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Characterization of central amygdala circuits activated by social transfer of fear

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Abstract

Emotional contagion is a process of sharing affective reactions as a result of observation. Typically it is studied in dyads, in which one of the subjects, called 'demonstrator', reacts to emotionally relevant stimuli – for example with expression of fear if the stimuli signal threat. The second subject (called 'observer'), although does not receive similar stimulation, through observation mimics reactions of the demonstrator on behavioral and physiological levels. Currently the most popular explanations of this phenomenon are formulated in the 'shared circuits' framework. The common assumption of this group of explanations is that observing emotional reactions activates similar brain structures to the ones activated in demonstrators. On the behavioral level this results in mimicry, that is, automatic copying of demonstrators' behavior. In this thesis an animal model of emotional contagion was used to verify the claims formulated by the 'shared circuits' framework.

In the model of emotional contagion, the observer rats displayed freezing reaction, similarly as the demonstrators subjected to aversive somatosensory stimulation. Time course analysis did not detect synchronization between freezing of demonstrators and observers. In other words, no evidence for behavioral mimicry were found. On the neuronal level, the central amygdala circuits activated by the fear contagion were characterized on the level of molecular markers, anatomical connections and behavioral function. The collected data was used to verify the hypothesis that social transfer of fear activates similar neural circuits as the ones which control freezing in single animals subjected to aversive stimulation. Double immunostainings did not detect in observers high co-localization of c-Fos protein with protein kinase Cδ nor with corticotropine releasing factor. Optogentic reactivation of the central amygdala cells which were previously activated by the social transfer of fear provoked passive defensive responses (including freezing), but did not influence social behaviors. Active projections mapping demonstrated that the central amygdala cells activated during social transfer of fear most probably receive input from basolateral amygdala. Furthermore, chemogenetic inhibition of this projection increased active behaviors executed by rats during novel environment exploration. To sum up, the results indicate that social transfer of fear activates similar, but not exactly the same circuitry as first-hand aversive experience.

Abstract in Polish

Społeczny transfer emocji (ang. *emotional contagion*) to proces polegający na współdzieleniu reakcji emocjonalnych w wyniku obserwacji. Typowo bada się go w parach, gdzie jeden z osobników, nazywany „demonstratorem", reaguje na nacechowane emocjonalnie bodźce - na przykład ekspresją strachu, jeśli bodźce sygnalizują zagrożenie. Drugi osobnik (czyli „obserwator") chociaż sam nie otrzymuje podobnej stymulacji, zaczyna w wyniku obserwacji naśladować reakcje demonstratora na poziomie behawioralnym i fizjologicznym. Obecnie najbardziej popularne wyjaśnienia tego zjawiska są formułowane w nurcie „wspólnych obwodów" (ang. *shared circuits*). Wyjaśnienia te zakładają, że obserwacja emocjonalnych reakcji wywołuje aktywację struktur mózgu podobnych do tych, które są aktywne u demonstratorów. Na poziomie behawioralnym prowadzi to do mimikry, to znaczy automatycznego kopiowania zachowań demonstratora. W niniejszej rozprawie posłużono się zwierzęcym modelem społecznego transferu emocji w celu weryfikacji hipotez stawianych w nurcie wspólnych obwodów.

Model społecznego transferu strachu pozwolił zaobserwować u szczurów-obserwatorów reakcję zamierania, podobną do tej wykonywanej przez demonstratorów otrzymujących awersyjne bodźce czuciowe. Analiza przebiegu zachowań w czasie nie potwierdziła synchronizacji pomiędzy zamieraniem demonstratorów i obserwatorów – innymi słowy, nie znaleziono dowodów na występowanie mimikry behawioralnej. Na poziomie neuronalnym zbadano obwody neuronalne w jądrze środkowym ciała migdałowatego aktywowane przez społeczny transfer strachu. Scharakteryzowano je pod względem markerów molekularnych, połączeń anatomicznych oraz funkcji w celu weryfikacji hipotezy, że społeczny transfer strachu aktywuje podobne obwody neuronalne do tych, które kontrolują reakcję zamierania u zwierząt poddanych bezpośrednio awersyjnej stymulacji. Podwójne barwienia immunohistochemiczne nie potwierdziły u obserwatorów wysokiego współwystępowania białek c-Fos z kinazą białkową C δ ani z czynnikiem uwalniającym kortykoliberynę. Reaktywacja metodami optogenetycznymi tych komórek z jądra środkowego, które były aktywowane przez społeczny transfer strachu pozwoliła zaobserwować u szczurów pojawienie się pasywnych zachowań obronnych (w tym zamierania) i nie miała wpływu na zachowania społeczne. Mapowanie aktywnych połączeń wykazało, że podczas

transferu strachu neurony jądra środkowego są najprawdopodobniej pobudzane przez projekcję z jądra podstawno-bocznego ciała migdałowatego. Dodatkowo, zahamowanie tej projekcji metodami chemogenetycznymi doprowadziło do podwyższenia liczby aktywnych reakcji wykonywanych przez szczury podczas eksploracji nowego środowiska. Podsumowując, uzyskane wyniki wskazują, że społeczny transfer strachu prowadzi do aktywacji podobnych, ale nie identycznych obwodów neuronalnych w jądrze środkowym, co bezpośrednia ekspozycja na bodźce awersyjne.

List of abbreviations

ACC - anterior cingulate cortex

AI - anterior insular cortex

AP - anteroposterior

BLA - basolateral amygdala

CA1 - CA1 field of hippocampus

ChR2 - channelorhodopsin-2

CNO - clozapine-*N*-oxide

CRF - corticotropine-releasing factor

CS – conditioned stimulus

CTB - cholera toxin subunit B

d - days

DREADDs – designer receptors exclusively activated by designer drugs

DV - dorsoventral

GPCRs - G-protein coupled receptors

hM3 - muscarinic acetylcholine receptor 3

IL - infralimbic cortex

LC - locus coeruleus

ML - mediolateral

NGS - normal goat serum

NpHR – halorhodopsin

PAG - periaqueductal gray

PFA - paraformaldehyde

PKCδ - protein kinase C δ

PL - prelimbic cortex

PVT - paraventricular nucleus of the thalamus

SOM – somatostatin

US – unconditioned stimulus

USVs - ultrasonic vocalizations

wks - weeks

1. Introduction

1.1. What is emotional contagion and motor mimicry?

Obtaining fast and accurate information about the environment is critical for animals survival. This is perhaps best illustrated by the example of prey species, which integrate cues from all available modalities in order to detect predators and execute appropriate defensive reactions (Pereira & Moita, 2016). When not enough cues are perceived, animals typically display exploratory behaviors to sample more information (Blanchard & Meyza, 2019; Thompson et al., 2018). Additionally, many species can learn about the environment by observing behavior of conspecifics (Olsson et al., 2020). For example, witnessing a group member that executes a sudden escape might signal the presence of predator, even if no direct cues indicating the threat are detected.

The ability to detect threats based on social cues is now well-documented in multiple species. For example, zebrafish can detect chemicals that are released when the skin of their conspecifics is damaged (Mathuru et al., 2012). Exposure to this chemical, chondroitin, increases speed of swimming and promotes escape behaviors. Birds exposed to predators often emit calls which are used as warning signals by their kin (Griesser, 2009). Monkeys born in captivity can learn to avoid snakes through observation of conspecifics which are already afraid of those animals (Mineka et al., 1984). These three examples illustrate that social information triggers defensive responses in multiple species and through various modalities (Pereira & Moita, 2016).

In the neuroscience literature such socially-triggered defensive responses are usually described in the context of a more general phenomenon, called ‘emotional contagion’. The term can be loosely defined as ‘sharing of the emotional states between individuals’ (Meyza et al., 2017). More descriptively, it is a process of triggering affective state not by some first-hand experience (receiving rewards or punishments), but rather by observing similar state in conspecifics. For example, exposure to stressed partners can in turn evoke stress in the observers, both in humans (Dimitroff et al., 2017) and rodents (Carnevali et al., 2020). Another related concept is ‘motor mimicry’ – which describes sharing similar behavior, but outside emotional domain (Chartrand & Lakin, 2013). A classic example of motor mimicry

is contagious yawning (Massen & Gallup, 2017). Although mechanisms of emotional contagion and motor mimicry might be very different (Massen & Gallup, 2017), they are often treated as closely related phenomena – perhaps due to the fact that in many real-life situations they can be hard to separate (De Waal & Preston, 2017). The next sections will briefly describe how contemporary theories explain both processes on the neuronal, behavioral and functional level.

1.2. The mechanisms of emotional contagion and mimicry

1.2.1. Theoretical context

Before describing in details how emotional contagion is explained causally, it might be useful to consider how this phenomenon is situated among other concepts related to social cognition. According to a very influential 'Russian-doll model', developed by Preston and de Waal (2002; 2017), interactions with conspecifics form a continuum, in which simple responses (e.g. mimicry) serve as foundation for much more complex processes (e.g. empathy). More specifically, the theory distinguishes three levels of empathy-related behaviors:

I. Motor mimicry and emotional contagion, which are largely automatic, evolutionary the oldest and present in many (or most) animal species
II. Emphatic concern and consolation, which describe actions taken to ameliorate the distress of conspecifics, present in highly social species like canines or voles
III. Perspective taking and targeted helping, also known as 'cognitive empathy', which require some form of theory of mind and are present only in animals with high degree of encephalization, such as dolphins, apes and humans.

The key assumption of the 'Russian-doll' model is that the lowest level (I) forms the foundation for the higher levels, both in the onto- and phylogenetic sense. In other words, although the most sophisticated empathy-related behaviors are present only in a small subset of species (predominantly during adulthood), they could not be developed without some core skills possessed by most mammals (often from birth). In this thesis I will focus only on these most rudimentary forms of behavior:

emotional contagion and motor mimicry. The next section will describe what is their neuronal basis.

1.2.2. Neuronal correlates

To understand how contemporary neuroscience explains motor mimicry and emotional contagion, it is essential to describe the discovery of 'mirror neurons'. Originally found in ventral premotor cortex of macaque brain, these neurons respond both to execution of motor acts and observing similar actions executed by other individuals (di Pellegrino et al., 1992). Mirror neurons display different sensitivity to physical characteristics of the action. For example, grasping a peanut could be executed either with two fingers or whole palm, and some mirror neurons would respond only to one of these movements (Rizzolatti & Craighero, 2004). However, most of them are activated by various motor acts which share the same goal (Rizzolatti et al., 2009). Furthermore, activity of mirror neurons is typically not sensitive to such factors as whether the action is rewarded or not, what species executes it (human or monkey in monkey studies) or how far away from observer it takes place (Rizzolatti & Craighero, 2004). Neuroimaging studies showed that some areas of human premotor and parietal cortex display very similar properties, being activated by both executed and observed actions (Fabbri-Destro & Rizzolatti, 2008). Finally, single-unit recordings from neurological patients provided direct evidence that mirror neurons exist in humans, also in areas not traditionally related to motor system, like hippocampus and amygdala (Mukamel et al., 2010).

The unique response properties of mirror neurons lead to hypothesizing that they play causal role in motor mimicry (for review, see Iacoboni, 2009). There is some evidence confirming this view, coming mainly from studies using transcranial magnetic stimulation (TMS) in humans. This non-invasive technique allows to transiently block or stimulate parts of the cortex which typically show mirror responses (Walsh & Cowey, 2000). The studies demonstrated that blocking parts of 'mirror neurons system' can disrupt automatic imitation, while not interfering with self-initiated movements (Catmur et al., 2009; Heiser et al., 2003). Stimulating similar brain regions, on the other hand, improves imitation (Hogeveen et al., 2015; Restle et al., 2012).

It is worth noting that the exact role of mirror neurons in social behaviors is a very controversial topic. According to some interpretations, mirror neurons play crucial role in understanding actions of others and social learning (Iacoboni, 2009; Rizzolatti & Craighero, 2004). More critical authors point out that it is not possible to infer the function of mirror neurons from their response properties, as the observed activation patterns could be explained by simple visuo-motor plasticity also outside the context of social interactions (Heyes, 2010; Hickok, 2009; Keysers & Perrett, 2004). However, the controversies seem to concern mainly high level social cognition, corresponding to the levels II and III in the Russian-doll model. When basic motor mimicry is considered, there seems to be much more universal agreement that mirror neurons play at least some role in this process (Heyes & Catmur, 2020; Thompson et al., 2019).

The research on mirror neurons lead some scholars to ask if similar mechanisms could account also for emotional contagion (Keysers & Gazzola, 2009). According to this hypothesis, the same neurons are activated during first-hand experience and observation of affective states (Bastiaansen et al., 2009). The results of human neuroimaging studies, although they cannot provide cellular resolution, are in general consistent with the hypothesis for such states as pain (Keysers et al., 2010) and disgust (Jabbi et al., 2008; Wicker et al., 2003). A recent rodent study provided also direct evidence that the same neurons in anterior cingulate cortex are active during experience and observation of pain, proving the existence of ’emotional mirror neurons’ (Carrillo et al., 2019).

Collectively, all these findings had major influence on social neuroscience and inspired or enriched various theoretical approaches: simulation theory (Gallese & Goldman, 1998), perception-action model (Preston & de Waal, 2002), shared circuits model (Hurley, 2008), neurocognitive model of emotional contagion (Prochazkova & Kret, 2017) and others. Although there are some important differences between these frameworks, all of them claim that activation of similar brain circuitry during first-hand and vicarious (i.e. observed) experience can serve as a mechanism for emotional contagion, motor mimicry or both. Due to this reason, in the following sections they will be collectively referred to as ‘shared circuits approaches’.

Importantly, the shared circuit approaches claim that the vicarious and first-hand experience activate similar, but not necessarily the same brain areas. In fact, there is now compelling evidence that some brain regions might be activated preferentially by observing states of others (Adolphs, 2009). However, the shared circuits approaches generally tend to stress the similarities between vicarious and first-hand brain activity. They also point out that the vicarious activity can be triggered very quickly, in apparently automatic manner (Iacoboni, 2009; Preston & de Waal, 2002). This simple mechanism is believed to promote execution of similar behaviors to the ones which are observed – which will be the topic of next chapter.

1.2.3. Behavioral characteristics

Most data on behavioral mimicry comes from human studies. People have well-documented tendency to copy the reactions observed in partners, for example yawning (Helt et al., 2010), changing body posture (Tia et al., 2011) or face touching (Chartrand & Bargh, 1999). Behaviors indicating emotional state are also contagious, which was demonstrated for facial expressions of all basic emotions (Hess & Blairy, 2001; Lundqvist, 1995; Mui et al., 2018), cry (Simner, 1971) and laughter (Provine, 1992). The matching of responses usually occurs with a short delay – for example, up to five seconds – which is a prerequisite for classifying it as 'mimicry' (Chartrand & Lakin, 2013). Importantly, experimental data suggest that this kind of basic behavioral matching is often triggered without conscious awareness and can occur independently from the goals of the observer (Chartrand & Lakin, 2013; Lakin & Chartrand, 2003).

Some evidence indicates that behavioral mimicry occurs also in other animal species, including non-primates. Perhaps the best documented case is vicarious freezing in rats and mice, which will be described more thoroughly in the next sections (for review, see Meyza et al., 2017). It is also known that mice subjected to nociceptive stimulation display more pain-related behaviors when they are tested with a partner also experiencing pain then when tested alone (Langford et al., 2006; Martin et al., 2015). Other examples include mimicking: posture and facial expressions by different monkey species (Anderson & Kinnally, 2020; Davila Ross et al., 2008; Mancini et al., 2013) vocalizations by dolphins (Reiss & McCowan,

1993) or yawning by dogs (Romero et al., 2013) and chimpanzees (Campbell & de Waal, 2011).

To sum up, motor mimicry can occur both within and outside the context of emotional contagion. Found among many species, it is often described as an automatic, evolutionary old mechanism (De Waal & Preston, 2017). Of course, the fact that mimicry can be found both among humans and animals does not necessarily mean that in both cases it serves the same function. This topic will be more thoroughly discussed in the next section.

1.2.4. Functions

Although motor mimicry and emotional contagion are believed to be simple, evolutionary conserved processes, many theoretical approaches predict that their main function is supporting much more complex social cognition (Chartrand & Lakin, 2013; De Waal & Preston, 2017; Iacoboni, 2009). Firstly, mutual mimicry between infants and their caregivers is hypothesized to play important role in development of communication skills (Meltzoff & Moore, 1994). Secondly, direct matching of emotions and behaviors could potentially serve a mechanism through which humans are able understand intentions of others (Iacoboni, 2009). Thirdly, experimental data strongly suggests a bidirectional relationship between mimicry and affiliation (Preston & de Waal, 2002). People are generally more likely to copy behavior of individuals who belong to the same group or trigger positive emotions, while mimicry can improve forming affiliation between strangers (Lakin et al., 2003). Finally, mimicry is one of possible ways to achieve synchrony, which might in turn improve the efficiency of communication between individuals (Chartrand & Lakin, 2013).

Although all of the mechanisms presented above might play important role in human social interactions, they do not explain what is the proximate function of sharing behaviors described in the introduction – that is, mimicking defensive responses of conspecifics. Intuitively such reactions increase the chance of avoiding predator, but that might be hard to prove experimentally. Computational work suggests that in noisy environments – that is, under conditions when it is sometimes

not evident if the defensive response should be executed or not – a tendency to mimic behavior of a partner can improve accuracy of threat detection (Han et al., 2019). As expected, this advantage is particularly strong if the animal can mimic a partner which has access to more reliable (less noisy) information than itself. Interestingly, this scenario does not generate any significant costs for the better informed partner, as long as the level of coupling between the animals is not very high (Han et al., 2019). Recent work suggests that similar conclusions may apply also to appetitive context – rats in groups find food quicker than when tested alone, and this effect is probably achieved through a simple tendency to follow each other (Nagy et al., 2020).

1.3. Rodent defensive responses and their social regulation

The previous sections provided a general introduction into the research on emotional contagion and motor mimicry, with the special focus on shared circuit approaches. Majority of data presented so far was collected on humans or other primates. This type of research – although very fruitful – in general does not allow to study specific neural circuits, that is, populations of individual neurons selected based on their molecular markers or projection targets. Such detailed experiments are performed in mammals mainly with the use of rodent (mice or rats) models. The following sections will summarize data on emotional contagion collected from these species, focusing on fear contagion. First, basic types of defensive responses executed by single rodents will be introduced. Next, it will be presented what neural circuits control these behaviors, with the special focus on central amygdala (CeA). The final section will summarize most important findings on fear contagion in rodents.

1.3.1. Types of defensive responses

When rodents detect danger, they execute various types of defensive responses, which can be selected based on the context. If the potential threat is very distant – for example, a movement was noticed far away – rodents often perform additional risk-assessment behaviors to estimate the level of danger (Blanchard et al., 2011).

Typical examples are approach with stretched posture (Dielenberg & McGregor, 2001) or rearing, i.e. standing on the hind paws, which allows to scan bigger area (Lever et al., 2006). If this additional information sampling confirms that a predator might be nearby, rodents typically retreat to a safer environment (Blanchard & Blanchard, 1989). In some cases the perceived cues indicate an imminent threat – for example, a looming shadow might signal that a bird of prey will soon reach its target on the ground (Yilmaz & Meister, 2013). In this situations the retreat of the animal often takes form of a rapid escape (Evans et al., 2018). Although for human observer such behaviors might look like uncoordinated panic reactions, rodents have the ability to continuously track their home base and usually flee in the direction which offers maximum safety (Coimbra et al., 2017; Vale et al., 2020). Finally, if there is low probability that the escape will be successful – for example, when the animal is trapped in some confined space - typically a freezing reaction is executed (Fanselow & Lester, 1988). It is characterized by sudden cessation of any movement and – opposite to flight - decrease of the heart rate (Roelofs, 2017). Such response might enhance the probability of not being noticed by the predator; it is displayed by various species of animals, including rats (Blanchard & Blanchard, 1971), mice (Valentinuzzi et al., 1998), fruit flies (Zacarias et al., 2018) and humans (Hagenaars et al., 2014). When freezing does not help and the predator is still approaching, sometimes rodents 'desperately' attack the animals which are much bigger and stronger than them, including human experimenters (Guimarães-Costa et al., 2007).

The examples presented above illustrate how diverse is the repertoire of defensive behaviors presented by rodents in nature. Laboratory studies typically use paradigms that promote one type of these responses, which makes behavior easier to quantify. The most popular approach is to provide mild foot-shocks to a rodent closed in a small chamber, which very reliably evokes freezing (Fanselow & Lester, 1988). Importantly, such procedure leads to robust learning – the animal placed again in the same chamber will typically recognize the environment as threatening and display freezing even in the absence of any foot-shocks (Maren, 2001). This procedure, called 'classical fear conditioning', or more specifically 'contextual conditioning' is routinely used to study memory (where high level of freezing indicates efficient learning). If the foot-shocks are proceeded by sounds, typically

the animals will freeze after presentation of the auditory cue even in a different testing context (Maren, 2001). Such variant of the paradigm is called ‘auditory cue conditioning’.

Alternatively, the animals can be tested in an environment where escape route (i.e., another chamber) is available. This kind of environment promotes active reactions to the foot-shocks (running to the other chamber) and is used by different types of ‘active avoidance’ tests (Diehl et al., 2019). Finally, some environments can be used to detect both active (rearing, jumping, escaping) and passive (freezing) defensive responses from the same animals during a single session (Fadok et al., 2017). All these behavioral paradigms allow to study under laboratory conditions what brain circuitry controls defensive reactions – which will be topic of the next section.

1.3.2. Neural circuits controlling defensive responses

Detecting threats and responding to them depends on a broad network of rodent brain structures (Pereira & Moita, 2016; Vetere et al., 2017). Visual and auditory cues which signal danger evoke rapid activation of superior and inferior colliculus (respectively), thalamus and cortical sensory areas (Branco & Redgrave, 2020; Herry & Johansen, 2014; Xiong et al., 2015). Olfactory cues about threats are processed by both main and accessory olfactory bulb and transmitted to piriform cortex (Takahashi, 2014). Sensory signals from all modalities converge (through different subnuclei) to amygdala, which is considered to be one of the major hubs orchestrating selection of appropriate defensive responses (Herry & Johansen, 2014; Knapska et al., 2007). Amygdala (and other, parallel inputs) in turn activate a wide set of subcortical structures, which either directly trigger defensive responses through their motor outputs (e.g., periaqueductal gray, PAG) or increase arousal through neuromodulatory and autonomic mechanisms (e.g., locus coeruleus, LC; see Herry & Johansen, 2014 and Fadok et al., 2018, respectively). At the same time high-order, associative structures (e.g., medial prefrontal cortex or hippocampus) mediate defensive reactions by adjusting them to the context (that is, state of the animal or previous learning; Maren, 2005). Describing the interactions between all these structures is beyond the scope of this thesis. Instead,

the next paragraphs will focus on one region and briefly summarize how central amygdala circuits (CeA) mediate the selection of appropriate defensive response.

CeA is a forebrain subcortical structure, composed predominantly of inhibitory GABA-ergic cells (Ehrlich et al., 2009). It is typically divided into centrolateral (CeL) and centromedial (CeM) parts, which harbor most of the input and output projections, respectively (Haubensak et al., 2010). The third, capsular part (CeC) is usually studied jointly with the CeL. In comparison to other amygdala nuclei, CeA receives less projections from classic sensory areas (Knapska et al., 2007). Instead, it gets strong projections from associative cortical regions (Knapska et al., 2007) and basolateral amygdala (BLA), which is a well-described hub for plastic changes during fear conditioning (Sah et al., 2020). It is also reciprocally connected with multiple brainstem, basal forebrain and hypothalamic areas, effectively targeting all major neuromodulatory systems (Knapska et al., 2007). Due to its unique connectivity, CeA was for long described as a 'relay station' between the BLA and the brainstem, receiving highly-processed information about salient events and triggering emotional reactions (LeDoux, 2000). However, literature published during the last decade clearly indicates that instead of passively transmitting activation, CeA plays critical role in selecting the behavioral output (Fadok et al., 2018).

The seminal experiments studying this process focused on two types of CeA neurons – either expressing protein kinase C δ (PKCδ+) or not (PKCδ-). They mutually inhibit each other through recurrent, monosynaptic connections (Haubensak et al., 2010). In the context of fear conditioning, the PKCδ- population promotes freezing, while PKCδ+ promotes movement (Ciocchi et al., 2010). The effects cannot be explained by simple regulation of anxiety, because during novel environment exploration these cells play opposite roles: PKCδ- increase, while PKCδ+ decrease locomotion (Botta et al., 2015). At least some of the PKCδ- neurons express somatostatin (SOM+; Hunt et al., 2017). Consistently with the fear conditioning data, the SOM+ cells induce freezing, most probably through their long range inhibitory projections to PAG and paraventricular nucleus of the thalamus (PVT; Li et al., 2013; Penzo et al., 2014; Yu et al., 2016). Different parts of PAG are believed to directly trigger defensive responses – not only freezing, but also flight – through their projections to pre-motor neurons in medulla, which in

turn activate spinal cord (Deng et al., 2016; Evans et al., 2018; Tovote et al., 2016). Finally, it was shown that corticotropine-releasing factor (CRF+) cells within CeA promote active reactions to threats (rearing, escapes), while CRF- neurons promote freezing (Fadok et al., 2017). Importantly, these two types of cells form a similar recurrent inhibition circuit as the PKCδ+/- neurons, strongly suggesting that competitive inhibition is a common mechanism through which CeA selects the behavioral output (Fadok et al., 2017). Although this topic is generally beyond the scope of this thesis, all the mentioned CeA cell populations – PKCδ, SOM and CRF – are known to regulate also other aspects of behavior, such as feeding, anxiety or addiction (Fadok et al., 2018; Janak & Tye, 2015).

Although the CeA circuits are interconnected according to regular motifs, their dynamics is far from fixed and can be substantially changed by synaptic plasticity. For example, fear conditioning potentiates synapses from BLA to SOM+ cells, which in turn promotes freezing (Li et al., 2013). Interestingly, external inputs to CeA can simultaneously target groups of cells which mutually inhibit each other. This is true for projections from insula, which depolarize equally strongly PKCδ+ and PKCδ- cells (Zhang-Molina et al., 2020). Similarly, connections from BLA to CeA excite both CRF+ and CRF- cells (Hartley et al., 2019). As a result of fear conditioning, the projections to CRF- cells are preferentially strengthened, which leads to their higher activation and freezing (Hartley et al., 2019).

Taken together, the data strongly suggests that recurrent circuits in CeA control defensive responses through a ‘winner takes it all’ mechanism. Molecularly defined populations of cells which promote opposite behaviors form mutual inhibitory connections. When they are activated by external inputs – for example, related to threat detection – one of them typically dominates over the other, triggering autonomic and motor components of a respective defensive behavior. The balance between the competing cell populations can be changed by learning, which increases the probability of one reaction type (for example, active vs. passive). Furthermore, the local circuit activity is regulated by the inputs from high-order cortical areas and BLA, which converge in CeA. Because these projections sometimes target multiple neuronal populations, the same projection could theoretically support opposite functions depending on how the balance within CeA circuits is currently shifted (Fadok et al., 2018; Zhang-Molina et al., 2020). This

prediction is consistent with some recent experimental data – for example, stimulation of projection from insula to CeA can evoke either approach or avoidance, depending on whether the object is threatening or not (Rogers-Carter et al., 2018; see also Ponserre et al., 2020).

1.3.3. Fear contagion in rodents – behavioral paradigms

The last sections briefly summarized findings on defensive responses in single rodents, with the special focus on CeA. Although less is known about how similar reactions are controlled in the context of fear contagion, there is now some substantial literature on this topic as well (for review, see Meyza et al., 2017). The following sections will summarize the most important experimental results.

There are multiple behavioral procedures which allow to observe fear contagion in laboratory rodents (Meyza et al., 2017). Most of them study pairs of animals: 'demonstrator', which at some point receives electric foot-shocks and 'observer', which witnesses the reactions of the demonstrator. In the simplest scenario, the observer is exposed to the demonstrator during the process of fear-conditioning (i.e., while the other animal is receiving foot-shocks) in a small chamber (Atsak et al., 2011). The shocks can be also proceeded by auditory cues (Yusufishaq & Rosenkranz, 2013). The animals are separated with a perforated wall, so they can contact using all modalities except from touch; the separation protects the observer from getting the electric shocks (Kondrakiewicz et al., 2019). This kind of procedure evokes freezing not only in demonstrators, but also observers, which was demonstrated for rats (Atsak et al., 2011; Yusufishaq & Rosenkranz, 2013) and some strains of mice (Chen et al., 2009; Jeon et al., 2010; Keum et al., 2016). When later tested alone, the observers usually show some degree of cue or contextual memory (Twining et al., 2017). Although the effect can be observed in experimentally naïve animals (Han et al., 2019; Yusufishaq & Rosenkranz, 2013), before the vicarious conditioning the observers are often subjected to foot-shocks. Such pre-exposure is known to significantly increase their freezing levels related to fear contagion (Allsop et al., 2018; Atsak et al., 2011; Cruz et al., 2020; Han et al., 2019).

In another variant, the demonstrator is separated and subjected to auditory fear conditioning alone (Cruz et al., 2020; Pereira et al., 2012). Afterwards it is tested for fear recall (by being exposed to the auditory cue) in the presence of the observer. In pre-exposed, but not naive observers such procedure leads to vicarious freezing accompanied by learning. When tested alone 24 hours later, the observers still react to the auditory cue with freezing (Cruz et al., 2020; Pereira et al., 2012).

In both variants in the control group the demonstrators do not receive any foot-shocks. An alternative approach, called 'fear conditioning by proxy', studies triads of animals, in which there are two observers: one experimental and one control (Bruchey et al., 2010; Jones et al., 2018). First, the demonstrator is separated and fear conditioned to a tone. Then the experimental observer is exposed to the demonstrator in a dyadic, free social interaction (no partition is introduced). During the interaction the auditory cue paired with foot-shocks is presented, evoking defensive responses in the demonstrator. Finally, all three animals can be tested separately for fear memory. The demonstrators and experimental observers – but not control observers – display freezing after presenting the auditory cue (Bruchey et al., 2010). This procedure allowed to show that efficiency of the fear contagion depends on social hierarchy – submissive observers learn from reactions of dominant demonstrators much more robustly than the other way round (Jones & Monfils, 2016).

All the procedures described above focus on passive defensive reaction, i.e. freezing. Alternatively, the observer can be exposed in the home cage to a demonstrator that had just received foot-shocks in another room (Knapska et al., 2006; Kondrakiewicz et al., 2019; Meyza et al., 2015). Interaction with the recently stimulated partner evokes active reactions: the observers explore environment more than controls, which is manifested especially by rearing (Andraka et al., 2020; Knapska et al., 2006).

1.3.4. Fear contagion in rodents – neuronal correlates

The behavioral paradigms described in the previous section allowed to collect seminal data on how brains of observers process social cues about threats. In brief,

majority of experimental work focused on amygdala, medial prefrontal cortex and thalamus (Meyza et al., 2017).

The brain structure which was most thoroughly studied in the context of fear contagion is anterior cingulate cortex (ACC). Multiple reports confirm that this structure is active during vicarious freezing paradigms (Allsop et al., 2018; Ito et al., 2015; Jeon et al., 2010; Jones & Monfils, 2016), similarly as other regions of the medial prefrontal cortex (Knapska et al., 2006; K. Meyza et al., 2015; Mikosz et al., 2015). Furthermore, blocking ACC interferes selectively with observational fear conditioning, but not with fear recall or with first-hand conditioning (Allsop et al., 2018; Carrillo et al., 2019; Y. Han et al., 2019; Jeon et al., 2010; Jones & Monfils, 2016). A study investigating lateralization reported that blocking only the right (vs. left) ACC disrupts fear contagion (Kim et al., 2014). Finally, ACC contains some 'emotional mirror neurons', that is – cells which are activated both by own pain and observation of painful stimulation of the demonstrator (Carrillo et al., 2019).

Research on anatomically defined circuits indicate that the projection from ACC to basolateral amygdala (BLA) is especially important for the observational fear. Vicarious freezing paradigm increases the strength of synaptic connections from ACC to BLA (Ito et al., 2015) and augments the synchrony of neuronal oscillations recorded in these two structures (Jeon et al., 2010). Recently it was shown, with combination of electrophysiological and optogenetic techniques, that the projections from ACC to BLA are preferentially activated by auditory cues during observational conditioning (Allsop et al., 2018). Blocking these projections disrupts observational fear learning and other social behaviors, but not first-hand conditioning (Allsop et al., 2018).

Activity mapping experiments suggest that different subnuclei of amygdala also play important role in fear contagion (Ito et al., 2015; Jones & Monfils, 2016; Knapska et al., 2006; Meyza et al., 2015). In rats interaction with recently fear conditioned partner activates all nuclei of amygdala to similar extent as first-hand conditioning; in fact the CeA is activated in observers stronger than in demonstrators (Knapska et al., 2006). In mice the results are less clear and it seems that mainly BLA is activated in the observers (Meyza et al., 2015). Furthermore,

the strength of synaptic connections from LA to MeA allows to predict freezing levels during recall – but only in rats subjected to observational, not first-hand conditioning (Twining et al., 2017). Blocking this projection disrupts the vicarious freezing, both during observational training and memory test (Twining et al., 2017).

Much less is known about implication of other brain structures in fear contagion. The thalamic nuclei which process affective, but not sensory aspects of pain were shown to be necessary for vicarious freezing (Jeon et al., 2010; Kim et al., 2012). Ventral hippocampus is activated by contact with a fearful partner, but blocking it does not interfere with social conditioning (Jones & Monfils, 2016).

To sum up, there are several behavioral models which allow to study fear contagion in well-controlled, rodent behavioral models. Using them allowed to show that in general similar brain structures that the ones involved in first-hand defensive reactions – amygdala, medial prefrontal cortex and thalamus – are activated also in observers, in line with shared circuit approaches. This was demonstrated on the level of brain regions as well as single neurons. At the same time, it is clear that some circuits are necessary for observational, but not first-hand fear conditioning. To verify how well fear contagion can be explained by the shared circuits approach, it is necessary to perform more experiments on molecularly and anatomically defined cell populations. This seems especially promising for such structures as CeA, where a lot is already known about what circuitry controls single-subject defensive behaviors.

1.4. Methods used to study fear contagion

Before proceeding to describing the aims of the thesis, it seems beneficial to provide some introduction into the methods that will be later referred to in the experimental part. The next sections will briefly describe three techniques that can be used to study fear contagion in rodents: c-Fos expression mapping, optogenetics and chemogenetics. The introduction will focus on basic mechanisms, typical applications and most important limitations of each method.

1.4.1. c-Fos as a marker of neuronal activation

The *c-fos* gene belongs to the family of immediate-early gens (IEG), which are transiently activated by some form of cell stimulation (Minatohara et al., 2016; Sheng & Greenberg, 1990). The baseline level of c-Fos protein detected in most brain regions is very low. It can be transiently increased, for example *in vitro* by introducing neurotransmitters (Kaczmarek et al., 1988) or *in vivo* by exposing the animal to novel stimuli (Nikolaev et al., 1992). The increase in mRNA level can be detected as quickly as 15 min after the stimulation, reaching its peak after 30-60 min (Curran & Franza, 1988; Kaczmarek et al., 1988). The maximum protein level, on the other hand, is typically detected 1.5-2 hours after the stimulation and goes back to baseline after about 6 hours (Nikolaev et al., 1991).

Although the mechanism of c-Fos action is not completely understood, the protein is implicated in neuronal plasticity (Minatohara et al., 2016; Yap et al., 2020). After translation in the cytoplasm c-Fos is transported to cell nucleus, where together with products of another IEGs it forms a dimer AP-1 (Morgan & Curran, 1991). The AP-1 is a transcription factor which regulates many biological processes, including increasing the number and strength of synaptic connections (Sanyal et al., 2002). Blocking *c-fos* expression in auditory cortex (using interfering RNA) disrupts tone discrimination learning – which is manifested both on behavioral and electrophysiological level (de Hoz et al., 2018).

Expression of *c-fos* is triggered by novel stimuli and decreases with habituation or training in behavioral tasks (Anokhin & Rose, 1991; Nikolaev et al., 1992). At the same time, memory recall in the context of fear conditioning can increase level of c-Fos in some brain structures even if the animal was trained 30 days ago (Frankland et al., 2004). Furthermore, in some tasks *c-fos* expression allows to predict efficiency of learning across animals (Martinez et al., 2013). Finally, some data indicates that *c-fos* expression correlates with firing rates (Yassin et al., 2010), but not necessarily calcium levels (Peter et al., 2013) measured in individual neurons.

Taken together, the data indicates that c-Fos protein is a marker of neuronal plasticity. It has several properties of a very effective measurement tool: it can be reliably detected with immunohistochemistry, has low baseline levels and it is

located in cell nuclei, which provides single-cell resolution. These desirable characteristics made c-Fos mapping a very popular method, in practice used to probe which brain structures are activated by behavioral task (Boulton et al., 2003). Despite obvious advantages, this approach still has some limitations. First, performing immunohistochemistry requires sacrificing the animal, which in turns allows to measure expression only in one time point after the experiment. Second, it is likely that any novel stimulus will induce c-Fos in laboratory animals, so it is essential to habituate them really well to such non-specific factors as handling by humans (Nikolaev et al., 1992). Third, one should keep in mind that although neuronal plasticity is almost always accompanied by changes in activity, the opposite might not be true (Fauth & Tetzlaff, 2016). In other words, positive results are easier to interpret than the negative ones: presence of c-Fos seems to be a reliable indicator of neuronal activation, but lack of it does not allow to safely conclude that the neuron was not active (Peter et al., 2013).

1.4.2. Optogenetics

Optogenetics is an experimental technique which allows to control neuronal activity with light (Guru et al., 2015). It utilizes the fact that many proteins existing in nature are light-sensitive (Govorunova et al., 2017; Sakmar et al., 2002). A classic example is channelorhodopsin-2 (ChR2), discovered in green alga *Chlamydomonas reinhardtii*, in which it supports phototaxis (Foster et al., 1984; Hou et al., 2012). ChR2 is an ion channel that opens after being illuminated with blue light, permitting flow of such cations as H^+, Na^+, Ca^{2+}, and Mg^{2+} (Schneider et al., 2015). This property can be used to control neuronal activity: when expressed in neurons and stimulated with light, ChR2 will evoke membrane depolarization and action potentials (Lin, 2011). Early works demonstrated that ChR2 can be used not only in brain slices *ex vivo* (Boyden et al., 2005), but also *in vivo* – introducing it to the brain together with optic fibers allows to influence animal behavior in a relatively straightforward manner by switching the laser illumination on (Arenkiel et al., 2007).

Similar logic can be applied also to inhibit neuronal activity. This can be achieved for example with halorhodopsin (NpHR), a proton pump powered by

orange light (Gradinaru et al., 2008). Under typical physiological conditions its activation leads to introducing more chloride ions into the cell, which hyperpolarizes it and blocks spiking (Raimondo et al., 2012). ChR2 and NpHR are only two examples from a very broad range of proteins used for optogenetics. Currently multiple other opsins are available, which allows to select the most appropriate tool based on such characteristics as mechanism of action, time course of activation, current efficiency or sensitivity to a given light color (Govorunova et al., 2017; Lin, 2011; Tye & Deisseroth, 2012). The applications of the technique reach beyond changing the membrane potential – for example, some solutions enable to control intracellular signaling cascades or even expression of single genes (Ellwardt & Airan, 2018; Repina et al., 2017).

The two most important advantages of optogenetics are cell-type and timing specificity. Opsins are typically expressed in the brain using viral vectors, which allows to limit their delivery only to the population of neurons defined by a molecular marker (Nectow & Nestler, 2020). This strategy is especially straightforward if transgenic Cre or Flp lines are available (De La Crompe et al., 2020). Alternatively, the neuronal population might be selected based on input or output projections (Tye & Deisseroth, 2012). Because majority of neurons do not endogenously express photoreceptors, the stimulation of the selected population does not affect neighboring cells as long as the power of light is not high enough to generate heat (Owen et al., 2019). Furthermore, light can be switched on and off almost immediately, which under some conditions allows to control neuronal activity with a milliseconds resolution, for example for the purpose of a closed-loop stimulation (Grosenick et al., 2015). Importantly, the technique can be used both *in vitro* and *in vivo* in combination with another methods (e.g. electrophysiology, imaging, pharmacology), which dramatically broadens the range of possible applications (Kim et al., 2017).

The main pitfalls of optogenetics are related to some undesired, off-target effects (Allen et al., 2015). For example, some opsins require high power of light, which in turn might cause tissue heating and interfere with neuronal activity in non-specific manner (Owen et al., 2019). What is more, disrupting the physiological flow of ions can lead to unpredictable side effects (Allen et al., 2015). A well-known example is a ‘rebound from inhibition’ – a rapid increase of neuronal activity

(above the baseline level) shortly after inhibitory opsin is deactivated by switching the light off (Arrenberg et al., 2009). To avoid these off-target effects, it is highly recommended to electrophysiologically test the stimulation protocol used for each experiment (Allen et al., 2015; Kim et al., 2017).

1.4.3. Chemogenetics

In principle, chemogenetics works similarly to optogenetics – it utilizes artificially introduced trans-membrane proteins to control neuronal activity (Boesmans et al., 2018). The difference is that the receptors used in chemogenetics are not activated by light, but by chemical compounds (Atasoy & Sternson, 2018). The receptors (as well as their ligands) should not endogenously occur in the nervous system to avoid off-target effects, hence they are known as DREADDs - Designer Receptors Exclusively Activated by Designer Drugs (Roth, 2016). One of the most popular DREADDs was produced by subjecting the muscarinic acetylcholine receptor 3 (hM3) to random mutagenesis and selecting the outputs with most desired properties (Armbruster et al., 2007). After several rounds of this process it was possible to obtain a receptor which shows only minimal responses to acetylocholine, but instead can be activated by clozapine-*N*-oxide (CNO), a metabolite of a neuropsychiatric drug clozapine (Armbruster et al., 2007). The new protein (called hM_4D) belongs to the family of G-protein coupled receptors (GPCRs) and enables to inhibit mammalian neurons through opening potassium channels (Armbruster et al., 2007). Another variant of the receptor, hM_3Dq, allows to stimulate neurons, though the exact mechanisms of cell depolarization are in this case more complicated (Alexander et al., 2009; Atasoy & Sternson, 2018).

Similarly as with optogenetics, initial findings stimulated development of many new DREADDs – not only GPCRs, but also ion channels (Atasoy & Sternson, 2018). Available tools have different mechanisms of actions, which in some extent allows to limit the effects only to the specific aspects of neuronal activity or plasticity (Roth, 2016). For example, receptor hM_4D blocks release of synaptic vesicles from the axon terminals, while only moderately depolarizing soma (Stachniak et al., 2014).

Chemogenetics provides similar cell-type specificity to optogenetics, enabling to control neuronal populations defined by molecular markers or projection targets. Importantly, it does not require to provide light into the brain, which makes the surgeries less invasive and allows animals to move freely without any tethering (Boesmans et al., 2018). For most applications, the ligand can be delivered to the animals with a simple systemic injection shortly before the experiment, and acts sufficiently long to perform typical behavioral or electrophysiological procedures (Roth, 2016).

Two most important disadvantages of DREADDs are poor temporal resolution and potential non-specific effects of their ligands. In contrast to optogenetics, it usually takes several minutes before DREADDs are activated and much longer (on the order of hours) before the effect is washed out (Jendryka et al., 2019). Although such prolonged course of action might be desired for some applications, in practice it does not allow to perform typical within-subjects experiments (for example, to inhibit neurons only during presentations of some stimuli). Moreover, it was recently shown that in the brain CNO can be converted back to clozapine, which potentially activates endogenous receptors (MacLaren et al., 2016; Manvich et al., 2018). Because other ligands (for example, C21) can often activate the same DREADDs, systematic research will probably allow to select the drugs which are optimal for chemogenetic applications (Jendryka et al., 2019). Nevertheless, it is essential to always inject control groups with the same ligand as experimental groups (opposed to saline), to exclude the possibility of non-specific effects.

2. Research aims

As discussed in the previous paragraphs, there are now numerous studies supporting the idea that emotional contagion might be explained by shared circuit activation and behavioral mimicry. However, most of the arguments come from non-invasive human studies, which do not allow to directly study single cells. Animal models, on the other hand, enable to target populations of neurons precisely defined by their molecular markers or connectivity patterns. Furthermore, a lot is already known about neuronal circuits that control fear conditioning in single rodents. This allows to formulate some explicit predictions regarding which cell populations should be activated by the fear contagion in rats, assuming that the shared circuit approaches are true. Verification of these hypotheses is the main scientific aim of this thesis.

On the behavioral level, the shared circuit approaches often predict that observers copy reactions of demonstrators through mimicry. If this is true, during fear contagion the responses of observers should occur shortly after similar responses of demonstrators, which leads to the following hypothesis:

1. **During fear contagion paradigm, the freezing of observers can be predicted by the previous freezing of demonstrators.**

On the neuronal level, the CeA circuits activated by the fear contagion should closely resemble the circuits described in the literature on single subjects fear conditioning. More specifically, it can be hypothesized that:

2. **Central amygdala neurons activated in observer rats have similar characteristics to the cells activated by fear conditioning, as defined by:**

    a) molecular markers - low expression of CRF and PKCδ
    b) connectivity - input projections from BLA
    c) behavioral function - promoting freezing and/or anxiety.

3. Methods

3.1. Animals

All research was conducted on experimentally naïve Wistar rats. Adult animals (250-300g) were provided by the Center of Experimental Medicine in Białystok, Poland (total *n* = 280). They were housed in pairs under 12h light/dark cycle, in standard cages of size 43 x 25 x 18.5 cm. All experimental procedures were carried during the day, which corresponded to the light phase. The water and food were provided *at libitum*. One of the animals (randomly selected in each pair) was marked on the tail with a permanent marker to distinguish observers from demonstrators. All experimental procedures were approved by the Local Ethical Committee (permission LKE 126/2016).

3.2. Behavioral testing

3.2.1. Habituation

All the animals were handled two weeks before any testing to get used to the experimenter. The procedure involved lifting them up, placing on the forearm and petting for about 3 minutes. Additionally, before any behavioral tests the rats were habituated to the transportation and experimental room, by being placed there for 20 minutes for 3 consecutive days.

3.2.2. Fear contagion paradigm

After habituation the rats were tested using identical procedure as the ‘imminent threat paradigm’ described in Kondrakiewicz et al., 2019. For the sake of simplicity, in this thesis the procedure will be referred to as the ‘fear contagion’ paradigm. It was performed using a custom made behavioral apparatus, split into two compartments with a perforated, transparent plexiglass wall (Fig. 1). All of the outer walls (except from the front one) were covered with black material, to facilitate

visibility of the white rats on video footage. One of the compartments was equipped with a metal grid floor, which allowed to provide foot-shocks to the animal using electrical stimulator (ENV-414SA, *Med Associates*). The rat placed in this compartment will be referred to as the 'demonstrator'. The 'observer' will refer to the animal in the second chamber.

The demonstrator and observer were placed in the testing chambers immediately after each other (in random order). After 2 min habituation period, demonstrator received 10 electric foot-shocks (1mA, 1 sec long) separated with inter-trial interval (ITI) of 59 sec. No shocks were delivered to the observer at any point. In the control group, pair of rats was placed in the same cage for the same amount of time (12 min), but without delivering foot-shocks to any of the animals. Because in this case both rats underwent identical treatment, they will be referred to jointly as 'controls'. After testing each pair of animals, the cage was cleaned with 70% ethanol.

In the auditory cue variant, the foot-shocks were paired with sounds (20 sec, pure tone 2kHz, 70dB, ITI 60 sec). The shocks were presented either during the last second of the sound ('CS-first' group) or terminated immediately before the sound ('US-first').

The whole procedure was recorded with a camera (1080P OV2710, *ELP*) placed in front of the testing apparatus. The foot-shock delivery was controlled by a micro-controller (*Arduino Uno*), triggered from Bonsai software (*https://bonsai-rx.org/*) using custom-written scritpts. In a subset of animals, ultrasonic vocalizations emitted during the test were also recorded using a specialistic microphone (*UltraSoundGate, Avisoft)*.

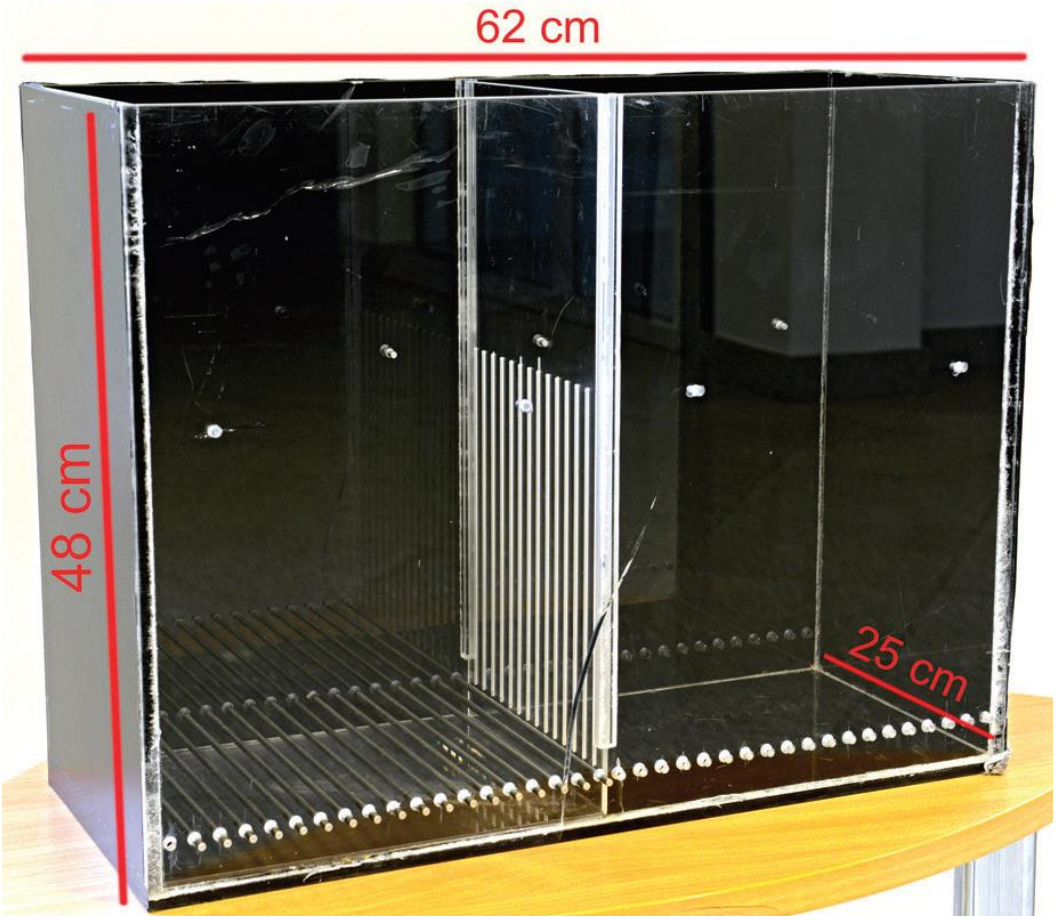


Figure 1. Behavioral apparatus used for the fear contagion paradigm. Photograph by Anna Mirgos (modified from Kondrakiewicz et al., 2019)**.**

3.2.3. Exploration test

For exploration test, a modified version of an open-field test was used (Fig. 2). The apparatus was a square arena (1m x 1m x 40cm) constructed from gray plastic. One of its corners was brightly illuminated. In the opposite corner a square plastic shelter was placed; it was big enough for rats to enter it (15cm x 15cm) and use as a cover. The whole arena was placed in a sound-attenuated, dimly lighted room (~630 Lux). It was additionally illuminated with infra-red light and recorded with a camera (1080P OV2710, *ELP*) from above.

During testing the rats could freely explore the arena for 6 min. After each animal testing it was cleaned with 70% ethanol.

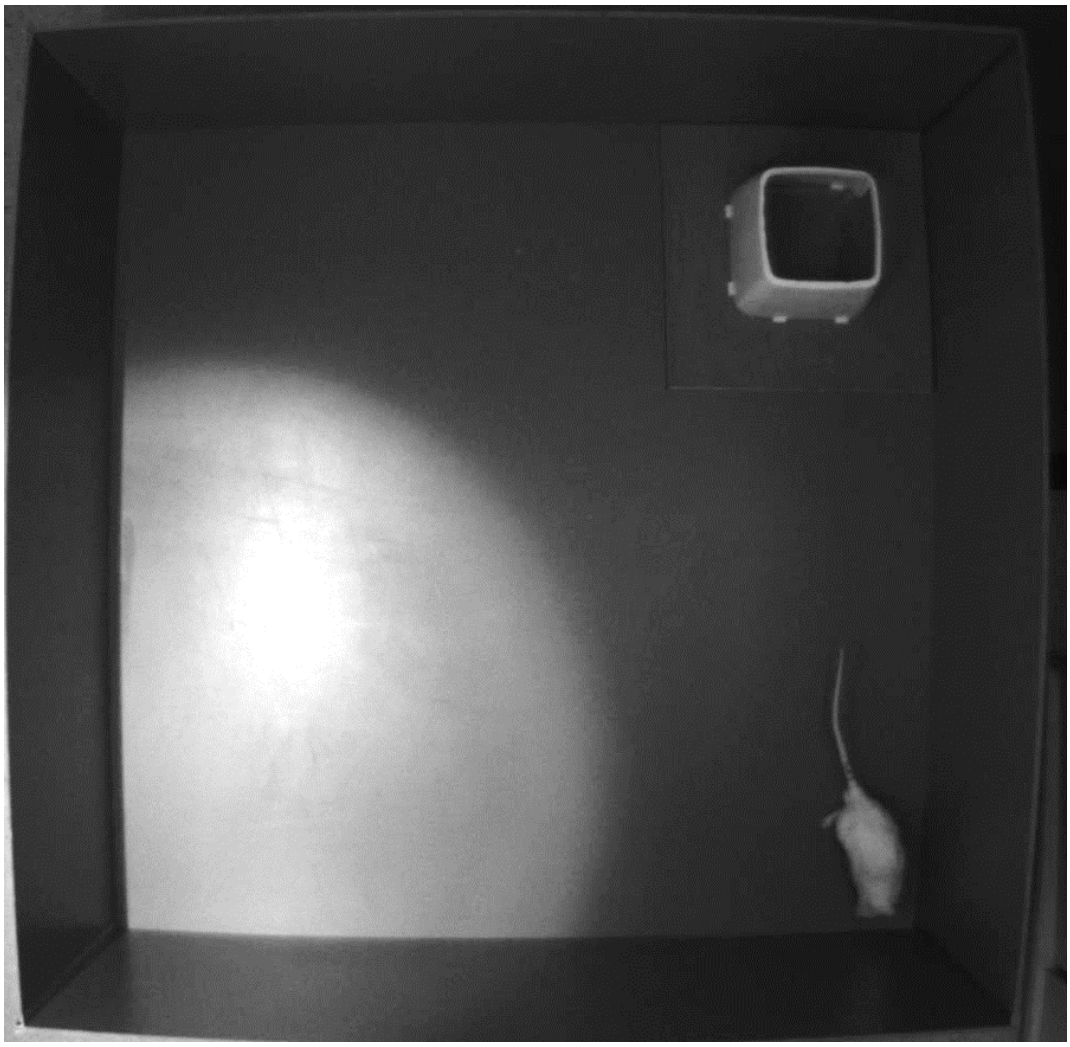


Figure 2. Exploration test. Bottom left – brightly illuminated corner, upper right – the shelter, bottom right – adult Wistar rat.

3.2.4. Social interaction test

To measure social interactions, rats were transported in their home cages (43 x 25 x 18.5 cm) to the experimental room. One of the animals (demonstrator or control) was removed from the cage and placed in a single cage with fresh bedding for 15 min. After that time it was returned to the observer, while the interaction between the rats was recorded for 12 min.

3.2.5. Fear recall test

To measure freezing after the fear contagion paradigm, 24 hours later the observers were returned to the same experimental chamber (Fig. 1). Their behavior was recorded for 8 min. In the auditory cue variant, after 2 minutes of habituation the sound (CS) was presented 3 times with a 1-minute long inter-trial interval.

3.3. Stereotactic surgeries

Before optogenetic, chemogenetic and tracing experiments (see below) rats underwent stereotactic surgery. All the surgical instruments were sterilized prior to the procedure. For anesthesia, isoflurane was used (5% induction, 1% maintenance), followed by additional injection of analgesic butorfanol (1mg/kg subcutaneously). Anesthetized animal was placed in stereotactic apparatus (*David Kopf Instruments*) on a heating pad (*Stoelting*). The scalp was shaved and the eyes were lubricated with an ocular gel Vidisic (*Dr Mann Pharma*). After being disinfected with 70% ethanol, the skin on the skull was incised, moved to the sides and temporally fixed in position using surgical clamps (*Fisher Scientific*). The skull was cleared and two holes were drilled in it. The holes allowed to introduce a 1 μL NanoFil syringe needle (33 GA, *World Precision Instruments*) to the CeA bilaterally according to the following stereotactic coordinates: anteroposterior (AP), -1.8mm; mediolateral (ML), ± 3.8mm; dorsoventral (DV), −7.5 mm relative to bregma (Paxinos & Watson, 2007). In case of chemogenetic experiments, the needle was introduced to BLA (bilaterally), using the following coordinates: AP, -2.4mm; ML, ± 5.0mm; DV, −7.6 mm (Paxinos & Watson, 2007). In all cases the viral vector or tracer (see next section) was then delivered using UMP3 UltraMicroPump (*World Precision Instruments*) with the speed of 100nL/min. The needle was retracted 5 minutes after finishing the injection process to allow for diffusion.

In case of optogenetic surgeries, 2 skull screws (*Bilaney*) were additionally placed in the parietal and frontal areas of the skull, followed by implantation of optic cannulas (see section 'Optogenetics') 0.3 mm above the place of viral vector delivery. The cannulas were then secured with dental cement Duracryl Plus

(*SpofaDental*). In case of tracing and chemogenetic experiments, after injection the skull was sutured using dissolvable stitches (*Peters Surgical*). In all cases after the surgery rats received subcutaneous injection of an analgesic (Tolfedine; 4 mg/kg), antibiotic Baytril (4 mg/kg) and NaCl (0.95%, 2mL).

For chemogenetic experiments, a double-construct strategy was used to limit the DREADD expression only to BLA cells which send projections to CeA. A second surgery was performed 2-3 weeks after the first one. The procedure was identical as described above, with the exception that the Cav-2 vector (see next section) was delivered to CeA (using the same coordinates as for the other surgeries).

3.4. Viral vectors and tracers

During the surgeries, the following vectors/tracers were used:

For optogenetic experiments:

a) AAV-c-Fos-ChR2(H134R)-EYFP - titre 10^7, 400 nL/site or

b) AAV-c-Fos-NpHR-EYFP - titre 10^8, 350 nL/site.

Both constructs were developed, produced and validated in the Nencki Institute of Experimental Biology PAS (Andraka et al., 2020). They carry ChR2 or NpHR gene (variant eNpHR3.0) under *c-fos* promoter, allowing to tag population of neurons which express endogenous c-Fos in response to behavioral stimulation (Knapska et al., 2012). That enables to reactivate or deactivate (using ChR2 or NpHR variant, respectively) the activity-defined neuronal population 24 hours after the behavioral stimulation (Andraka et al., 2020).

For tracing experiments:

- retrograde tracer cholera toxin subunit B (CTB), Alexa Fluor 488 conjugate (C34775, *Life Technologies*) – 200nL/site, 1.0 mg/mL, dissolved in 0.1 M sodium PBS, pH 7.4.

For chemogenetic experiments:

a) For the BLA injection (first surgery): AAV-hSyn-DIO{hCAR}off-{hM4Di-mCherry}on-W3SL – 10^9 titre, 350 nL/site. The plasmid was purchased from *AddGene* (#111397) and the viral vector was produced by the Laboratory of Animal Models, Nencki Institute of Experimental Biology PAS.

b) For the CeA injection (second surgery): Cav2-CreGFP (*Plateforme de Vectorologie de Montpellier*) – 10^{12} titre, 150 nL/site.

3.5. Tissue preparation

After completing behavioral testing, the animals were injected with a lethal dose of Morbital (1ml/kg intraperitoneal, 133.3 mg/ml sodium pentobarbital, 26.7 mg/ml pentobarbital). In case of experiments which involved immunohistochemistry, this step was performed 120 min from the start of behavioral test. After confirming that the rats do not react to tail-pinch, they were transcardially perfused with ice-cold 0.1 M PBS (pH 7.4, *Sigma*), followed by 4% paraformaldehyde (PFA, *POCh*) in PBS (pH 7.4). The brains were removed and stored in the PFA for additional 24 hours at 4°C. After this time they were transferred to 30% (wt/vol) sucrose at the same temperature and stored in it until sinking. Finally, they were frozen to -21°C and cut with cryostat into 40 μm coronal sections.

For immunohistochemistry experiments, two brain sections were selected for each animal. The sections had to include intact CeA (bilaterally) and corresponded to the range 1.5-2.5mm posterior to Bregma, as defined by the stereotactic atlas (Paxinos & Watson, 2007).

3.6. Immunohistochemistry

3.6.1. c-Fos

To detect c-Fos protein, immunohistochemical fluorescent staining was performed on free-floating sections. They were first washed with PBS (0.1 M, pH 7.4, *Sigma*, 3 x 5min), which was followed by blocking with 5% (vol/vol) normal goat serum (NGS) in PBST for 1.5 hours (room temperature). Then the sections

were incubated for 24 hours (at room temperature) in anti-c-Fos rabbit antibody (*Millipore*, 1:1000), diluted in NGS solution (3% in PBST). After this time the sections were rinsed with PBST (3 x 5 min) an incubated for 2 hours (at room temperature) with the secondary antibody (Alexa 555 made in rabbit, *Invitrogen*, 1:500). Finally, they were washed in the PBS (3 x 5 min) and mounted onto glass slides. Then they were covered with a cover glass using Fluoromont G Medium (*ThermoFisher Scientific*).

3.6.2. c-Fos & CRF

To detect c-Fos and CRF simultaneously, double staining was performed. The sections were first washed (PBS, 3 x 5 min), which was followed by blocking with 5% (vol/vol) NGS in PBST for 1.5 hours (room temperature). Then they were incubated in 2 antibodies: anti-c-Fos rabbit (1:1000, *Millipore*) and anti-CRF chicken (1:1000, *ABCAM*), diluted in NGS solution (3% in PBST), for 24 hours in room temperature. After this time the sections were rinsed with PBST (3 x 5 min) and incubated for 2 hours (at room temperature) with the secondary antibodies: Alexa Fluor 488 made in chicken (1:500, *Invitrogen*) and Alexa 555 made in rabbit (1:500, *Invitrogen*). The sections were washed in the PBS (3 x 5 min), mounted onto glass slides and covered with a cover glass using Fluoromont G Medium (*ThermoFisher Scientific*).

3.6.3. c-Fos & PKC δ

The procedure was identical as described above, with one exception: the antibody used on the first day was not anti-CRF, but anti-PKC δ mouse antibody (1:1000, *BD Transduction Laboratories*).

3.7. Optogenetics

Before the stereotactic surgeries, 2 optical cannulas per animal were prepared by gluing multimodal optic fiber (200 μm, NA = 0.39, # FT200UMT *Thorlabs*) into ceramic ferrule (1.25 mm OD, # CFLC230, *Thorlabs*) with epoxy (Hysol 0151,

Loctite). After that the fiber was cut with ruby knife to desired length (~8.5 mm). The part of the fiber extending from the ferrule was polished using a set of polishing papers with gradually decreasing grit size (#F30D, LF6D, LF3D, LF1D, LFCF, *Thorlabs*) and a disc holder (D50-F, *Thorlabs*) until at least 80% light transmission efficiency was achieved. Finally, the ferrule was glued inside a custom-made protective metal tube, which allowed to connect it securely to the input optic fiber with an M3 thread.

To provide light during experiments, 2 lasers were used: a blue (for CHR2 - 472nm, # MGL-FN-473-100mW, *CNI*) or an orange one (for NpHR - 589 nm, # MGL-FN-589-100mW, *CNI*). In each case the laser was connected to an optical commutator & splitter (*Doric Lenses*), and delivered bilaterally to both optical cannulas on the animal head through two metal-shielded optical patchcords (*Doric Lenses*). Before testing each animal, the power of the laser was adjusted using an LED driver (*CNI*) to obtain ~10mW at the cannula tip.

Before the optogenetic experiment, the rats were subjected to the fear contagion paradigm to induce the opsin expression (see section 'Viral vectors and tracers'). In the control group, the animals were exposed to the same chamber, but no foot-shocks were provided. 24 hours later the observers and controls (but not demonstrators) were tested in one of the three behavioral paradigms: exploration test (both ChR2 and NpHR groups), social interaction (ChR2) or fear recall (ChR2). In each case, the paradigm was divided into two, 3-minutes long parts (in counterbalanced order), during which the light was either provided (light ON) or not (light OFF). For the fear recall experiment, this was additionally proceeded by a 2-min long habituation period. In case of NpHR experiments, the light was provided continuously during the whole ON period (10mW). In case of ChR2, the light was provided in pulses (5ms long, 30Hz, 10mW), controlled by an Arduino Uno microcontroller connected to the LED driver. Identical light stimulation was provided both for observers and controls.

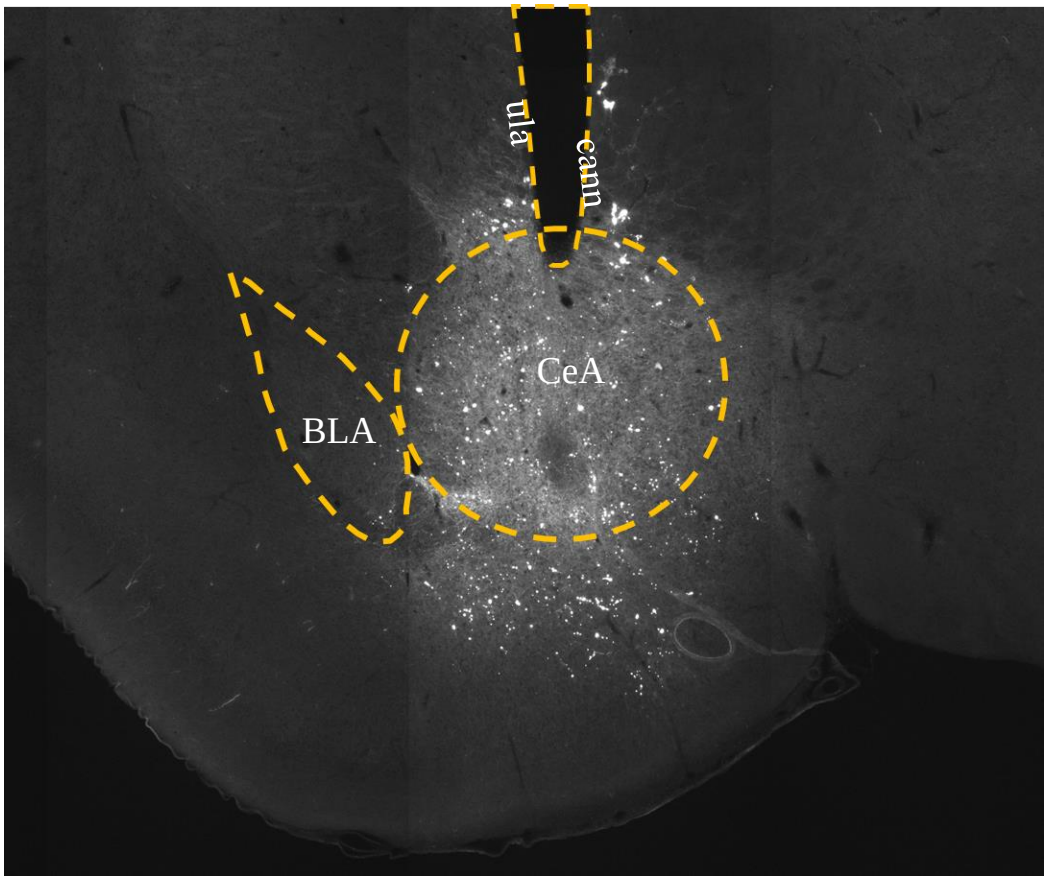


Figure 3. Expression of the optogenetic construct (ChR2). The optogenetic construct was expressed in CeA. The optic fiber was implanted in the upper part of this structure.

3.8. Chemogenetics

Three weeks after the second chemogenetic surgery, the animals were transported to the experimental room and received an intraperitoneal injection of water-soluble C21 (#HB6124, *HelloBio*) diluted in NaCl (3mg/kg, 10mg/ml). 30 min after the injection the animals were subjected to the exploration test. The control group was injected with the same dose of the ligand, but it previously did no undergo any surgery.

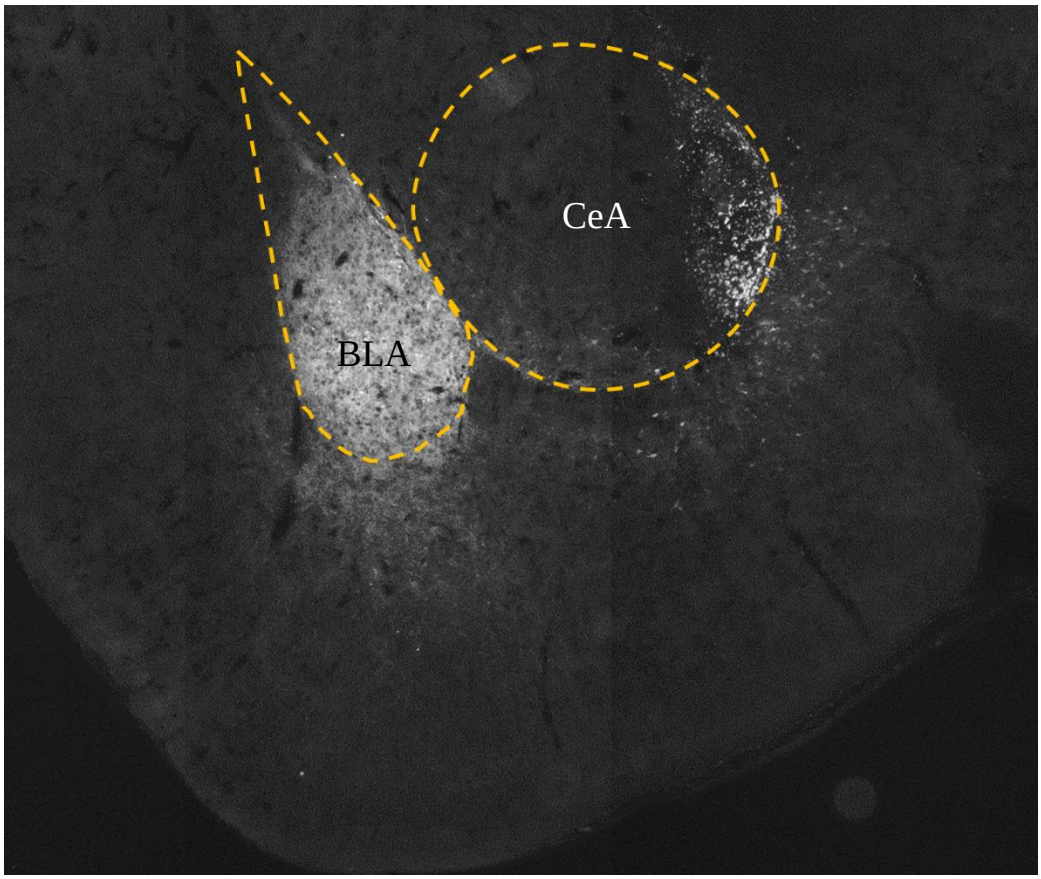


Figure 4. Expression of the chemogenetic construct. The chemogenetic construct was expressed in BLA cells which sent projections to CeA.

3.9. Image collection and analysis

To analyze the results of the immunohistochemical stainings and/or assess the quality of stereotactic surgeries, coronal brain sections were photographed with a Nikon Eclipse Ni-U fluorescent microscope. It was equipped with a color camera (QImaging QICAM Fast 1394) and two lasers allowing to emit the following wavelengths: 467 or 488nm (Ar laser) and 568 nm (Kr laser). Multiple images were acquired using the 10x objective and tailed together by the Image-Pro Plus software (v. 7.0.1.658, *Media Cybernetics*), to include whole area of interest in a single image. Then they were saved as monochromatic 16-bit .tiff files, with a separate file for each wavelength in case of the double-staining experiments.

To perform manual image analysis, ImageJ software was used. The following parameters were calculated:

For c-Fos staining:

The number of c-Fos positive nuclei in two main divisions of CeA: CeL and CeM, divided by the area of each nucleus (to obtain cell density / mm^2).

For double stainings (c-Fos + CRF or PKC δ):

The images with two fluorescence colors were overlaid on each other. This allowed to count (for CeL and CeM) the number of cells which were c-Fos positive and double positive (c-Fos & CRF/PKC δ). For the analysis, the mean ratio of c-Fos positive to double positive neurons was calculated for each animal (using 4 samples – 2 from left and 2 from right CeA).

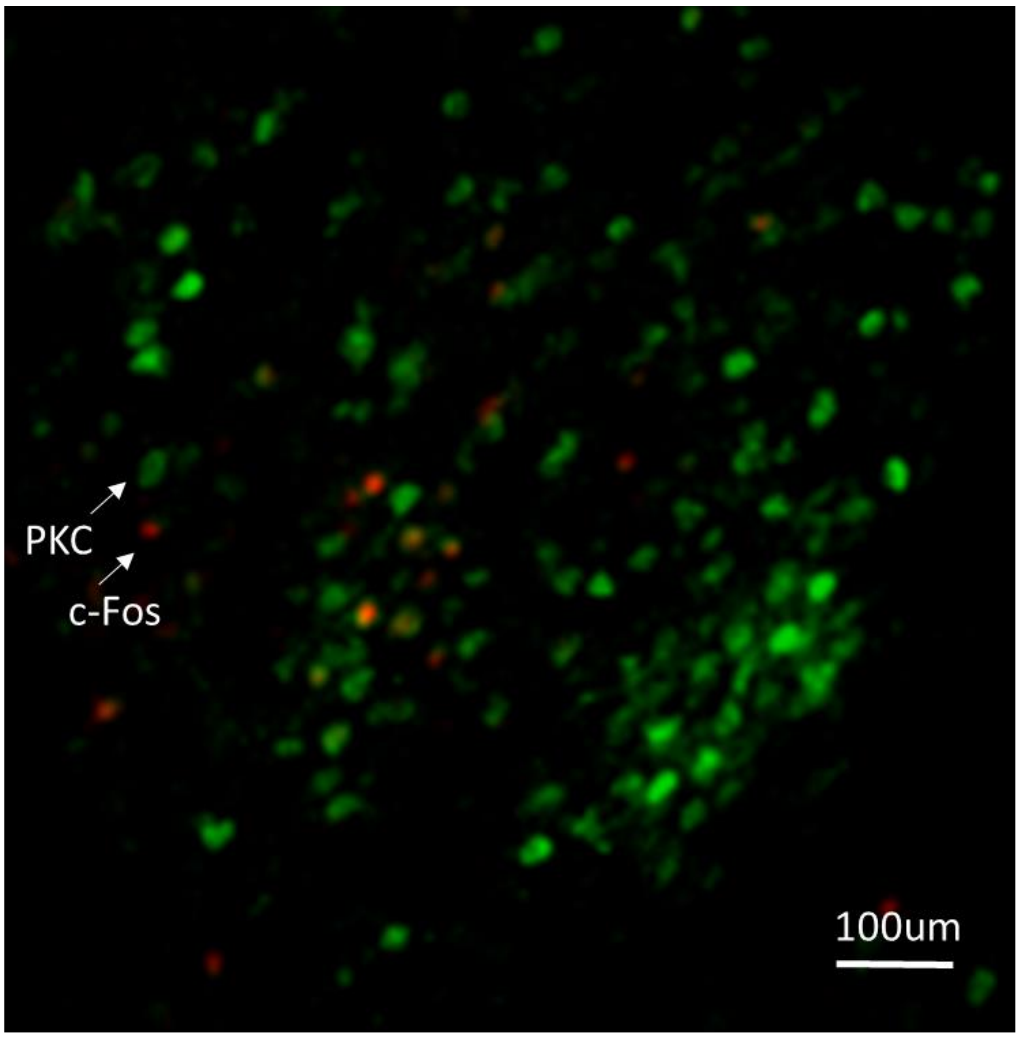


Figure 5. Double staining example. In green - PKC δ, in red – c-Fos.

For CTB tracing experiment:

Similarly as for the double stainings, the two images were overlaid. The number of CTB positive and double positive (c-Fos & CTB) cell bodies was counted for several brain structures which are known to send projections to CeA: anterior cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL), anterior insular cortex (AI), basolateral amygdala (BLA) and CA1 field of hippocampus (CA1). For the analysis, the mean ratio of c-Fos positive neurons to double positive neurons was calculated, using 4 samples for each rat (2 bilaterally). Only data from the animals in which the injection of the tracer was limited to CeA was used.

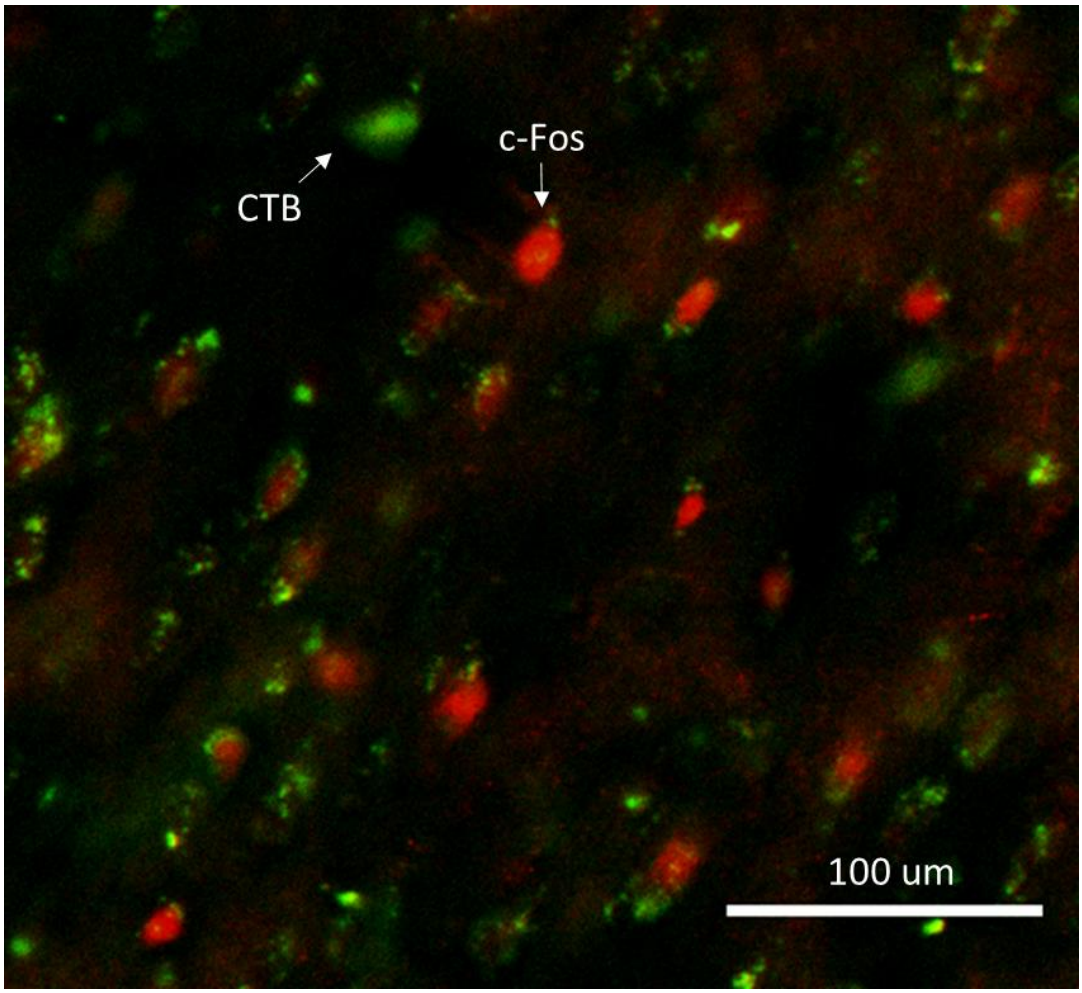


Figure 6. Functional tracing example. In green - CTB, in red – c-Fos.

For optogenetic experiments:

The images were used to decide which animals should be excluded from behavioral data analysis, based on unsuccessful stereotactic surgery. The criteria for exclusion were: 1) no expression of the construct or 2) expression not limited to CeA or 3) optic cannula not localized in the CeA.

For chemogenetic experiment:

Similarly as for the optogenetic experiments, the images were used for exclusion of some animals. The criteria for exclusion were: 1) no expression of the construct or 2) expression not limited to BLA or 3) the needle tract localized outside CeA, indicating that delivery of the Cav-2 was not limited to axon terminals within CeA.

3.10. Behavioral data analysis

3.10.1. Manual scoring

The videos acquired during behavioral tests were analyzed manually with single-frame temporal resolution, using the software BehaView (http://www.pmbogusz.net/?a=behaview, P. Boguszewski). The list of scored behaviors included:

For the fear contagion paradigm: exploration (locomotion and head movements), freezing (defined as no movement except from respiration for at least ~1sec), rearing

For the exploration test: exploration (which included: locomotion, head movements, exploring the bright corner, rearing) and avoidance (which included: being in the dark shelter, running away from the bright corner, immobility)

For the social interaction test: exploration (locomotion and head movements), rearing, social contact (any non-violent physical contact between the animals).

3.10.2. Automatic analysis

To analyze the fear contagion and fear recall experiments, freezing was automatically detected using BehaActive software (P. Boguszewski). The threshold for the frame-to-frame change was adjusted to be crossed by any movement except from respiration (Kondrakiewicz et al., 2019). The binary movement data (with 5Hz resolution) was then post-processed to filter out all the cases in which cessation of movement was shorter than 1 second. All the remaining no-movement data points were interpreted as freezing.

To calculate distance travelled by rats during the exploration test, EthoVision XT9 (*Noldus*) was used. The software tracked animal position during the experiment. All the obtained tracks were manually curated by a human observer to exclude estimation errors.

3.10.3. Ultrasonic vocalizations

Ultrasonic vocalizations (USVs) were analyzed semi-automatically using RatRec software (M. Kursa, A. Hamed). The recorded sound was displayed as a spectrogram. An amplitude threshold was manually adjusted to discard as much noise as possible, while at the same time preserving all the vocalizations in the spectrogram. The software then selected all the candidate USV episodes (continuous sounds within frequency range of 18-100kHz). The selection was manually curated to discard any sounds which were not USVs. Finally, the software calculated mean frequency of each USV episode.

3.11. Statistical analysis

3.11.1. Between- and within-group comparisons

All the statistical testing was performed in GraphPad Prism 8 software (*GraphPad*). To compare behavior of observers, demonstrators and controls, ANOVA followed by post-hocs (t-tests with FDR correction for multiple comparisons) was used. If the distribution of the data was different from the normal

distribution (as assessed with Shapiro-Wilk test), a Kruskall-Wallis test was used instead, followed by Dunn's tests (with FDR correction). The p values after FDR correction for multiple comparisons are reported as 'p(q)'.

If two groups were compared during one time point, unpaired t-test or U-Mann Whitney test was used, respectively. To verify the effect of the light stimulation the during optogenetic experiments, a difference score was calculated for each rat. The score indicated how much a given parameter changed as a result of the light stimulation relative to baseline (light ON – light OFF). The distribution of the difference scores was tested against null hypothesis that mean equals 0 using one-sample t-test.

To compare behavior of 2 groups of rats ('CS-first' vs. 'US-first') in different time points, a 2x2 mixed ANOVA followed by Sidak post-hoc tests was used. The freezing during different experiment periods (before, during and after CS) was compared within-subject to the baseline (habituation) period. Additionally, for each period a between-group comparison was performed.

3.11.2. Synchronization analysis

To verify the hypothesis that observers mimic behavior of demonstrators, 3 different methods were used on data from several experiments pulled together. All the analyses were performed using custom-written Matlab scripts (*MathWorks*).

Spearman rank correlation was calculated to test for correlation between general amount of time spent on freezing by demonstrators and observers. This non-parametric method was chosen because the distribution of freezing was deviated from normal.

Cross-recurrence profile was plotted to asses if freezing of observers occurred with some regular interval after freezing of demonstrators. First, the binary freezing data was down-sampled to 1Hz. Then a diagonal cross-recurrence profile was calculated according to procedure implemented by (Coco & Dale, 2014), similarly as in (Richardson & Dale, 2005), using a custom-written Matlab function (David Lopez-Perez). Briefly, for each pair of rats it was computed how often the freezing of an observer co-occurred with the freezing of a demonstrator, while

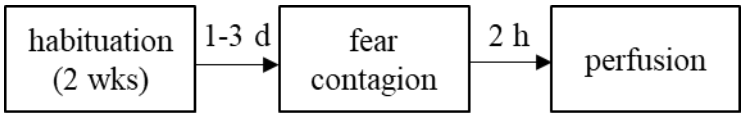
systematically shifting one of the time series from -20 to +20 seconds relative to the other. To obtain distribution of control profiles, the same calculation was performed 100 times after shuffling randomly the demonstrators freezing in time. The 100 control profiles were averaged, which allowed to obtain one real and one control profile from each pair and compare them on a group level with paired t-tests, separately for each time point (with FDR correction). To illustrate the results, mean profile and standard errors of the mean were calculated for the whole group, both for the real data and the shuffled control.

Granger causality was used to verify the conclusions derived from the cross-recurrence profiles. It was performed according to the procedures described by (Han et al., 2019), using the Matlab Multivariate Granger Causality Toolbox (Barnett & Seth, 2014). The binary freezing time series (1Hz) was smoothed with a Gaussian kernel (width = 300s, sigma = 1.5) to get numeric data. The toolbox automatically verified that the data from each pair passed stationarity assumption. Then it determined the optimal model order - that is, number of previous data points used for prediction – as 19 seconds. Finally, a group of regression models was built to calculate how well the past freezing of demonstrator predicts the freezing of observer and vice versa. To summarize the quality of prediction mathematically, the G-causality F values were calculated. To obtain control distributions of the F values, the same procedure was repeated 1000 times, while shuffling the predictors in time (demonstrators freezing if the observers freezing was predicted and vice versa). The distributions allowed to calculate for each pair (and each direction of prediction) the *p* value of prediction quality.

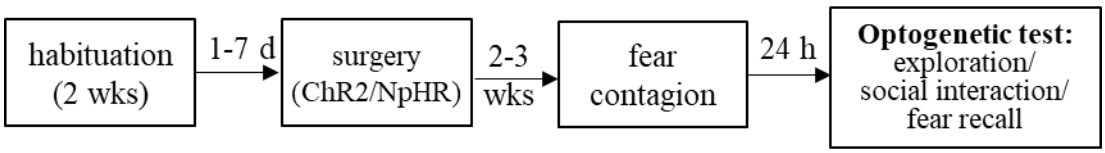
3.12. Experimental timeline & sample sizes

The following schemes summarize the timeline of all the experiments:

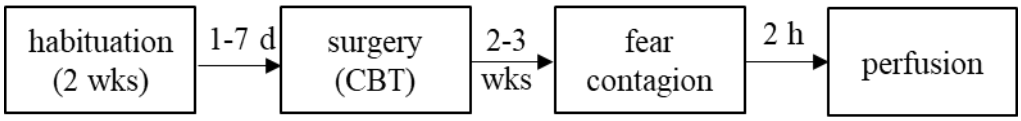
Behavioral and immunohistochemical:



Optogenetic:



Functional tracing:



Chemogenetic:

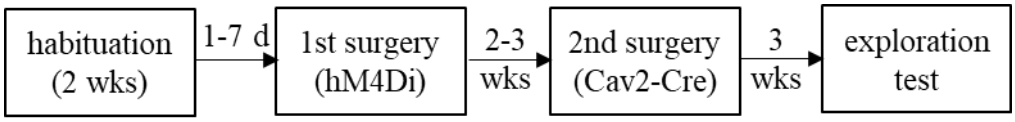


Table 1 summarizes sample sizes in all the experiments, reporting how many animals were subjected to the experiment procedures (total) and how many were included in the final analysis (used). The reasons for exclusion were: a) unsuccessful surgery b) data loss (tissue damage or data acquisition software malfunction). If demonstrators were not tested in a given experiment (that is, they were used solely as behavioral stimulus for the observers), they are marked as '-' in the column 'used'.

Table 1. Summary of sample sizes.

	Demonstrators		Observers		Controls	
Experiment type	total	used	total	used	total	used
Behavioral (contextual)	10	10	10	10	10	10
USVs recordings	10	9	10	9	10	9
Behavioral (cued)	16	16	16	16	-	-
Immunohistochemical – c-Fos staining	8	-	8	7	10	10
Immunohistochemical - double stainings	8	-	8	8	8	8
Optogenetic - exploration test, ChR2	10	-	10	8	10	8
Optogenetic - exploration test, NpHR	11	-	11	7	11	10
Optogenetic – social interaction, ChR2	9	-	9	8	8	8
Optogenetic – fear recall, ChR2	6	-	6	5	4	4
Functional tracing	4	-	4	3	4	4
Chemogenetic	-	-	10	7	11	11

4. Results

4.1. Behaviors observed in fear contagion paradigm

To characterize the behavioral effects of fear contagion, the responses of demonstrators, observers and controls were compared (Fig. 7). Between-group differences were observed for all the investigated behaviors: freezing [$F(2, 27) = 14.41$, $p < 0.001$, Fig. 7A], rearing [$F(2, 27) = 4.373$, $p = 0.023$, Fig. 7B] and walking [$H(2) = 17.06$, $p < 0.001$, Fig. 7C]. Further post-hoc tests revealed that – as expected – demonstrators stimulated with foot-shocks displayed more freezing than controls [$t(18) = 5.368$, $p(q) < 0.001$] and also more than observers [$t(18) = 2.751$, $p(q) = 0.015$, Fig. 7A]. Observers displayed more freezing than controls as well [$t(18) = 2.617$, $p(q) = 0.015$, Fig. 7A]. The time spent on rearing was similar between observers and demonstrators [$p(q) = 0.695$, ns.], but both groups spent less time on it than controls [$t(18) = 2.736$, $p(q) = 0.011$ and $t(18) = 2.340$, $p(q) = 0.014$, respectively, Fig. 7B]. Finally, both observers and demonstrators were walking in the testing chamber less than controls [$M_{diff} = -9.3$, $p(q) = 0.009$ and $M_{diff} = 16.2$, $p(q) < 0.001$, respectively]; demonstrators were walking also significantly less than observers [$M_{diff} = 6.9$, $p(q) = 0.028$, Fig. 7C]. To sum up, during fear contagion paradigm observers display similar behaviors as demonstrators, but not necessarily with the same intensity.

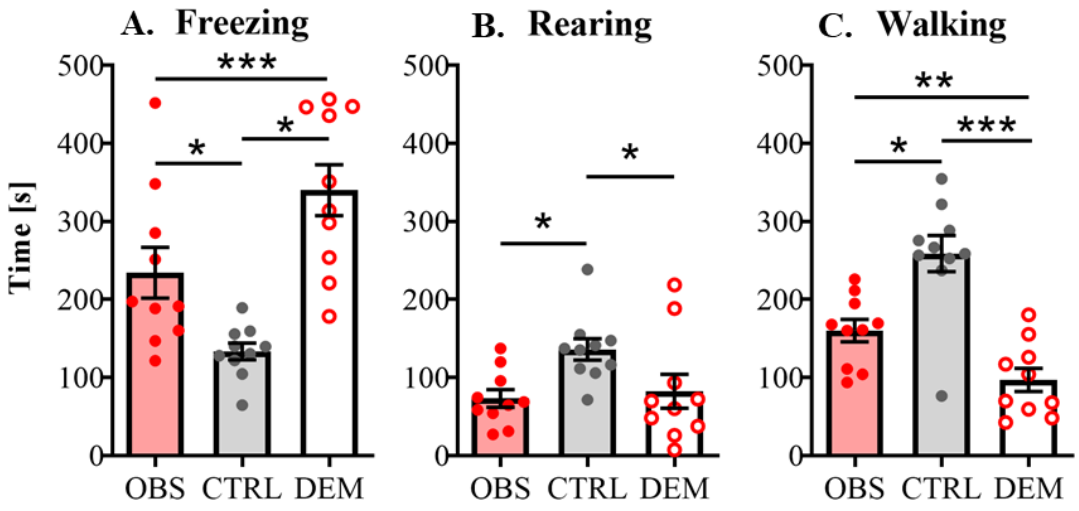


Figure 7. Behavioral results of the fear contagion experiment. OBS – observers, CTRL – controls, DEM – demonstrators. Bars indicate the mean and error bars

indicate standard error of the mean (SEM). * - p(q) < 0.05, ** - p(q) < 0.01, *** - p(q) < 0.001

4.2. Ultrasonic vocalizations

Analysis of USVs revealed that rats emitted different sounds in experimental and control condition during the fear contagion paradigm. Manual inspection demonstrated that experimental pairs emitted mainly characteristic alarm calls (long, flat sounds with low frequency, ~22kHz, Fig. 8A), whereas control pairs emitted much more diverse, high frequency USVs (~50kHz, Fig. 8B). Semi-automatic quantification confirmed that the vocalizations recorded from experimental pairs were on average lower in frequency than the ones from control pairs [U = 0, p < 0.001]. Furthermore, experimental pairs emitted more episodes of vocalizations than control pairs [U = 12, p = 0.009].

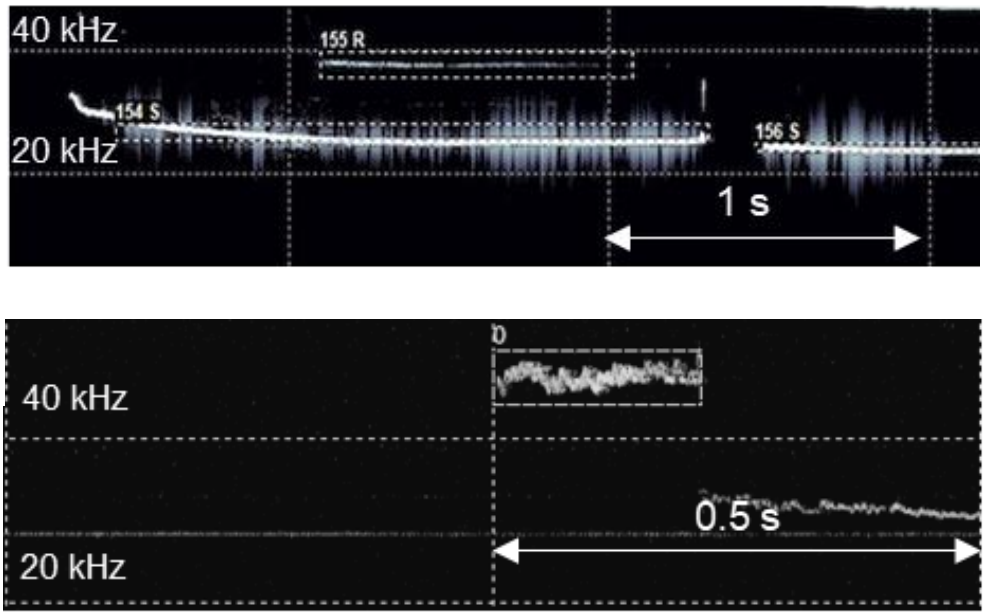


Figure 8. Representative vocalizations emitted by experimental (A) and control pairs (B). Please note that two USVs (annotated as '155R' and '154S', panel A) were emitted simultaneously, that is – by two different rats (observer and demonstrator).

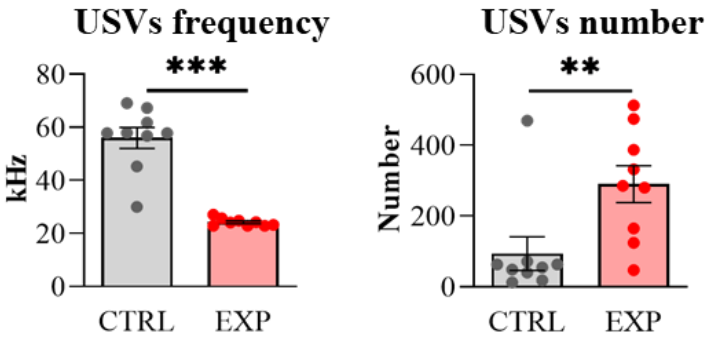


Figure 9. Frequency (left) and number (right) of ultrasonic vocalizations. CTRL – control pairs, EXP – experimental pairs. Bars indicate the mean and error bars indicate SEM. ** - $p < 0.01$, *** - $p < 0.001$

Based on spectrograms it was not possible to determine which rats – observers or demonstrators – emitted the USVs. However, some rare instances of two vocalizations emitted simultaneously were detected in 3 out of 9 experimental pairs (for example, see Fig. 8A). These cases prove that at least some of the alarm calls were emitted by the observers and not exclusively by the demonstrators.

4.3. Behavioral synchrony

To verify if during the fear contagion paradigm observers mimic freezing of demonstrators, three different analyses were performed. To increase the sample size, data from several behavioral experiments (described in the next sections) was pulled together (n = 27 pairs). First, Spearman rank correlation was calculated between the general percentage of time spent on freezing by demonstrators and observers. A statistically significant, positive relationship was observed [rho = 0.44, $p < 0.05$, Fig. 10].

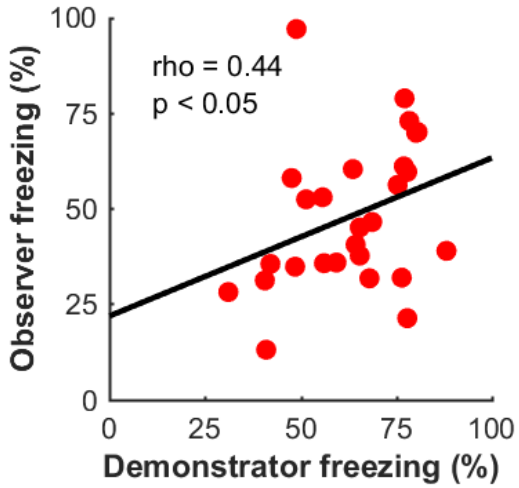


Figure 10. Correlation between time spent on freezing by demonstrators and observers.

Next, a cross-recurrence profile (see Methods) was plotted (Fig. 11). Briefly, it illustrates what is the probability of demonstrators freezing relative to detection of observers freezing within a +/- 20 seconds window (n = 27 pairs). A clear peak on the left side of the plot would indicate that freezing of observers was usually proceeded by freezing of demonstrators with some regular time interval. No such peak is visible. In fact, the profile is relatively flat and does not significantly differ from the shuffled control (see Methods), indicating that freezing of observers and demonstrators was weakly synchronized on the group level.

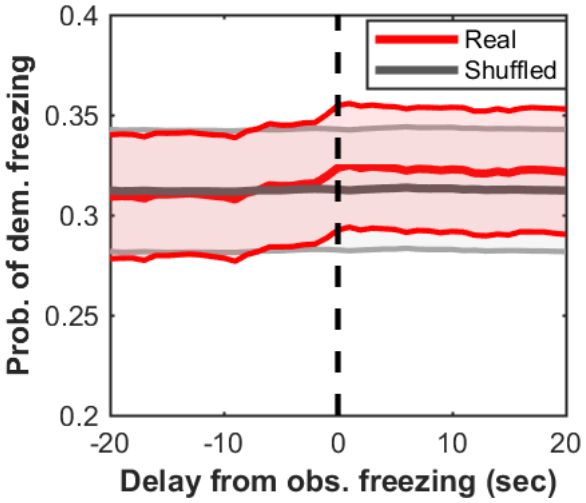


Figure 11. Cross-recurrence freezing profile. Solid thick lines indicate group-average profile for real data (red) and control shuffled condition (gray). Shaded areas between thinner lines indicate SEMs.

Finally, to verify the conclusions a Granger causality analysis was performed. The regression models constructed for each pair if rats were summarized with G-causality (F) values, indicating how well freezing of the demonstrator allowed to predict freezing of the observer ('From DEM to OBS', Fig. 12) and vice versa ('From OBS to DEM'). The obtained G-causality values were subtracted from mean control values, calculated by repeating the analysis 1000 times for each pair on temporally shuffled data (see Methods). Such difference score indicates how much the prediction improved relative to random (shuffled condition). The distribution of the score ('G-causality – shuffled', Fig. 12) is centered around 0, indicating that taking into account freezing of one animal does not allow to better predict freezing of the partner (in both directions). When individual scores for each pair were compared to respective control distributions, only in 3 out from 54 cases (27 pairs x 2 directions) the obtained p values crossed conventional significance threshold (<0.05, uncorrected for multiple comparisons). Thus, on the group level the Granger causality analysis does not allow to predict when the observers displayed freezing based on freezing of the demonstrators (or vice versa). To sum up, the 3 methods of analysis revealed that there was a positive correlation between the general amount of time spent on freezing by both animals, but the synchrony (correlation across time) could not be detected.

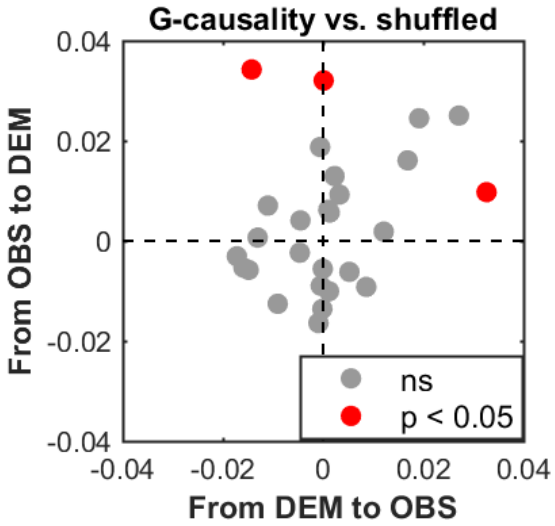


Figure 12. Granger causality analysis. The points illustrate for each pair of rats how much the prediction improved relative to shuffled control in two directions – from demonstrators to observers (x axis) and from observers to demonstrators (y axis). Statistically significant improvements ($p < 0.05$) are marked in red; no correction for multiple comparisons was applied.

4.4. Auditory cue conditioning

To verify if naïve rats could learn through observation, animals were tested in a modified version of the fear contagion paradigm, in which the shocks (US) were associated with auditory cues (CS). In the first variant ('CS first' group) the auditory cue proceeded the foot-shock given to demonstrator, while in the second variant ('US first' group) the order was reversed. To examine responses to the sound, the freezing data was averaged separately for 4 different phases of the experiment: baseline (the two minutes long period before the first shock), pre-CS (20 seconds before presentation of each sound), CS (20 seconds during the sound), post-CS (20 seconds after each sound).

As expected, behavior of demonstrators changed across time [$F(2.441, 34.17) = 151.0$, $p < 0.001$; Fig. 13], as they were freezing more during all examined periods than during the baseline [all $p < 0.001$]. Overall, the level of freezing between the two groups was similar [ns.]. However, there was a significant

interaction effect between examined period and experimental group [$F(3, 42) = 12.58$, $p < 0.001$]. Demonstrators from the 'US-first' group – in which the sound was a safety cue, signaling end of shock – displayed higher freezing during post-CS period than demonstrators from the 'CS-first' group [$p < 0.05$].

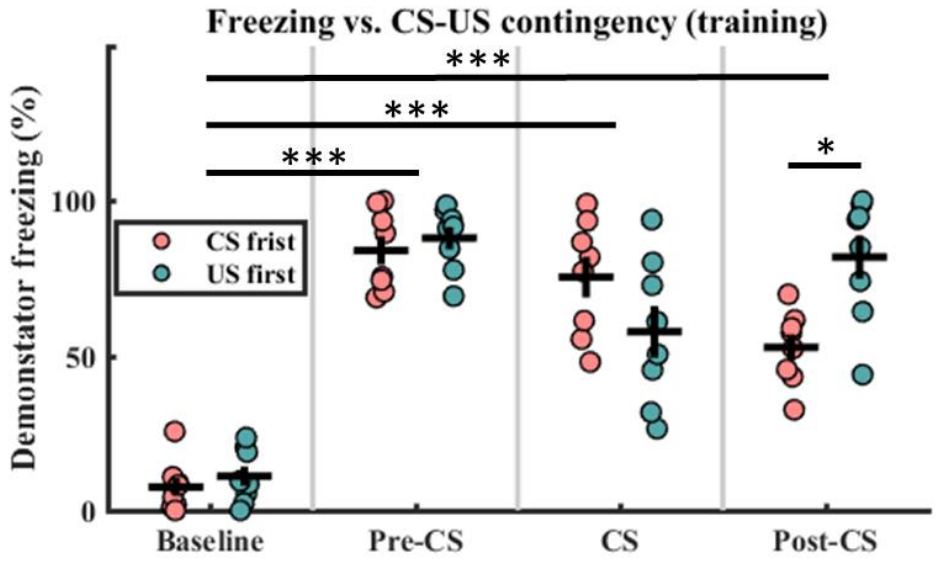


Figure 13. Freezing of demonstrators during different phases of auditory cued conditioning. Horizontal lines indicate mean and vertical lines indicate SEM. CS – conditioned stimulus. * - $p < 0.05$, *** - $p < 0.001$

Observers also reacted with an increase of freezing relative to baseline [main effect of time: $F(1.271, 17.79) = 31.26$, $p < 0.001$; Fig. 14], which was significant for all the three examined periods [all $p < 0.001$]. The effects of group and interaction were not detected [ns.]. Unlike demonstrators, observers from 'CS-first' and 'US-first' groups displayed very similar level of freezing before, during and after the sounds [ns.].

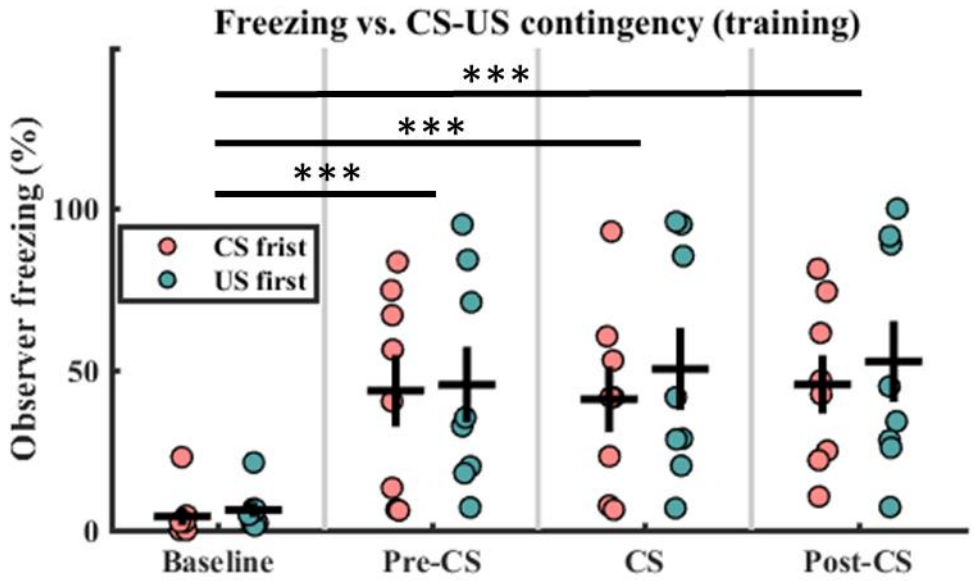


Figure 14. Freezing of observers during different phases of auditory cued conditioning. Horizontal lines indicate mean and vertical lines indicate SEM. CS – conditioned stimulus. *** - $p < 0.001$

Observers from both groups were tested also in a fear recall paradigm 24 hours later. The fear contagion resulted in learning, which was indicated by significant effect of time [$F(2.077, 29.08) = 10.64$, $p<0.001$; Fig. 15]. Specifically, rats increased their freezing relative to baseline during CS [$p = 0.016$] and post-CS [$p = 0.004$], but not during the pre-CS period [ns.]. Efficiency of learning was similar between paradigm variants, as the effects of group and interaction were not detected [ns.]. Observers from 'CS-first' and 'US-first' groups displayed similar freezing during all examined phases of test [ns.].

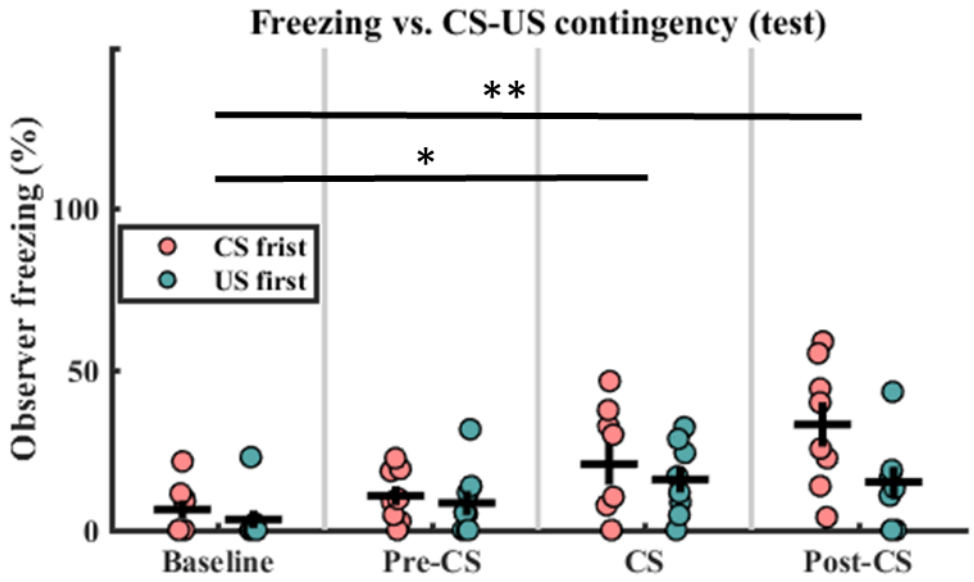


Figure 15. Freezing of observers during different phases of auditory fear recall. Horizontal lines indicate mean and vertical lines indicate SEM. CS – conditioned stimulus. * - $p < 0.05$, ** - $p < 0.01$

4.5. Activation of CeA neuronal populations

Immunohistochemical staining for c-Fos was performed to verify if the fear contagion paradigm activates CeA. Because significant expression was expected in the control animals – in the response to the novel environment of behavioral chamber – an additional 'home cage' group was also tested (n = 6). This group did not undergo any behavioral procedures before the perfusion and immunohistochemical detection of c-Fos. Fig. 16 illustrates the results obtained from observers, controls and home-cage animals. A statistically significant difference between groups was detected [$H(2) = 10.47$, $p = 0.005$]. Post-hoc tests demonstrated that both observers and controls had higher c-Fos density than home-cage animals [$M_{diff} = 7.98$, $p(q) = 0.036$ and $M_{diff} = 11.28$, $p(q) = 0.003$, respectively]. No difference between observers and controls was found [ns.].

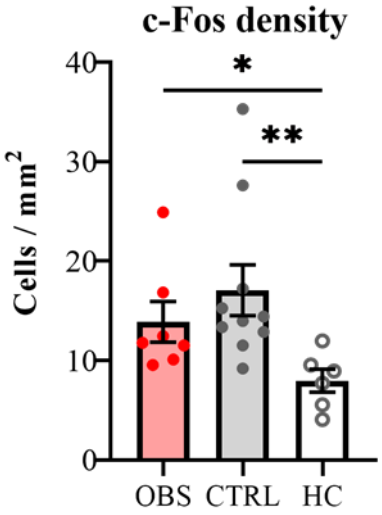


Figure 16. Expression of c-Fos in CeA. OBS – observers, CTRL – controls, HC – home cage. Bars indicate the mean and error bars indicate SEM. * - p(q) < 0.05, ** - p(q) < 0.01

The fact that similar number of neurons expressed c-Fos in observers and controls did not exclude possibility that the cells activated in each case were characterized by different molecular markers. To test this, double stainings for c-Fos + PKCδ and c-Fos + CRF were performed. The results (Fig. 17) are illustrated separately for two main divisions of CeA: CeM and CeL. No significant differences between the groups (observers vs. controls) were found for the tested cell populations: neither in PKCδ+ nor CRF+, in any part of CeA [all p > 0.05].

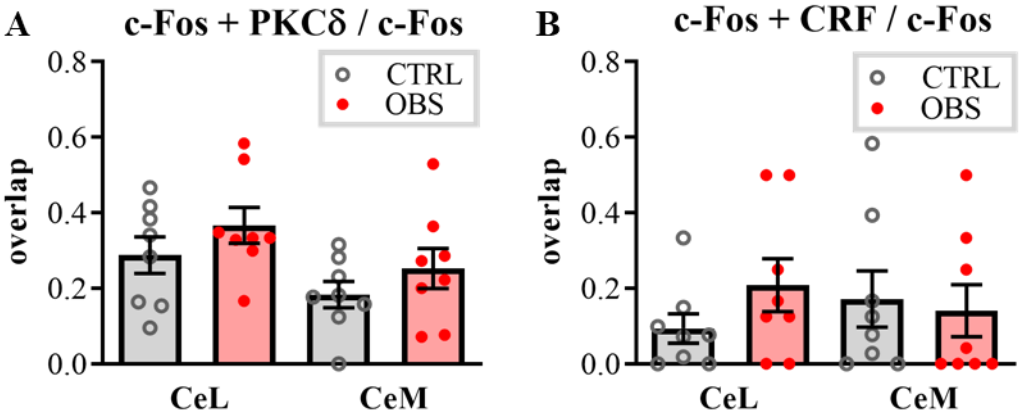


Figure 17. Activation of (A) PKCδ+ and (B) CRF+ neuronal populations. CeL – centrolateral amygdala, CeM – centromedial amygdala, CTRL – controls, OBS – observers. Bars indicate the mean and error bars indicate SEM.

4.6. Optogenetic manipulations

4.6.1. Exploration test – activation

Despite negative results of immunohistochemical experiments, it was still possible that fear contagion activated in observers a population of CeA cells which was either heterogeneous or characterized by different markers than PKCδ or CRF. To manipulate a population of cells without a known molecular marker, an activity-dependent approach was used. A viral vector carrying ChR2 under c-Fos promoter (see Methods) allowed to tag CeA cells activated during fear contagion in observers and controls, and reactivate these neurons optogenetically 24 hours later during exploration test. The light stimulation increased time spent by observers on avoidance [t(7) = 3.636, p = 0.008, Fig. 18A] and decreased time spent on exploration [t(7) = 3.044, p = 0.019, Fig. 18B]. It did not influence distance travelled [ns. , Fig. 18C]. No effects were observed in the control group (Fig.18A-C), which underwent identical treatment as observers (viral vector injection and light stimulation) with the exception that no foot-shocks were provided to any of the rats during the fear contagion paradigm 24 hours earlier.

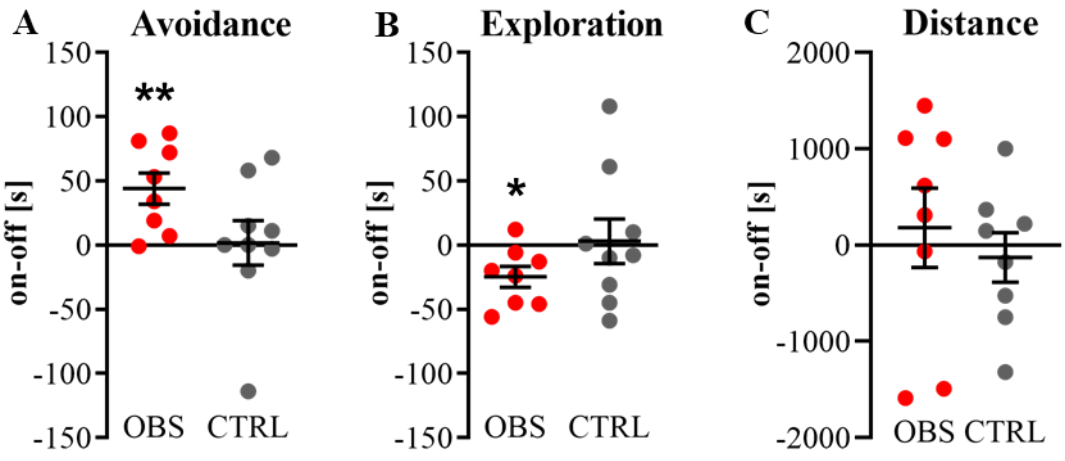


Figure 18. Effects of light stimulation (ChR2 group) during the exploration test. (A) Avoidance (B) Exploration (C) Distance travelled. The results are shown as a difference score between time spent on respective behavior during light ON and light OFF periods (in seconds). OBS – observers, CTRL – controls. Horizontal lines indicate the mean and error bars indicate SEM. * - $p < 0.05$, ** - $p < 0.01$.

4.6.2. Exploration test - inhibition

To complement the results of the ChR2 experiment, the same procedure was repeated, but this time with eNpHR3.0 in order to inhibit the neuronal population activated by fear contagion. Although the mean effects of light stimulation were shifted in the opposite direction as for the ChR2 experiment (Fig. 19A-B), the effects were significant neither for exploration nor avoidance [ns.]. Instead, the orange light stimulation increased the distance travelled by the observers [$t(6) = 2.656$, $p = 0.0377$, Fig. 19C], but not by the control group [ns.].

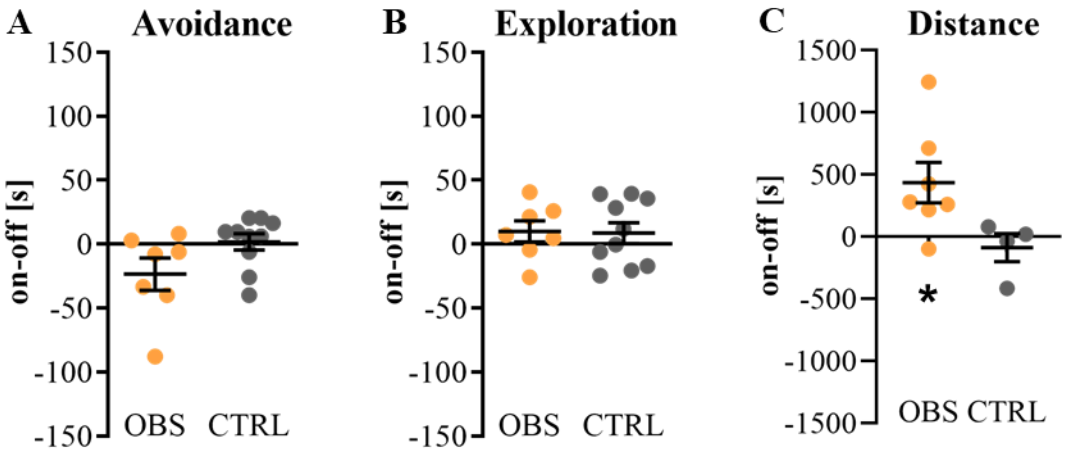


Figure 19. Effects of light stimulation (NpHR group) during the exploration test. (A) Avoidance (B) Exploration (C) Distance travelled. The results are shown as a difference score between time spent on respective behavior during light ON and light OFF periods (in seconds). OBS – observers, CTRL – controls. Only 4 points are shown in panel C for CTRL due to data loss. Horizontal lines indicate the mean and error bars indicate SEM. * - $p < 0.05$, ** - $p < 0.01$.

4.6.3. Social interaction

The results of the optogenetic experiments suggested that the population of CeA cells activated by the fear contagion promotes passive behaviors and decreases exploration. To investigate further if this population regulates also social behaviors,

the ChR2 experiment was repeated, but this time instead of exploration test, a social interaction assay was used. Stimulation of neurons activated by the fear contagion paradigm influenced neither social interaction [ns., Fig. 20A] nor exploration of the home cage [ns., Fig. 20B]. Instead, it decreased time spent on rearing by the observers [$t(7) = 2.379$, $p = 0.049$, Fig. 20C]. No effects for controls were observed [ns., Fig. 20A-C].

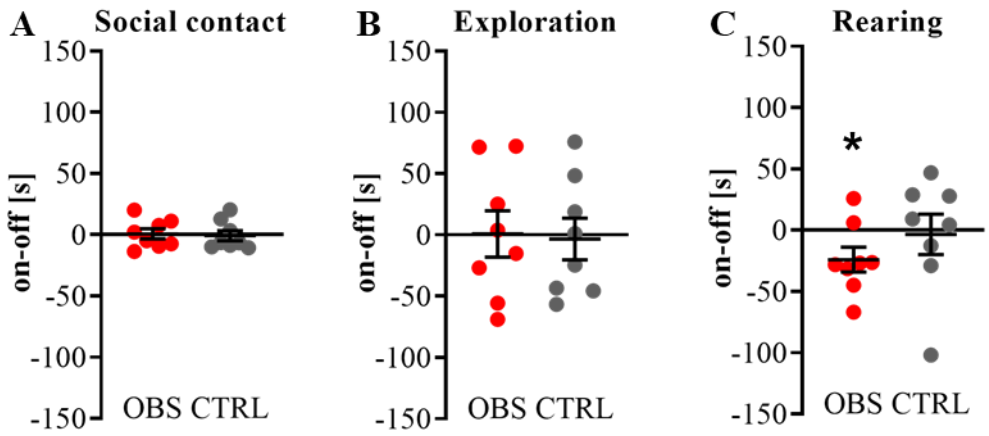


Figure 20. Effects of light stimulation during social interaction. (A) Social contact (B) Exploration (C) Rearing. The results are shown as a difference score between time spent on respective behavior during light ON and light OFF periods (in seconds). OBS – observers, CTRL – controls. Horizontal lines indicate the mean and error bars indicate SEM. * - $p < 0.05$.

4.6.4. Fear recall

The results of the optogenetic experiments suggested that the population of CeA cells controlled defensive/exploratory behaviors rather than social interactions. However, in both cases the spacious testing environment did not allow to reliably detect freezing. To verify if the optogenetic reactivation in a different environment could evoke freezing typical for the fear contagion paradigm, the experimental design was modified. As previously, the animals were subjected to the fear contagion paradigm; 24 hours later they were tested optogenetically in the same, confined chamber, normally used for the fear conditioning. Under these conditions,

light stimulation increased freezing in the observers [Z = 36, p = 0.048, one-sided, Fig. 21] but had no effects in the control group [ns.].

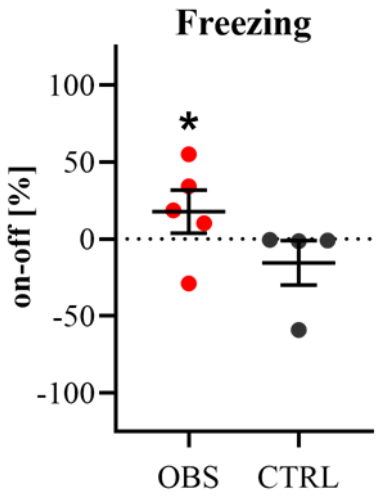


Figure 21. Effects of light stimulation during fear recall. The results are shown as a difference score between time spent on respective behavior during light ON and light OFF periods (in freezing %). OBS – observers, CTRL – controls. Horizontal lines indicate the mean and error bars indicate SEM. * - $p < 0.05$.

4.7. Functional tracing

A functional tracing approach was used to characterize which inputs drive activity of CeA during fear contagion (see Methods). Staining for c-Fos allowed to get a proxy for neural activation in multiple brain structures of observers and controls: ACC, IL, PL, BLA, AI and CA1. (Fig 22A). In combination with the retrograde tracer CBT, it enabled to determine also how many of the active cells were sending projections to CeA (Fig. 22B). Although the small sample sizes (n = 3 observers & 4 controls) did not allow for robust statistical analysis, three observations could be made. First, the functional tracing results (proportion of active cells which send projections to CeA) differentiated observers and controls more than just the activity measure (c-Fos; Fig. 22 A vs. B). Second, the control stimulation activated proportionally more neurons which project from IL, PL, AI and CA1 to CeA than the fear contagion (Fig. 22B). Third, the only structures for which the proportion of active projections was higher for observers than for controls

were BLA and ACC (Fig. 22B). Furthermore, for BLA also higher expression of c-Fos was observed (Fig. 22A). Although the results were not statistically significant, they suggested that the BLA-CeA projection could be especially important for the control of defensive responses during the fear contagion.

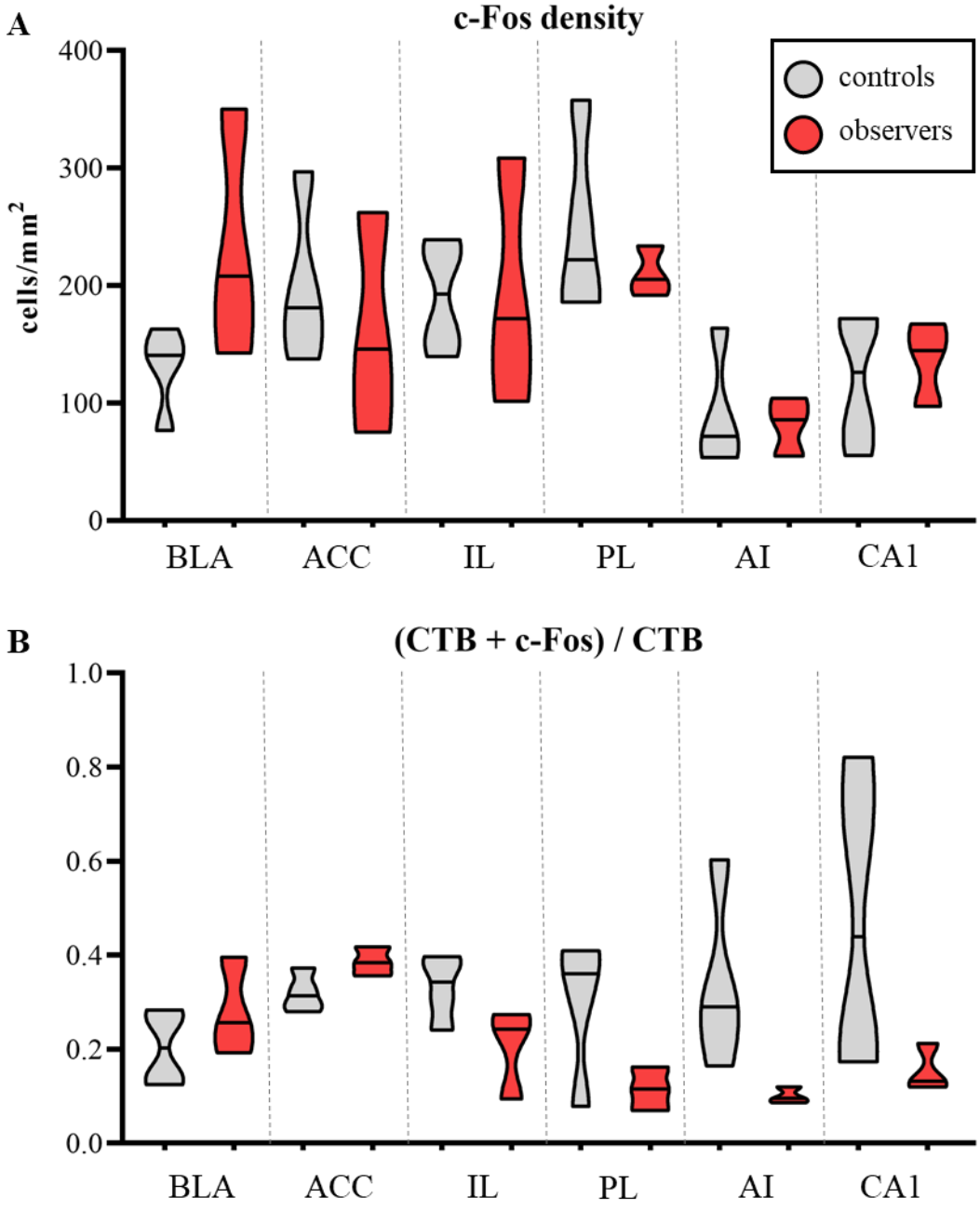


Figure 22. Functional tracing results. (A) Density of c-Fos positive neurons. (B) Proportion of c-Fos positive neurons which send projections to CeA. BLA – basolateral amygdala, ACC – anterior cingulate cortex, IL infralimbic cortex, PL – prelimbic cortex, AI – anterior insula, CA1 – CA1 field of hippocampus. The upper

and lower borders of violin plots indicate the highest and lowest individual data points; the horizontal line indicates median. The thickness of individual plots represents density of data points. * - p(q) < 0.05.

4.8. Chemogenetic manipulation

Based on results of the functional tracing experiment, it was hypothesized that CeA neurons which drive passive defensive responses during fear contagion are driven by inputs from BLA. To verify the role of this projection, a chemogenetic approach was used. A double construct system (see Methods) allowed to express inhibitory receptor hM4Di specifically in the neurons projecting from BLA to CeA. To probe the general function of these neurons, they were inhibited during the exploration test. In comparison to the control group, inhibition of the BLA-CeA pathway did not change the time spent on avoidance nor distance travelled [ns.; Fig. 23A-B]. However, it increased the time spent by rats on one of the exploratory behaviors - rearing [t(16) = 2.537, p = 0.022; Fig. 23C].

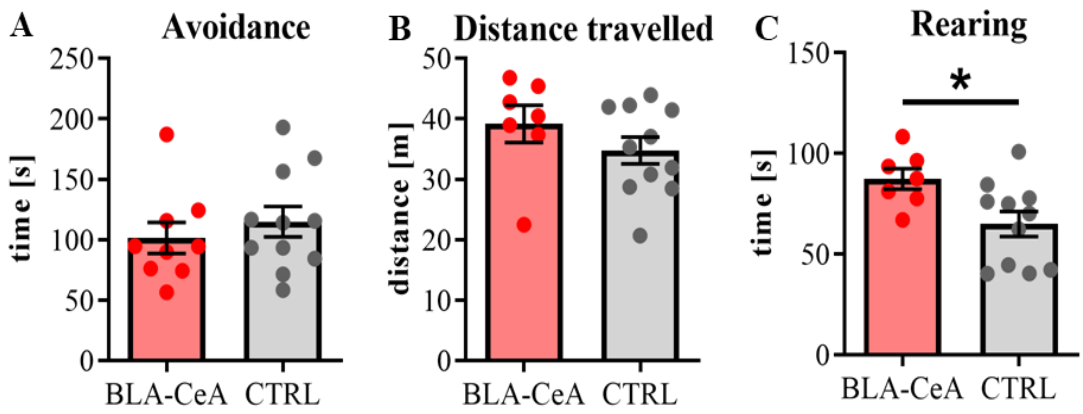


Figure. 23. Results of chemogenetic manipulation on (A) Avoidance (B) Distance travelled and (C) Rearing. BLA-CeA – group with the chemogenetic receptor expressed in the projection from BLA to CeA, CTRL – controls. Bars indicate the mean and error bars indicate SEM. * - p < 0.05.

5. Discussion

5.1. Behavior of observer rats

5.1.1. Evidence for fear contagion

During fear contagion paradigm, observers displayed similar behaviors as demonstrators (Fig. 7). Their responses were characterized by increased freezing (compared to control group) and decreased exploration (both locomotion and rearing). Furthermore, the experimental pairs emitted low-frequency USVs (Fig. 8-9), which are typically interpreted as alarm calls (Knutson et al., 2002). Although it is not possible to determine which rat was the source of each USV, some rare example of simultaneous vocalizations indicate that at least some low-frequency sounds were emitted by the observers (Fig. 8A). Taken together, the data indicates that the fear contagion paradigm was efficient: the observers exposed to fearful partners displayed passive defensive behaviors similar to demonstrators. As expected, the level of freezing was not as high in observers as in demonstrators (Fig. 7) – which is a typical result and could be easily explained by the fact that receiving foot-shocks is presumably a much stronger stimulation that observing a partner who gets them (Atsak et al., 2011; Twining et al., 2017).

It is worth noting that all the observers were experimentally naïve, that is – they were never subjected to foot-shocks themselves. The fact that in the current study it was possible to detect robust responses in naïve rats might be related to relatively strong stimulation – 10 repetitions of the foot-shock delivered to demonstrator. In some studies fewer trials are used, but usually observers are pre-exposed to foot-shocks before the fear contagion procedure contagion (Allsop et al., 2018; Atsak et al., 2011; Cruz et al., 2020; Y. Han et al., 2019). According to some reports, otherwise the fear contagion does not occur (Cruz et al., 2020; Sanders et al., 2013) or is weaker (Han et al., 2019). The drawback of using experienced observers is that it might lead to generalization of the fear memory – that is, to freezing not caused by the fear contagion itself, but by the previous aversive experience. The behavioral paradigm used here avoids this confound, allowing to study neural circuits activated strictly by observation.

5.1.2. Behavioral mimicry assessment

To test if the freezing of observers could be explained by automatic mimicry, additional analysis were performed on behavioral data. The level of freezing displayed by demonstrators and observers was correlated across the pairs, suggesting that observers indeed copy behaviors of their partners (Fig. 10). On the other hand, the results of more detailed analysis were not consistent with this straightforward explanation. First, the cross-recurrence profile did not show any characteristic delay between freezing of two animals, indicating that observers displayed freezing independently from demonstrators across time (Fig. 11). Second, the Granger causality analysis did not allow to predict behavior of the observers based on previous behavior of the demonstrators better than random (Fig. 12). In some rare cases (3/27 pairs), it allowed for the opposite (to predict behavior of demonstrators based on behavior of observers), which could point out to some individual differences between pairs in regard to synchronization. Nevertheless, on the group level the results of both analyses (cross-recurrence and Granger) were highly consistent – there was no evidence for behavioral synchronization within pairs of rats.

Taken together, the results seem puzzling – if there was no synchrony between demonstrators and observers, why the total amount of time spent by them on freezing was correlated? One possible explanation is that the measurements based on freezing do not offer enough time resolution to detect fine temporal regularities, because freezing initiated by some instantaneous event can be continued un-interruptedly for many seconds (Fanselow & Lester, 1988). This is highly unlikely, as the same analysis method allowed to detect bi-directional synchrony between demonstrators and observers by another group in a similar fear contagion paradigm (Han et al., 2019). However, it should be pointed out that the cited results were collected using rats pre-exposed to foot-shocks, which displayed significantly stronger coupling than naïve animals (Han et al., 2019). A more plausible explanation is that the observers copied behavior of demonstrators, but after very long or highly irregular (within animal) time delays. In this case their responses could no longer be classified as behavioral mimicry in the strict sense, because the

latter requires responding to the behavior of demonstrator within some short lag (Chartrand & Lakin, 2013). This explanation is consistent with work on mice, indicating that during fear contagion observers and demonstrators display maximal freezing levels during different phases of the experiment (Gonzalez-Liencres et al., 2014).

How these results could contribute to understanding the mechanisms of emotional contagion? They indicate that behavioral mimicry – which seems to be favored by shared circuit approaches – is not the only possible process underlying fear contagion (De Waal & Preston, 2017; Dezecache et al., 2015; Iacoboni, 2009; Prochazkova & Kret, 2017). It is worth noting that observer rats most probably receive not only visual (freezing) and auditory (USVs), but also olfactory cues from the demonstrators (Inagaki et al., 2014; Jeon et al., 2010; Pereira et al., 2012; Pereira & Moita, 2016). Odorants, which are known to play important role in the rodent social interactions (Arakawa et al., 2011; Wyatt, 2003), by nature provide information that propagates relatively slowly and is poorly specified in time. Such non-specific signals about threat could increase the arousal of observers without causing them to copy the freezing of demonstrators in a moment-to-moment manner. In this scenario the shared state – and not automatic mimicry – would explain occurrence of similar behaviors in demonstrators and observers. The implications of this approach will be considered in next paragraphs.

5.1.3. Learning

According to some theoretical approaches, behavioral mimicry facilitates social learning – as both subjects react to incoming stimuli in a similar, synchronized manner (Chartrand & Lakin, 2013). No mimicry detected in the fear contagion paradigm could suggest that the naïve rats, although reacted with freezing to aversive signals from the demonstrators, were not able to learn through observation (Allsop et al., 2018; Atsak et al., 2011; Cruz et al., 2020; Han et al., 2019). To examine this, two additional groups of animals were tested in a modified version of the fear contagion paradigm, in which foot-shock were paired which auditory cues (conditioned stimuli - CS). In one group the cue allowed to predict occurrence of the shock (‘CS-first’), while in the other it signaled that the shock was terminated

('US-first'). As expected, demonstrators reacted to these two paradigms differently, indicating that their freezing was at least to some degree regulated by the contingency between US and CS (Fig. 13). No such difference could be found for observers, which responded to the cues with very high variability (Fig. 14). Importantly, the observers did learn to react to the cue presentation with moderate level of freezing, as indicated by the results of the fear recall test performed 24h later (Fig. 15). However, the pattern of freezing was similar between rats from the 'CS-first' and 'US-first' groups. Taken together, the experiment demonstrated that even naïve rats were able to associate a discrete cue with aversive value. However, the data suggests that they did not perceive the CS-US contingency in the same way as the demonstrators. These results are consistent with the hypothesis that during fear contagion behavior of observers is driven by signals which are poorly specified in time.

5.2. Characterization of neurons activated by fear contagion

5.2.1. Molecular markers

Based on literature, it was hypothesized that fear contagion would activate similar populations of the CeA cells to the ones activated by first-hand experience (that is, contextual fear conditioning). Specifically, it was expected to find less PKCδ+ and CRF+ neurons which co-expressed c-Fos in observers than in controls (Ciocchi et al., 2010; Fadok et al., 2017; Hartley et al., 2019; Haubensak et al., 2010). None of this predictions was confirmed, as for both divisions of CeA (CeM and CeL), the proportion of active cells characterized by each marker was equal between observers and controls (Fig. 17). Nevertheless, it is worth noting that the overlap between c-Fos and the selected markers was low (especially for the CRF, ~20%), consistently with the expectations. The lack of clear negative results could be explained by several factors: methodological discrepancies with previous studies, limitations of immunohistochemistry or genuine differences between neuronal activity in observers and demonstrators. The three possibilities will be discussed in more details below.

The experimental work which allowed to describe the role of PKCδ and CRF circuits in CeA was performed almost exclusively on mice (Ciocchi et al., 2010; Fadok et al., 2017; Hartley et al., 2019; Haubensak et al., 2010). The research described in this thesis, on the other hand, was carried with the use of Wistar rats. Although it is commonly assumed that data from these two model organisms can be used jointly, there are numerous behavioral and neuronal differences between the two species (Ellenbroek & Youn, 2016). For example, when exposed to stressed cage-mates, they react with different patterns of brain activation measured with c-Fos (Knapska et al., 2006; Meyza et al., 2015; Mikosz et al., 2015). It cannot be excluded that the PKCδ and CRF circuits – which are well described in mice – have different function in rats. What is more, the cited literature used transgenic Cre mouse lines, which allow to achieve vsery high expression of fluorescent reporters and/or opsins in the selected population of cells (Nectow & Nestler, 2020). This approach is usually not possible when working with rats, because very few transgenic lines of these animals are available. Instead, it was necessary to use double staining approach, which comes with its own limitations.

Immunohistochemical stainings – although they are an extremely useful technique – as every method have only limited accuracy (de Matos et al., 2010). Although it is challenging to measure this accuracy systematically, some instructive examples can be found in the literature (Sfanos et al., 2019). For example, detecting the same molecular marker in neurons using transgenic mice and immunohistochemistry usually gives slightly different results (Alon et al., 2009; Haubensak et al., 2010; Pomrenze et al., 2015). When two proteins need to be detected simultaneously – as in case of PKCδ and c-Fos – the probability of error is multiplied. It is worth noting that observers had relatively low level of c-Fos expression in CeA – higher from the home-cage animals, but not from the controls (Fig. 16) - which could additionally decrease accuracy of the double measurement. One cannot also exclude the possibility that a significant proportion of CeA cells was activated in the observers, but did not express c-Fos (Peter et al., 2013; Yap et al., 2020).

Finally, the lack of detected differences between observers and demonstrators could be related not to methodological, but strictly biological reasons. Observers displayed more freezing than controls, but less than demonstrators (Fig. 7), which

suggests that the PKCδ- and CRF- circuits could be activated in observers only to a moderate degree. It is also possible that the population of CeA neurons activated by fear contagion is very heterogenous or defined by a different molecular marker. One obvious candidate is SOM - an attempt was made to detect also this marker, but the sensitivity of antibody was too low to draw any conclusions from the results (data not shown). Further experiments – preferably comparing directly tissue from observers and demonstrators – are required to definitely resolve this issue.

5.2.2. Behavioral function

Because the population of CeA cells activated by the fear contagion could not be defined by molecular markers, it was not possible to use typical manipulation approaches. Instead, to probe the behavioral function of these neurons, an activity-dependent strategy was used. The AAV-c-Fos viral vectors, developed in the Nencki Institute (Andraka et al., 2020), enabled to tag population of CeA neurons which expressed endogenous c-Fos during fear contagion. This made possible to reactivate (or inhibit) them 24 hours later during a different behavioral test and measure the responses of the rats. For the sake of simplicity, in the following sections this neuronal population will be referred to as ‘fear contagion’ neurons.

Optogenetic stimulation of the ‘fear contagion’ neurons during novel environment exploration increased time spent by rats on avoidance, while decreasing time spent on exploration (Fig. 18). In contrast, it did not influence the overall distance travelled (Fig. 18C). Inhibiting this population, on the other hand, increased the distance, but did not change time spent on approach nor avoidance (Fig. 19). The results suggested that the ‘fear contagion’ neurons hampered active exploration. However, it was not clear if they control specifically exploration or a wider class of responses (for example, through increasing anxiety). To check if they would influence also social behaviors, they were stimulated optogenetically during free interaction with the partner. The manipulation did not have any effect on the social contact; instead, it reduced time spent on rearing (Fig. 20). The results suggested that the function of the studied population was controlling defensive/exploratory rather than social behaviors. However, it was still not clear if activation of the ‘fear contagion’ neurons could promote freezing in a more

threatening, enclosed environment. To verify this, the rats were tested optogenetically during the fear recall paradigm. Such manipulation indeed increased time spent by rats on freezing (Fig. 21).

A limitation of this approach, which could potentially influence conclusions, was the fact that the expression of optogenetic constructs was not conditioned by any other factors than the promoter sequence. In other words, after the surgery expression of ChR2 or NpHR could be triggered in CeA by any novel events that led to endogenous c-Fos transcription, and not exclusively by the fear contagion paradigm. This risk could be controlled better, at least theoretically, through implementing a conditional system in which the construct expression would be temporally limited by an additional factor, such as tetracycline (Lewandoski, 2001). However, when the project was started, no solution described in the literature allowed to use such system in rat CeA (Schönig et al., 2012; Sørensen et al., 2016). Instead, the baseline c-Fos expression was limited by careful habituation of the animals, which is not a universally efficient strategy, but is known to work well for rat amygdala (Knapska & Maren, 2009; Yassin et al., 2010).

The strongest argument indicating that this approach succeeded is the fact that no stimulation effects were detected in the control animals. They received the same viral vector and light stimulation as the observers – the only difference between the groups was lack of foot-shocks provided to the partner 24 hours before the optogenetic testing. Due to almost identical treatment, the control was well suited to detecting potential effects of random construct expression. At this point it is also worth noting that no differences could be found between observers and controls regarding the number of cells expressing endogenous c-Fos (Fig. 16). Although this observation alone could initially suggest lack of CeA engagement in the fear contagion, together with optogenetic results it leads to opposite conclusion: populations of neurons activated in observers and controls were similar in size, but functionally different.

Another point that should be discussed is the asymmetry of excitation and inhibition results. For example, if stimulation of ‘fear contagion’ neurons increased avoidance, why blocking them did not influence this behavior? One cannot exclude the possibility that NpHR was simply less effective than ChR2. Robust optogenetic

inhibition is generally believed to be much harder to achieve than excitation, as it requires constant delivery of energy through light, optimal expression levels and can lead to unexpected effects on membrane potential (Allen et al., 2015; Mahn et al., 2016; Raimondo et al., 2012; Wiegert et al., 2017). Keeping this limitation in mind, it should be noted that the AAV-c-Fos-NpHR construct was validated electrophysiologically previously, which demonstrated that under typical testing conditions it inhibits spiking in CeA *in vitro* (Andraka et al., 2020, Suppl. Mat.). Furthermore, the optogenetic inhibition influenced the distance travelled by rats, which indicates that it was efficient enough to influence at least some behavioral parameters. That suggests that the manipulation results reflect some non-symmetric action of the studied neuronal population. The latter could be observed for example if the exploration test caused too low anxiety in the animals to detect its further decrease (Gehrlach et al., 2019). Further experiments would be needed to resolve this issue definitely.

Taken together, the results of the optogenetic experiments provide evidence that the 'fear contagion' neurons reduce exploration and promote defensive behaviors. Although these effects could be interpreted simply as increase of anxiety, it should be pointed out that the manipulation did not influence social interactions, which are typically highly dependent on the arousal level (File, 1985; Lezak et al., 2017). Thus, the function of 'fear contagion' neurons should be perhaps summarized more specifically as promoting passive exploratory strategy. The word 'strategy' reflects the fact that stimulating the population did not always evoke one stereotypical behavior, for example freezing. Instead, depending on the testing environment, different responses to the light stimulation could be detected – such as avoidance, decrease of rearing or freezing. To sum up, the 'fear contagion' neurons seem to promote different forms of reducing active environment exploration.

The findings correspond well to theoretical approaches which stress the modulatory role of CeA in regulating single animals behavior. For example, it was proposed that while BLA encodes specific sensory features of emotional events, CeA processes their general motivational value and triggers preparatory physiological responses (Balleine & Killcross, 2006). According to this approach, activation of CeA alone does not initiate specific reactions, but rather modulates the gain of ongoing behaviors (Robinson et al., 2014). For example, stimulation of

insula terminals in CeA during social interaction can evoke both approach or avoidance, depending on whether the conspecific is threatening or not (Rogers-Carter et al., 2018). Such divergent effects could be potentially explained by increasing arousal during some behaviors, as CeA innervates all the major brain neuromodulatory systems (Balleine & Killcross, 2006; Knapska et al., 2007). On the other hand, there is now evidence that this brain structure can also trigger some stereotypical motor programs by activating premotor brainstem nuclei. For example, both freezing and flight are controlled by parallel pathways through the following structures: CeA – PAG – medulla – spinal cord (Deng et al., 2016; Evans et al., 2018; Tovote et al., 2016). Similarly, in the context of hunting the pathway CeA – PAG – mesencephalic motor region – spinal cord triggers prey chasing, while the pathway CeA – reticular formation – trigeminal nucleus controls prey biting (Han et al., 2017). The crucial difference between those two modes of CeA function is that while the first one is highly context dependent (eg., CeA stimulation will cause avoidance only if the partner is threatening), the second one triggers reactions even under very 'unnatural' conditions (eg., stimulation will cause robust jaw movements in the absence of food, Han et al., 2017). The optogenetic results suggest that the 'fear contagion' neurons control defensive behaviors, such as freezing, through the first type of mechanism – that is, in a context-dependent manner.

5.2.3. Input connectivity

Functional tracing enabled to gather preliminary data on which pathways to CeA could activate 'fear contagion' neurons (Fig. 22). Qualitatively, the input projections can be divided into two groups: the ones activated stronger in controls than in observers (AI, CA1, IL and PL) and the ones activated preferably in observers (ACC and BLA). The two groups will be discussed below separately.

The results indicating stronger activation of some pathways in controls than in observers seem more difficult to interpret. These projections could potentially regulate the active exploration of the novel cage; however, the literature data on this topic is extremely scarce. The pathway from insula to CeA is known to promote anxiety (Gehrlach et al., 2019), approach or avoidance to social partners (depending

if they are a threat or not; Rogers-Carter et al., 2018) and aversive alimentary conditioning (Schiff et al., 2018; Zhang-Molina et al., 2020). Some data suggests that it might be implicated also in controlling active defensive responses, which would explain its low recruitment during freezing (Andraka et al., 2020). The CA1 activity is expected during any task involving novel environment exploration (Zemla & Basu, 2017), while PL/IL are known to bi-directionally regulate anxiety based on such factors as previous learning (Giustino & Maren, 2015). However, specific functions of projections from these structures to CeA are currently not known.

The activation of the ACC-CeA projection is not surprising, as the former structure is a crucial hub for fear contagion and observational conditioning (Allsop et al., 2018; Han et al., 2019; Jeon et al., 2010; Lavin et al., 2013). On the other hand, the majority of projections from this brain structure to amygdalar complex target BLA (Knapska et al., 2007), and emotional contagion studies so far focused on this specific pathway (Allsop et al., 2018; Smith et al., 2021). The tracing results suggest that direct pathway from ACC to CeA can also play role in fear contagion.

Finally, projections from BLA to CeA were also characterized by higher activation in observers than in controls. To verify their possible function in fear contagion, they were inhibited chemogenetically during novel environment exploration (Fig. 23). The manipulation did not influence general level of avoidance or distance travelled, but increased time spent on a specific type of exploratory behavior – that is, rearing. The result was generally consistent with previous optogenetic experiments, confirming that the neuronal population activated in CeA by fear contagion downregulates some aspect of exploration.

Comparing the findings on BLA-CeA pathway to the literature is not an easy task, as the literature itself is ambiguous. The classic view assumed that the two structures work serially: BLA receives majority of inputs and associates them through synaptic plasticity, while CeA reacts to BLA activity by engaging the autonomic and motor outputs (LeDoux, 2000). Although some modern experiments confirm that this might be indeed the dominant direction of information flow (Hartley et al., 2019), other works prove that the two structures might work also in a more parallel manner (Balleine & Killcross, 2006; Killcross et al., 1997), and that

plasticity within CeA is important for selection of behavioral outputs and learning (Fadok et al., 2018). It was shown that projections from BLA to CeA are selectively strengthened during fear conditioning (and shifted from CRF+ to SOM+ neurons), and in this context drive freezing (Hartley et al., 2019; Li et al., 2013). Stimulating this pathway in classic anxiety tests, based on novel environments exploration, somewhat surprisingly decreases anxiety and increases locomotion (Tye et al., 2011). Others have found that during active avoidance task the BLA-CeA projection inhibits freezing and promotes escapes (Terburg et al., 2018). Finally, in the context of exploring non-threatening, well-known environment the pathway controls sudden locomotor arrests (Botta et al., 2019). Such diverse findings are probably best explained by the fact that different types of BLA neurons preferentially reach different molecularly defined CeA targets - stimulating some of these circuits has appetitive, while others aversive consequences (Kim et al., 2017). Additionally, learning can partially rewire BLA projections to other outputs, drastically changing their function (Hartley et al., 2019).

To sum up, the 'fear contagion' population in CeA is most probably driven by inputs from BLA (which are in turn activated by ACC, as shown by multiple studies). This pathway inhibits active exploratory behaviors (rearing), consistently with the behavioral pattern observed during fear contagion. The projections from BLA which are activated by the fear contagion most probably target preferentially some type of CeA neurons. The results of double staining, together with clues from literature, suggest that the target population is largely CRF negative, but its other molecular characteristics are not yet known.

5.3. Conclusions

The main aims of this thesis were to verify predictions on fear contagion formulated in the shared circuits framework. On behavioral level, this included hypothesis that freezing of observers would be explained by mimicry. The data did not support this claim, suggesting that behavior of the observers and demonstrators is only weakly synchronized. On the neuronal level, it was predicted that the fear contagion would activate similar CeA circuits as the ones described in the literature on single subjects fear conditioning. More specifically, it was expected that the

activated population would a) not express PKCδ and CRF b) control freezing c) receive projections form BLA. The first prediction was not confirmed (due to lack of difference relative to the control group), but it is worth noting that the proportion of active CRF+ neurons was indeed low (~20%). The second prediction was verified positively, with the reservation that the control over freezing seemed highly context-dependent. Finally, the data was consistent also with the third prediction. The general implications of the findings for understanding the mechanisms of emotional contagion will be discussed in the following paragraphs.

First, it should be noted that the neural circuits activated by fear contagion were generally similar to the ones described in the context of single subjects fear conditioning. Although some discrepancies were also found, they could be partly explained by methodological issues and/or the fact that the observers received presumably much weaker stimulation than the fear conditioned animals. Taken together, the neuronal data is broadly consistent with the shared circuits approaches.

On the other hand, behavioral data was less consistent with this framework. Shared circuit approaches typically stress the automatic nature of emotional contagion, and predict that the observers would mimic the behavior of demonstrators in a moment-to-moment manner (Iacoboni, 2009; Prochazkova & Kret, 2017). The behavioral data – both from correlational and manipulation experiments – suggests that the responses of observers are initiated in a more complex processes. Firstly, the freezing during fear contagion was executed in observers and demonstrators independently across time. Secondly, reactivation of 'fear contagion' neurons did not evoke only one type of response – freezing – but different passive behaviors, depending on the testing context. Together, both observations suggest that contact with the fearful demonstrator does not lead to freezing automatically, but through some intermediate process. A potentially good candidate for such intermediate step is change of affective state in the observers. This kind of mechanism could lead to more flexible responses to threats than behavioral mimicry – it would increase vigilance in observers without narrowing down the behavioral outcome to only one type of defensive response. To confirm this hypothesis, it would be necessary to study in more details the targets of 'fear contagion' neurons and verify if they act mainly through stimulating neuromodulatory responses rather than triggering specific motor programs.

It should be stressed that all the experiments described in this thesis were focused on CeA, which undoubtedly controls only some aspects of the fear contagion. More specifically, based on literature it could be described as the 'output structure', which mediates selection of the appropriate defensive response and triggers its execution. It is less likely to be crucial for threat detection or processing social signals. Due to this properties, one could expect that similar CeA circuits would be activated in observers and demonstrators, as both groups of animals displayed similar defensive behaviors. That does not exclude the possibility that fear contagion activates in observers some unique brain circuitry, specialized in processing social signals about threats. However, such distinct circuits are probably more likely to be found upstream to CeA, for example in the medial prefrontal or olfactory cortex.

Finally, although the experiments described in this thesis allowed to partially characterize CeA circuits activated by fear contagion, an important future step would be comparing them directly to the circuits engaged in first-hand fear conditioning. An optimal way for that would involve recording activity of single neurons from the same animal, tested consecutively in the role of observer and demonstrator. Only this strategy would allow to definitely answer if first-hand and vicarious experience is supported by the same or just similar circuits. Although such experiments are extremely challenging to design, recent work indicates that they may be a promising line of future inquires (Carrillo et al., 2019).

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