.4; Invitrogen, Molecular Probes) into the ACC (AP), -1,8; (ML), ±0.8; (DV), −1.8) or the OFC (AP), +3.7; (ML), ±2,1; (DV) -4.2. All surgical instruments were sterilized before the surgery. I anaesthetized rats with isoflurane (5% induction, 2% for maintenance), and I gave them a subcutaneous injection of an analgesic (Butorfanol, Butomidor, 3 mg/kg). The ocular lubricant was used to moisten the eyes, and the scalp was shaved. After being placed into the stereotaxic apparatus (David Kopf Instruments), the scalp was disinfected with 70% (vol/vol) alcohol, incised, and retracted. Two small burr holes were drilled to allow for a 1 µL NanoFil syringe needle (World Precision Instruments) to be lowered into the desired part of the brain. All coordinates for stereotaxic surgeries were obtained from the rat brain atlas (Paxinos and Watson, 2006) with anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) positions referenced from Bregma. I kept the rats on a heating pad until they recovered from anesthesia. Then I placed them in a single cage.

3.4.2. Surgical procedures for chemomanipulation experiments

For chemoinhibition experiments I used pAAV-hSyn-DIO-{hCAR}off-{hM4Di-mCherry}on-W3SL plasmid with efficient retrograde spread in long-range projections, purchased from Addgene (Li et al., 2018). Following isolation using EndoFree Maxi Prep, the pAAV-hSyn-DIO-{hCAR}off-{hM4Di-mCherry}on-W3SL plasmid was used to generate recombinant AAV vectors serotype 9 with 10^9 titre. For the control group I used AAV9-hSyn-DIO-mCherry with10^9 titre (AddGene). To initiate expression of DREADDs receptors, CAV2-CreGFP (IGMM, Montpellier, France) was used. For chemoactivation experiments I used the pAAV-hSyn-frt-hM3D(Gq):mCherry plasmid, a generous gift from Roger A. Adan, Utrecht University, Netherlands (Kakava-Georgiadou et al., 2019). Following isolation using EndoFree Maxi Prep, the pAAV-hSyn-frt-hM3D(Gq):mCherry plasmids were used to generate recombinant AAV vectors serotype 5-10^8 titre. In the control groups, I used AAV5-EF1a-fDio-mCherry (AddGene). To initiate expression of DREADDs receptors, I used CAV-FLExloxP-Flp (IGMM, Montpellier, France).

For chemoinhibition experiments, the I injected the Wistar rats with AAV9-hSyn-DIO-{hCAR}off-{hM4Di-mCherry}on-W/3SL, or AAV9-hSyn-DIO-mCherry (AddGene) in the control groups. For chemoactivation of the dopaminergic projections, I injected the Th-Cre rats with AAV5-hSyn-frt-hM3D(Gq):mCherry, or AAV5-EF1a-fDio-mCherry (AddGene) in the control rats (300 nL/site). For chemoinhibition experiments, the infusion was made into one of the brain regions (bilaterally): the ACC (AP), -1,8; (ML), ±0.8; (DV), −1.8), OFC (AP), +3.7; (ML), ±2,1; (DV), −4.2, or CeA (AP) -1,8; (ML), ±3,8; (DV), −7.7 (Fig. 8-10).

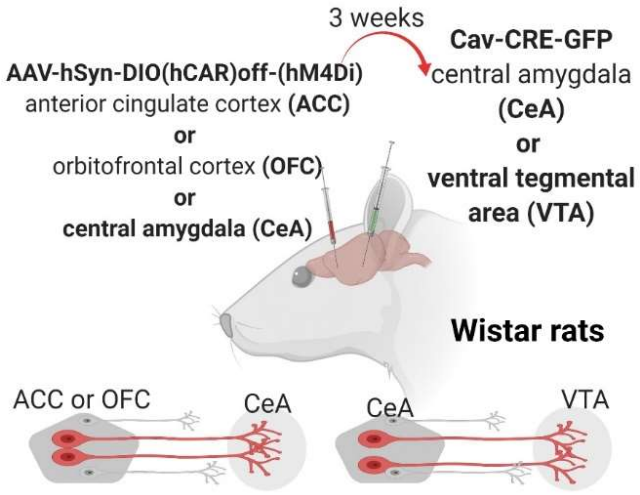

Fig. 8. The diagram illustrating the viral vectors injections scheme for the projections chemoinhibition.

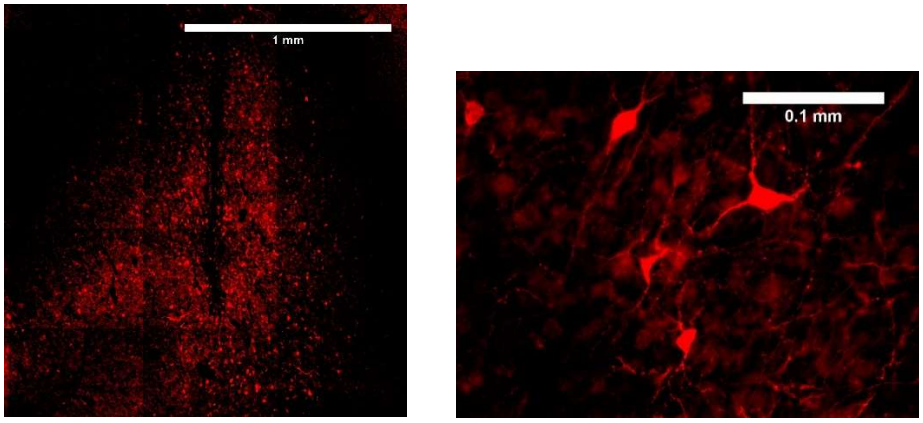

Fig. 9. The example OFC cells sending projections to the CeA that express the chemogenetic construct.

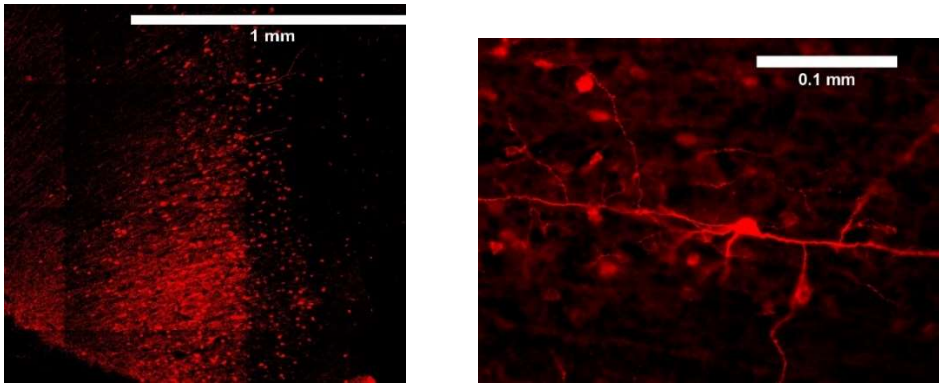

Fig. 10. The example ACC cells sending projections to the CeA that express the chemogenetic construct.

For chemogenetic manipulation of the CeA-VTA projection, rats were injected with AAV-hSyn-DIO-{hCAR}off-{hM4Di-mCherry}on-W3SL to the CeA and, after three weeks, with CAV-Cre-GFP to the VTA: (AP), -5.35; (ML), ±1.1; (DV), −8.0). For chemoactivation of the dopaminergic projections, I injected the rats with with AAV-hSyn-frt-hM3D(Gq):mCherry to the VTA, and, after three weeks, with CAV-FlexFlp into the ACC or OFC (using the same coordinates as described above, Fig. 11). The surgical procedures were identical as those described above. wI conducted the behavioral experiment after three weeks of social separation that started after the second surgery.

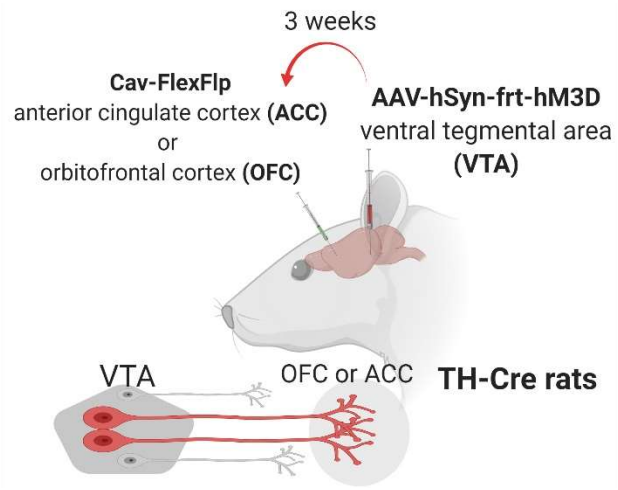

Fig. 11. The schematic of the injections of viral vectors for chemoactivation of dopaminergic projections.

3.4.3. Surgical procedures for photomanipulation experiments

Rats received intracranial injections of c-fos-ChR2 (400 nL/site) or c-fos-NpHRR3 (350 nL/site) viral vectors 3 weeks before the behavioral experiment. The surgical procedures was identical as described above. The coordinates used for the CeA were: anteroposterior (AP), -1.8mm; mediolateral (ML), ±3.8mm; dorsoventral (from dura) (DV), −7.7mm (Fig. 12 implanted Optic cannulas (200 µm in diameter) bilaterally 0.2 mm above the injection sites and afterwards secured them with two skull screws and dental cement. The animals were administered an analgesic (Tolfenamic acid, Tolfedine; 4 mg/kg; s.c.) and an antibiotic (Enrofloxacin, Baytril 2.5 mg/kg; s.c). To avoid dehydration, I gave the animals 1

mL of 0.95% NaCl/100 g of body weight by s.c. Injection. I kept them on a heating pad until they recovered from anaesthesia before placing them in a single cage.

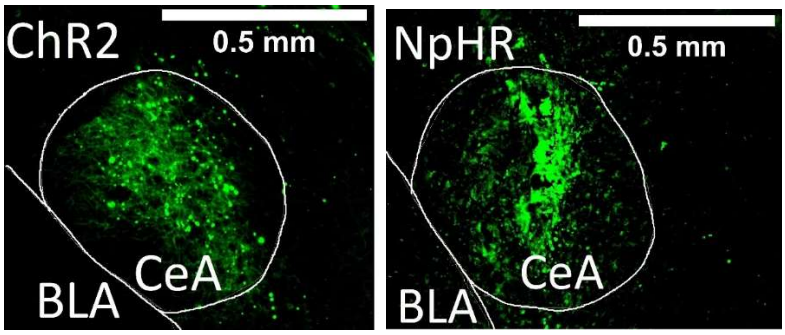

Fig. 12. Expression of the optogenetic constructs (left: ChR2, right: NpHR) in the CeA.

3.5. Behavioral data analysis

The data from social interactions were manually scored by trained observers, with frame-to-frame temporal resolution using BehaView open-source. (P. Boguszewski, http://www.pmbogusz.net/?a=behaview). Social interaction test experiments were additionally analyzed with Bonsai open-source software (Lopes et al., 2015) to extract locomotor trajectories. The number of the lever presses were automatically collected with Med-PC® V Software Suite (SOF-736).
The USVs were recorded with a ultrasound microphone coupled with UltraSoundGate system (Avisoft Bioacoustics, Germany) and then analyzed with RavenPro 1.5 interactive sound analysis software (Cornell Lab of Ornithology).

3.6. Immunohistochemistry

After completing behavioral testing, rats received a lethal dose of Morbital (133.3 mg/ml sodium pentobarbital, 26.7 mg/ml pentobarbital), and were transcardially perfused with ice-cold PBS (pH 7.4, ThermoFisher Scientific) and 4% (wt/vol) paraformaldehyde (POCh,) in PBS (pH 7.4). The brains were removed and stored in the same fixative for 24 h at 4°C, and subsequently

immersed in 30% (wt/vol) sucrose at 4°C. The brains were then fast frozen on dry ice and sectioned at 40 µm on a cryostat.

3.6.1. c-Fos immunohistochemistry

Immunofluorescence staining for c-Fos was performed on free-floating sections. I washed the sections with PBS (ThermoFisher Scientific, 3 x 5 min) and blocked with 3% (vol/vol) normal goat serum (NGS, Abcam) in PBST (0,2% Triton X-100, Polysciences) for 1.5-h at room temperature. Subsequently, I incubated sections with anti-c-Fos rabbit antibody (1:1000, Millipore) diluted with 5% NGS in PBST at room temperature for 24 h. The next day, sections were rinsed with PBST (3 x 5 min), before 2-h incubation at room temperature with a secondary antibody Alexa 594 made in rabbit (Invitrogen, 2h). After several washes, I mounted the sections onto glass slides, overlaid them with the Fluoromount G Medium (Merck), and covered them with a glass coverslip (Fig. 13).

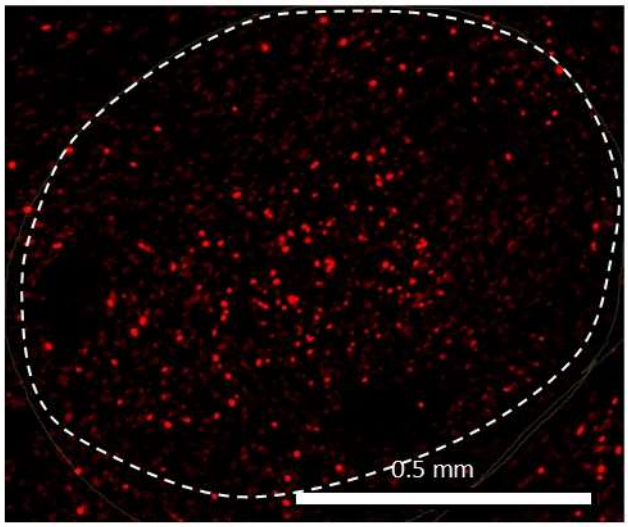

Fig. 13. An example of c-Fos staining in the CeA.

3.6.2. GFP immunohistochemistry

I performed the GFP fluorescent staining on free-floating sections. The sections were washed with PBS (ThermoFisher Scientific, 3 x 5 min) with 0.2% Triton X-100 (Polysciences), blocked with 5% (vol/vol) normal goat serum in PBST, and incubated overnight at 4°C with anti-GFP rabbit antibody [1:500, Millipore) diluted with 3% normal goat serum (NGS, Abcam)] in PBST. The next

day, I rinsed sections with PBST (3 x 5 min) before 1 h incubation at room temperature with a secondary antibody conjugated to Alexa Fluor 488 (1:500; Invitrogen). After several washes, I mounted the sections onto glass slides, overlaid with the DAPI Fluoromount-G (SouthernBiotech), and covered them with a glass coverslip (Fig. 14).

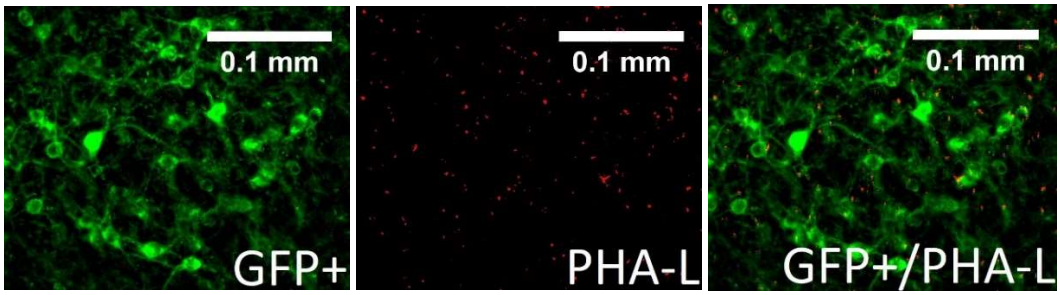

Fig. 14. An example of functional tracing. In green – GFP, in red – PHA-L.

3.6.3. Th immunohistochemistry

I performed the Th-Cre fluorescent staining on free-floating sections. The sections were washed with PBS (ThermoFisher Scientific, 3 x 5 min) with 0.2% Triton X-100 (Polysciences), blocked with 5% (vol/vol) normal goat serum (NGS, Abcam) in PBST, and incubated overnight at 4°C with anti-Th mouse antibody (1:500, *Millipore*) diluted with 3% normal goat serum in PBST. The next day, I rinsed sections with PBST (3x 5 min) before 1 h incubation at room temperature with a secondary antibody conjugated to Alexa Fluor 488 (1:1000; Invitrogen). After several washes, I mounted the sections onto glass slides, overlaid with the Fluoromount G Medium (Merck), and covered them with a glass coverslip (Fig 15).

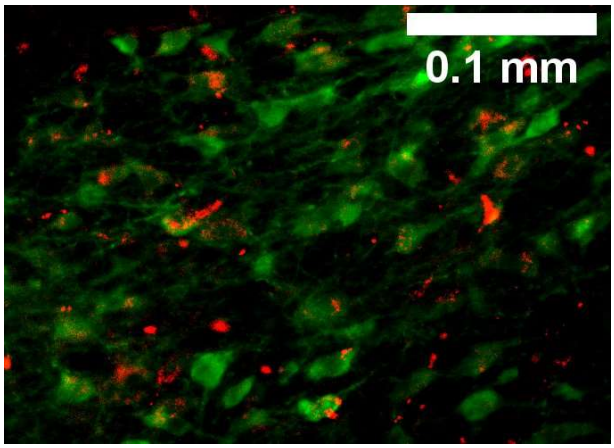

Fig. 15. An example of Th staining in theVTA. In green – Th positive cells, in red – mCherry expression.

3.6.4. GAD67 and mCherry immunohistochemistry

I performed the GAD67 and mCherry fluorescent stainings on free-floating sections. The sections were washed with 0.1 M TRIS (pH: 7,6, Millipore, 3 x 5 min), blocked with 5% (vol/vol) normal goat serum (NGS, Abcam) in 0.1 M TRIS-BSA (0.005% BSA, ThermoFisher Scientific) for 1h, and incubated overnight at 4°C with Anti-GAD67 rabbit antibody (1:500, Abcam) and anti-mCherry chicken antibody (1:500, Novus Biologicals) diluted in TRIS-BSA (0.005% BSA diluted in 0.1 M TRIS pH 7.6). The next day, I rinsed sections with TRIS-BSA (3x 5 min) before 1 h incubation at room temperature with a secondary antibodies conjugated to Alexa Fluor 647 (1:500; ThermoFisher Scientific) and Alexa Fluor 555 (1:1000; ThermoFisher Scientific). After several washes, I mounted sections onto glass slides, overlaid with the DAPI Fluoromount-G (SouthernBiotech), and covered them with a glass coverslip (Fig. 16).

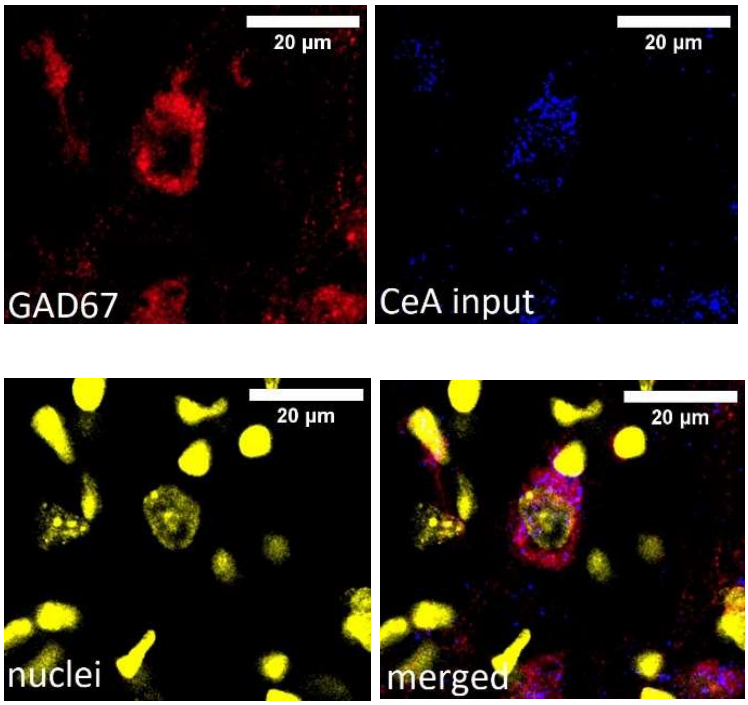

Fig. 16. An example of GAD67 staining in the VTA. Representative images of (from the left): the GABA positive cells (in red-anti-GAD67 ab), projections from the CeA (in blue), nuclei of cells (in yellow), and the merged image of all the former.

3.7. Image Capture and Analysis

3.7.1. Anterograde tracing data visualization

I analyzed the double-labelling results with Olympus VS110 fluorescent microscope. The images were then processed with ImageJ software by experimenters who were blind to the treatment during image acquisition as well as during cell counting. PHA-L images were merged with Venus-stained cell bodies and proximal dendrites to analyze the presence of close appositions between PHA-L and Venus positive neurons in ROIs. The ratio of the Venus neurons receiving projections to the whole number of Venus positive neurons was calculated.

3.7.2. CeA-VTA projections visualization

I analyzed The double-labelling results with Celldiscoverer 7 high-content phase-contrast microscope. The images were then processed with ImageJ software by experimenters who were blind to the treatment during image acquisition as well as during cell counting. The CeA inputs images were merged with GAD67 cell bodies to analyze the presence of close appositions between neuronal terminals from the CeA and GAD67 positive neurons in the VTA. The ratio of the GAD67 positive neurons receiving projections from the CeA to the number of non GAD67 positive neurons that receive projections was calculated.

3.7.3. Verification of viral expression

The correct expression of viruses was assessed with the aid of a Nikon Eclipse Ni-U fluorescent microscope equipped with a QImaging QICAM Fast 1394. Two objectives (20× and 10×) were used to capture samples with the aid of Image-Pro Plus 7.0.1.658 (Media Cybernetics) software. Structures were localized with the use of the atlas, “The Rat Brain in Stereotaxic Coordinates” by Paxinos and Watson (Paxinos and Watson, 2006).

3.7.4. HPLC analysis

The rats were sacrificed by decapitation immediately after the Progressive Ratio test with optomanipulations of the CeA circuits. Brain tissues were frozen in isopentane held on dry ice, and stored at −76 °C for neurochemical analysis. Frozen brains were cut into sections with a cryostat (−20 °C). Then, the samples of tissue underwent homogenization and neurotransmitter analysis.

3.8. Sample sizes

	Total # of animals	#of repetitions	social interaction (# of animals)		food reward (# of animals)	
Experiment			total	Used in the analysis	total	Used in the analysis
Functional tracing	4	1	2	2	2	2
ACC-CeA inhibition(NaCl/c21)	14	3	7/7	6/6	6/8	6/6
OFC-CeA inhibition(NaCl/c21)	12	1	6/6	6/6	6/6	6/5
ACC-CeA, OFC-CeA , CeA-VTA Control (NaCl/c21)	10	2	5/5	5/5	5/5	5/5
Immunohistochemical double stainings	3	1	1	1	1	1
Optogenetic, social cells, ChR2	7	3	-	-	10	7
Optogenetic, social cells, NpHR	12	3	-	-	14	12
Optogenetic,, social cells, Control	7	3	-	-	8	7
Optogenetic, food cells, ChR2	7	3	-	-	11	9
Optogenetic, food cells, NpHR	11	5	-	-	12	11
Optogenetic, food cells, Control	7	2	-	-	8	7
HPLC, social cells, ChR2		2	-	-	4	4
HPLC, social cells, NpHR		2	-	-	6	6
HPLC, food cells, ChR2		2	-	-	6	6

HPLC, food cells, NpHR		2	-	-	6	6
CeA-VTA inhibition	10	1	5/5	5/5	5/5	5/5
VTA-ACC activation	12	3	6/6	6/6	6/6	6/6
VTA-OFC activation	14	3	7/7	7/7	6/8	6/8
VTA-ACC/OFC Control	12	1	6/6	6/6	6/6	6/6
Total animal used in experiments: 142						

Table 1: Sample sizes for all experiments, including the total number of animals subjected to the experimental procedures, and the number of animals included in the final analyses (used). The reasons for exclusion were: a) a misplaced injection/cannulation; b) loss of data (tissue damage or data acquisition software malfunction).

3.9. Statistical analysis

I performed all the statistical analyses in GraphPad Prism 9 software (GraphPad). If two groups were compared during one-time point, an unpaired t-test was used. If the distribution of data was different from normal (as assessed with the Shapiro-Wilk test) U-Mann Whitney test was used. When comparing data for the same animals, I used a t-test for pairs.

For comparisons of more than two groups, I used one-way ANOVA followed by Holm-Sidak post-hoc tests. If the data distribution was different from normal (as assessed with Shapiro-Wilk test), I used a Kruskall-Wallis test followed by Dunn's tests (with FDR correction). To compare more than two groups in different time points, I used a two-way ANOVA followed by Holm-Sidak post-hoc tests. The names of tests are given together with the results of the analyses in the Result section.

4. Results

4.1. Functional characterization of the ACC-CeA and OFC-CeA projections activated during social interaction

4.1.1. Direct social interaction activates the CeA and its ACC and OFC inputs

Continuing the research from our laboratory, which showed that the central nucleus of the amygdala (CeA) is involved in social interaction after a 3-week separation in rats, I identified its inputs activated by direct social contact. I focused on the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC), two cortical regions implicated in social behavior (Rudebeck et al., 2006).

I used the c-fos-PSD95-Venus rats, which express a green fluorescent Venus protein under the control of the *c-fos* promoter. I injected an anterograde tracer (PHA-L: Phytohemagglutinin-L) into the ACC or OFC of these rats (**Methods**, **Fig. 4)**. I found that the cells in the CeA that express a reporter protein after social interaction (i.e., cells activated by social interaction, from here on social cells, **Fig. 17 A**) receive inputs from both the ACC and OFC **(Fig. 17 B**). Additionally, I observed that the ACC innervates more social CeA cells than the OFC [**Fig. 17, Methods, Fig. 14**, unpaired t-test ($t(5)=6,917$, $p=0.001$, ACC-CeA: $n=3$, OFC-CeA: $n=4$].

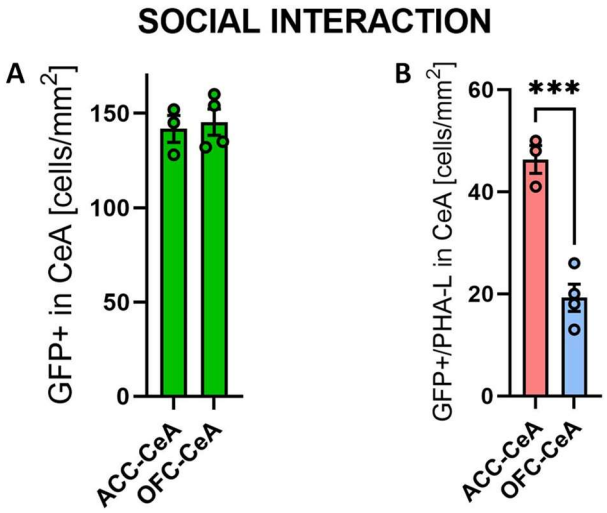

Fig. 17. Quantification of CeA neurons receiving ACC or OFC projections activated by social interaction. A: The number of neurons in the CeA activated by social interaction measured as the GFP fluorescence (Venus reporter protein was detected using anti-GFP antibody). **B**: The number of active cells receiving projections from the ACC (red) and the OFC (blue). All data are shown as mean ± s.e.m., and dots represent individual data points, *** $p < 0.001$.

Additionally, since the CeA, ACC, and OFC have been earlier implicated in food motivation (Douglass et al., 2017; Hart et al., 2020; Hong et al., 2019), I investigated whether the ACC and OFC neurons project onto the CeA cells activated by lever pressing for food. I employed a test used earlier in similar studies, namely operant responding under a progressive ratio schedule of reinforcement that allows assessing food motivation (food cells, **Fig. 18 A, B**). I found that the cells in the CeA that express a reporter protein after lever pressing for food receive inputs from both the ACC and OFC [**Methods, Fig 4,** ACC-CeA: n=3, OFC-CeA: n=3].

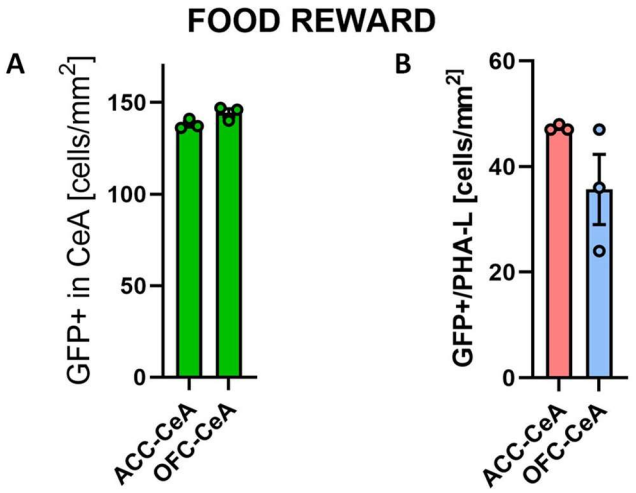

Fig. 18. Quantification of CeA neurons activated by a food reward. A: The number of neurons in the CeA activated by lever pressing for food, measured as the GFP fluorescence (Venus reporter protein was detected using anti-GFP antibody). **B**: The number of active cells receiving projections from the ACC (red) and the OFC (blue). All data are shown as mean ± s.e.m., and dots represent individual data points.

4.1.2. Inhibition of the ACC-CeA and OFC-CeA projections disturbs diverse aspects of social interaction

In the next step, I performed a loss-of-function experiment to investigate the functional role of the ACC-CeA and OFC-CeA projections in social interaction. Using the AAV-DIO-hM4Di vector injected into the ACC or OFC, and Cav-Cre construct injected into the CeA, I inhibited specific projections with the DREADD agonist c21 (Compound 21, **Methods, Fig 8, 9, 10**). To evaluate social interaction, I divided behaviors into three categories. The behavior that starts social interaction was marked as "initiation" and measured as the number of social approaches. The "maintenance" of social interaction was defined as the ability to respond to social signals that lead to maintaining social contact and was measured as a positive reaction to the partner's approach, including sniffing, crawling, allogrooming and crossing. Finally, the "blocking" of social contact was measured as the number of avoiding or pushing the partner back **(Methods, Fig. 2)**. The behavioral analysis included 10 minutes of social interaction after 24 hours (baseline) or 3-week (test)

separation from a familiar rat **(Methods, Fig. 1)**. The baseline of the social interaction was measured in the same environment as the final test but the injections were not given. After 3 weeks of separation, 30 min before the test, one rat from the pair was injected with c21, and the partner received NaCl injection **(Methods, Fig. 5)**. I compared the behavior of tested rats with their partners and control rats without DREADD expression injected with c21 before the social reunion. The analysis of social behavior revealed that the ACC-CeA and OFC-CeA differentially control various components of social contact.

Inhibition of the OFC-CeA projection affected initiating (i.e., social approach**, Fig. 19A**) and maintaining (i.e., positive reaction to the partner's approach, **Fig. 19B**) of social contact. On the other hand, inhibition of the ACC-CeA projection had a more limited effect. It decreased the ability to maintain social contact (**Fig. 19B**). Inhibition of both projections increased attempts to block social interaction (**Fig. 19C**).

[Social contact initiation: one-way ANOVA group effect: $F(5,28) = 4.941$, $p=0.0023$) followed by Holm-Sidak post-hoc tests. Social contact maintenance: one-way ANOVA (group effect: $F(5,28) = 12.19$, $p<0.0001$) followed by Holm-Sidak post-hoc tests. Social contact blocking: one-way ANOVA (group effect: $F(5,28) = 21.65$, $p<0.0001$) followed by Holm-Sidak post-hoc tests. Ctrl: NaCl/c21 n=5/5, ACC-CeA: NaCl/c21 n=6/6, OFC-CeA: NaCl/c21-n=6/6.]

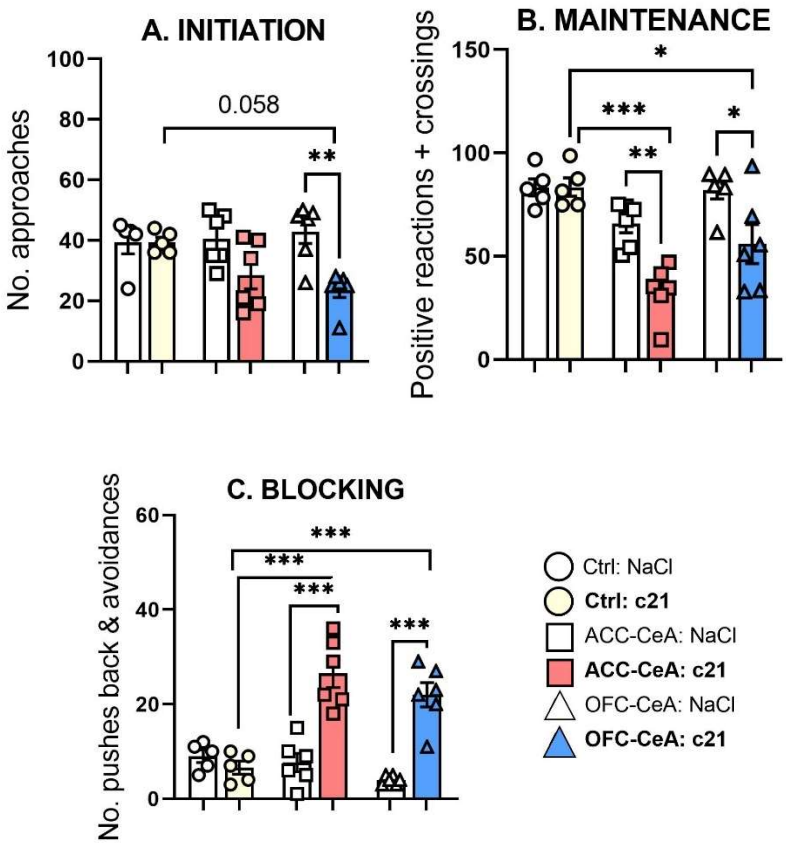

Fig. 19. Inhibition of the cortical-CeA projections revealed different involvement of the ACC-CeA and OFC-CeA pathways in specific components of social interaction. (A) Inhibition of the OFC-CeA pathway disturbed social contact initiation. Inhibition of the OFC-CeA or ACC-CeA projections disturbed maintaining of social contact **(B)** and increased blocking of social interaction **(C).** The white bars represent the partners of the tested rats. Ctrl: rats without DREADD expression. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The within-subject comparison revealed more profound deficits in initiating social contact than in maintaining it during inhibition of the OFC-CeA pathway [**Fig. 20**, one-way ANOVA (group effect: $F(3,20) = 5.037$, $p=0.0092$) followed by Holm-Sidak post-hoc tests; ACC-CeA: c21 n=6, OFC-CeA: c21 n=6].

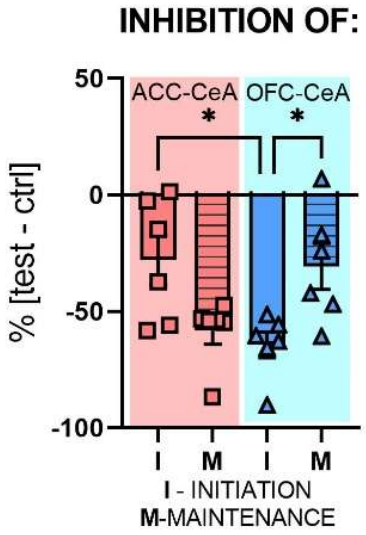

Fig. 20. Change in the initiation and maintenance of social interaction, computed as a percentage of baseline (performance after 24h of separation), during inhibition of the ACC-CeA or OFC-CeA projections. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$.

Then, I compared initiation and maintenance of social interaction during the baseline social interaction (after 24h of social separation) and during inhibition of the ACC-CeA or OFC-CeA projections. This comparison confirmed a decrease in initiation of social interaction when the OFC inputs to the CeA were inhibited (**Fig. 21**) and reduction of social contact when either the ACC-CeA or OFC-CeA projections were inhibited [**Fig. 21**, **22,** social contact initiation: two-way ANOVA (test x group effect: $F(5,28) = 8.376$, $p<0.0001$) followed by Holm-Sidak post-hoc tests, social contact maintenance: two-way ANOVA (test x group effect: $F(5,28) = 4.849$, $p=0.026$) followed by Holm-Sidak post-hoc tests].

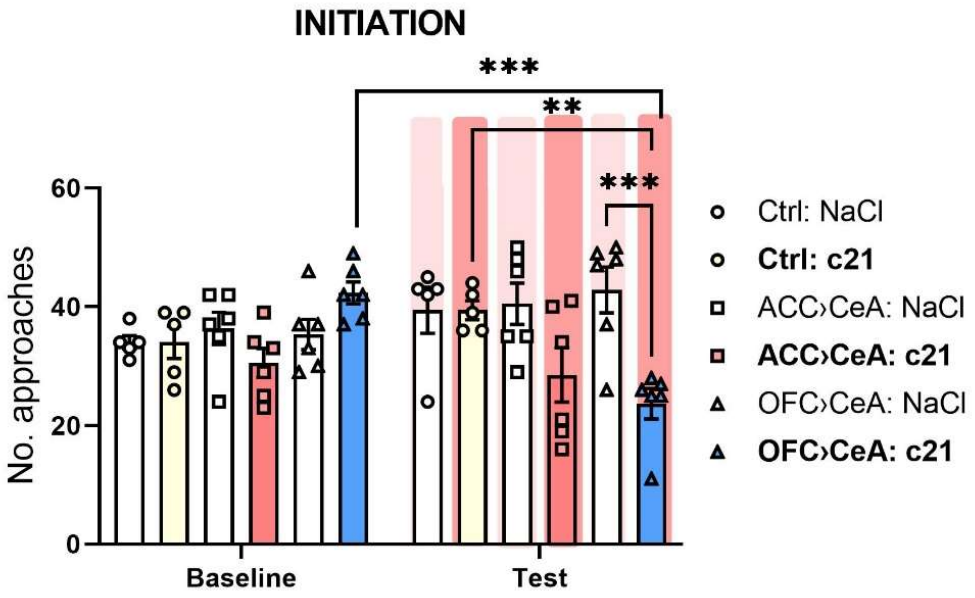

Fig. 21. Number of social approaches (social contact initiation) during social interaction after 24-h social separation (Baseline) and 3-week social separation (Test). Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test. Ctrl: rats without DREADD expression injected. All data are shown as mean ± s.e.m., and symbols represent individual data points, ** $p < 0.01$, *** $p < 0.001$.

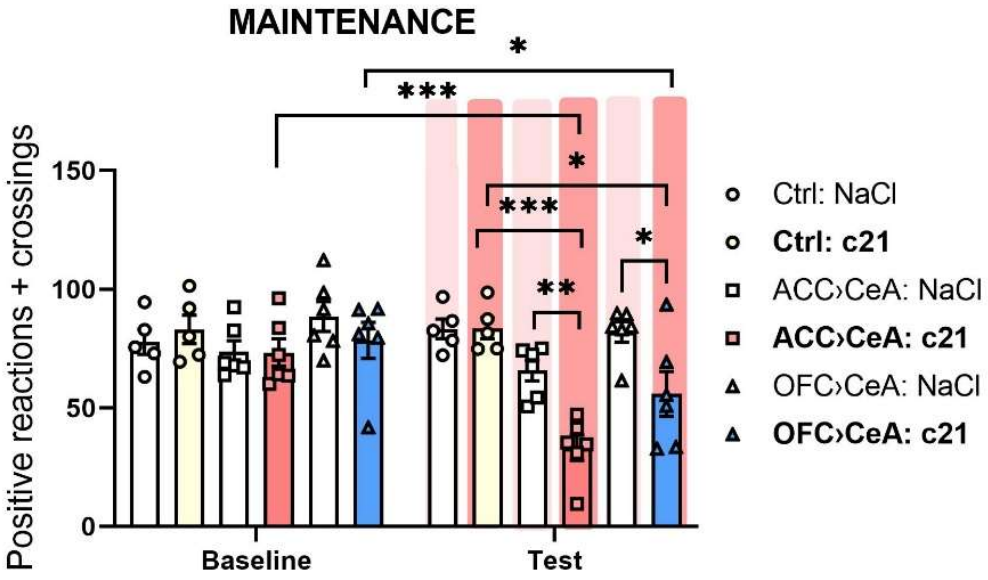

Fig. 22. Maintenance of social contact during social interaction after 24-h of social separation (Baseline) and 3-week social separation (Test). Pink background: rats injected with C21 before the test, light pink background: rats injected with NaCl before the test. Ctrl: rats without DREADD expression. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$.

Next, I analyzed general locomotor activity. The analysis revealed no changes in locomotor activity during the social interaction test in either of the groups (**Fig. 23**).

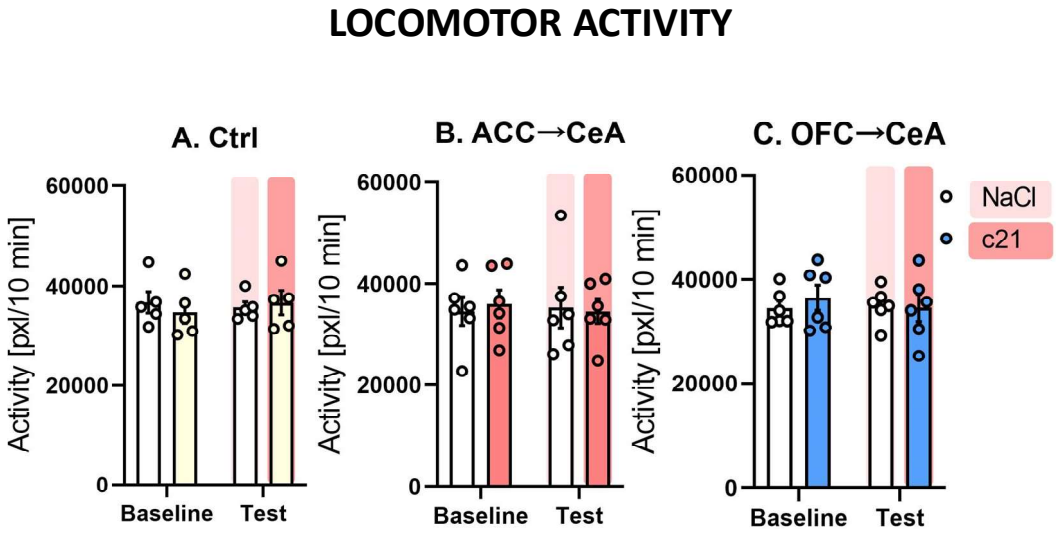

Fig. 23. Chemogenetic inhibition does not change general locomotor activity. A. Locomotor activity in the control group, rats without DREADD expression. B. Locomotor activity in the ACC-CeA group. C. Locomotor activity in the OFC-CeA group (pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test). All data are shown as mean ± s.e.m., and symbols represent individual data points.

Since the CeA has been implicated in food motivation and my tracing experiment showed that the CeA cells activated by food reward receive the ACC and OFC projections, I investigated the functional role of the ACC-CeA and OFC-

CeA projections in food motivation in the chemogenetic experiment. This experiment was designed to verify the selectivity of the ACC-CeA and OFC-CeA projections in social interaction. The rats from the previously described experiments were additionally trained in pressing a lever to be rewarded with a sucrose pellet. They were trained to have a high stable level of responding. Before social separation, I established a baseline level of motivation for a food reward. Then, the food motivation test was performed one day after the social interaction test (**Methods, Fig 5).** I found that inhibition of the ACC-CeA and OFC-CeA inputs decreases the number of operant responses to food rewards [**Fig. 24**, two-way ANOVA (test x group effect: $F(5,27) = 4.319$, $p=0.0051$), followed by Holm-Sidak post-hoc tests; Ctrl: NaCl/c21 n=5/5, ACC-CeA: NaCl/c21 n=6/6, OFC-CeA: NaCl/c21 n=6/5].

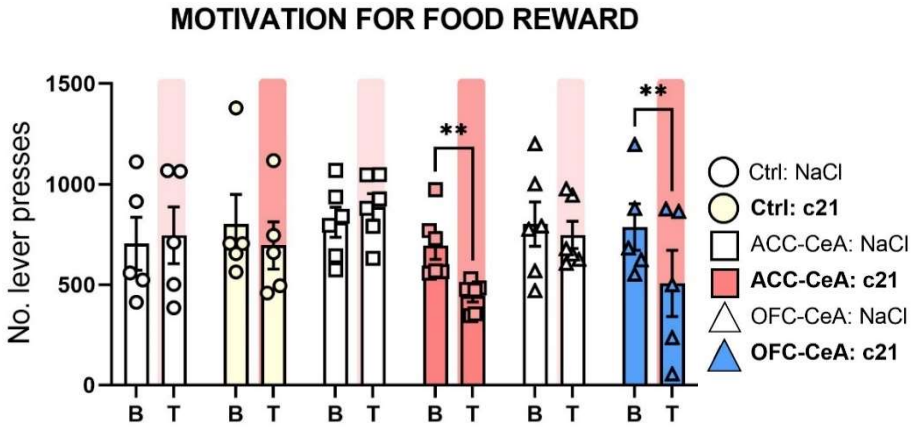

Fig. 24. Motivation for food reward during inhibition of the cortical-CeA projections. Inhibition of either the ACC-CeA or OFC-CeA projections decreased the number of lever presses for food. Comparisons were made between the test: with c21 (pink background) or NaCl injection (light pink background) and the baseline. Ctrl: rats without DREADD expression. All data are shown as mean ± s.e.m., and symbols represent individual data points, ** $p < 0.01$.

4.2. Functional characterization of the CeA outputs activated by social interaction: optogenetic manipulation of the CeA social cells

4.2.1. Inducing expression of *c-fos*-dependent constructs in the CeA social cells and food cells

The CeA has been implicated in both social and food behaviors (Andraka et al., 2021; Douglass et al., 2017; Ferretti et al., 2019; Knapska et al., 2013). In line, my data (presented in Section 1) showed that the ACC and OFC inputs to the CeA play a role in both social interaction and food motivation. Thus, in the next step, I investigated whether the neuronal populations in the CeA activated by social interaction functionally overlap with those activated by working for a food reward. I aimed at localizing the circuits that are specific, or at least preferentially involved in, social behavior. To that end, I used *c-fos*-dependent opsins (c-fos-NpHR and c-fos-ChR2), whose expression was induced by social interaction (social cells) or lever pressing for food (food cells). The next day, I activated (ChR2) or inhibited (NpHR) the social and food cells optogenetically during the test that assessed motivation for a food reward. I hypothesized that if the populations of social and food cells overlap, I will see similar effects of manipulating their activity.

First, I confirmed that both, the social interaction and lever pressing for food, induced robust expression of c-Fos in the CeA [**Fig. 25, Methods Fig. 13,** unpaired t-test: social interaction vs. social separation ($t(4)=6{,}360$, $p=0.031$, unpaired t-test: lever pressing for food vs. social separation ($t(4)=10{,}23$, $p=0.0005$, social separation: n=3, s + social interaction: n=3, s + lever pressing for food: n=3].

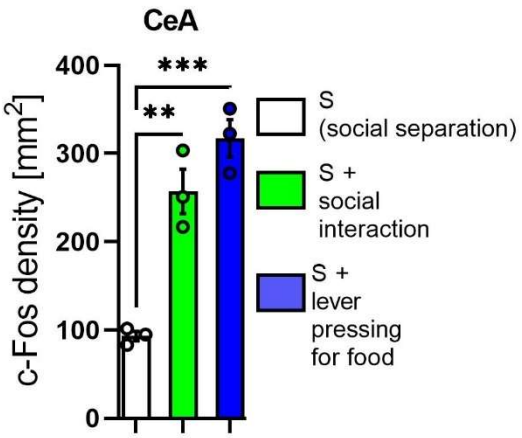

Fig. 25. C-Fos expression in the CeA after 3-week social separation (S), social interaction after the separation, and lever pressing for food after the separation. All data are shown as mean ± s.e.m., and symbols represent individual data points, ** $p < 0.01$, *** $p < 0.001$.

Second, I confirmed that endogenous c-Fos expression colocalizes with behaviorally induced opsin expression in both social and food cells. Further, I found that the number of social and food cells was comparable. Also, I confirmed that social separation did not induce significant expression of opsins (**Fig. 26, Methods, Fig. 12**).

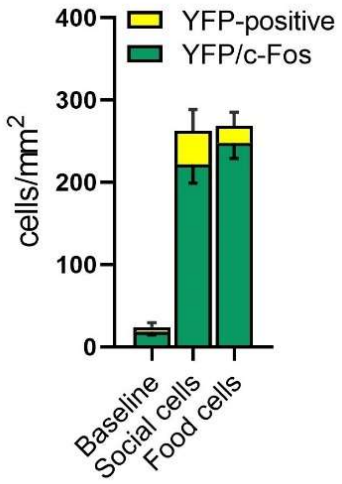

Fig. 26. Expression of the optogenetic constructs in the CeA, measured as the YFP fluorescence colocalizing with the endogenous c-Fos after 3-week social separation (Baseline), social interaction after the separation (Social cells), and lever pressing for food after the separation (Food cells). All data are shown as mean ± s.e.m.

Next, I randomly assigned the animals into three groups: the activation, inhibition, and control. In the control group, animals were subjected to the same experimental protocols as in the other two groups, except for applying laser light during the test (**Methods, Fig 6, 7)**. In the activation and inhibition groups, the social or food cells were activated or inhibited with light, respectively. First, I confirmed that the groups had comparable social interactions inducing opsin expression; they did not differ in the level of social approach or positive ultrasonic vocalizations (50 kHz) during social interaction (**Fig. 27**).

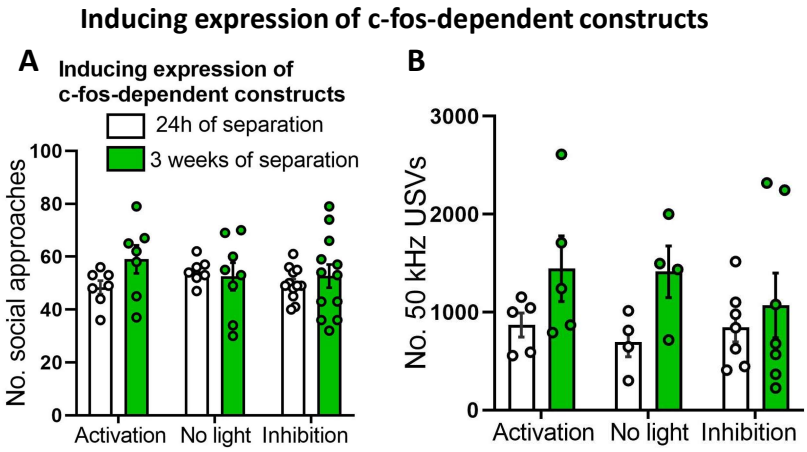

Fig. 27. There was no significant difference in the number of social approaches (A) and appetitive ultrasonic vocalizations (B) during social interaction inducing expression of *c-fos*-dependent opsins between the groups. All data are shown as mean ± s.e.m., and dots represent individual data points.

Similarly, there was no difference in the number of lever presses between the groups during lever pressing for food that induced the expression of *c-fos*-dependent constructs (**Fig. 28**).

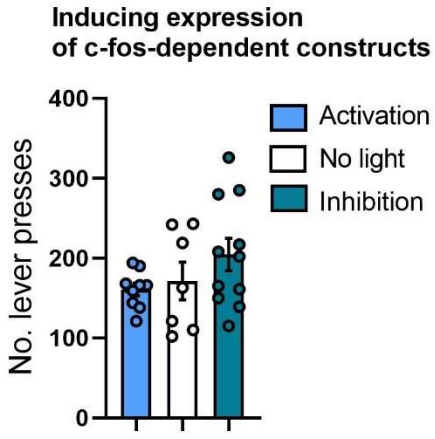

Fig. 28. The number of lever presses during lever pressing for a food reward inducing expression of c-fos dependent opsins did not differ between the groups. All data are shown as mean ± s.e.m., and dots represent individual data points.

Next, I tested behavioral responses of the animals with optoactivation or optoinhibition of the social or food cells. The photomanipulation started after a 2-min adaptation without light stimulation. The light was delivered in 1-min ON-laser periods, followed by 2-min OFF-laser periods. Since the laser stimulation effects were visible also after the laser was switched off, I analyzed jointly the behavior during the on and the following off periods ("ON-OFF batch").

4.2.2. Optogenetic activation

I observed that optogenetic activation of food cells in the CeA increased the number of lever presses during the first two stimulations. Subsequent laser activations introduced fluctuations in lever pressing, most likely due to overstimulation of the neurons. Thus, to avoid non-specific effects of laser stimulation, I decided to analyze only the effects of the first two “ON-OFF” periods of activation of the social and food cells.

I found that optogenetic activation of the food cells increased food motivation, which confirmed the previously published results (Douglass et al., 2017; Warlow et al., 2017) the method’s validity. In contrast, activation of the social cells suppressed lever pressing for food as compared to the baseline. The control groups showed no difference between the baseline and the test [**Fig 29,** two-way ANOVA (time x group effect: $F(6,52) = 8.052$, $p<0.0001$), followed by Holm-Sidak post-hoc tests. Ctrl: Social cells: n=7, Ctrl: Food cells: n=7, Social cells: activation n= 7, Food cells: activation n=9].

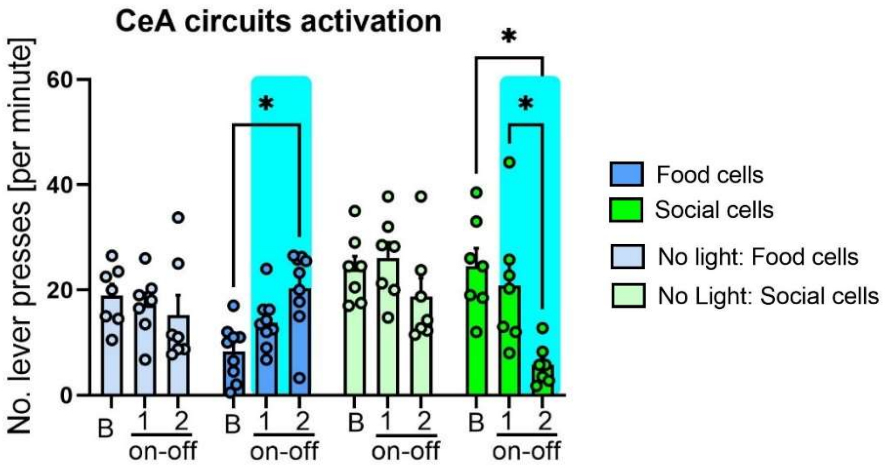

Fig 29. Activation of the food cells increased lever pressing for food, while activation of the social cells decreased lever pressing as compared to the baseline. The controls showed no difference between the baseline and the test (blue background: optogenetic activation, B – 2-min baseline). All data are shown as mean ± s.e.m., and dots represent individual data points, * $p < 0.05$.

A direct comparison between the effects of photoactivation of the social and food cells showed a difference between the groups [**Fig 30,** two-way ANOVA (time x group effect: $F(1,14) = 13.52$, $p<0.0025$].

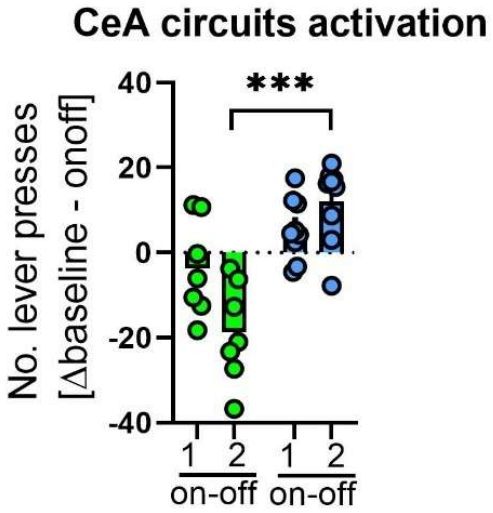

Fig. 30. Activation of the CeA social and food cells had different effects. Change in the number of lever presses, computed as the difference between the baseline (first two minutes of the test) and the following two "ON-OFF" periods when the CeA social cells (green) or food cells (blue) were activated. All data are shown as mean ± s.e.m., and dots represent individual data points, *** $p < 0.001$.

Interestingly, I observed the results described above only when the social cells were activated by positive social interaction. In rare cases (2 out of 10 rats tested in the optogenetic experiments) when rats showed signs of negative affect, including social avoidance and aversive 22 kHz USVs, stimulation of the CeA cells activated by such interaction did not affect food motivation [**Fig. 31,** Baseline vs 1-2 on-off: two-way ANOVA (time effect: $F_{(1,13)} = 10.11$, $p=0.0072$), followed by Holm-Sidak post-hoc tests. Baseline vs 1-10 on-off: two-way ANOVA (timexgroup effect: $F_{(2,13)} = 4.606$, $p=0.0307$), followed by Holm-Sidak post-hoc tests. Ctrl: Social cells n=7, Social cells: activation: n=7, Aversive social cells: activation: n=2].

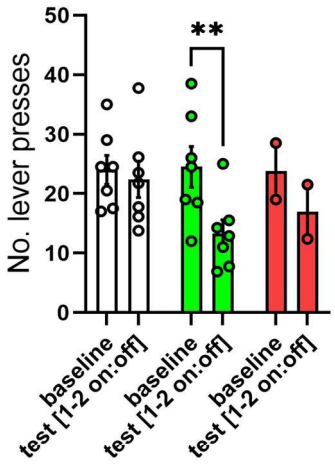

Fig. 31 Optoactivation of the CeA cells activated by aversive social interaction did not affect the lever pressing for food in contrast to stimulation of the CeA cells activated by positive social interaction. The number of lever presses during the first two stimulations of the CeA social cells. The social cells in the CeA previously activated by: positive social interaction (green bars) or aversive social interaction (red bars). All data are shown as mean ± s.e.m., and dots represent individual data points, ** $p < 0.01$, *** $p < 0.001$.

4.2.3. Optogenetic inhibition

Inhibiting either the food cells or social cells activated by positive interaction decreased lever pressing compared to the baseline [**Fig 32,** time effect: $F(1.460,48,17) = 12.40$, $p=0.0002$), followed by Holm-Sidak post-hoc tests. Ctrl: Social cells: n=7, Ctrl: Food cells: n=7, Social cells: inhibition: n=12, Food cells: inhibition: n=11].

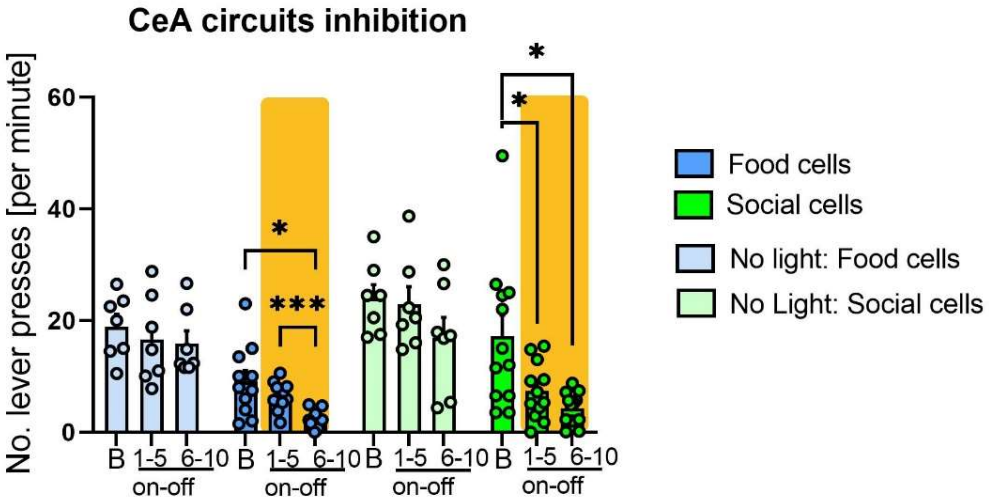

Fig. 32 Inhibition of food and social cells decreased lever pressing for a food reward. The controls showed no difference between the baseline and the test (orange background: optogenetic inhibition, B – 2-min baseline). All data are shown as mean ± s.e.m., and dots represent individual data points, * $p < 0.05$, *** $p < 0.001$.

4.2.4. Mapping of neurotransmitters and neuromodulators release

Next, I collected the brain tissue from the optomanipulation experiments to investigate which brain structures are activated when the CeA social or food cells are activated or inhibited. This step allowed me to identify brain structures preferentially involved in social interaction (i.e., whose activity changed after manipulation of the social but not food cells). I used the HPLC method to identify different neurotransmitters and their metabolites released in several brain structures. I focused on the following structures: the anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), prefrontal cortex (PFC), nucleus accumbens (NAc), basolateral amygdala (BLA), the hippocampus (Hipp), and ventral tegmental area (VTA; **Fig. 33)**.

I found that manipulation of the CeA social cells but not the food cells affected the levels of glutamic acid (Glu) in the cortical regions including the OFC and PFC, and gamma-aminobutyric acid (GABA) in the OFC, BLA, VTA, and NAcc. In addition, activation of the social cells increased dopamine level in the

ACC compared to inhibition of the social cells. On the other hand, manipulation of the CeA food cells had a more profound effect on the noradrenaline release in the ACC, OFC, PFC, BLA, and Hipp. Also, manipulation of the CeA food cells but not social cells led to changes in the level of Glu in the Hipp (**Fig. 33).**

[Fig. 33, ACC; social cells-DA; Kruskal-Wallis test (p=0.0039) followed by Dunn's post-hoc tests.
Food cells-NA; one-way ANOVA (group effect: F(2,13) = 8.344, p=0.0047) followed by Holm-Sidak post-hoc tests.
OFC: social cells-Glu; one-way ANOVA (group effect: F(2,11) = 6.258, p=0.0153) followed by Holm-Sidak post-hoc tests. Social cells-GABA; one-way ANOVA (group effect: F(2,11) = 4.583, p=0.0357) followed by Holm-Sidak post-hoc tests. Social cells-NA; one-way ANOVA (group effect: F(2,11) = 13.59, p=0.0011) followed by Holm-Sidak post-hoc tests.
Food cells-NA; one-way ANOVA (group effect: F(2,13) = 18.88, p=0.0001) followed by Holm-Sidak post-hoc tests.
PFC: social cells-Glu; one-way ANOVA (group effect: F(2,11) = 7.530, p=0.0087) followed by Holm-Sidak post-hoc tests. Social cells-NA; one-way ANOVA (group effect: F(2,11) = 5.326, p=0.0241) followed by Holm-Sidak post-hoc tests.
Food cells-NA; one-way ANOVA (group effect: F(2,13) = 6.979, p=0.0087) followed by Holm-Sidak post-hoc tests.
BLA: Social cells-GABA; one-way ANOVA (group effect: F(2,11) = 10.75, p=0.0026) followed by Holm-Sidak post-hoc tests. Social cells-NA; one-way ANOVA (group effect: F(2,11) = 10.45, p=0.0029) followed by Holm-Sidak post-hoc tests.
Food cells-GABA; Kruskal-Wallis test (p=0.0352) followed by Dunn's post-hoc tests. Food cells-NA; one-way ANOVA (group effect: F(2,13) = 17.06, p=0.0002) followed by Holm-Sidak post-hoc tests.
VTA: Social cells-GABA; one-way ANOVA (group effect: F(2,11) = 19.91, p=0.0002) followed by Holm-Sidak post-hoc tests.
NAc: Social cells-GABA; Kruskal-Wallis test (p=0.046) followed by Dunn's post-hoc tests. Social cells-5-HT; Kruskal-Wallis test (p=0.0215) followed by Dunn's post-hoc tests.

Hipp: Food cells-Glu; one-way ANOVA (group effect: $F(2,13) = 10.42$, $p=0.002$) followed by Holm-Sidak post-hoc tests. Food cells-NA; Kruskal-Wallis test ($p=0.0023$) followed by Dunn's post-hoc tests. Ctrl: n=4, Social cells: Activation/Inhibition: n=4/6, Food cells: Activation/Inhibition: n=6/6.]

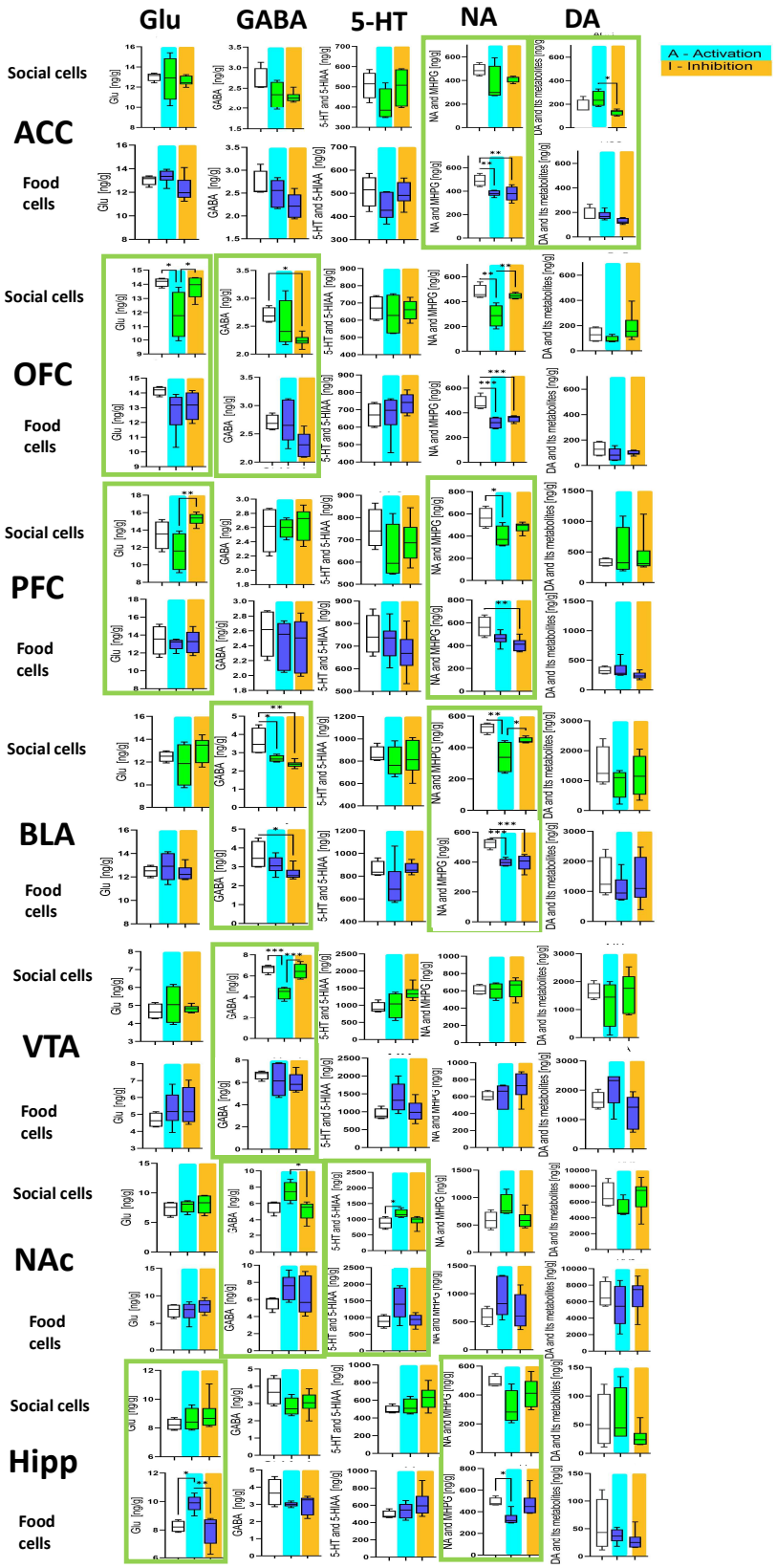

Fig. 33 Changes in neurotransmitter levels in different brain structures after photomanipulation of the CeA social cells or food cells. Activation (blue

background) or inhibition (yellow background) of the CeA social cells. Green frames indicate the difference in neurotransmitter levels and their metabolites after manipulating the CeA social vs. food cells. White background: control group. Boxplots showing median, quartiles, and the lowest and highest data points of the dependent variables, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

I found the most significant differences between manipulating the CeA social cells and food cells in the level of DA, Glu and GABA in the ACC, OFC, and VTA (**Fig. 34**). Activation of the CeA social cells increased the level of DA and its metabolites in the ACC compared to the inhibition of the CeA social cells (**Fig. 34 A**). On the other hand, I did not observe a change in the DA level in the ACC after manipulating the CeA food cells. Also, activation of the CeA social cells reduced the level of Glu and its metabolites in the OFC (**Fig. 34 B)**. Further, activation of the CeA social cells but not food cells decreased the level of GABA in the VTA (**Fig 34 C**); [Social cells: ACC-DA: Kruskal-Wallis test ($p=0.0039$) followed by Dunn's post-hoc tests. OFC; one-way ANOVA (group effect: $F(2,11) = 6.258$, $p=0.0153$) followed by Holm-Sidak post-hoc tests. VTA; one-way ANOVA (group effect: $F(2,11) = 19.91$, $p=0.0002$) followed by Holm-Sidak post-hoc tests. Ctrl: n=4, Social cells: Activation/Inhibition: n=4/6, Food cells: Activation/Inhibition: n=6/6.]

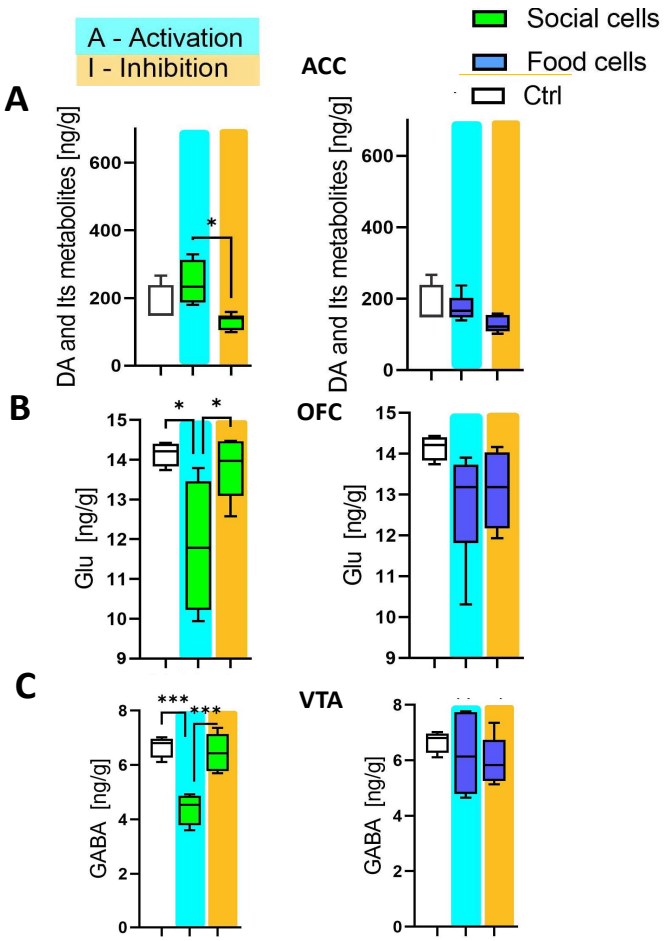

Fig. 34. Activation of the CeA social cells specifically changes the neurotransmitter level in the ACC, OFC, and VTA. (A) Activation of the CeA social cells increased DA and its metabolites (DOPAC, HVA, 3-MT) in the ACC compared to inhibition of the CeA social cells. (B) Activation of the CeA social cells decreased the level of Glu in the OFC. (C) Activation of the CeA social cells decreased the level of GABA in the VTA. Boxplots showing median, quartiles, and the lowest and highest data points of the dependent variables, * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$.

4.3. Role of the CeA-VTA projection in social interaction

Guided by the results described in the previous section, I focused on the dopaminergic projection to the cortex. The experiments carried out earlier in the laboratory showed that the ventral tegmental area (VTA), the primary source of DA in the cortex, is activated in rats subjected to social interaction after separation

(unpublished data). Thus, I tested the role of the CeA-VTA projection in social interaction.

Using the chemogenetic approach (DIO-hM4Di and Cav-Cre constructs) activated by DREADD agonist c21, I found that inhibition of the CeA-VTA projection disrupted social interaction. Rats with inhibited CeA-VTA pathway showed decreased initiation **(Fig. 35 A),** and maintenance **(Fig. 35 B)** of social contact and increased blocking of social contact initiated by their partners **[Fig. 35 C,** Initiation of social contact: one-way ANOVA (group effect: $F(3,16) = 7.364$, $p=0.0026$) followed by Holm-Sidak post-hoc tests; Maintenance of social contact: one-way ANOVA (group effect: $F(3,16) = 9.563$, $p=0.0007$) followed by Holm-Sidak post-hoc tests; Blocking of social contact: one-way ANOVA (group effect: $F(3,16) = 6.018$, $p<0.0060$) followed by Holm-Sidak post-hoc tests; Ctrl: NaCl/c21 n=5/5, CeA-VTA: NaCl/c21 n=5/5].

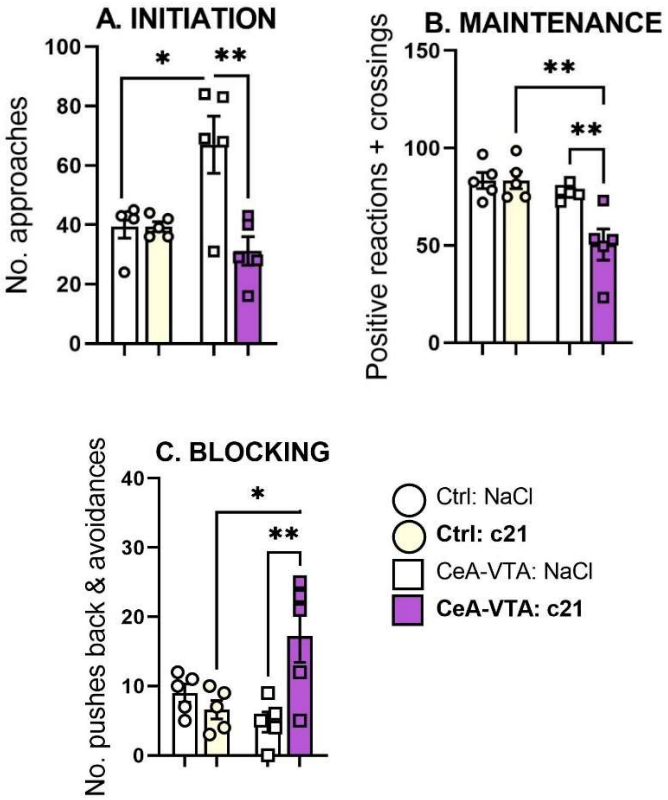

Fig 35. Inhibition of the CeA-VTA pathway disturbs initiation and maintenance of social contact and increases blocking of social contact. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$, ** $p < 0.01$.

Direct comparison between change in the initiation and maintenance of social contact during inhibition of the CeA-VTA projection revealed no differences (the data were compared as a percentage of initiation and maintenance of the baseline of social interaction, **Fig. 36).**

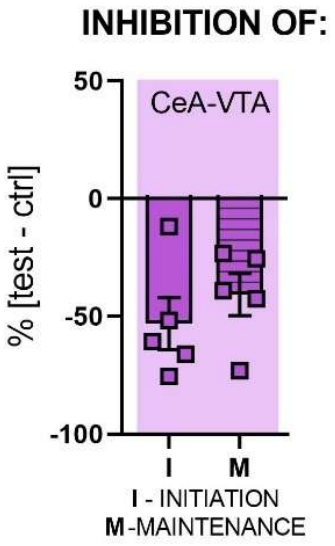

Fig. 36. Change in initiation and maintenance of social interaction shown as the percentage of baseline (test performed after 24-h social separation) during inhibition of the CeA-VTA projection. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$, ** $p < 0.01$.

Then, I compared the change in the Initiation and maintenance of social interaction to the baseline (measured after 24-h social separation) and to behavior of the partner rats. I found decreased Initiation of social interaction in rats with inhibited CeA-VTA projection compared to their partners. However, I also noticed a significant increase in Initiation of social contact in the partners as compared to the baseline **[Fig. 37,** Initiation of social contact: two-way ANOVA (test x group effect: $F(3,16) = 5.865$, $p=0.0067$) followed by Holm-Sidak post-hoc tests].

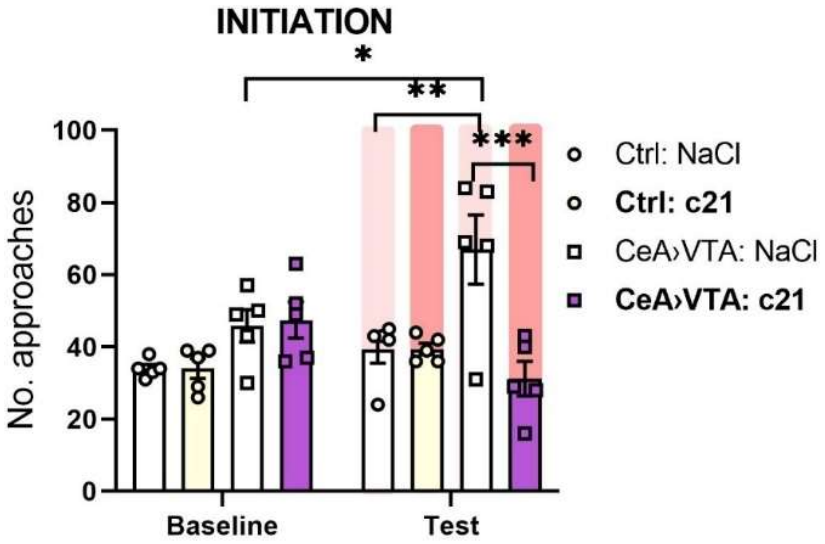

Fig. 37. Inhibition of the CeA-VTA affects partners' motivation to interact. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test. Baseline - social interaction after 24-h social separation. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

Next, I directly compared the number of approaches during the baseline and the test. The analysis revealed a decrease in social approach to the partners when the CeA-VTA projection was inhibited [**Fig. 38**, paired t-test $t(4)=3.3322$, $p=0.0291$].

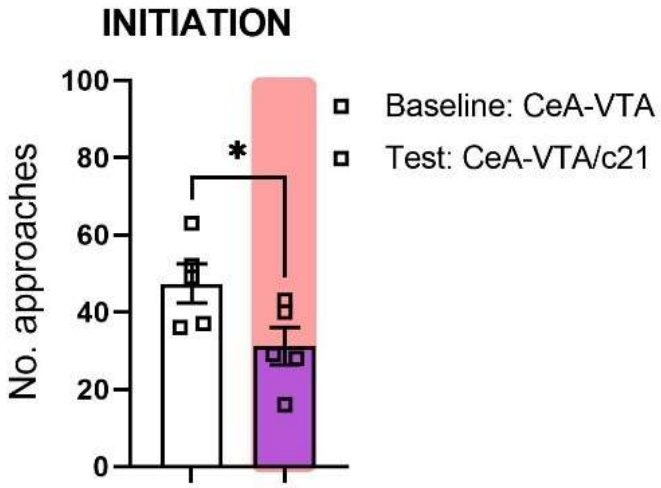

Fig. 38. Number of social approaches to the partner decreased during inhibition of the CeA-VTA pathway. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$.

Analysis of the maintenance of social interaction revealed that it is decreased during the CeA-VTA inhibition compared to the baseline [**Fig. 39,** Maintenance of social contact: two-way ANOVA (test x group effect: $F(3,16) = 6.220$, $p=0.0053$) followed by Holm-Sidak post-hoc tests].

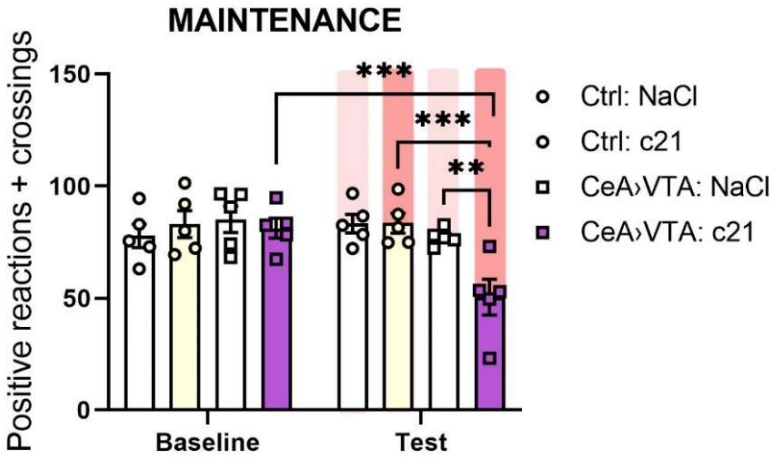

Fig. 39. Maintenance of social interaction is decreased in rats with inhibited CeA-VTA projection. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test. All data are shown as mean ± s.e.m., and symbols represent individual data points, ** $p < 0.01$,*** $p < 0.001$.

Inhibition of the CeA-VTA pathway did not affect the general activity level during the social interaction test **(Fig. 40).**

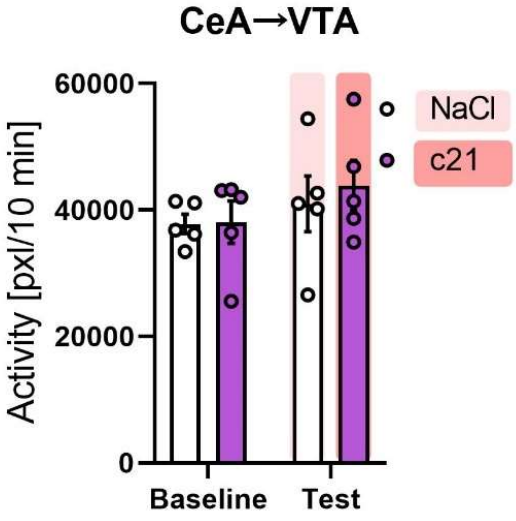

Fig. 40. Locomotor activity during the CeA-VTA inhibition. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test, B -baseline, T - test. All data are shown as mean ± s.e.m., and dots represent individual data points.

Further, inhibition of the CeA-VTA projection did not affect food reward motivation (**Fig. 41**).

Fig. 41. Number of lever presses for food during inhibition of the CeA-VTA pathway was not changed. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test, B – baseline, T – test. All data are shown as mean ± s.e.m., and symbols represent individual data points.

Further, I found that the cells in the VTA that receive projections from the CeA are predominantly GABA-ergic neurons **(Fig. 42, Methods Fig. 16**, paired t-test $t(4)=4.028$, $p=0.0158$); CeA-VTA: n=5).

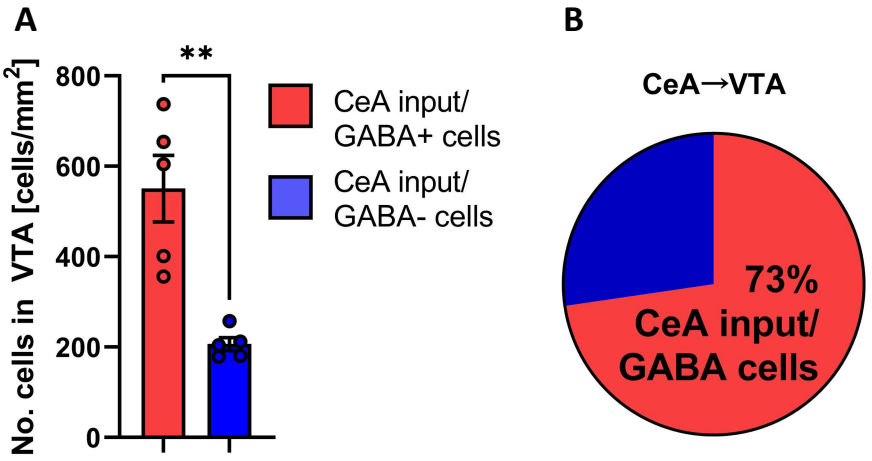

Fig. 42. The CeA-VTA projection mainly innervates GABAergic cells in the VTA. (A) The number of GABA-positive cells in the VTA receiving projections from the CeA (red) and the number of the other types of cells receiving projections from the CeA (blue). **(B)** Percentage of the GABA cells in the VTA that receive projections from the CeA. The data are shown as mean ± s.e.m., and dots represent individual data points, ** $p < 0.01$.

4.4. Role of the VTA-ACC/OFC dopaminergic projections in social interaction

Next, I studied the functional role of the projections from the VTA to the cortex focusing on the dopaminergic neurons (TH-positive) in the VTA that send projections to the ACC or the OFC. I chose the ACC and OFC based on the results of the HPLC analysis (described in section 3.7.4), in which I found changed levels of dopamine, noradrenaline, and their metabolites in these cortices after manipulation of the CeA social cells activity. Dopamine and noradrenaline largely overlap in domains such as shared biosynthetic pathway, convergent innervations, non-specificity of receptors and transporters, and shared intracellular signaling pathways (Ranjbar-Slamloo and Fazlali, 2020). Thus, I decided to investigate the dopaminergic projections to both, the ACC and OFC. To activate dopaminergic projection, I injected TH-Cre rats with AAV-frt-hM3D(gq) vector to the VTA and Cav-FlexFlp construct to the ACC or OFC. I activated the VTA-ACC/OFC

dopaminergic projections by DREADD agonist c21 delivered 30 min before the test (**Methods, Fig. 11, 15)**.

I observed that activation of either the VTA-ACC or VTA-OFC dopaminergic projections resulted in hypersociality. Activation of the VTA-ACC increased the initiation of social contact (**Fig. 43 A**). In contrast, activation of the VTA-OFC increased the behavior aimed at maintenance of social interaction as compared to behavior of the interaction partners (**Fig. 43 B**). Notwithstanding, the over-social behaviors during activation of the VTA-ACC/OFC projections led to the active blocking of social contact by the partners [**Fig. 43 C**, Initiation of social contact: one-way ANOVA (group effect: $F(5,32) = 5.362$, $p=0.0011$) followed by Holm-Sidak post-hoc tests; Maintenance of social contact: one-way ANOVA (group effect: $F(5,32) = 4.468$, $p=0.0044$) followed by Holm-Sidak post-hoc tests; Blocking of social contact: one-way ANOVA (group effect: $F(5,32) = 6.295$, $p=0.0004$) followed by Holm-Sidak post-hoc tests; Ctrl: NaCl/c21 n=6/6, VTA-ACC: NaCl/c21 n=6/6, VTA-OFC: NaCl/c21 n=7/7].

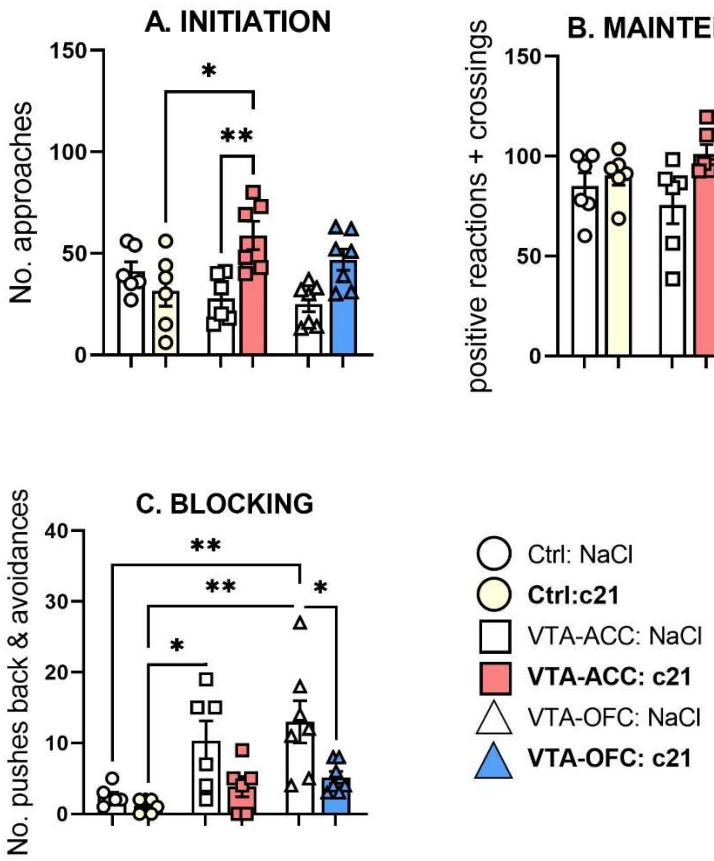

Fig. 43. Activation of the VTA-ACC and VTA-OFC dopaminergic projections affects social interaction. (A) Activation of the VTA-ACC pathway increases social approaches to the partner. **(B)** Activation of the VTA-OFC projection enhances contact maintenance. **(C)** Activation of both pathways results in the active blocking of social contact by the partners. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$, ** $p < 0.01$.

The within-subject comparison (with the baseline) confirmed an increase in initiating social contact during activation of the VTA-ACC pathway [**Fig. 44,** one-way ANOVA (group effect: $F(3,22) = 5.621$, $p=0.0051$) followed by Holm-Sidak post-hoc tests; Ctrl: c21 n=6, VTA-ACC: c21 n=6, VTA-OFC: c21 n=7].

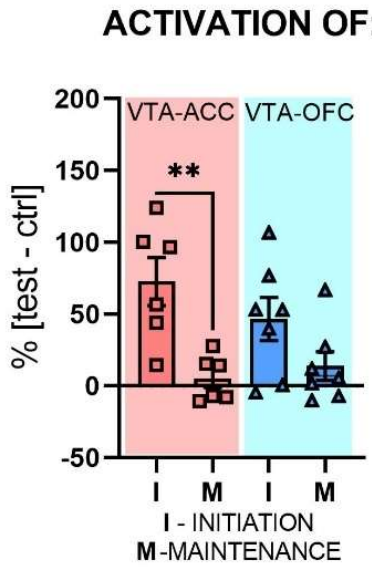

Fig. 44.Increase in initiation compared to maintenance of social interaction expressed as a percentage of baseline during activation of the VTA-ACC projection. All data are shown as mean ± s.e.m., and symbols represent individual data points, ** $p < 0.01$.

Further, comparison with the baseline and the partners' behavior confirmed an increase in social approaches during activation of the VTA-ACC dopaminergic projection. Also, activation of the VTA-OFC dopaminergic projection resulted in an increase in social approach as compared to the partners [**Fig. 45,** Initiation of social contact: two-way ANOVA (test x group effect: $F(5,32) = 5.119$, $p=0.0015$) followed by Holm-Sidak post-hoc tests].

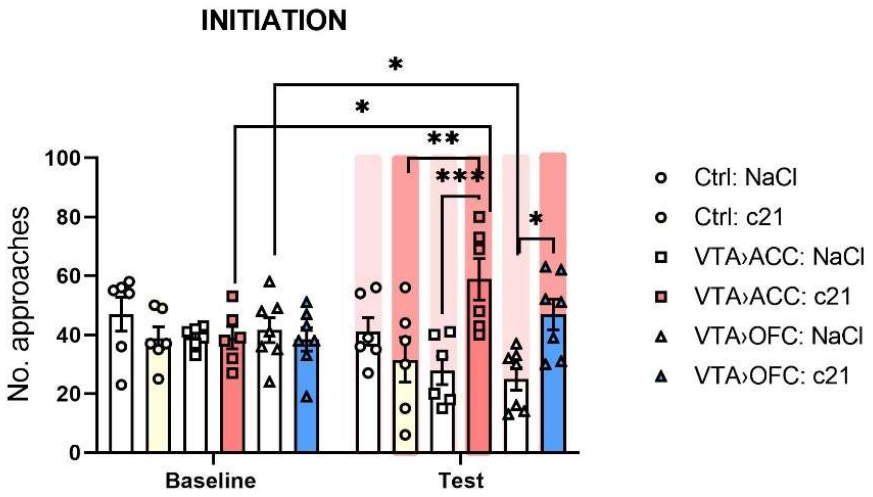

Fig. 45. Number of social approaches during social interaction when the VTA-ACC dopaminergic projection is activated increases compared to the baseline social interaction. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test. Baseline: social interaction after 24h of social separation. All data are shown as mean ± s.e.m., and symbols represent individual data points, * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

However, the direct comparison between the baseline and test in the VTA-OFC group did not show a significant difference [**Fig. 46,** paired t-test $t(6)=1.601$, $p=0.1604$].

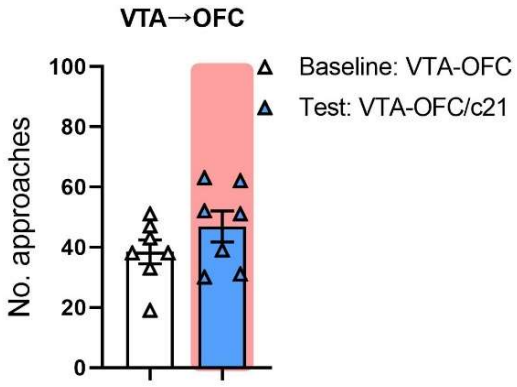

Fig. 46. Activation of the VTA-OFC dopaminergic projection did not increase the number of social approaches to the partner compared with the baseline. Baseline: social interaction after 24h of social separation. Pink background: rats injected with c21. All data are shown as mean ± s.e.m., and symbols represent individual data points.

Comparison of social interaction maintenance between the baseline and test revealed no differences **(Fig 47).**

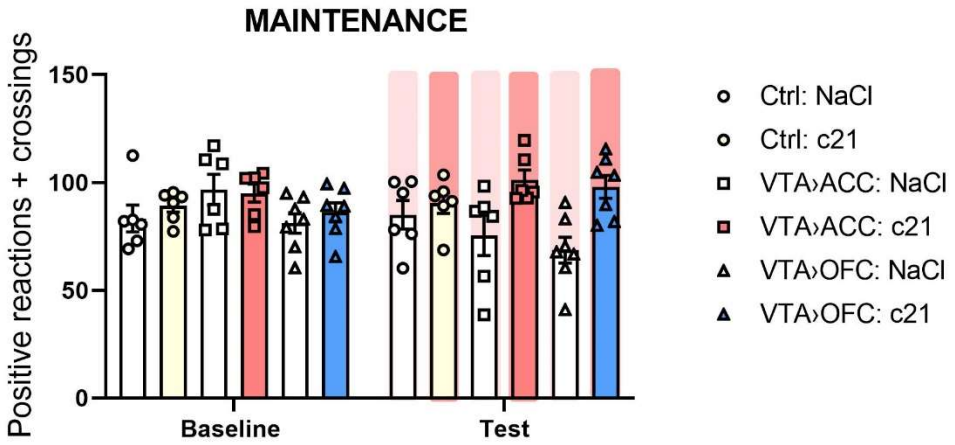

Fig. 47. Activation of the VTA-ACC or VTA-OFC dopaminergic projections did not affect the maintenance of social interaction compared to the baseline. Baseline: social interaction after 24h of social separation. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test. All data are shown as mean ± s.e.m., and symbols represent individual data points.

Notably, the partners of rats with activated dopaminergic VTA-ACC and VTA-OFC projections displayed freezing behavior during the test **[Fig. 48,** Freezing: VTA-ACC/c21 vs VTA-ACC/NaCl, Mann-Whitney test: p=0.012; VTA-OFC/c21 vs VTA-ACC/OFC, Mann-Whitney test: p=0.037].

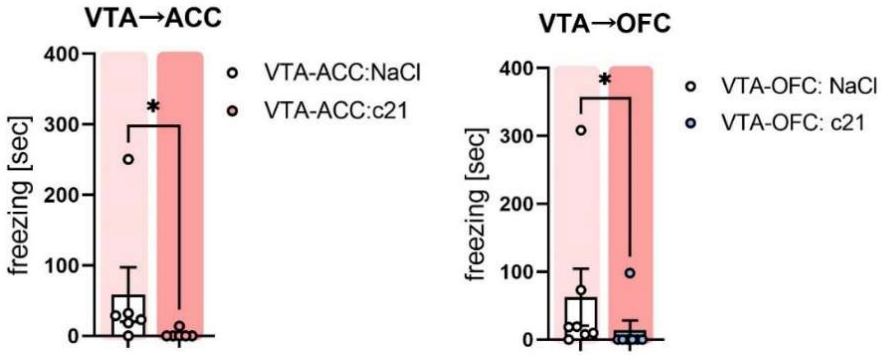

Fig. 48. Freezing recorded in the partners of the VTA-ACC and VTA-OFC rats during the test. The white bar present level of freezing of the partners of the tested rats. All data are shown as mean ± s.e.m.,and symbols represent individual data points, * $p < 0.05$.

Activation of neither the VTA-ACC nor VTA-OFC affected general locomotor activity during social interaction (**Fig 49).**

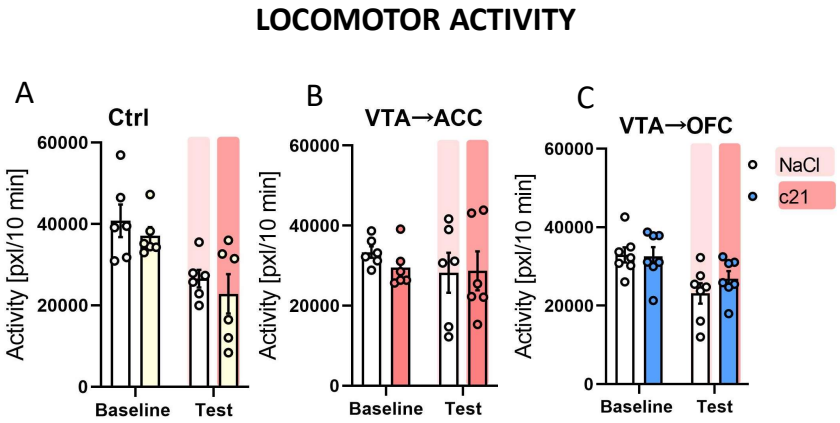

Fig 49. General activity of rats measured after 24h of social separation (Baseline) and after 21-day separation (Test) when the VTA-ACC or VTA-OFC dopaminergic projections were activated. Pink background: rats injected with c21 before the test, light pink background: rats injected with NaCl before the test, B - baseline, T - test. All data are shown as mean ± s.e.m., and symbols represent individual data points.

Further, activation of neither the VTA-ACC nor VTA-OFC affected food motivation (**Fig 50**).

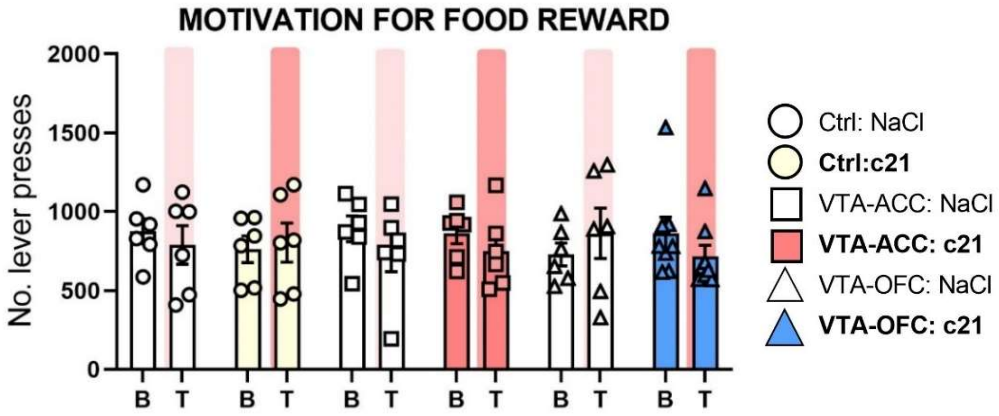

Fig. 50. Number of lever presses for food during activation of the VTA-ACC and VTA-OFC pathways. Pink background: rats injected with c21 before test, light pink background: rats injected with NaCl before test, B - baseline, T - test. All data are shown as mean ± s.e.m., and symbols represent individual data points.

5. Discussion

In this study, I utilized projection-specific and neuron-specific manipulations to investigate the role of the ACC/OFC-CeA-VTA-ACC/OFC circuitry in social interaction. Positive social interaction requires the motivation to initiate social contact, the capacity to transfer social information, and the ability to interpret social cues. For this reason, I have separated social interaction into initiation (i.e., the motivation for approaching a partner) and maintenance (positive responses to the partner's attempts to initiate contact). In addition, I implemented the third category, blocking, to illustrate aversion or unwillingness to engage in social interaction. It accounted for the number of social interactions avoided in response to the partner initiating social interaction. Also, I investigated the selectivity of social neuronal circuits by testing their role in the motivation for a food reward using a progressive ratio lever pressing test.

I demonstrated that manipulations of particular projections selectively affect the initiation and/or maintenance of social contact. I asserted that the OFC-CeA, CeA-VTA, and VTA-ACC projections are involved in initiating social interaction, while the ACC-CeA, OFC-CeA, and CeA-VTA projections are involved in maintaining social interaction. Moreover, I demonstrated that the activity of CeA-VTA projection, and dopaminergic VTA-ACC/OFC projections mediate social interaction but not food motivation. My findings describe the novel neural circuitry of the social brain, distinguishing between circuits underlying motivation to initiate social contact and capacity to maintain social interaction. In addition, I showed that the identified social circuitry only partially overlaps with the previously described food reward circuitry.

5.1. Experimental model

As I wanted to study the social interaction and its components, I chose rats as a model because they have a shorter latency to start social interaction and a higher total frequency of interactions compared to mice (Ellenbroek and Youn, 2016). I used a 21-day social separation to increase rats' motivation to interact and decrease the variability of their response (Hamed et al., 2015). In the optogenetic experiments, in which I used *c-fos* dependent constructs, the 21-day separation

period allowed for not disturbing rats for a time sufficient to achieve stable expression of the construct at the same time maintaining very low basal level of *c-fos* expression, as confirmed in the control groups. For consistency, all other animals were separated for the same amount of time. In each experiment I compared social behavior of rats after 24-hour and 21-day separation and I did not observe a decrease of social interest during social interaction after the prolonged separation.

Importantly, social separation for up to 21 days does not induce depressive-like behavior in rats (Gorlova et al., 2018). In my experiments, upon reuniting with a partner increased production of 50-kHz USVs, which are a marker of positive affective states in rats. In line, it has also been shown that reunion after the 21-day separation activates the mesolimbic reward system, comparing to non-separated animals (Brudzynski, 2013; Hamed et al., 2015; Knutson et al., 1999).

5.2. ACC-CeA and OFC-CeA pathways inhibition disturbs social interaction

In my studies, I focused on the networks identified earlier by Bickart et al. (Bickart et al., 2014) as important for social interaction in humans, including the amygdala, ACC, and OFC. This study showed that the ACC is a part of the affiliation network, while the OFC is involved in the perception of social stimuli. In animals, it has been shown that the brain structures that mediate positive social interaction include the ACC and OFC (Guo et al., 2019; Hung et al., 2017; Jennings et al., 2019), but the role of their connections with other parts of the reward system in mediating social interaction and its components was unknown.

I demonstrated that the ACC and OFC are functionally connected to the neural circuits of the CeA during social contact or lever pressing for food. In addition, I found that the ACC innervates more social cells in the CeA than the OFC, while the food CeA cells receive the similar number of inputs from the ACC and OFC, which indicates that the CeA social cells receive different input from the cortex than the CeA food cells. This finding also suggests that the ACC-CeA projection may have a more extensive role in social behavior modulation. However, the weaker connectivity between the OFC and CeA may be also attributed to the

anterograde tracer injection site in the OFC. The tracer was injected to the ventral part of the OFC that is less connected with the CeA than the medial part of the OFC. The mOFC targets the amygdala, VTA, and substantia nigra, whereas the vOFC is more connected to the ACC, sensorimotor, and temporal cortices (Hoover and Vertes, 2011). The connectivity pattern suggests the mOFC is involved in goal-directed behavior and the vOFC in directed-attention decisions (Hoover and Vertes, 2011). Thus, the further studies are needed to fully understand the observed difference.

Social interactions are a form of an incentive reward, and their rewarding properties depend on social contact reciprocity. Successful social interaction requires motivation to approach a partner, and coordinated responses to partner's approach (Chevallier et al., 2012; Trezza et al., 2011). To examine motivation to initiate social contact and the ability to maintain social interaction, I segmented the social behavior of rats into components including active approaches to the partner and the capacity to respond to the partner's attempts to initiate contact.

I found that inhibition of the ACC-CeA projection disrupts social interaction maintenance. The inability to maintain interaction with a partner combined with an increase in blocking behavior in response to the partner's approach suggest impairments in social information processing. The ACC has been implicated in experience-based decision-making (Rudebeck et al., 2008; Yamagishi et al., 2020), processing emotional reactions of others (Carrillo et al., 2019; Hernandez-Lallement et al., 2020; Lamm et al., 2011), and social distress (Eisenberger et al., 2003). In particular, impairments of the ACC-amygdala connection has been linked to high levels of social inhibition and the tendency to avoid social stimuli and withdraw from social contacts in humans (Blackford et al., 2014). According to my findings, the ACC projection to the CeA is crucial for efficient processing of social information required to maintain social interaction.

As the ACC-amygdala projection has been shown to be involved in the interpretation of aggression signals sent by facial expressions of others (Passamonti et al., 2008), inhibition of this projection could also result in an inability to distinguish between positive and negative social signals. Thus, the behavioral changes accompanying inhibition of the ACC-CeA projection might result either from an inability to understand social information or interpreting partner's signals

as negative. However, as I observed that social motivation did not change, it is unlikely that rats interpreted their partners' behavior as threatening. In contrast, an increase in blocking social interaction in response to a partner's attempt to initiate it may indicate coping with the ambiguous situation. Thus, the former explanation seems to be more likely.

Importantly, I observed only deficiencies in maintaining social contact, whereas the social approach has remained unchanged. These findings suggested that the projection from the ACC to the CeA modulates recognition and interpretation of social signals, but not motivation to start the interaction. These findings show that the initiation and maintaining social interaction indeed differ at the neural level.

My results show that inhibition of the OFC-CeA projection has a more profound impact on social interaction than inhibition of the ACC-CeA projection, as it reduced both the initiation and maintenance of social interaction and increased the blocking of social contact. The OFC is the region involved in forming stimulus-reward associations and reward value estimation (Hong et al., 2019). The OFC regulates emotions evoked by rewards facilitating adaptation to unpredictable situations (Kringelbach, 2005; Reekie et al., 2008). Thus, decreased motivation to initiate social contact and problems with its maintenance that I observed in rats with inhibited OFC-CeA pathway resonate well with the role of the OFC in the reward system. The manipulation of the OFC-CeA pathway might affect the social reward processing.

Consistently with my results showing involvement of the OFC-CeA pathway in social interaction, inactivation of the OFC increases impulsive aggression (Kuniishi et al., 2017). Further, individuals with ASD who lack knowledge about appropriate social behaviors show a deficit in the OFC activation (Weston, 2019). In particular, ASD subjects showed a reduction in the OFC-amygdala connectivity during resting-state imaging (von dem Hagen et al., 2013). Rodent research shows that disruption of the OFC-amygdala connection maturation may result in social anxiety (Li et al., 2021). Together, these studies suggest involvement of the OFC-amygdala connection in regulation of social behavior, which is consistent with my results.

To investigate the specificity of the ACC/OFC-CeA circuits in social interaction, I tested their role in food motivation, using operant responding under a progressive ratio reinforcement schedule. Both the ACC and OFC regions have been implicated in reward decision-making (Aly-Mahmoud et al., 2017; Hart et al., 2020, 2017; Hong et al., 2019; Rudebeck et al., 2006; Zhong et al., 2017). Single-photon imaging of the ACC revealed that ACC neurons are more active when food rewards are presented with alternatives, i.e., when the alternative food reward was available (Hart et al., 2020). Deactivation of the ACC decreased lever pressing for sucrose reward when an alternative food reward was present. The results indicate that the ACC may play a crucial role in discerning the use of various options in effort-based decision-making (Hart et al., 2020, 2017). Similarly, activation of the OFC neurons depends on reward value (Hong et al., 2019). Together, these results show that both the ACC and OFC regions play an important role in food motivation. However, it was unknown whether their projections to the CeA, essential for social interaction, are also involved in food motivation. My findings demonstrate that inhibiting either projection reduces lever pressing for food reward, implicating the ACC/OFC-CeA pathways in food motivation. Interestingly, inhibition of the ACC-CeA projection did not affect motivation to start social interaction but diminished food motivation. However, as the experimental protocols were not designed to directly compare social and food motivation, and the latter involved instrumental learning, I will not compare the involvement of the ACC-CeA projection in social and non-social motivation here.

Together, my data suggest that the ACC-CeA and OFC-CeA circuits are involved in social interaction as well as pursuit and consumption of food reward (Fig. 51).

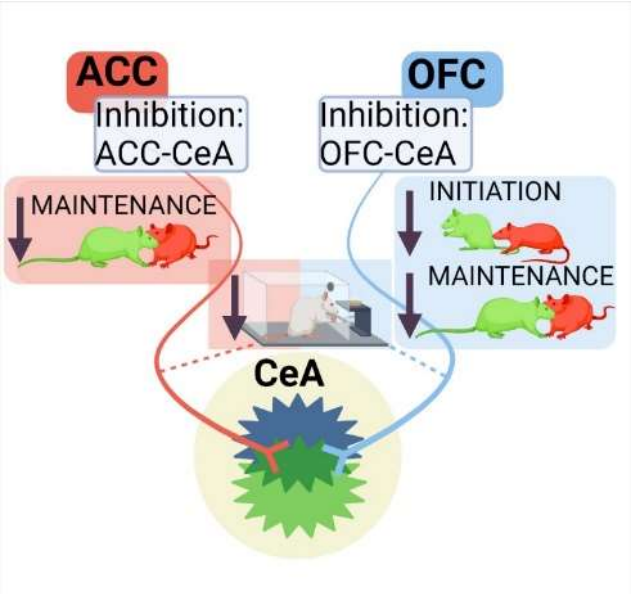

Fig. 51. Ilustration of the ACC-CeA and OFC-CeA projections' role in controlling initiation and maintenance of social contact.

5.3. Optogenetic manipulation of CeA social cells activates VTA, ACC, and OFC

As the ACC-CeA and OFC-CeA pathways appeared to be involved both in social interaction and food motivation, next I asked the question whether the CeA circuits and their downstream targets for social interaction and food motivation also overlap. The CeA is an important part of the reward system and is involved in processing of both food and drug rewards (Douglass et al., 2017; Knapska et al., 2013; Warlow et al., 2020), and social information (Andraka et al., 2021; Ferretti et al., 2019). Also, the amygdala has long been thought of as a component of the "social brain," and abnormalities in this area have been associated with deficiencies in social behavior (Baron-Cohen et al., 2000). However, the structure is heterogeneous, with a cortex-like basolateral part and a striatum-like part that includes the CeA. Previous research found that basolateral projections inhibit social interaction (Elorette et al., 2020; Felix-Ortiz and Tye, 2014). On the other hand, less was known about which circuits promote social contact.

First, I determined that both social interaction and food-motivated lever pressing activates the CeA, as measured with c-Fos expression. Next, I used *c-fos*-dependent genetic constructs that enabled me to express opsins in the activated, *c-fos*-expressing neurons (Andraka et al., 2021). Inducing their expression in the

social and food cells in the CeA allowed to investigate their functional role. The results show that the social and food CeA cells overlap only partially. Whereas inhibition of the social cells inhibited motivation for food, activation of the social and food cells had the opposite effects.

These findings may indicate that social and food-related information is processed in the CeA in a hierarchical manner. As often social interaction is more urgent than food consumption, social cells may inhibit food cells. This hypothesis is consistent with the findings in the OFC showing that indeed social cells can inhibit feeding behavior (Jennings et al., 2019). The previous studies revealed two non-overlapping populations of the CeA neurons that control feeding behavior. Activation of the neurons expressing serotonin receptors increases food intake, whereas activation of the PKCδ-positive neurons decreases food intake (Cai et al., 2014; Douglass et al., 2017). Thus, taking into account my results, PKCδ-positive cells might be involved in inhibition of feeding and promotion of social interaction. However, the previous study from the laboratory showed that these neurons are active when an animal witnesses fear conditioning of a partner, which inhibits its activity, in particular social interaction (Andraka et al., 2021). As I did not investigate the neuronal markers of the social and food CeA cells in my experiments, this is the hypothesis that requires further studies. Importantly, photoactivation of the CeA cells activated by aversive social interaction had no effect on food motivation, suggesting that not only stimulus type (social vs. non-social) but also stimulus value (positive vs. aversive) is important for the effect.

Next, to identify the brain structures targeted by the CeA social but not food cells I mapped neurotransmitters and neuromodulators release in different brain structures of the rats after the CeA social and food cells photoactivation and photoinhibition. The results were consistent with the notion that these populations overlap only partially. They helped me to identified the target structures of the CeA social cells, which include the VTA, ACC, OFC, and PFC. In my further, functional studies I focused on the CeA-VTA-ACC/OFC circuits.

One interesting observation was a decrease in the level of GABA in the VTA after the activation of social cells in the CeA. The involvement of VTA DA neurons in signaling social prediction error has been suggested to be a crucial neural substrate for social learning and social interaction (Solié et al., 2022). GABAergic

interneurons provide inhibitory synaptic inputs to dopaminergic neurons, modulating their activity and dopamine secretion (Morales and Margolis, 2017). Increased levels of GABA in the VTA may lead to disrupting the perception of interval timing and impair reward-seeking, as well as induce conditioned place aversion (Bouarab et al., 2019; Shields et al., 2021; Tan et al., 2012). My findings suggest that regulation of DA activity in the VTA by the CeA inputs plays an important role in social interaction.

Another potentially interesting circuit that was suggested by these experiments included the inhibitory neurons in the basolateral amygdala (BLA). Activation of the CeA social but not food cells decreased GABA levels in the BLA. Taking into account an important role of the BLA in social behavior (Elorette et al., 2020; Felix-Ortiz and Tye, 2014), local inhibitory circuits in this structure are likely to be an important regulator of social interaction (Ehrlich et al., 2009). However, the CeA has a very sparse, if any, direct projection to the BLA, thus the observed effects are likely involving other brain pathways, and thus I did not further investigate them in my studies.

The other part of the amygdala that has not been investigated in my studies but is potentially important for social interaction is the medial amygdalar nucleus. The medial amygdala-hypothalamus circuit has been recently linked to social reward (Hu et al., 2021). Because the CeA receives projections from the medial amygdala (Pitkänen et al., 2006), the CeA may integrate information flowing from the cortex and medial amygdala.

The majority of previous research on the CeA circuits focused on neuronal populations identified by the molecular markers they express. This method revealed neuronal circuits that are mutually connected and control specific, and often opposing behaviors (Fadok et al., 2018). However, unlike inputs and outputs, markers rarely define the function of the neuronal population. Notably, marker-defined populations are frequently heterogeneous and can control various behaviors (Fadok et al., 2018). Thus, my study defined cell populations based on their functional connectivity rather than the markers they express (Fig. 52).

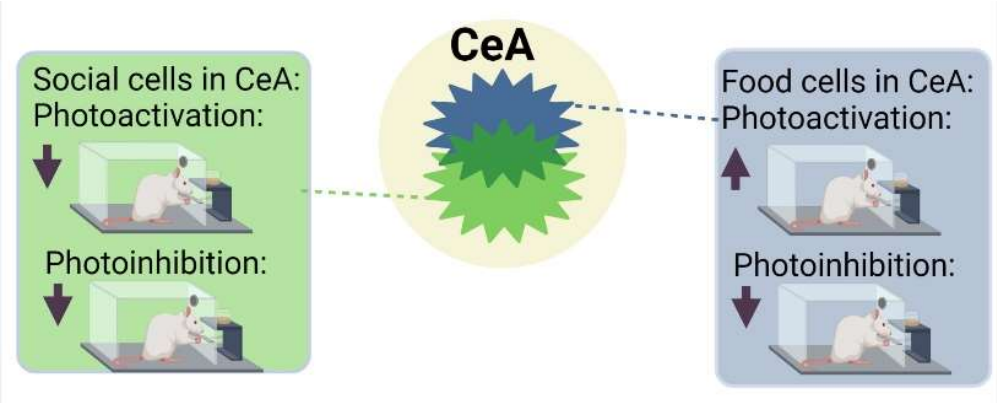

Fig. 52. Effect of social and food CeA cells activation and inhibition on food motivation.

5.4. CeA-VTA, VTA-ACC, and VTA-OFC projections are critical for initiation and maintenance of social interaction

The VTA is the primary source of dopamine in the brain, which plays a crucial role in a variety of motivated behaviors, including social interaction (Bariselli et al., 2018, 2016; Solié et al., 2022). The activation of discrete VTA circuits, comprising DAergic and GABAergic populations, is responsible for generating approach and avoidance behaviours (Bouarab et al., 2019; Lammel et al., 2014, 2012; Shields et al., 2021; Tan et al., 2012). I discovered that inhibition of the CeA neurons projecting to the VTA disrupts social interaction. These neurons innervate primarily GABA-ergic VTA cells. It has been earlier shown that the VTA GABA-releasing neurons are activated by cues signaling the absence of reward (Root et al., 2020), and diazepam, a GABA agonist, injection into the VTA increases social competition in non-familial rats (van der Kooij et al., 2018). As the projections from the CeA are mostly inhibitory, inhibition of the GABA-ergic cells in the VTA most likely disinhibits dopaminergic cells promoting social interaction. The hypothesis about the inhibitory nature of the CeA-VTA projection is consistent with the results of neurotransmitters release discussed in the previous section, which showed that activation of the CeA social cells results in a decrease of GABA in the VTA. It is thus plausible that facilitation of social interaction is achieved through disinhibition of the dopaminergic cells.

Rats with inhibited projection from the CeA to the VTA performed fewer social approaches and had reduced ability to maintain social contact. In addition, they blocked their partners' attempts to initiate social interaction. Their behavior also affected behavior of their partners, who more often initiated social contact. In addition, food reward motivation was unchanged in animals with inhibited CeA-VTA pathway. This finding is consistent with the lack of change in GABA level in the VTA after photoactivation of the CeA food cells.

The disparate effects of the CeA-VTA pathway inhibition on social interaction and food motivation show that the underlying neural circuitry is, at least to some extent, different. As social withdrawal is one of the most common symptoms of depression (Häfner et al., 2005), which also involves changes in non-social reward processing, understanding the causes of different symptoms may help to develop better treatments. Perception of social interaction as rewarding is crucial for the development of the social brain (Gunaydin et al., 2014). The dark side of rewarding nature of social interaction is that it can contribute to the high rate of using drugs which increase motivation to establish and maintain social contacts. For instance, drugs with a high affinity to the dopamine transporter (DAT) increase the positive effects of social contact (Sharp and Smith, 2022).

My data show that increased activity of the VTA-ACC dopaminergic projection enhances initiation of social contacts, even if the partner displays avoidance or aversion. These findings show the role of the VTA-ACC projection in social behavior, in particular in initiating social contact. Earlier studies demonstrated that lesion of dopaminergic terminals in the ACC significantly reduces conditioned place preference produced by intra-VTA injection of u-opioid receptor agonist (Narita et al., 2010), suggesting the role of the VTA-ACC projection in reward processing. The VTA releases DA in response to social stimuli, and the magnitude of DA release is proportional to the social contact duration (Gunaydin et al., 2014). Thus, the VTA provides a bottom-up modulating signal to the ACC, which may be involved in stimuli evaluation and decision making (Elston et al., 2019, 2018).

Repeated initiation of social contact, despite the symptoms of distress of the interaction partner (visible e.g., as freezing), suggests that the VTA-ACC also plays a role in processing social information. These findings are consistent with the

observation that the ACC dysfunction leads to diminished sensitivity to others' pain (Allsop et al., 2018; Carrillo et al., 2019; Hernandez-Lallement et al., 2020).

Further, my data show that activation of the VTA-OFC dopaminergic projection enhances maintenance of social contact regardless of the partners' behavior which signaled high levels of stress (e.g., via freezing). Consistently with this result, the OFC has been implicated in adaptation to unpredictable dynamic events (Sarlitto et al., 2018; Schoenbaum et al., 2009), such as social interaction in which fast responses that are well-aligned to the partner's behavior are required. In particular, the OFC dopamine neurons have been shown to signal prediction errors (Takahashi et al., 2009). Further, the aberrant dopamine signaling in the OFC has been associated with social anxiety disorder (Plavén-Sigray et al., 2017), characterized by impaired processing of social stimuli.

Activation of neither the VTA-ACC nor VTA-OFC dopaminergic projections induced aggressive behavior toward the partners. However, even when partner rats signaled that the interaction is aversive for them and tried to avoid social contact, the rats with activated VTA-ACC or VTA-OFC dopaminergic projection did not stop their attempts to maintain social contact. Interestingly, in contrast to partners of rats with inhibited ACC/OFC-CeA projections, partners of rats with inhibited CeA-VTA projection were more likely to initiate social interaction. This result suggests that social impairments have different effects on interaction partners, depending on their nature, such as withdrawal from social contact or hypersociality.

Activation of the VTA-ACC or VTA-OFC dopaminergic projection did not affect food motivation, which suggests their primary role in modulation of social contacts. Social stimuli processing has been shown to involve DA receptors in the ACC and OFC (Kim et al., 2014; Plavén-Sigray et al., 2017). Notably, the DA level had been increased to social but not to non-social stimuli (Sotoyama et al., 2022). In line with these findings, specific stimulation of the dopaminergic VTA-ACC or VTA-OFC projections enhanced social interaction, revealing their crucial role in modulating social behavior. However, the successful social interaction involves reciprocal contact with other individuals and requires well-orchestrated responses from interaction partners. Overactivation of the dopaminergic projections to the ACC and OFC apparently disrupted processing of social information and, as a result, social interaction. Unpredictable behavior and insensitivity to social signals

in rats with activated dopaminergic projections resulted in stress and aversion to social interaction in their interaction partners.

In sum, my research demonstrated that specific neuronal circuits initiate social contact (CeA-VTA, VTA-ACC), while others join when the partners are already interacting or when the interaction partner initiates the contact (CeA-VTA, VTA-OFC). According to the results, different neuronal circuits are sequentially involved, and their proper function is required to elicit a response from the interaction partner. In addition, I demonstrated that destabilization in neuronal projections involved in the initiation and/or maintenance of social interaction could influence the blocking of social contact in tested animals and their interaction partners. Moreover, I demonstrate that manipulating specific projections (CeA-VTA-ACC/OFC) disrupts the ability to engage in positive social interaction without altering the motivation for food reward, revealing these projections' significant and selective role in social behaviors (Fig. 53).

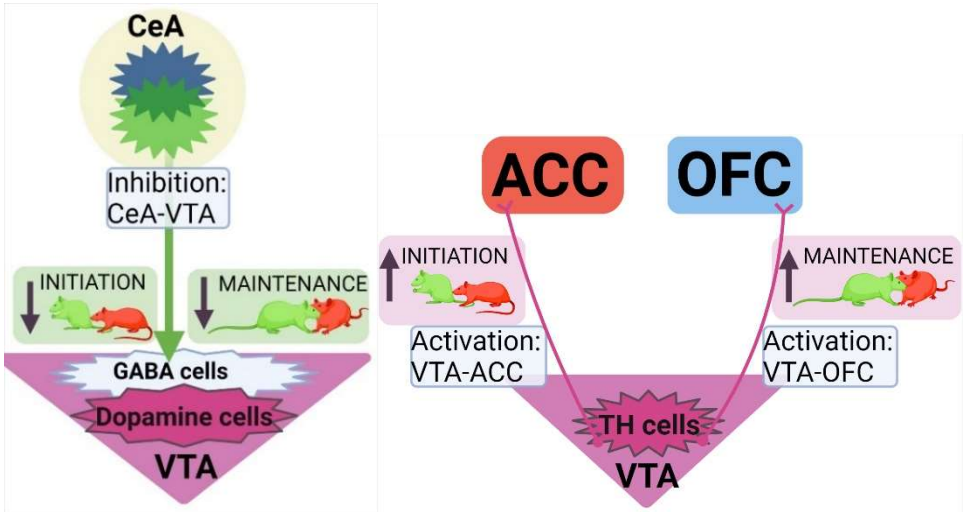

Fig. 53. Role of the CeA-VTA-ACC/OFC circuitry in initiation and maintenance of social contact.

5.5. Limitations and future studies

My research is a first attempt to characterize the role of the cortical-CeA-VTA circuits in social interactions. My results encourage further investigations but many unanswered questions remain. For instance, we do not know whether the same neuronal circuits are involved during initiation and maintenance of social interaction with an unfamiliar rat or among female rats. Also, to obtain complete picture of the neuronal circuits involved, more precise description of the neurons involved is needed. The latter includes the dynamics of their activity, as well-coordinated activation of different circuits is most likely necessary for successful social interaction.

To obtain a basic understanding of the role of the cortical-CeA-VTA circuits in social interaction, I focused on interaction of freely moving animals. Additional experiments, testing for sociability or social motivation, e.g., using three-chamber sociability test, instrumental conditioning with a social reward, or place preference test could provide additional information about the role of the cortical-CeA-VTA circuits in social behavior. Interestingly, inhibition of the ACC/OFC-CeA projections affected both social and food-related behaviors, while modulation of the activity of the CeA-VTA-ACC/OFC projections changed only social behavior. Nonetheless, it remains unclear whether the same neuronal populations are involved in control of social and food related behaviors. As I blocked the projecting neurons in a manner non-specific to their behavioral involvement, it is possible that these projecting neurons contain different subgroups involved in social and food related behaviors. This problem requires further studies.

Blocking of the dopaminergic VTA-ACC projection increased initiation of social contact but the partner rats tried to avoid interaction. I hypothesize that rats with blocked VTA-ACC projection had problems with interpreting social signals emitted by their partners and adjusting their responses accordingly. However, additional tests are needed to understand this phenomenon. Such tests could assess, e.g., fear contagion or social buffering which rely on interpretation of partner's signals.

Finally, I tested the role of the cortical-CeA-VTA circuits in social interaction and motivation for a food reward measured in the progressive ratio level

pressing test. To systematically compare the role of these circuits in social and food related behaviors, additional experiments are needed. They should test the role of the cortical-CeA-VTA circuits in social motivation using behavioral tests employing instrumental responses, and in food consumption using test measuring food intake.

6. Summary and conclusions

The previous studies did not address the problem of the neuronal circuits underlying the initiation and maintenance of social interaction. I demonstrated that the OFC-CeA, CeA-VTA, and VTA-ACC projections are involved in the initiation of social interaction, while the ACC-CeA, OFC-CeA, and CeA-VTA, projections are involved in its maintenance. These findings describe novel neural circuitry in the social brain and support my hypothesis that initiation and maintenance of social interaction recruit different neuronal circuits.

Previous studies linked subpopulations of neurons within the CeA to pursuing and consuming food rewards (Douglass et al., 2017; Hardaway et al., 2019; Knapska et al., 2013; Robinson et al., 2014). In addition, it has been demonstrated that optogenetic activation of the CeA increases the motivation to pursue food and drug rewards (Robinson et al., 2014; Warlow et al., 2017) or induces maladaptive attraction to painful stimuli (Warlow et al., 2020). I showed that the neuronal population in the CeA that mediates social interaction is distinct from the neuronal population involved in food motivation. In particular, these populations are distinct in their functional connectivity with other brain structures as I observed by mapping the neurotransmitters release after manipulating their activity. Intriguingly, activation of the CeA population involved in social interaction diminishes food motivation, which suggests that these populations are interrelated functionally.

The specific neuronal circuits involved in social interaction and food motivation were previously unexplored. I showed that manipulating the CeA-VTA-ACC/OFC projections disrupts the ability to initiate and maintain positive social interaction. However, the manipulation does not change motivation for food reward, suggesting its selective role in social behavior. In sum, my results describe projection-specific

engagement of neuronal populations involving the ACC, OFC, CeA, and VTA in initiation and maintenance of social interaction and show that these circuits only partially overlap with the food reward system (Fig. 54).

Conclusions:

1. Different neuronal circuits underly social interaction initiation and maintenance.
2. Social interaction and motivation for food reward recruit different populations of cells in the CeA.
3. Manipulation of some of the tested neuronal circuits affects social interaction but not motivation for food reward suggesting that the circuits are specific to social behavior.

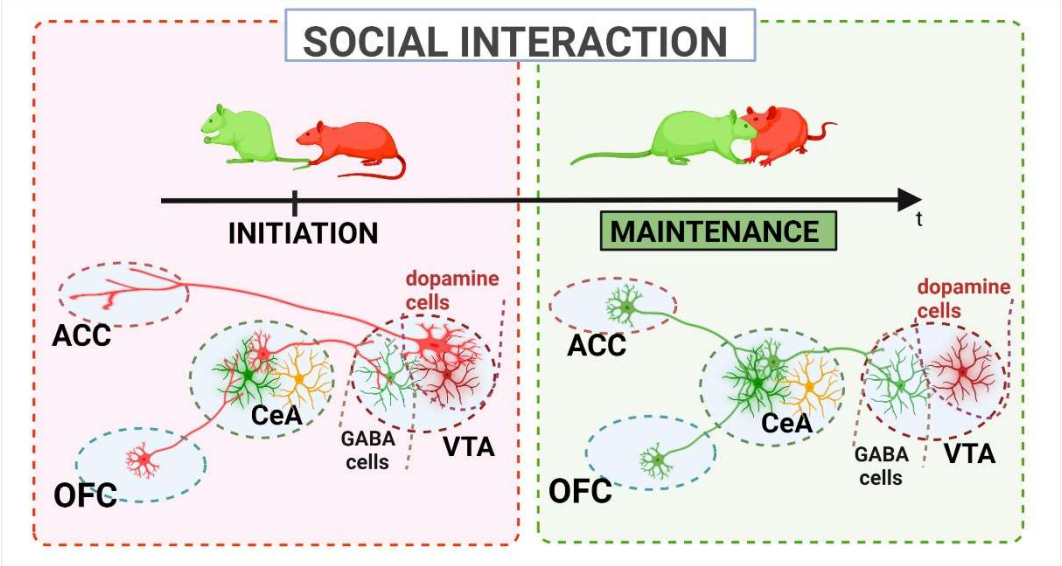

Fig. 54. Graphical summary of the ACC/OFC-CeA-VTA-ACC/OFC projections role in the initiation and maintenance of social interaction

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