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Emotional contagion in *Fmr1* knockout mice, a model of Fragile X Syndrome

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Abstract

Lately, we can observe a steady increase in the number of autism spectrum disorder (ASD) diagnoses. The ASD population is characterised with deficits in social interactions and communication, as well as the presence of stereotyped behavior. Lack of social skills often manifests itself through empathy impairment. Until recently, empathy was thought to occur only in humans, but a growing body of research indicates that emotional contagion - the simplest form of empathy, is widely found in nature, including in primates, marine mammals, birds and rodents. Despite the importance of the phenomenon, there is still little data on the neuronal basis of sharing emotions.

Aim of my PhD project was to assess the empathic abilities and the activity pattern within the amygdala and the prefrontal cortex of Fmr1KO(FVB) mice (both males and females) - commonly used mouse model of ASD. To study this phenomenon, I employed the Remote Transfer of Fear paradigm, in which mice are housed in pairs for three weeks, one labelled an Observer, and the other a Demonstrator. In the test session, the Demonstrator is subjected to aversive stimuli outside of the home cage, while the Observer remains there undisturbed. Then, the Demonstrator returns to the home cage, where it can freely interact with the Observer and the first nine minutes of interactions are recorded. The activity of the amygdala and prefrontal cortex was assessed using immunohistochemistry against c-Fos protein, a standard neuronal novelty marker. Behavior was measured by using software utilizing machine learning, for automatic pose estimation (DeepLabCut) and automatic classification and recognition of animal behavior patterns (simBA).

Behavioral and c-Fos activation pattern results indicated the existence of deficits in emotional contagion in Fmr1KO(FVB) mice. Furthermore, data obtained during this study points to differences in response to stressed partner between females and males, both on behavioral and c-Fos levels. The behavior recognition model created during this study made it possible to study behavior with great accuracy, and after short re-training, can be successfully used in another study.

Streszczenie

W ostatnim czasie obserwujemy stały wzrost liczby diagnoz zaburzeń ze spektrum autyzmu (ASD). Osoby w spektrum charakteryzują deficyty w interakcjach społecznych i komunikacji, a także obecność stereotypowych zachowań. Brak umiejętności społecznych często manifestuje się u nich także upośledzeniem empatii. Do niedawna uważano, że empatia występuje jedynie u ludzi, natomiast rosnąca liczba badań wskazuje, że zarażenie emocjonalne - najprostsza forma empatii, występuje powszechnie w przyrodzie, w tym u naczelnych, ssaków morskich, ptaków i gryzoni. Pomimo znaczenia tego zjawiska, wciąż istnieje niewiele danych na temat neuronalnych podstaw dzielenia się emocjami.

Celem mojego projektu doktorskiego była ocena zdolności empatycznych i wzorca aktywności w ciele migdałowatym i korze przedczołowej myszy Fmr1KO(FVB) (zarówno samców, jak i samic) - powszechnie stosowanego mysiego modelu ASD. Aby zbadać to zjawisko zastosowałem paradygmat Pośredniego Transferu Strachu, w którym myszy są trzymane w parach przez co najmniej trzy tygodnie przed eksperymentem. Jedna z myszy zostaje oznaczona jako Obserwator, a druga jako Demonstrator. W sesji testowej Demonstrator jest poddawany awersyjnym bodźcom poza klatką domową, podczas gdy Obserwator pozostaje w klatce domowej. Następnie Demonstrator zostaje umieszczony z powrotem w klatce domowej, gdzie może swobodnie wchodzić w interakcje z Obserwatorem, a pierwsze dziewięć minut interakcji jest nagrywane. Aktywność ciała migdałowatego i kory przedczołowej oceniano za pomocą barwień immunohistochemicznych przeciwko białku c-Fos, standardowemu neuronalnemu markerowi nowości. Zachowanie zanalizowano przy użyciu oprogramowania wykorzystującego uczenie maszynowe do automatycznego szacowania pozycji (DeepLabCut) oraz automatycznej klasyfikacji i rozpoznawania wzorców zachowań zwierząt (simBA).

Wyniki behawioralne i wzorce aktywacji c-Fos wskazały na istnienie deficytów w zarażaniu emocjonalnym u myszy Fmr1KO(FVB). Co więcej, dane uzyskane podczas tego badania pokazały różnice w reakcji na zestresowanego partnera między samicami i samcami, zarówno na poziomie behawioralnym, jak i w poziomie białka c-Fos. Model analityczny stworzony podczas tego badania umożliwił badanie zachowania z dużą dokładnością, a co więcej, po krótkim przetrenowaniu, powodzeniem może być wykorzystywany w innych badaniach.

List of abbrevations

ACC - Anterior Cingulate Cortex

ACO - the of *c-fos* antisense oligonucleotide

AP - anterior posterior

AP-1 - Activator Protein-1

ASD - autism spectrum disorder

BLA - basolateral nucleus of the Amygdala

CeA - central nucleus of the Amygdala

Dem - Demonstrator

DLC - DeepLabCut

ERK1/2 - extracellular signal-regulated kinases 1 and 2

FMPR - Fragile X Messenger Ribonucleoprotein 1

FXS - Fragile X Syndrome

HPA axis - Hypothalamus-Pituitary-Adrenal axis

Hth - Hypothalamus

IEG - immediate-early genes

IL - Infralimbic Cortex

KO - Knockout

LA - lateral nucleus of the Amygdala

LTP - Long Term Potentiation

MeA - medial nuclei of the Amygdala

mPFC - medial Prefrontal Cortex

Obs - Observator

OFC - Orbitofrontal Cortex

OFL - Observational Fear Learning

PAG - Periaqueductal Grey

PBS - Phospate Buffer Saline

PrL - Prelimbic Cortex

SHANK3 - SH3 and multiple ankyrin repeat domains 3

SimBA - Simple Behavioral Analysis

SNP - single nucleotide polymorphism

STS - Superior Temporal Sulcus

TC - Temporal Cortex

Thal - Thalamus

TPJ - Temporo-Parietal Junction

TSC1/2 - Tuberous sclerosis proteins 1 and 2

USV - Ultrasonic vocalization

vmPFC - ventro medial Prefrontal Cortex

VTA - Ventral Tegmental Area

WT - Wildtype

1. Introduction

1.1. Emotions

Description of what an emotion is presents a difficult task. There are many definitions used by different research groups (Kret et al., 2022). Damasio (Damasio, 2004) pp. 50, 52 describes emotions as 'bioregulatory reactions', which can be measured by physiological measures and whose role is to modify our behavior in order to adapt to the changing environment. They are not necessarily conscious or can cause feelings. Anderson & Adolphs (Anderson & Adolphs, 2014) propose to define emotions as internal states of the brain, 'Emotion primitives', which can be expressed through simple behaviors like approach or avoidance and can be found even in fruit flies. In search of neuronal correlates of emotions LeDoux (LeDoux, 2017) recently suggested to study 'survival circuits' - neural circuits found in subcortical areas, governed by the amygdala and responsible mainly for defensive and appettitive behaviors crucial for species survival. In case of 'feelings' or what we call emotions in humans, LeDoux (LeDoux, 2021) considers them not directly caused by activity of the survival circuits but a parallel phenomenon merely correlated with emotions. A similiar perspective is presented by Barrett (Barrett, 2006). She presents a view that emotions are predictively constructed by the brain, postulating focus on these mental representations ('feelings') rather than the physiological state of the organism during a given emotion. According to her view, to be capable of experiencing feelings, a highly evolved neocortex is necessary as well as an ability to verbally report subjective experiences, which by definition is near impossible to study in species different from humans. On the other hand, Paul Ekman suggests that basic emotions are universal for every human despite differences in language, ethnicity, culture or origins (Ekman et al., 1999) pp. 301-320. Already in XIX century Darwin (Ekman, 1998) observed that many species exhibit similar emotional reactions (e.g. defecating in presence of great danger, infant weeping, piloerection during arousal or obliquity of eyebrows while suffering, and suggested that these may exist abundantly in animal kingdom.

For a long time scientists thought that animals do not possess the capacity to have mixed emotions (Grandin & Johnson, 2006) and that they only display few, very basic ones (McFarland, 1982). There is, however, growing evidence this is not the case. For example a frightened chimpanzee was seen poking a snake, simultaneously displaying hesitation and curiosity, a clear exhibit of more sophisticated emotions (Menzel, 1974). The ability to turn

from hostile to friendly attitude towards a conspecific (reconciliation) also indicates the existence of more complex emotional states rather than just straightforward ones. For example, reconciliation was observed in many social species such as primates, dolphins, hyenas and goats (Aureli & De Waal, 2000).

Debate about emotions and semantics is still ongoing, but consensus is that there are present internal (interoceptive) states that arise in response to the various stimuli, which then influence the behavior. Such states can be measured also in animals. For the basic emotional states, such as fear and anxiety, the neuronal background is well-defined. Many studies carried out in rodents and in human subjects consistently point to a high activation of the amygdala (M. Davis, 1992; LaBar et al., 1998; LeDoux, 2003) during tasks evoking these states. Stimulating rat amygdala elicits fight and flight response which confirms its important role in processing defensive responses. Notably, akin physiological reactions are noticed across many species in response to threat. Increased heart rate and blood pressure was observed in birds, rats, cats, dogs, and primates after stressful stimuli (Halász & Shepherd, 1983). Thus, even if we cannot ask animals what they feel, animal research can still give us insight into how aversive and appetitive stimuli are processed in the brain. While appreciating the fact that the debate about emotions is still ongoing, I will refer to the aversive and appetitive responses of animals as emotions throughout my thesis for the sake of simplicity.

1.1.1. Neuroanatomy of emotions

One of the first hypotheses describing the structures responsible for emotion recognition and social interaction was the one proposed by James Papez, called the Papez Circuit. Brain areas presumably controlled emotions included the thalamus (Thal), hypothalamus (Hth), anterior cingulate cortex (ACC) and the hippocampus (Papez, 1937). At that time, research done by Kluver and Bucy, showed that damaging temporal lobe can lead to abnormal emotional reactions, such as: loss of fear, hyperactivity, hyper-sexuality or coprophagy (Klüver & Bucy, 1937). Subsequent studies conducted on monkeys demonstrated that bilateral lesion of the amygdala is sufficient to produce a similar effect, which confirmed the role of this structure in emotional processing (Weiskrantz, 1956). Another brain region included was the neocortex, whose function is to control cortical and subcortical regions previously mentioned. This set of brain areas was later called the limbic system (MacLean, 1990). This term is still functioning

as a concept although some scientist postulate that due to emotions being controlled by distinct neural substrates, it should become obsolete (Calder et al., 2001). Ongoing research points to additional structures such a orbitofrontal cortex (OFC), temporal cortex (TC), ventral tegmental area (VTA) and medial prefrontal Cortex (mPFC) as playing a role in controlling emotions (Porcelli et al., 2019). The most studied brain region in context of emotions, mainly connected with fear, is still the amygdala which is described as the hub of all stimuli coming from different modalities (Herry & Johansen, 2014; Knapska et al., 2007). Amygdala is a complex structure with distinct parts and is characterized by multiple connections with other structures such as the hippocampus, PFC or brainstem (LeDoux, 2000). Conditioned Fear experiments revealed that neural circuits including lateral (LA) and central nuclei of amygdala (CeA) are necessary for acquisition, storage and expression of fear responses (Davis & Whalen, 2001; Goosens & Maren, 2001; Kapp et al., 1992). A study performed on mice by Karalis (Karalis et al., 2016) indicates the importance of reciprocal connection between the prefrontal cortex and the amygdala in fear expression, and that freezing response correlates with internally generated 4-Hz oscillations in the prefrontal-amygdala circuit. Optogentically induced oscillation of that frequency was sufficient to evoke a freezing response. It was also observed that the activity of the prelimbic cortex (PrL) recorded by multichannel unit recording was synchronized with the freezing behavior after auditory fear conditioning (Burgos-Robles et al., 2009). (Cummings & Clem, 2020) provided data suggesting that microcircuits of somatostatin and parvalbumin interneurons in mPFC encode memory formation and expression of fear.

Modern neuroscience suggests that emotion processing is done by dynamic and hierarchical set of neural circuits dispersed throughout the whole brain. They are involved and specialize in various aspects of emotions processing and are collectively referred to as the Social Brain (Adolphs, 2009). Scientists distinguished five different, large-scale networks responsible for various aspects of emotion-related processes. Three of them, social perception network, social affiliation network and social aversion network are engaged in noticing, interpreting and reacting towards social stimuli. Social perception networks' main task is to detect social stimuli, which facilitates engagement in social interaction. It is controlled by the amygdala and its connections with the hippocampus, orbitofrontal cortex and temporal pole (Bickart et al., 2014). Another important brain area with strong connection to the amygdala is the fusiform face area, which is selectively activated by face stimuli (Gobbini & Haxby, 2006). Both social affiliation and social aversion networks are processing social stimuli and decide whether to act pro-social or stay away from these stimuli. The amygdala plays a crucial role in both systems through its

connections with the VTA, ventromedial prefrontal cortex (vmPFC) and the ACC. It is thought that these brain areas govern pro-social aspects of the social affiliation network (Aron et al., 2005; Bickart et al., 2012; Inagaki et al., 2016; Moll et al., 2006). On the other hand, aversion network is formed by the amygdala, caudal ACC, the insula and its connections to ventrolateral striatum, the hypothalamus and the brainstem (Buckholtz et al., 2008; Moll et al., 2005).

Another proposed neural network related to processing of social stimuli is the mirroring network, responsible for the capacity to learn and understand others through imitation. It consists of mirror neurons found in the superior temporal sulcus (STS) and the ventral premotor cortex. These cells belong to the visuomotor neurons group that is characterized with being active during both execution and observation of task (Rizzolatti & Craighero, 2004). The most recent addition to the circuitry is the system called the mentalizing network, in charge of our ability to detect mental states of others and differentiate them from our own. Main brain regions forming this network are the posterior STS, the temporo-parietal junction (TPJ), the anterior temporal poles and the mPFC (Frith & Frith, 2006). Through perspective taking, the mentalizing and mirroring systems give us the capability to imagine and understand emotions, actions and intentions of others.

1.2. Empathy

Empathy can be described as a phenomenon, where one has a capacity to distinguish, share and respond to distress in others (de Waal, 2008). While living in social groups, communication is essential to thrive and for development of tightly knit societies. Perception of others' emotions helps us understand each other better, and interpret other peoples' behavior, make inferences about their intentions and motives which makes social interactions easier.

While empathy is often considered an only humans trait, the fact that emotions can spread onto conspecifics probably evolved even before our species formed (Eibl-Eibesfeldt, 1974; MacLean, 1985). It can be explained by a model proposed by Preston and de Waal (De Waal & Preston, 2017; Preston & De Waal, 2002) which divides empathy into three levels thus reminding a famous toy - the 'Russian-doll'. The most basic expression of empathy is known as *Emotional Contagion*. It serves as the foundation for more sophisticated process - *Empathic Concern*, which in turn is a foundation for the most complicated phenomena - *Perspective*

Taking and Targeted Helping. Importantly, according to this model, the highest levels of empathy cannot happen without the presence of the lower ones. Hatfield et al (Hatfield et al., 1994) defines *Emotional Contagion* as an ability to share emotional states between individuals, allowing for swift adjustment to changing environment and therefore giving better chance for survival. Hoffman (Hoffman, 1975) states that this process, due to its automatic and rapid nature, does not involve consciousness. In my research I will focus on this simplest form of empathy.

1.2.1. Emotional contagion

Emotional contagion has been observed in many species. In human newborns it can be seen at the maternity ward, when crying spreads between babies (Hoffman, 1975; Zahn-Waxler et al., 1984). Transmission of fear can be also seen in birds when they all suddenly take off due to one of them being startled. Another example of emotional contagion is when mother chimpanzees whimper when they heard their offspring cry (de Waal, 2008). It was also observed in rats and pigeons when they exhibited stress response after perceiving a conspecific in distress, which then influenced their behavior by temporary suppressing a previously learnt instrumental response (Church, 1959; Watanabe & Ono, 1986).

Emotional contagion can be also observed on a physiological level. In humans, cardiac activity was found increased, as compared with a control group, in people watching others experiencing stressful stimuli (Dimitroff et al., 2017). Emotional contagion is not limited to within-species occurrence which further reinforces its high prevalence in the animal kingdom. A study done on humans and horses has shown that when a person standing next to a horse became anxious both, horses and humans had reported elevated heart rate (Keeling et al., 2009). In our study it was also shown that after interactions of recently fear-conditioned human with rat it can elicit emotional arousal in rat measured by increased amygdala activation and occurrence of risk assessment behavior (Kaźmierowska et al., 2023).

Studies done on rodents showed also that distress can be transferred through social interaction between conspecifics. Rats who interacted with recently socially defeated partner exhibited social avoidance behavior and had increased heart-rate and elevated corticosterone levels (Carnevali et al., 2020). It was reported that prolonged observation of the social defeat

of conspecific by aggressor mouse can induce social avoidance in the Observer when confronted with novel social stimulus (Warren et al., 2013). In the same study when the Observer could not witness social defeat of the partner due to the opaque non-perforated divider social avoidance in the Observer was not elicited. Similar research done on female mice also confirms that blocking visual and olfactory cues inhibits development of social avoidance in animal witnessing social defeat (Iñiguez et al., 2018). Further investigation on sensory channels through which emotional information may transfer revealed that only hearing social defeat of conspesific is also not enough to induce social avoidance in witnessing Observer (Patki et al., 2015). These findings reinforce the view that emotional contagion cannot occur without multimodal cues or social interactions.

Experiencing pain can be modulated by social stimulus as well. Studies performed by Langford et al (Langford et al., 2006) revealed that when mice were subjected to a noxious stimulus in dyads their pain response was higher than in animals tested individually. Further research shows that just smelling and hearing others' pain can be enough to produce hyperalgesia in mice housed in the same facility room together with animals subjected to long-lasting painful stimuli (Smith et al., 2016).

Another phenomenon frequently linked with Emotional Contagion is known as 'motor mimicry'. It happens when a subject mimics the behavior of the conspecific and it is happening without a conscious effort (Van Baaren et al., 2009). Yawning is one such example (Helt et al., 2010). Interestingly contagious yawning was noticed in many species: non-human primates (Campbell & de Waal, 2010, 2011), canines (Romero et al., 2014) and rodents (Moyaho et al., 2015). To classify behavioral response as 'mimicry' the copied behavior has to occur with delay up to 5 seconds (Chartrand & Lakin, 2013). Other examples of such mirroring behavior, which comes mostly from human data, may include mimicking body posture (Tia et al., 2011), facial expressions representing emotions (Hess & Blairy, 2001; Lundqvist, 1995; Mui et al., 2018), cry (Simner, 1971) or laughter (Provine, 1992). Furthermore, there is growing evidence that such synchronization is not limited to motor mimicry, but it can occur in heart-rate or pupil-diameter during social interactions (Palumbo et al., 2017). Researchers suggest that emotional contagion may be actually a consequence of automatic mimicry and synchronization of behavior (Cacioppo et al., 2000).

Emotional contagion can be studied by employing various behavioral paradigms such as Fear Conditioning by Proxy, Observational Fear Learning or Remote Transfer of Fear. In Fear Conditioning by Proxy rats are interacting with a cage-mate who was recently fear conditioned

during fear memory retrieval. In this approach we can test the social fear learning process during which at some point transfer of emotional information between conspecifics should occur. Some tested rats are able to acquire condition response for a tone only by interacting with fear conditioned rat (Bruchey et al., 2010). Another often used paradigm is Observational Fear Learning. Research conducted on mice showed that by just observing another mouse being fear conditioned (pairing previously neutral stimuli, like a tone, with a footshock) mice can learn to freeze to the tone. Furthermore, when two animals (rats or mice) are present in the same chamber, divided only by a perforated wall, an electric shock to just one of them is sufficient to elicit fear response in both of them (Atsak et al., 2011; Q. L. Chen et al., 2009; Gonzalez-Liencres et al., 2014; Jeon et al., 2010). What is worth noticing, after repeated exposure to the Demonstrator being subjected to foot shock, Observer levels of freezing are decreasing (Carrillo et al., 2015). A different approach to study fear transfer is to observe interactions between recently stressed animal (Demonstrator) with naïve partner (Observer) in the safe conditions of home cage. Studies performed on rats (Knapska et al., 2006, 2010) and mice (Meyza et al., 2015) showed that emotions are socially transferred from the Demonstrator to the Observer and result with a change in the Observers' behavior (Remote Transfer of Fear). This paradigm will be described in more detail in the later chapters.

1.2.2. Automatization of analysis

Recently, due to overall development in the field of new learning algorithms, i.e. advanced theory and more user-friendly interface (sophisticated programming, and statistical skills are no longer necessary) machine learning based tools became more popular among scientists (Bordes et al., 2023; Glaser et al., 2019). The push for growth of this area came with the technological advances in methods used in neuroscience. Current state-of-the-art techniques produce big sets of data, which are getting harder and harder to analyze manually (Greener et al., 2022). Thus, machine learning approach is getting more and more popular in the analysis of such big sets of data.

Banos et al (Banos et al., 2024) conducted a meta-analysis of all literature which used machine learning approach to study Human Emotion Recognition. Based on 65 review publications, the study concluded that automatic emotion recognition is well established and can be used with great success. Unfortunately, due to the variability in autism research machine

learning tools usage in autism studies need further validation. Similar analyses can be carried out in animals studies. Team led by Dolensek (Dolensek et al., 2020) classified mouse facial expressions into emotion-like categories (neutral baseline, pain induced by tailshock, bitter after quinine lick, sweet after sucrose, malaise after lithium chloride, active and passive fear expression after footshock) that could be compared with human emotions. Similar reseach was done by Tanaka (Tanaka et al., 2023) where emotional states reflected by facial expression were classified into neutral, aversive (induced by tail pinching) and appetitive (induced by brushing). Another example was provided by Loos (Loos et al., 2014) who implemented machine learning tools to analyze huge sets of behavioral data coming from continuous observation of a few different strains of mice living in the safe environment of the home-cage. Results obtained in this study showed distinct behavioral patterns depending on the strain. Unprecedented opportunity to classify and recognize emotions and behaviors displayed by animals combined with neuroimaging and neuromanipulating techniques will provide new ways to study neuronal correlates of emotions.

1.2.3. Neuronal correlates of emotional contagion

Data obtained from human studies show that observing other people experiencing fear (Olsson et al., 2007) or being subjected to painful stimuli (Bernhardt & Singer, 2012) can trigger similar brain activation in the amygdala, insular and anterior cingulate cortex (ACC) as first-hand experience. Similar experiments were done on rats when the reaction of an individual towards a stressed mate was investigated. In this study animals were more interested in stressed conspecifics than in ones from the control group. Observer rats after social interactions with a Demonstrator who was recently subjected to an electric shock displayed almost identical c-Fos expression in the basolateral (BLA) and medial nuclei of the amygdala (MeA) and prelimbic (PrL) and infralimbic (IL) parts of the PFC as the Demonstrator (Knapska et al., 2006; Mikosz et al., 2015). Furthermore, encounters with a stressed partner elicited an activation of the central nucleus of the amygdala (CeA), while receiving footshocks did not. For my MSc project (Meyza et al., 2015) I employed the same behavioral paradigm on mice which confirmed empathic abilities in this species, although activation pattern differed from the one observed in the rat study. In the amygdala, interaction with a stressed cage mate activated only the BLA in Observer mice, while expression pattern in prefrontal cortices was similar in rats and mice. In

the Observational Fear Paradigm inactivation of ACC, parafascicular or medidorsal thalamic nuclei by lidocaine led to deficits in contagious freezing. Further investigations pointed to the role of Ca_v 1.2 type 1 Calcium channels, responsible for synaptic transmission in the ACC in this process (Jeon et al., 2010). Later studies showed also the involvement of dopamine and serotonin receptors in the Observational Fear Paradigm as well. Inhibiting dopamine D_2 receptor and 5-HT_{1A} and 5-HT_{2A} serotonin receptors in the ACC blocked the freezing response of the Observer in the Observational Fear Paradigm (Kim et al., 2014). Also, an induction of long-term potentiation in the dorsomedial prefrontal cortex-basolateral amygdala pathway was observed in animals observing fear in others (Ito & Morozov, 2019). Another paradigm used in emotional contagion research, Fear Conditioning by Proxy, showed that the lateral amygdala nucleus (LA) and the ventral CA1 region of the hippocampus are required for acquisition and retrieval of socially acquired fear response (Jones & Monfils, 2016). Studies performed on prairie voles also indicate the role of ACC and oxytocin signaling in emotional contagion (Burkett et al., 2016). Social Fear Conditioning paradigm which linked fear response with social interactions (every time when freely moving mice engaged in social interactions with a conspecific they were subjected to a footshock) indicated the involvement of somatostatin neurons in the prefrontal cortex in controlling fear expression. Xu et al (Xu et al., 2019) postulated that activation of somatostatin interneurons disinhibits pyramidal neurons in the dorsomedial prefrontal cortex, which through their projections to the Amygdala (LeDoux, 2000; Likhtik et al., 2014) and the periaqueductal grey (PAG) (Franklin et al., 2017; Rozeske et al., 2018) mediate fear response evoked in social conditions.

Another important phenomenon necessary for emotional contagion to occur and preceding it is the process of emotional recognition which gives an individual an ability to notice emotional expressions in others, which in turn provides essential information about their emotional state (Ferretti & Papaleo, 2019). Studies performed in mice showed that the prefrontal cortex plays a pivotal role in long-term social recognition memory. Mice were tested in the social recognition test, in which, with repeated exposure to a social stimulus animals interest in conspecific decreases. Interestingly in the PFC-lesioned group this was not observed (Sakamoto & Yashima, 2022). More detailed research focused on the hypothalamus and oxytocin signaling showed that endogenous oxytocin release from paraventricular nucleus of the hypothalamus to the central amygdala is required for discrimination of emotional states of other mice (Ferretti et al., 2019). The studies of Francesco Papaleo's group showed that the

prefrontal cortex is causally involved in emotional discrimination in mice (Scheggia et al., 2020).

It is often postulated that emotional contagion can occur due to the autonomic mimicry mechanism. Activation of the Hypothalamus-Pituitary-Adrenal axis (HPA axis) in response to stressful stimuli leads to an increase in stress hormone levels in the bloodstream, which is accompanied by physiological reactions such as blushing, pupil dilatation or sweating (Prochazkova & Kret, 2017). When the Observer unconsciously notices this set of symptoms, the superior collicus-pulvinar pathway to the amygdala is activated. This is turn, through locus coeruleus activation, triggers the HPA axis thus mimicking physiological reactions in the Observer (Tamietto & De Gelder, 2010). Simultaneously, through the amygdala and locus coeruleus pathway, the OFC and the ACC are activated which initiates the sensing of the stress response (Adolphs, 2001; Decety, 2011; Mutschler et al., 2013). Research performed on mice showed that socially transferred stress, similar to direct experience of the stressor, can induce plasticity in corticotropin-releasing hormone neurons found in paraventricular nucleus of the hypothalamus confirming its role in emotional contagion (Sterley et al., 2018).

It is also worth noticing that there is ongoing debate about the rodent prefrontal cortex nomenclature. Based on the meta-analysis of publications reporting findings related to the mouse prefrontal cortex, there are inconsistencies with regard to anatomical borders and the functional analogy to human PFC which makes it more difficult to draw conclusions from rodent prefrontal cortex data in the context of social interactions (Laubach et al., 2018).

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1.2.4. Sex differences

A major factor which may influence empathic abilities of an individual is the sex. Human research points to gender differences in empathy (Baron-Cohen & Wheelwright, 2004; Rueckert & Naybar, 2008; Schulte-Rüther et al., 2008) and the influence of the estrous cycle on women empathic behavior (Derntl et al., 2008; Guapo et al., 2009). Study conducted by (Mikosz et al., 2015) showed sex differences in the c-Fos expression pattern after social interactions with a stressed conspecific, even though there was no significant distinction in the behavioral response. Most experiments aimed at elucidating behavioral and neuronal correlates of emotional contagion are however done on males due to a common belief that menstrual cycle

variability will overwhelm results obtained from the study. Recent publication from Levy (Levy et al., 2023) suggests that every animal has an individual pattern of behavior, which the estrous cycle affects only minimally. Hopefully, this will encourage more research performed on both sexes to better understand its effect on empathy. Findings presented in this dissertation include results obtained from both male and female mice.

1.3. How and why empathy is being studied?

One of the ways to understand how a given phenomenon functions, and what the exact mechanism of it is, is to study the examples of impairment or even lack of the mentioned process in a model organism. An example would be to study emotional processing in people who may exhibit impairments in this area, e.g. in Autism Spectrum Disorder (ASD) patients. Since most of human research utilizes neuroimaging techniques, the results are often correlative in nature. To better understand neuronal correlates of this disease more detailed information can be obtained with the use of animal models.

1.3.1. Autism Spectrum Disorder

Living in a society requires an ability to communicate with conspecifics and any deficit in this domain may severely impact our quality of life. Emotion recognition is one of the skills which is crucial for social interactions and communication. A natural group to study deficits in this domain would be the people with ASD diagnosis. ASD is a neurodevelopmental disorder characterized by impairments in social communication and the presence of stereotypic behaviors as well as rigid behavioral patterns (Lai et al., 2014). The main causes of autism are not well understood. Research done on monozygotic twins and its high heritability indicate a strong genetic component of the disease (Freitag, 2007). Together with environmental factors during early development, such as prenatal exposure to toxins (e.g. a commonly used anti-epileptic drug, sodium valproate) or maternal stress during pregnancy (Varghese et al., 2017), the etiology of ASD is often difficult to pinpoint. Pregnant women treated with valproate have a trifold increased risk of having their child diagnosed with ASD (Bromley et al., 2008; Christensen et al., 2013). Additionally to its high heritability, as many as 30% cases can be

assigned to *de novo* mutations (De Rubeis & Buxbaum, 2015; Pinto et al., 2014). Some examples of most studied mutations are those found in single genes, such as, *TSC1/2* (lack of which causes tuberous sclerosis). It is a translational regulator and its malfunction can result in cerebellar deficits, brain enlargement and hyperactive mTOR signaling (Hampson & Blatt, 2015; Hevner, 2015; Vaughan et al., 2022). Another example is *SHANK3* gene. Protein encoded by this gene is a synaptic scaffolding molecule and incorrect levels of it may lead to striatal dysfunctions, lower Long Term Potentation (LTP) and F-actin dysregulation in PFC (Duffney et al., 2015; Jacot-Descombes et al., 2020; Schmeisser et al., 2012; Wang et al., 2011) or whole chromosome aberrations for instance 15q11-q13 deletions (Prader-Willi Syndrome), 15q13.3 microdeletion (Angleman syndrome), or 22q11.2 deletion (DiGeorge syndrome) (Varghese et al 2017). Mutations caused by CGG triplet expansion within the *FMR1* gene located on the X chromosome may lead to Fragile X Syndrome. The FMPR (Fragile X messenger ribonucleoprotein) is responsible for translational regulation and its deficiency manifest itself with enhanced protein synthesis, hypersensitivity to ERK1/2 pathway activation, higher dendritic spine density and altered synaptic plasticity (Kumari & Usdin, 2020; Lannom et al., 2021; Zhang et al., 2018). Up to 30% people suffering from Fragile X Syndrome are co-diagnozed with ASD.

Autism patients are also often diagnosed with immune system dysregulation, which was identified both in human subjects and in several mouse ASD models (Estes & McAllister, 2016; Malkova et al., 2012; Matta et al., 2019; Noriega & Savelkoul, 2014). Various immune response related genes were found to be upregulated in ASD subjects (Garbett et al., 2008) which suggests an ongoing inflammation state both in their brains and in the periphery. Lately it was also shown that through prevalent connections between gut microbiota and central nervous system bidirectional communication can occur (Agirman & Hsiao, 2021) and gut microbiota-brain axis started to be intensively studied, also in ASD research. It was noticed that people with autism often suffer from gastrointestinal issues, such as inflammatory bowel syndrome (Lee et al., 2018; McElhanon et al., 2014) and that their microbiome profile is distinct from the healthy individuals which is associated with abnormal metabolism activity (Dan et al., 2020; Hughes et al., 2018).

Another factor which might influence social and empathic abilities is the epigenetic makeup of an individual. Higher levels of oxytocin receptor promoter methylation were observed in ASD patients, which might affect their social skills (Baribeau et al., 2017; Kosaka et al., 2016). A mouse model with a knockout in the gene responsible for histone acetylation

was found to have deficits in social skills (Nott et al., 2016). Also, lower histone acetylation in the Prefrontal Cortex was observed in crab-eating monkeys after being prenatally exposed to valproic acid (Zhao et al., 2019). It is consistent with data obtained from human studies, showing that around 68% of people with ASD diagnosis have distorted histone acetylation profile (Sun et al., 2016).

Interestingly, some scientists point to mitochondria dysfunction as a cause for ASD. High energy demand of the brain and its deficiency during development may result in brain malfunction. Mitochondria regulate calcium homeostasis in the cell so any disturbance of that fragile equilibrium may lead to impairments in cell signaling pathways in neurons (Khaliulin et al., 2024).

Importantly, in most cases of ASD, the etiology is multifactorial (Varghese et al., 2017) and only 5-10% of cases can be associated with a single gene mutation or a SNP. With many factors converging in one individual it is often difficult to trace them all, and such cases are then referred to as idiopathic.

1.3.1.1. Neuroanatomy of ASD

The diverse and often unknown etiology of ASD complicates attempts to find precise neuronal correlates of the disorder. Recent findings from human neuroimaging studies describe few abnormalities found in ASD individuals compared with healthy control groups such as smaller neurons in several brain areas (archeocortex, cerebellum, brainstem) (Wegiel et al., 2014, 2015), as well as altered cortical structure and misplaced neurons as a result of incorrect neuronal migration and maturation (Casanova et al., 2013; Stoner et al., 2014).

Cerebral cortex is organized in a hierarchical way. At the bottom layers there are sensory and motor brain areas, then we have higher areas responsible for integrations of signals. At the top of cortical hierarchy structure, among others, there is prefrontal cortex which has a role in representation and execution of actions. Its medial and orbital parts are associated with controlling emotional behavior (Fuster, 2001). It was noticed that in young children diagnosed with ASD, the prefrontal cortex is bigger (Carper & Courchesne, 2005; Hazlett et al., 2011), and the number of parvalbumin interneurons in medial prefrontal cortex is lower than in their peers from control group (Hashemi et al., 2017). Also, spatial organization of microglia around

neurons found in dorsolateral prefrontal cortex is altered (J. T. Morgan et al., 2012) although Courchesne (Courchesne et al., 2011) showed that the number of other glia cells does not differ from the amount reported in control groups.

Since the main source of emotional information in humans comes from perception of others peoples' faces (Klingner & Guntinas-Lichius, 2023) researchers focused on the fusiform face area, a brain region belonging to the visual system which is selectively activated by faces (Kanwisher et al., 1997). Data from functional magnetic resonance shows lowered activity of this area during face processing in ASD patients (Bölte et al., 2006) which is attributed to less dense layer III neurons found in this structure. Other parts of the visual pathway seem to not differ compared to the control group (Uppal et al., 2014).

Another key structure in ASD research is the anterior insular cortex, an area involved in emotional processing and awareness of oneself and others (Gu et al., 2013). Von Economo cells are large bipolar cells found in the fifth layer of the ACC (Nimchinsky et al., 1999) and its postulated role is the involvement in social cognition and self-identity (Allman et al., 2005, 2010; Seeley, 2010). Interestingly, patients with ASD have been reported to have more von Economo cells in the ACC and their placement (in the sixth layer and white matter) was unusual (Allman et al., 2005).

The hippocampus and the amygdala are major areas involved in emotional processing and the formation of memory necessary for proper social behavior. People suffering from Fragile X Syndrome, prevailing single-gene cause of Autism, have been found to have focal thickening of hippocampal CA1 and altered neurons (cells were misplaced and had wrong orientation) in the dentate gyrus (Greco et al., 2011). ASD patients were reported to have more densely packed interneurons in the hippocampus (Lawrence et al., 2010) and swollen axon terminal in the entorhinal cortex and CA subfields (Weidenheim et al., 2001). In case of the amygdala, people with ASD have smaller amygdalar nuclei and at the same time higher cell density (Bauman & Kemper, 1985; Kemper & Bauman, 1993). More recent studies showed significant decrease in neuronal numbers in the amygdala, especially in the lateral nuclei (Morgan et al., 2014; Schumann & Amaral, 2006).

Due to small sample sizes, heterogeneity of groups, limitations of human imaging tools and correlational nature of these findings we must be careful with drawing conclusions. It is also important to note that the vast majority of studies performed on humans have been done *post mortem* which may not reflect organization of the living brain, but with advancing modern

imaging technologies scientists should soon be able to better pinpoint neuronal characteristic of this disease.

1.3.2. Mouse model used in this study

People with Fragile X Syndrome are often (up to 30% comorbidity) co-diagnosed with ASD. FXS is a result of an expanded CGG repeat in fragile mental retardation 1 gene which causes shortage or even lack of FMR1 protein. FMR1 protein is a RNA transporter, which regulates translation process during synaptic plasticity and development (Hagerman et al., 2010). People with FXS suffer, among others, from hyperactivity, repetitive behaviors, social difficulties and seizures - effects which can be reproduced in animal research using FMR1 KO mouse model, which is commonly used in research of neuronal correlates of autism (Chen & Toth, 2001; "Fmr1 Knockout Mice: A Model to Study Fragile X Mental Retardation. The Dutch-Belgian Fragile X Consortium.," 1994; Hayashi et al., 2007; Spencer et al., 2011; Yan et al., 2004). When trying to find brain mechanisms responsible for this disease scientists focused on synaptic connections, especially dendritic spines. What was found is that brains of FXS patients studied *post mortem* revealed less mushroom-shaped spines and higher number of long dendritic spines, usually associated with immature synaptic connections (Rudelli et al., 1985; Wisniewski et al., 1991). Similar abnormalities in spine structure were observed in FMR1 KO mouse models (Comery et al., 1997; Irwin et al., 2000). Interestingly, when researchers used pharmacological intervention aimed at maturation of long-filipodia shaped spines, along with more mature dendritic spines phenotypic deficits were rescued in knockout mice (Dolan et al., 2013; Puścian et al., 2022). Another common feature between human patients and the animal model is bigger relative volume of white matter in FMR KO mice, which may reflect altered connectivity found in people (Lai et al., 2016). Macroorchidism, a common trait found in males, especially children suffering from FXS is also replicated in FMR1 KO strain ("Fmr1 Knockout Mice: A Model to Study Fragile X Mental Retardation. The Dutch-Belgian Fragile X Consortium.," 1994). When it comes to the immune system FMR1 KO mice do not have a distinct profile of immune genes expression compared to WT strain (Hodges et al., 2017; Kong et al., 2014) but it was reported that microglia of FMR1 KO mice produces higher pro-inflammatory and phagocytic response as a result of lipopolysaccharide treatment (Parrott et al., 2021). Similar to ASD patients it was reported that in FMR1 KO2 strain mice had altered

gut micriobiome compared to their WT littermates (Altimiras et al., 2021). Examples provided in this chapter validate the use of FMR1 KO model in ASD research.

1.3.3. Autism Spectrum Disorder diagnosis

Since ASD is currently diagnosed only with observation-based criteria by qualified psychiatrists many researchers pursue a way to find a reliable and reproducible biological marker for ASD. Several research groups postulate that ASD may be diagnosed by specific profile of upregulated pro-inflammatory cytokines and downregulated anti-inflammatory cytokines found in the brain (Ashwood et al., 2011; Krakowiak et al., 2017; Masi et al., 2017). Schielen et al in their MRI-based research and meta-analysis of over 130 studies found that ASD identification done this way can be performed with 76% sensivity and 75,7% specifity (Schielen et al., 2024).

Screening for genetic mutations is still the most common attempt to find a biomarker of ASD. Usually, individuals with ASD have their genome sequenced, which is then compared with neurotypical (preferably someone of kin) genome to find differences. Next, identified mutations are being studied in animal models in a variety of ways.

1.4. How emotional contagion can be studied

1.4.1. c-Fos as a neuronal novelty marker

The *c-fos* gene is a member of a group of genes called the immediate-early genes (IEG). These genes are characterized by swift activation, in a matter of minutes, without the need of protein synthesis *de novo* after cell stimulation. Products of IEG are transcription factors that control expression of all kinds of genes which play a role in cell functioning (Gallo et al., 2018; Herdegen & Leah, 1998). Protein encoded by *c-fos* can be found in cell nuclei (Curran et al., 1984) and thanks to leucine zipper domain it can form heterodimers with other products of IEG. The most prominent example would be the combination with proteins from the Jun family which creates a transcription factor called Activator Protein-1 (AP-1) (Chiu et al., 1988; Pennypacker, 1995). The AP-1 transcription factor regulates a variety of target genes, such as

nerve growth factor, prodynorphin, neurotensin and neuropeptide Y, involved in cell functioning (Karin et al., 1997).

After discovering that rising levels of c-Fos are correlated with cell activity (J. I. Morgan et al., 1987), and knowing that baselines protein levels are low, induction of c-Fos began to be used as a marker for neuronal activation. Expression of c-Fos protein peaks at around 90 minutes and returns to baseline levels after about 6 hours (Nikolaev et al., 1991). Its expression is mainly induced by novel stimuli and during learning of behavioral tasks. During habituation to repetitive stimuli the responses are decreasing (Nikolaev et al., 1992). Interestingly, a change in context (different light conditions/scent) again triggers high c-Fos expression even in animals which had learned the task and their c-Fos levels have already decreased (Knapska et al., 2006; Nikolaev et al., 1992). Importantly, it was observed that just re-exposure to the cage where training took place can induce c-Fos expression even thirty-one days after training (Frankland et al., 2004). Martinez et al (Martinez et al., 2013) observed that animals which performed better in the active avoidance test had higher c-Fos levels in the amygdala and prefrontal cortex than conspecifics which carried the task out poorly. Although there is no clear correlation of c-Fos levels with spontaneous neuron activity measured, for example, by calcium imaging (Peter et al., 2013) it was shown that c-Fos can be induced by a variety of behavioral stimuli in a vast majority of brain structures: cortex, hippocampus, amygdala, cerebellum, striatum or thalamus (Carretta et al., 1999; Heurteaux et al., 1993; Kaczmarek & Nikołajew, 1990; Paban et al., 1999; Tischmeyer et al., 1990). Blocking of c-Fos expression in mouse auditory cortex, using short hairpin RNA sequences, can impair rodent ability to distinguish sounds while other learning tasks, independent of auditory cues, and basal neuronal activity remain intact (de Hoz et al., 2017). Suggesting that c-Fos expression is causally linked to the neuronal plasticity. Similar approach to test local c-Fos inhibition was taken by several research groups. They used ACO - the of *c-fos* antisense oligonucleotide which blocks *c-fos* expression. (Guzowski, 2002) and (Kemp et al., 2013) showed that inhibiting c-Fos in rat hippocampus can impair spatial long-term memory.

The data presented here indicate that measuring and manipulating c-Fos levels can be an useful tool for studying the involvement of a given brain structure in a task or process. Since many things can increase c-Fos levels it is crucial to include proper habituation prior to commencement of the experiment into the protocol, so that measured c-Fos levels reflect only the investigated process.

Nevertheless c-Fos is still one of the most prominent tools used in neuroscience to investigate various aspects of brain functioning (Appleyard, 2009). Another way to utilize *c-fos* is to establish various viral vectors working under *c-fos* promoter for opto and chemogenetic manipulation. Using optogenetics under *c-fos* promotor (Cowansage et al., 2014) showed that for acquisition and reactivation of contextual memories are responsible c-Fos expressing neurons in retrosplenial cortex, while (Liu et al., 2012) reported that c-Fos tagged neurons during fear learning when artificially activated in different context, evoked fear response. Recently with the use of before mentioned methods, differences in neural circuits controlling social interaction and food motivation in rats were discovered (Rojek-Sito et al., 2023).

2. Aims of the thesis

Neuronal correlates of empathy and Autism Spectrum Disorder, even though intensively investigated, are still unclear. The aim of this study was to enhance our understanding of these phenomena.

The first goal of the study was to assess emotional contagion abilities of mice with a full knockout of the Fragile X Messenger Ribonucleoprotein 1 (FMRP). This mouse strain is used as a mouse model for the most common monogenic form of ASD. To do that I employed Remote Transfer of Fear Paradigm which verifies whether the altered emotional state of Demonstrator, can be detected by the Observer through social interactions in the safe environment of the home cage. To verify whether such transfer is affected by the sex the studies were conducted on both males and females.

The second objective of the project was to establish novel, more accurate and efficient way to score behavioral data on freely moving animals. Until now, most of the automatic tracking used for assessment of emotional contagion was done on one animal at a time or in way that would prohibit the animals to freely interact with each other. This was mainly due to difficulties with establishing and maintaining proper identity of animals throughout prolonged recordings. To overcome these obstacles, state-of-the-art software which utilized machine learning approach for tracking (DLC) and creating classifiers (SimBA) were used.

The last aim of this dissertation was to check if potential changes in the behavior of Observers would be reflected in the neuronal activation pattern and whether it would resemble that of the Demonstrators. Brain activity of Demonstrators and Observers was assessed by measuring c-Fos levels in both amygdala and prefrontal cortex, structures involved in emotional processing.

3. Methods

3.1. Animals

Research was conducted on FMR1 KO mice of the FVB strain (FVB.129P-Fmr1tm1Rbd/J) and respective littermate controls which were bred at University of Warsaw breeding facility. Males and females of approximately 3 months of age were used. Animals were housed in standard conditions under 12h light/dark cycle with *ad libitum* access to water and food (standard laboratory chow). Prior to the habituation phase, mice stayed in cohorts consisting of 8-12 animals. Experimental procedures were conducted during the day in light phase. All of the experiments were carried out in accordance with the Local Ethical Committee (permission LKE 504/2018).

3.2. Behavioral paradigm

3.2.1. Habituation

Approximately three weeks prior to commencement of habituation phase mice were assigned to pairs mostly based on weight and familiarity (brothers or sisters from the same litter) to minimize the chance of aggression. Animals were checked daily and in case of aggression - separated and excluded from the experiment.

During the first three days of habituation the experimenter was putting hands in the home cage for about one minute and tried to gently lift the mouse up by the tail into the palm of the hand. Over the next 7 days the animals were transported to the experimental room and the procedure described in Kondrakiewicz (Kondrakiewicz et al., 2019) was followed. At first mice were left in the experimental room for about one hour while all of the necessary equipment such as the Conditioning chamber, PC and recording hardware were turned on. Then, one the first day, one of the mice was randomly assigned as a Demonstrator and had its tail marked by a marker consecutively on each day. After that the Demonstrator was taken out from the home cage and put into the new cage which was then placed in the conditioning chamber, while the remaining mouse in the home cage - the Observer was taken to the adjacent room. It is essential that the Observer is not able to hear, smell or see the Demonstrator while they are separated. After 5 minutes the Demonstrator was placed back into the home cage with the Observer and 9

minutes of their interactions were recorded. Later, mice were put in another room and this cycle was repeated for the next pair of mice. After all pairs were done with their habituation sessions they were left for approximately 30 mins in the room and then transported back into the housing facility.

3.2.2. Remote Transfer of Fear paradigm

On the Test Day half of the animals were assigned to the Control group in which animals were treated exactly as in previous days during habituation and the second half into the Experimental group in which Demonstrators were subjected to aversive stimuli while being separated from the Observer. In the Experimental group after the Demonstrator was taken from the home cage it was put into the conditioning apparatus and subjected to a series of aversive stimuli (90s habituation followed by ten 1-s, 0.6-mA shocks with 30-s intervals). Every step prior and after that was the same as in the Control group. Ninety minutes after the start of the interaction between mice (moment when the Demonstrator was put back into the home cage after separation/foot shocks) animals were sacrificed and standard perfusion procedure was carried out.

3.3. Scoring behavioral data

3.3.1. DeepLabCut and Simple Behavioral Analysis

Manual scoring of data is extremely laborious and prone to human error. For this reason, I decided to develop a way to make this process as automatic as possible. To do that I used software for markerless pose estimation based on deep neuronal network learning - DeepLabCut (DLC) (Mathis et al., 2018). Recent DeepLabcut updates offer a possibility to automatically quantify behavior from over 45 species with already pre-trained models (Ye et al., 2024). In my study I employed multi-animal pose estimation DeepLabCut, one of the very first pieces of software enabling markerless behavior tracking of at least two freely moving animals (Lauer et al., 2022). Another software used in my experiments was SimBA (Simple Behavioral Analysis), an open source toolkit for computer classification of complex social behaviors (Nilsson et al., 2020). It utilizes pose-estimation obtained by other tools to generate

supervised machine learning predictive classifiers of rodent social behavior. Both of these tools do not require specific video annotation, have user-friendly interface and can be used on almost any video quality. Importantly, the two models which I created for the purpose of this study can be quite easily used in different experiments employing different mouse strains. After only small re-teaching with the use of approximately 500 frames for DLC and from a hundred to a few thousands frames (depending on the behavior) for simBA both models started to work on video recordings of black mouse interactions, which I successfully used in another project.

In my experiments I focused on several behaviors namely:

1. Sniffing of the Demonstrator's anogential area by the Observer
2. Sniffing of the Demonstrator's body by the Observer
3. Rearing of the Demonstrator and the Observer
4. Digging in bedding by the Demonstrator and the Observer
5. Self-grooming of the Demonstrator and the Observer

Manually scored data was necessary for creation and validation of two models based on supervised machine learning. One for automatic pose estimation and second one for automatic classification and recognition of animal behavior patterns. These models are going to be thoroughly described in the following sections.

3.3.2. Manual scoring

To score data by hand Behaview software (created by Paweł Boguszewski) was used. In this scenario we assessed 8 different, previously mentioned, behaviors: Sniffing the Demonstrator's anogenital area by the Observer, Sniffing the Demonstrator body by the Observer, Rearing, Digging in bedding and Self-grooming for both animals. This method requires a manual review of the movie to check if desired behaviors were present. If so, start and end of behavior is marked by pressing the appropriate button on the keyboard. Movies were analyzed with the temporal precision of 1/5 second.

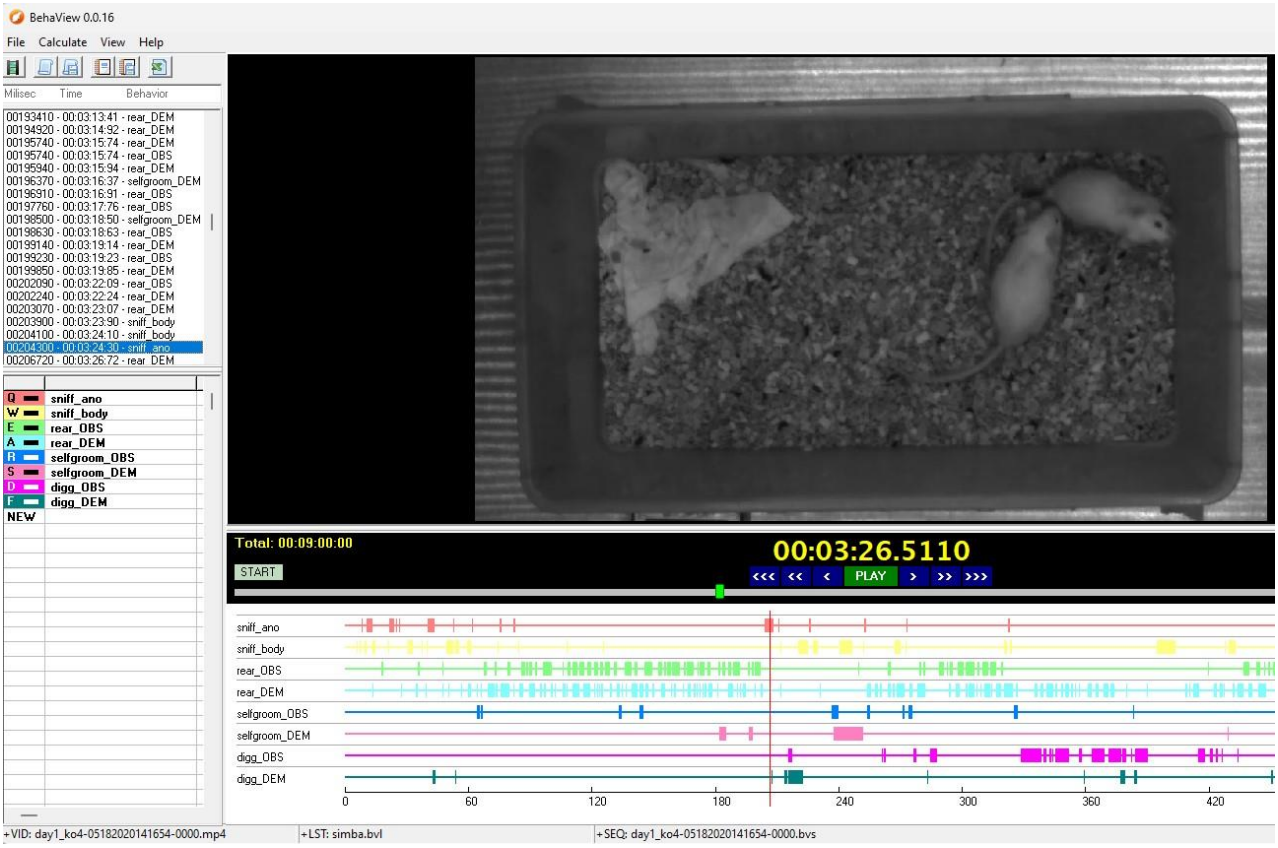

Figure 1. Screenshot of the BehaView software used for manual behavior annotation. Video depicts the Observer sniffing the Demonstrators anogenital area.

3.3.3. Automatic scoring

Since behavior was done over a prolonged period of time and various setups for recording were used, quality of movies differed between sets of experiments. At first movies had to be trimmed to have the exact same length of 9 minutes, cropped so that every movie showed only the home cage and its immediate surroundings (in case mice should climb the walls) and the movie format had to be unified. Trimming was done using the SimBA tool to pre-process videos while the other processes were done with the ffmpeg codecs package. After movies were made as uniform as possible and sets of example videos were loaded into the DLC the process, described thoroughly below, to make markerless post estimation began. After the DLC model was established, videos were analyzed with this model and resulting data was used for creating a SimBA model. Working SimBA model provides detailed behavioral data which was then analyzed using Excel and Prism software.

3.3.3.1. DeepLabCut

First step is to create the project with example data - a few chosen videos from the experiment. As mentioned in previous chapters I used a multi-animal project since it is suitable for at least two freely moving animals. All settings were stored in a config file (config.yaml). Example file can be found on GitHub (https://github.com/GozuNaz/phd) These include:

- identity set to “true” (so that model will learn that both mice are separate animals)
- individuals set to “Dem”, “Obs” (ID of animals)
- multianimalbodyparts set to (parts of the body which will be labelled)
    - “Ear_left”
    - “Ear_right”
    - “Nose”
    - “Center”
    - “Lateral_left”
    - “Lateral_right”
    - “Tail_base”
    - “Tail_end”
- Remaining parameters were left as default

Next step was to extract example frames from the movies on which relevant bodyparts were going to be manually marked by the experimenter. Frames were extracted with the automatic kmeans algorithm with 1 cluster step. Then, in the labelling step, on each frame every

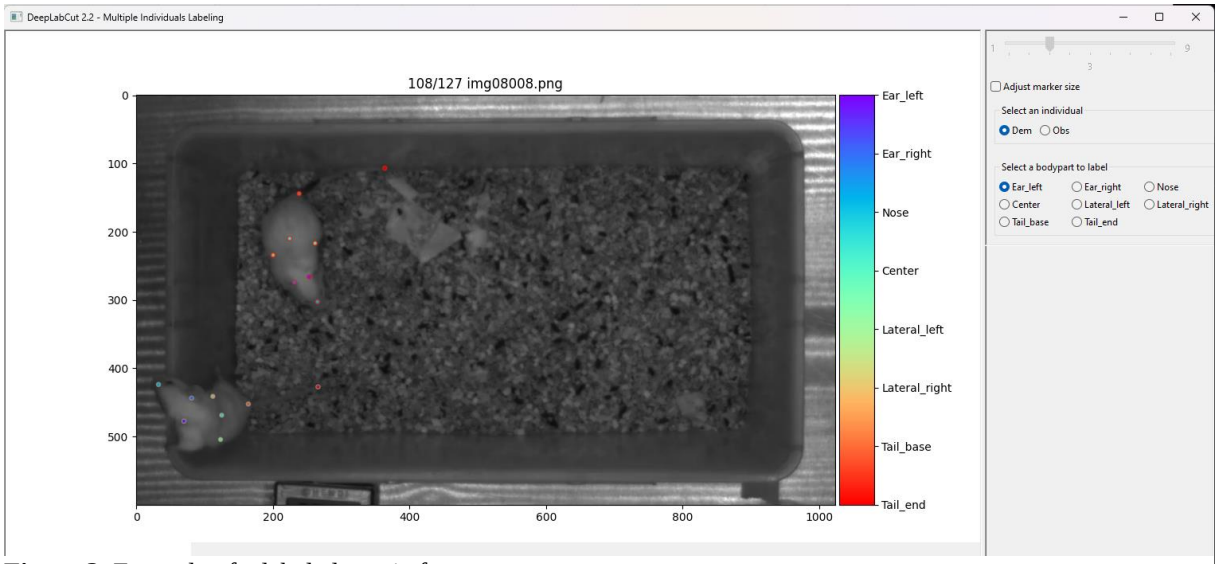

Figure 2. Example of a labeled movie frame.

body part had to be labeled for Demonstrator and Observer separately which is shown on **Figure 2.**

When all frames were labeled, a Training Dataset was created and then the training of the neuronal network started. Few parameters were tested to finally choose the following:

- Save number of iterations - **10000**
- Maximum iterations - **100000**
- Number of network snapshots to keep - **10**

Next step was to evaluate the trained model. To do that, DLC generated images with labels marked by the experimenter and ones predicted by model (see on the **Figure 3**). Average distance between them in pixels was also provided by the software. Then, in the next step, which was performed with default settings, DLC transformed predicted labels into pose estimation data which was then visualized on movies. This way, the final step of the evaluation was performed. If the data was not good enough, either parameters were changed, or more labeled data was given for training. In the end the model was established using 1600 frames total from 39 different example movies.

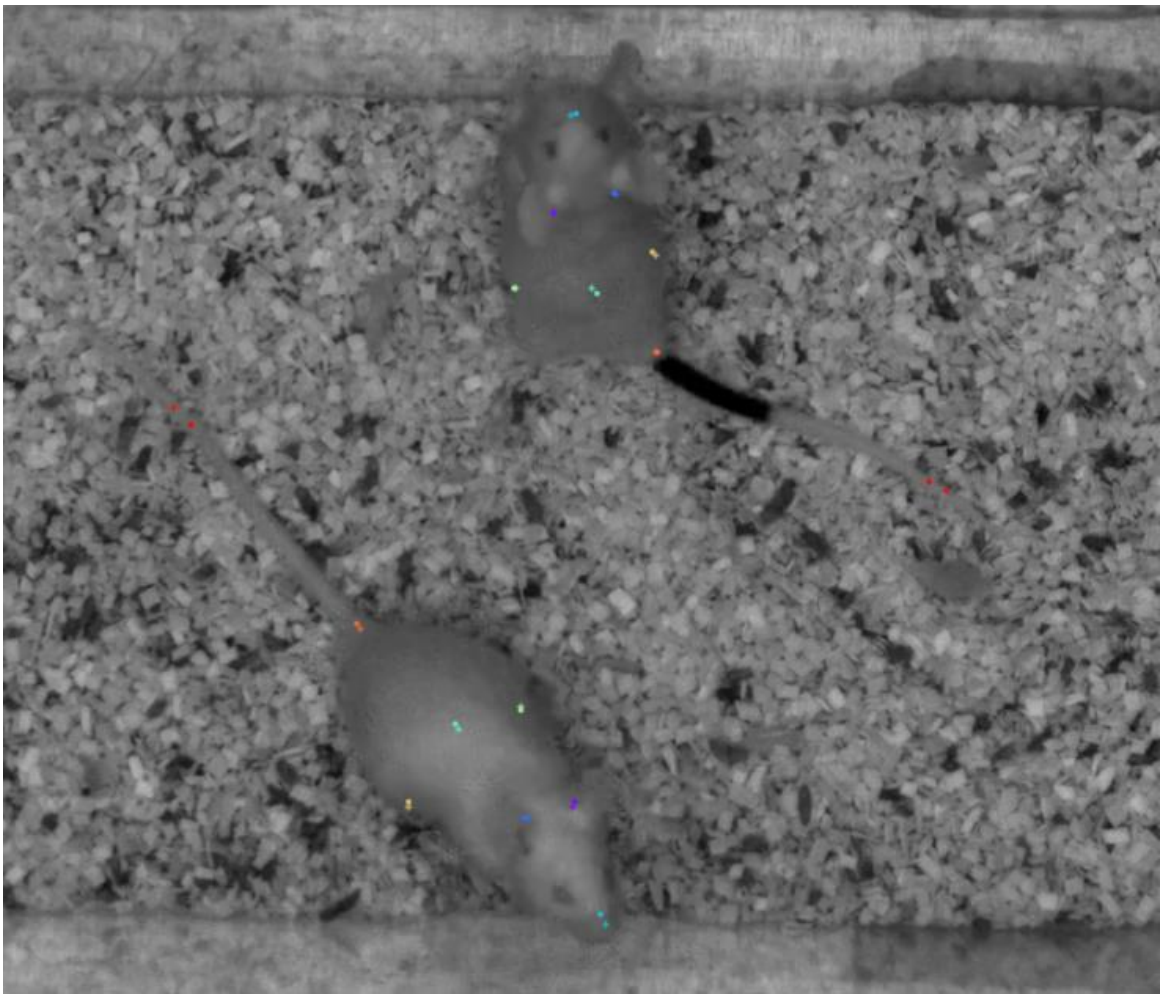

Figure 3. Example of comparison between manually labeled bodyparts and ones predicted by model.

After evaluation and validation, the model was run on all movies which resulted in pose estimation data for each video. After that, each movie was also manually inspected to check if DLC did not switch the ID of the animals or if there is any mislabeled data. In such cases, it had to be manually corrected. After completion of this process the movies with pose estimation tracking data were ready to be used in the second step - SimBA analysis.

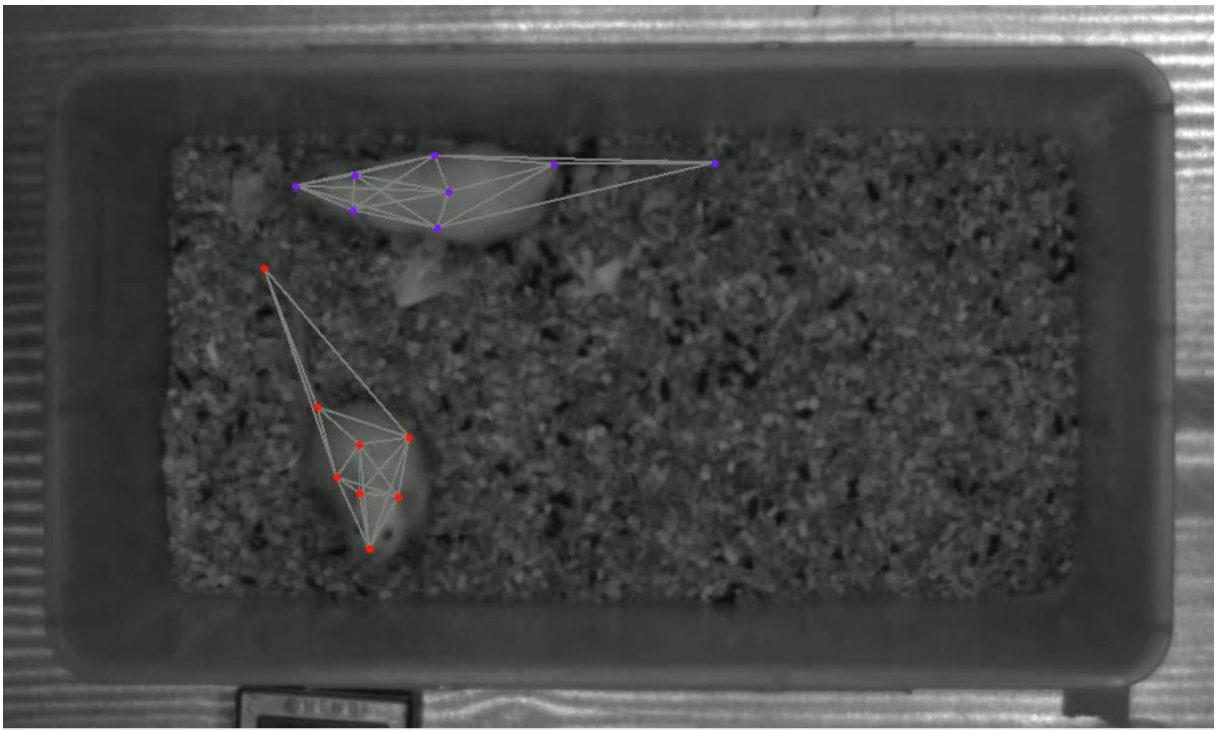

Figure 4. Frame from analyzed video with imposed tracking data.

3.3.3.2. SimBA

At first, example videos with their tracking data from DLC were loaded into a new SimBA project. The new project required the manual input of the eight predictive classifiers, Type of Tracking (chosen as Multi Tracking) and the number of bodyparts marked. It was crucial to choose the same points for bodypart labeling as the latter had to match the number used in the DLC analysis. Then, the width of the cage was provided into software for calibration of the ratio of millimeters per pixels for each video. Next, outliers correction was done by SimBA algorithm, with parameters:

Movement criterion: 1

Location criterion: 1.5

From nose to tail_end

Afterwards, for every movie an extraction of features was performed.

Then I proceeded to annotate the presence of a given behavior in every frame to build data for SimBA to learn from. To accelerate the process, all behaviors of interest were annotated

with the use of Behaview, and then with the script created by Konrad Danielewski (https://github.com/KonradDanielewski/SEBA), behavior data was made to fit SimBa dataset. Afterwards the Machine Models were trained and classifiers for each of eight behaviors were created. At the end, the model was trained on approximately 4000 to even 38000 frames depending on the behavior.

The process of validating the model consisted of comparing manually scored data with SimBA output. First, the data from the learning set was compared. After achieving compliance of about 98% movies which were not included in learning dataset were compared.

3.4. Tissue preparation

90 minutes after the onset of the test session standard perfusion procedure was conducted. At first, animals were anesthetized with isoflurane (5% in air) followed by an overdose of Morbital (ip, 133.3 mg/ml sodium pentobarbital, 26.7 mg/ml pentobarbital). Mice were then transcardially perfused with ice-cold Phospate Buffer Saline (PBS, pH=7.4) followed by 4% paraformaldehyde in PBS. After that the brains were removed and kept in 4% paraformaldehyde overnight in 4°C. Later brains were transferred to 30% sucrose solution in PBS and were kept in the fridge until sinking. The next step was to freeze brains with the use of dry ice and then store them in the -80°C freezer. Finally, the brains were cut at the cryostat into 40 μm coronal sections and then kept in the PBS with the sodium azide. For the immunostaining slices containing the amygdala (AP -1.58) and the prefrontal cortex (AP +1.70) were chosen (Paxinos & Franklin, 2019).

3.5. Immunostaining

The immunostaining was performed on free-floating coronal brain sections. Firstly sections were incubated in PBS (pH 7.45, Gibco #18912014) overnight at 4°C and then washed three times with fresh PBS. After that, sections were incubated in blocking solution consisting of 5% NGS (Normal Goat Serum, Vector Laboratories, #S-1000-20) in PBST (0.02% Triton X-100, Chempur #498418109) for 90 minutes. Next, slices were incubated with a primary antibody (anti-c-Fos, 1:1000, Millipore #ABE457) in PBST (0.02% Triton X-100, Chempur

#498418109) and 2% NGS for 48h at 4°C. Then, brain sections were washed 3 times with PBST and incubated with secondary antibody (anti-rabbit conjugated Alexa594, 1:1.000, Invitrogen #A32740) in PBST for 2h in Room Temperature. In the next step, sections were washed again three Times with PBS. Finally, brain slices were mounted on sides and coverslipped with Fluoromount (Sigma #F4680-25ML). Sections were imaged with the use of Nikon microscope (Nikon Eclipse Ni) and Image-Pro Plus 7.0.1.658 (Media Cybernetics) software.

3.6. c-Fos analysis

In order to make the process of counting less prone to error, a special protocol was established. At first images were duplicated. In the duplicate, with the use of the ImageJ tool for removing outliers (remove bright outliers) c-Fos positive cells (bright dots on the image) were removed leaving only the background. Then, using an image calculator tool in the ImageJ the background photo was subtracted from the original one, leaving just c-Fos cells with a uniform background. Next, the threshold was applied to mark only c-Fos cells (to get rid of some non-specific signal), desired ROIs were marked, and the ImageJ software counted the number of cells and the area of the given ROIs. To speed up the procedure, a special macro was written (with the help of Kacper Łukasiewicz) to make this process semi-automatic - only the ROIs were marked manually. Based on the calibration measuring ruler dimensions in pixels the area of ROIs was then converted to mm^2.

Here are the example steps of how macro worked and how parameters were checked that they are well chosen.

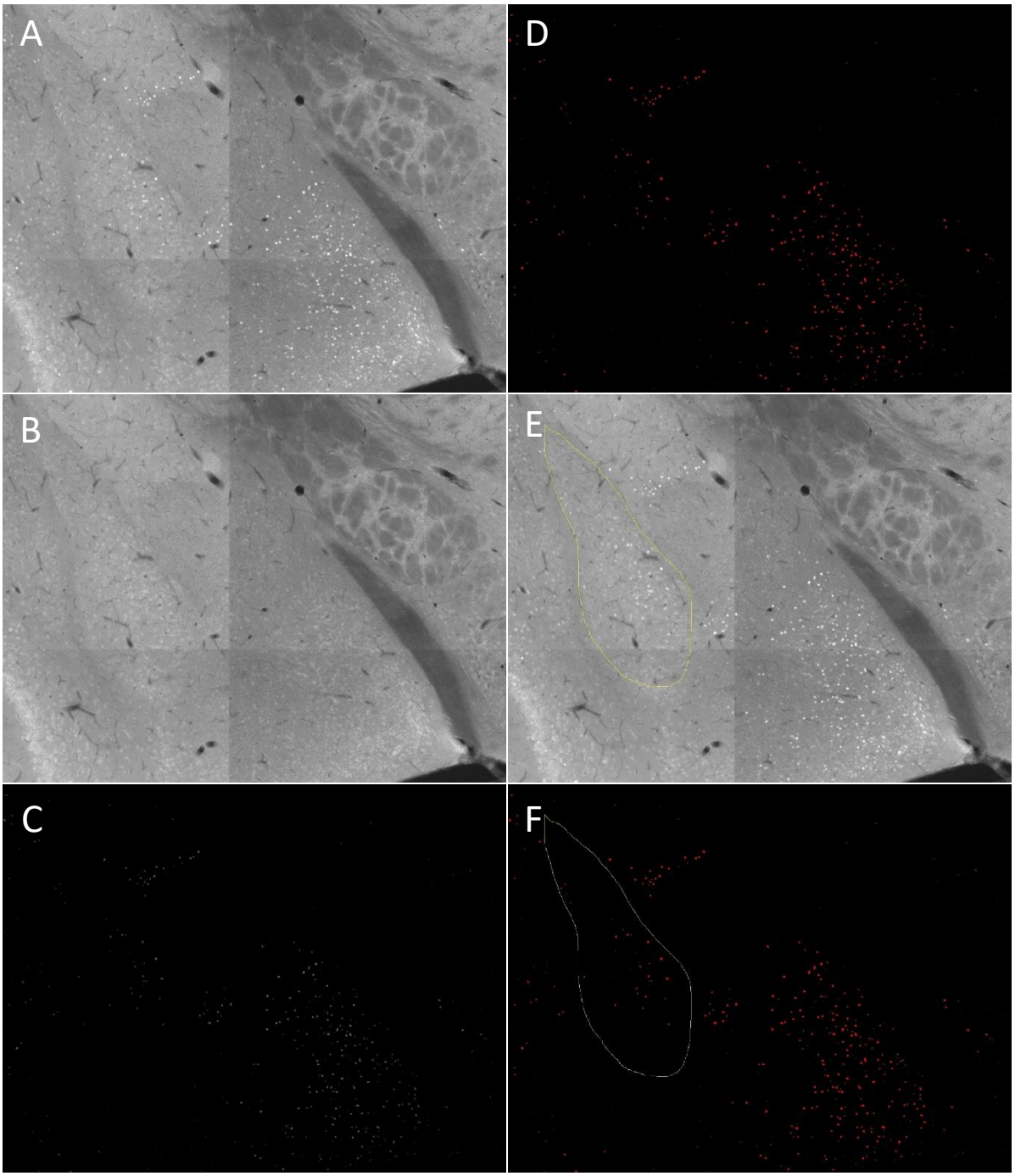

Figure 5. Figure represents steps of image analysis.

The steps described above are all presented in **Figures 5 and 6**. In panel A there is the original photo, while on panel B there is background photo. On panel C we can see only c-Fos positive cells, which are thresholded on panel D. To assess the desired ROI ImageJ sync tool was used, to simultaneously mark BLA on the original and the thresholded photo. On panel G, below, we can observe examples of the points the script marked as c-Fos positive nuclei.

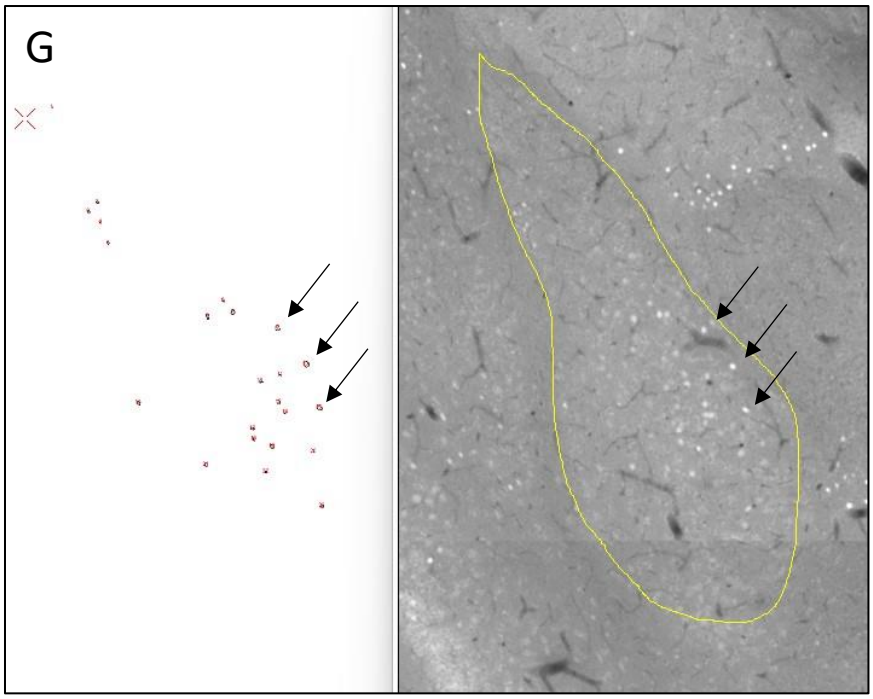

Figure 6. Final review of script.

3.7. Statistical analysis

3.7.1. Statisitical tests

Statistical analysis was done using the GraphPad Prism 10 software (*GraphPad*). All of the behavior data were checked for normal distribution with the Shapiro-Wilk test. Since the distribution of data was often not normal, non-parametric approach was chosen. Analysis of between genotype and experimental condition effects was done with the use of Kruskal - Wallis test with post-hoc Dunn's multiple comparison test. For c-Fos analysis Mixed-effects with post-hoc Tukey's multiple comparison test was used. Analyses were done within sex and Demonstrator or Observer groups. Analysis of the dynamics of behaviors was conducted using the Kolmogorov-Smirnov test. For correlation measurements between Demonstrator and Observer the Pearson coefficients were calculated.

Due to the technical reasons some of the brain tissue from males was lost and the experiment had to be repeated. Since there was no statistical difference between the two batches we decided to pool the behavioral data together for these groups.

Group	Males		Females	
	Behavior	Fos	Behavior	Fos
CTRL_KO	11 pairs	6 pairs	8 pairs	8 pairs
EXP_KO	13 pairs	7 pairs	8 pairs	8 pairs
CTRL_WT	10 pairs	4 pairs	7 pairs	7 pairs
EXP_WT	11 pairs	4 pairs	8 pairs	8 pairs

4. Results

4.1. A comparison of manual vs. automatic behavior analysis

Methods used in this thesis, namely DLC and SimBA analysis, were refined on the pre-trained models available in software packages and before they were used on the whole set of data, they had to be validated. To do that I compared behavior data scored manually with the data analyzed with DLC and SimBA. On the graph presented below we can observe that both methods resulted in similar outcomes regarding the number and duration of anogenital sniffing (**Figure 7 A** and **C**) and body sniffing (**Figure 7 B** and **D**) performed by Observers towards Demonstrators regardless of the genotype.

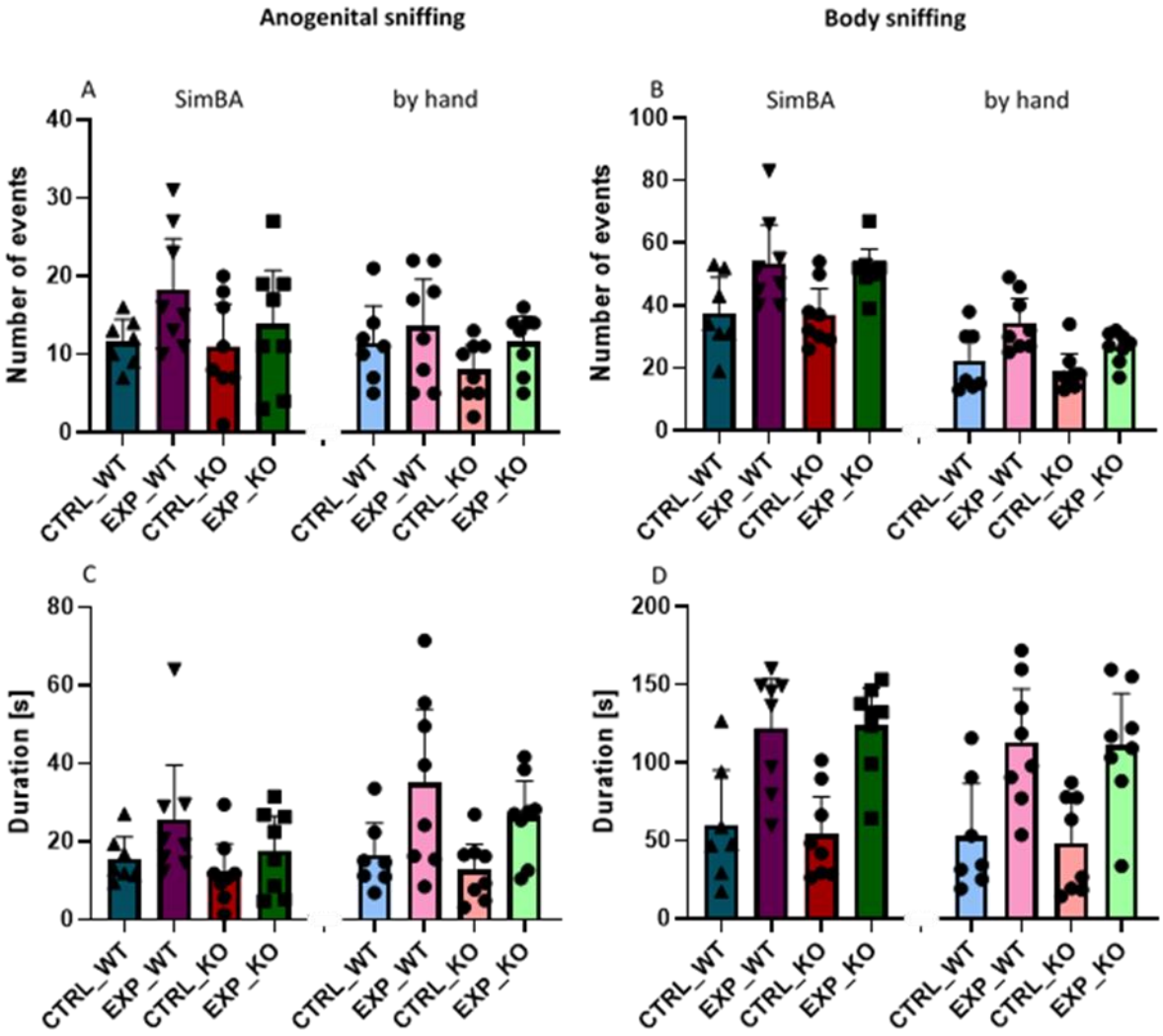

Figure 7. Comparison between behavioral results scored by SimBa and manually. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Sniffing behavior provided on graphs represents sniffing of Observers towards Demonstrators. All data are shown as mean ± s.e.m, dots represent each individual.

I also proved that both methods of scoring behavior resulted in similar outcomes regarding the number and duration of Demonstrator Rearing (**Figure 8 A** and **C**) and Observer Rearing (**Figure 8 B** and **D**) regardless of the genotype

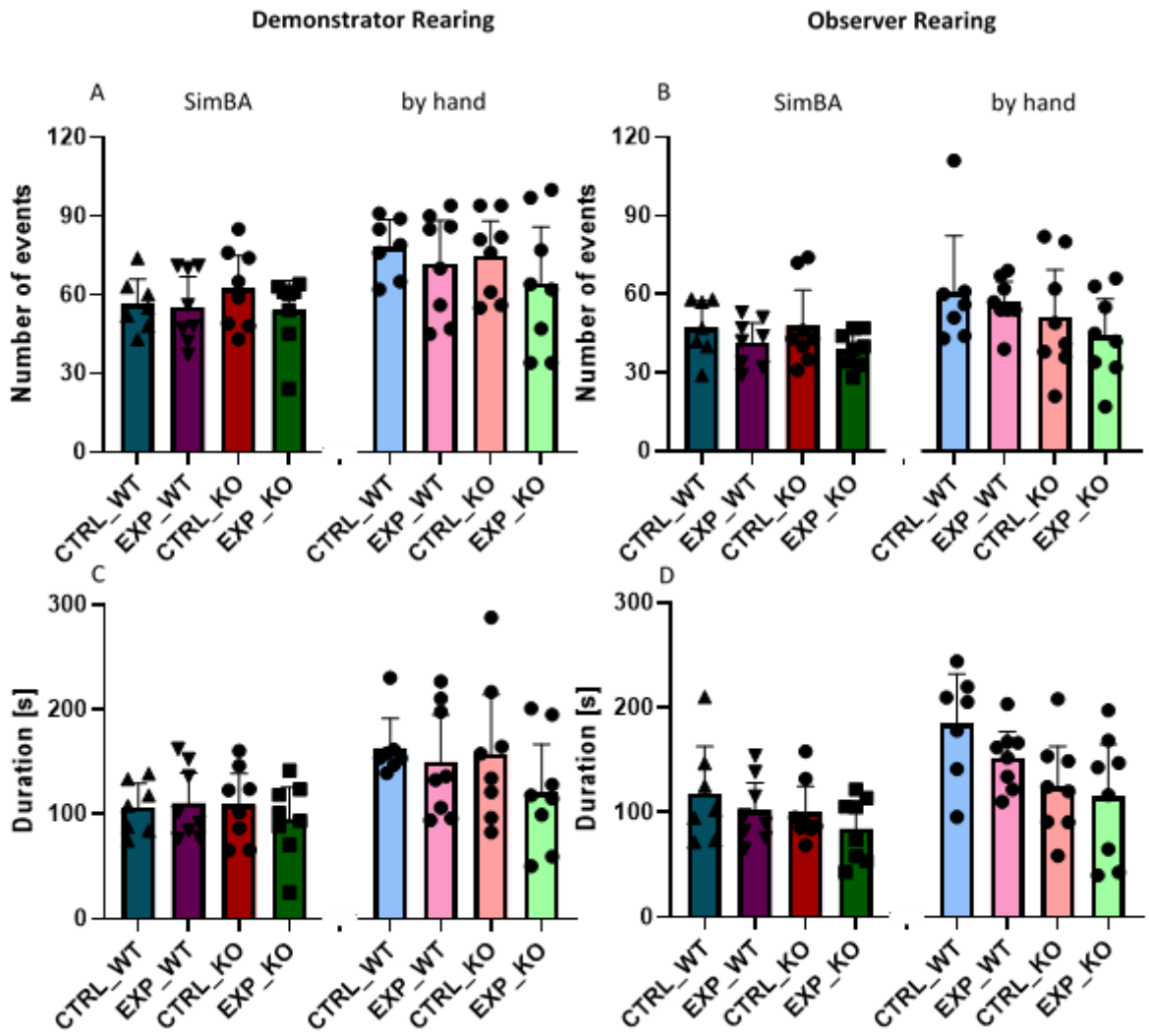

Figure 8. Comparison between behavioral results scored by SimBa and manually. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Exploratory behavior provided on graph represents Rearing of Demonstrators and Observers. All data are shown as mean ± s.e.m, dots represent each individual.

Similar results were shown for stereotypic behaviors (**Figure 9**), for the number and duration of Demonstrator Selfgrooming (**A** and **C**) and Observer Selfgrooming (**B** and **D**). Here as well obtained results were matching each other.

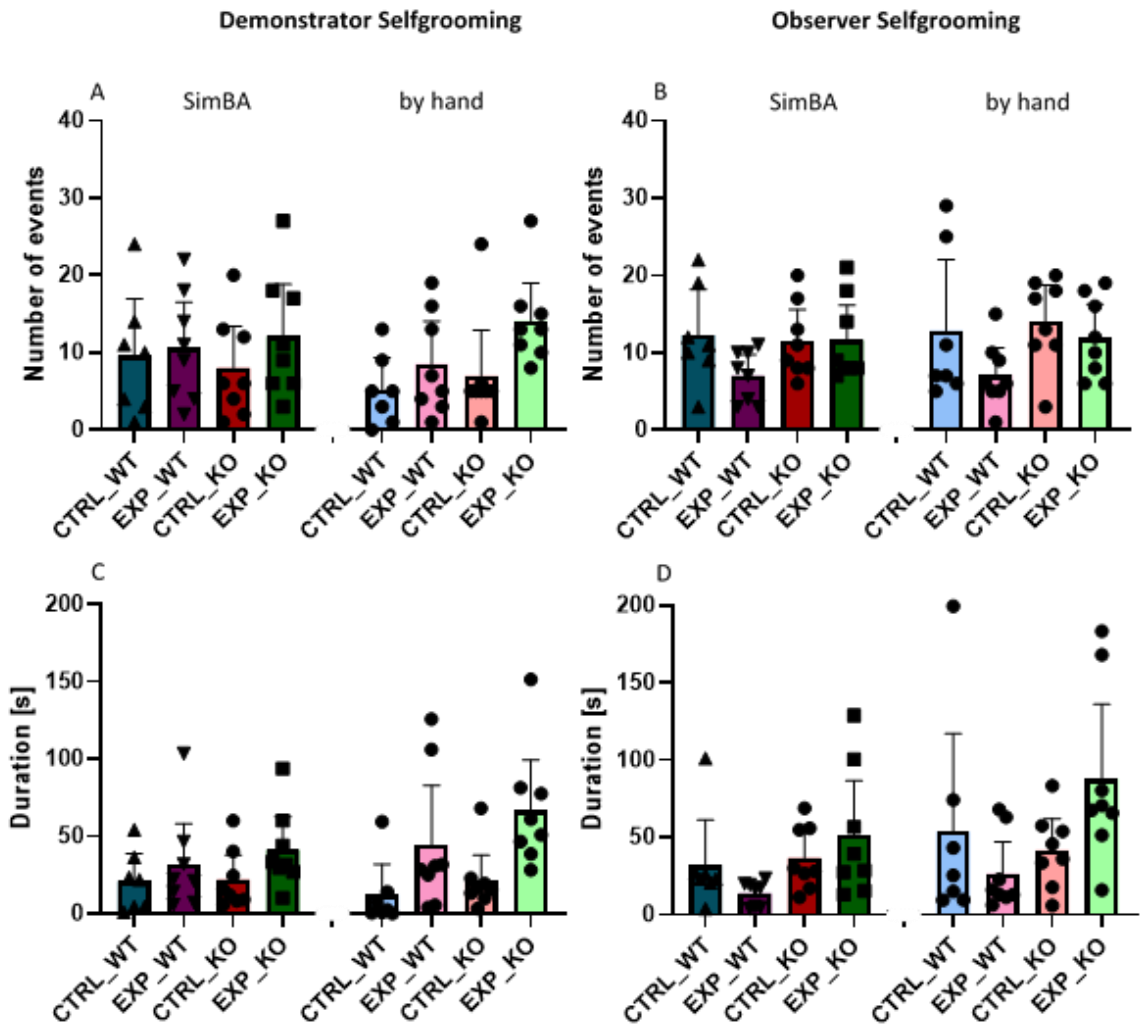

Figure 9. Comparison between behavioral results scored by SimBa and manually. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Stereotypic behavior provided on graph represents Selfgrooming of Demonstrators and Observers. All data are shown as mean ± s.e.m, dots represent each individual.

Figure 10 depicts data for another stereotypic behavior - digging in bedding. The number and duration of Demonstrator Digging (**A** and **C**) and Observer Digging (**B** and **D**) acquired by both methods was matching.

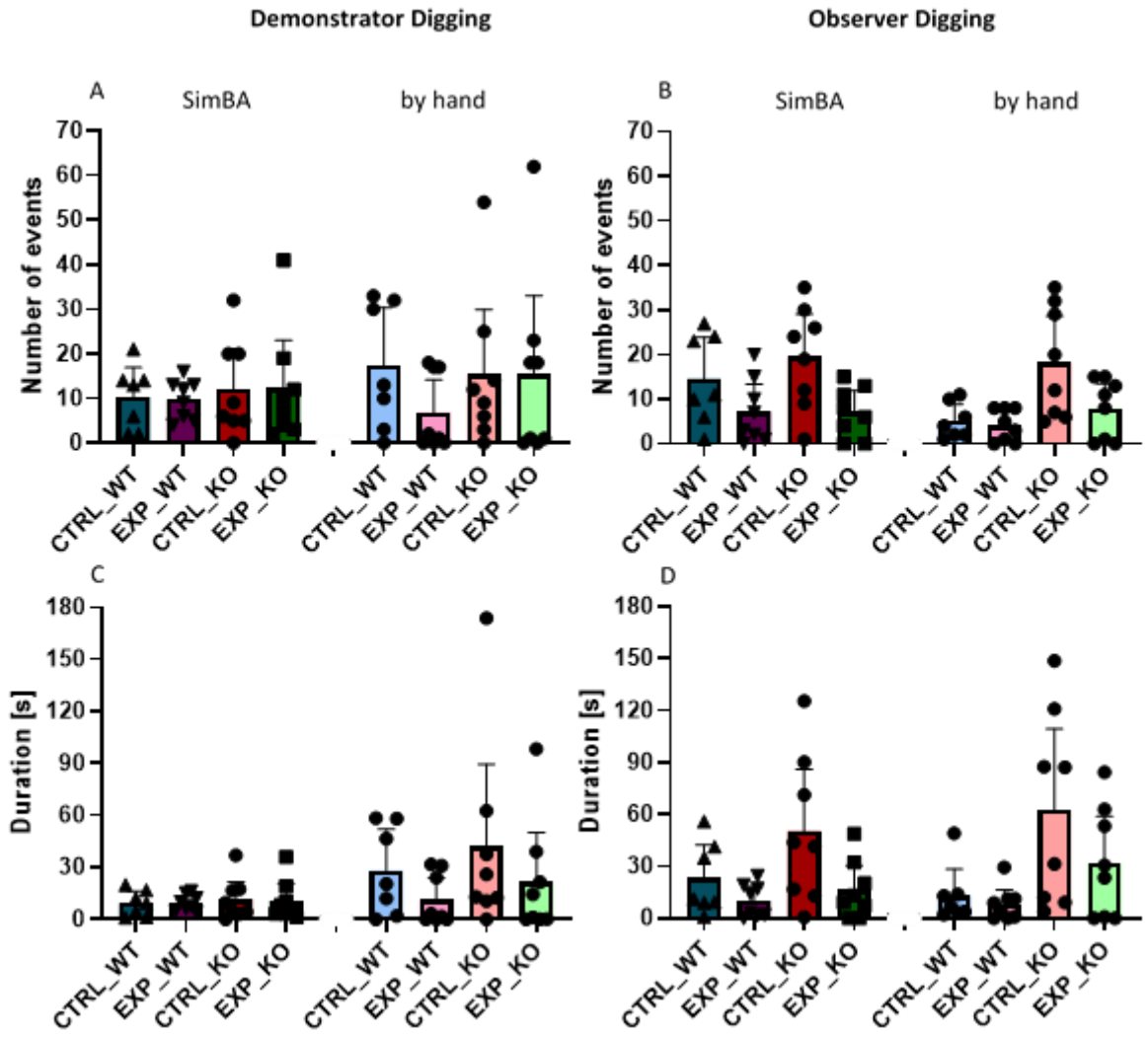

Figure 10. Comparison between behavioral results scored by SimBa and manually. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Stereotypic behavior provided on graphs represents digging of Observers towards Demonstrators. All data are shown as mean ± s.e.m, dots represent each individual.

4.2. Behavior

4.2.1. Social sniffing

In this and following sections presented data were obtained using SimBA software only. Graphs on **Figure 11** show data from social exploratory behavior, which may represent social interest in another conspecific. There was no significant difference in the anogenital sniffing in the number of events (**A)**, their total duration **(C)** and mean duration of a event **(E)** between the control and the experimental group regardless of genotype and sex. In the case of sniffing of the Demonstrator body by the Observer I noticed that the KO male Observers from the

Experimental group did not sniff more than their peers in the control group contrary to the WT males (**D**), in females I noticed an increase of interest towards the stressed mate in both genotypes (**D**). No significant differences were observed in the number of events (**B**) and the mean duration of an event (**F**). Analysis was done with the use of non-parametric Kruskal - Wallis test with post-hoc Dunn's multiple comparison test, * corresponds to $p<0.05$.

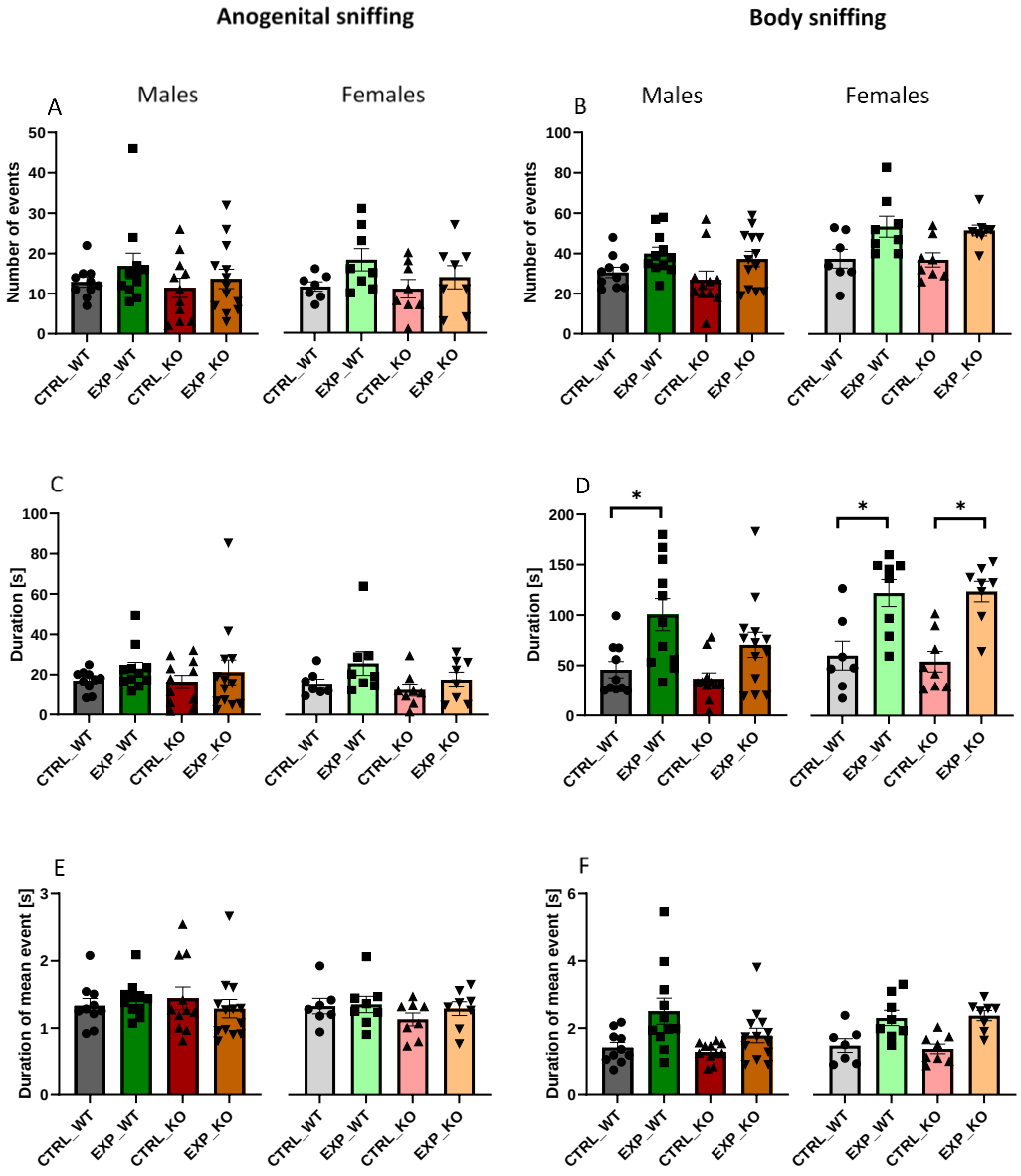

Figure 11. Social exploratory behavior. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Sniffing behavior provided on graphs represents sniffing of Observers towards Demonstrators. All data are shown as mean ± s.e.m, dots represent each individual.

4.2.1.1. Dynamics of social behaviors

Since there was little amount of anogenital sniffing and there were no differences in the mean amount of this behavior between groups I decided to investigate sniffing behavior more closely. One of the benefits of SimBA analysis over manually scored behavior is the possibility of plotting behavior over time with the resolution of one second. This approach gives us crucial information about the dynamics of the behavior, not only the total amount of it. On **Figure 12** The Observer Sniffing towards the Demonstrator is plotted over the whole duration of recorded interactions. Interestingly, we can observe that almost all of the anogenital sniffing behavior is happening right after the Demonstrator is put back into the home cage regardless of the experimental group, genotype or sex. We can also observe, that for body sniffing, right after anogenital sniffing ends, Observers that interacted with stressed demonstrator, except for KO males group, sniffed more.

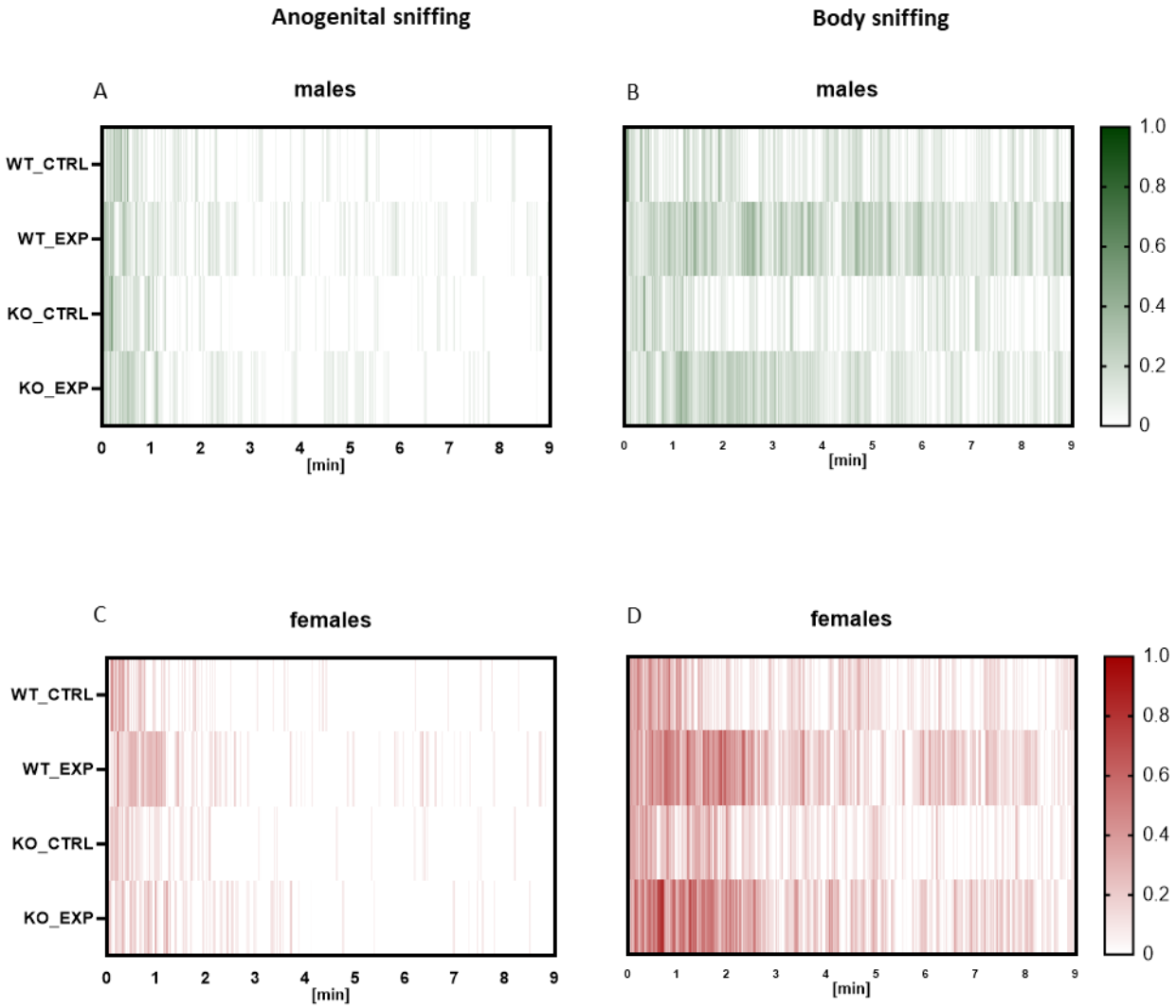

Figure 12. Distribution of sniffing behavior over time. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Sniffing behavior provided on graphs represents sniffing of Observers towards Demonstrators over nine minutes of recorded interactions. Each vertical line represents one second, while color intensity codes duration of measured behavior in a given second.

4.2.2. Exploration

When the mice do not engage in social contacts, they patrol the home cage in search for more clues which could inform them about the potential danger (especially pertinent in case of the EXP group). Graphs on **Figure 13** show data from rearing behavior, a widespread exploratory behavior which is used to gather information about the environment. There were

no significant differences in rearing of Demonstrators and Observers in the number of events (**A, B),** duration **(C, D)** and mean duration of each event **(E, F)** between control and experimental group regardless of genotype and sex. Analysis was done with the use of non-parametric Kruskal - Wallis test with post-hoc Dunn's multiple comparison test.

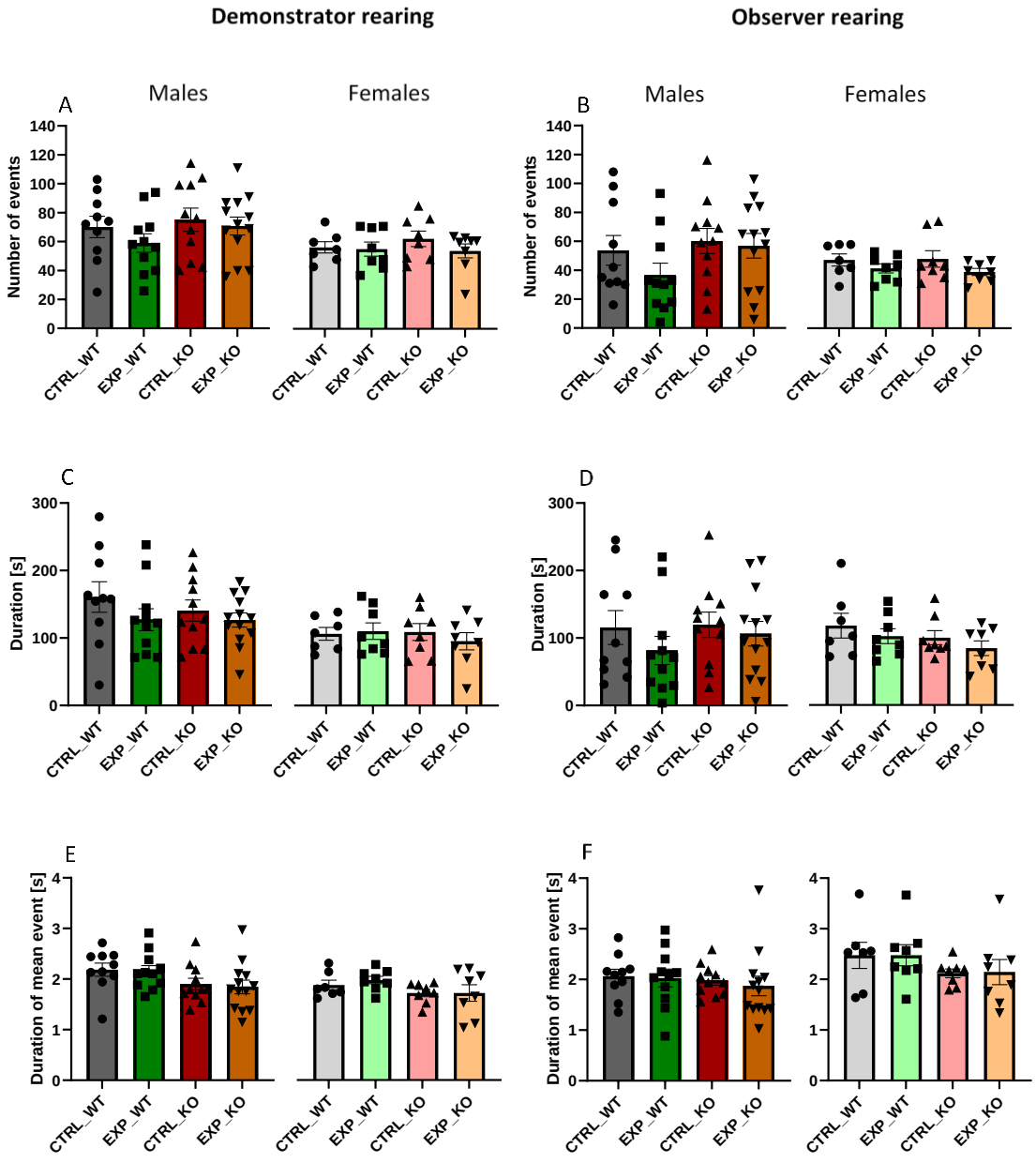

Figure 13. Exploratory behavior. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Rearing behavior provided on graphs represents rearing of Observer and Demonstrator. All data are shown as mean ± s.e.m, dots represent each individual.

4.2.2.1. Rearing dynamics

On **Figure 14** Rearing of Demonstrators and Observers is shown in the course of nine minutes of interactions. We can observe that among Observers males from WT group rear less at the beginning of social interactions (**B**), a similar effect can be noticed in females in both genotypes (**D**). What is worth noticing is that in females in WT experimental group (**D**) there is a peak of rearing activity after sniffing behavior is over, which may indicate that this is a reaction to information from the stressed cage mate. No such peak is observed in the KO females. Looking at Demonstrators rearing pattern (**A, C**) it looks similar in all groups.

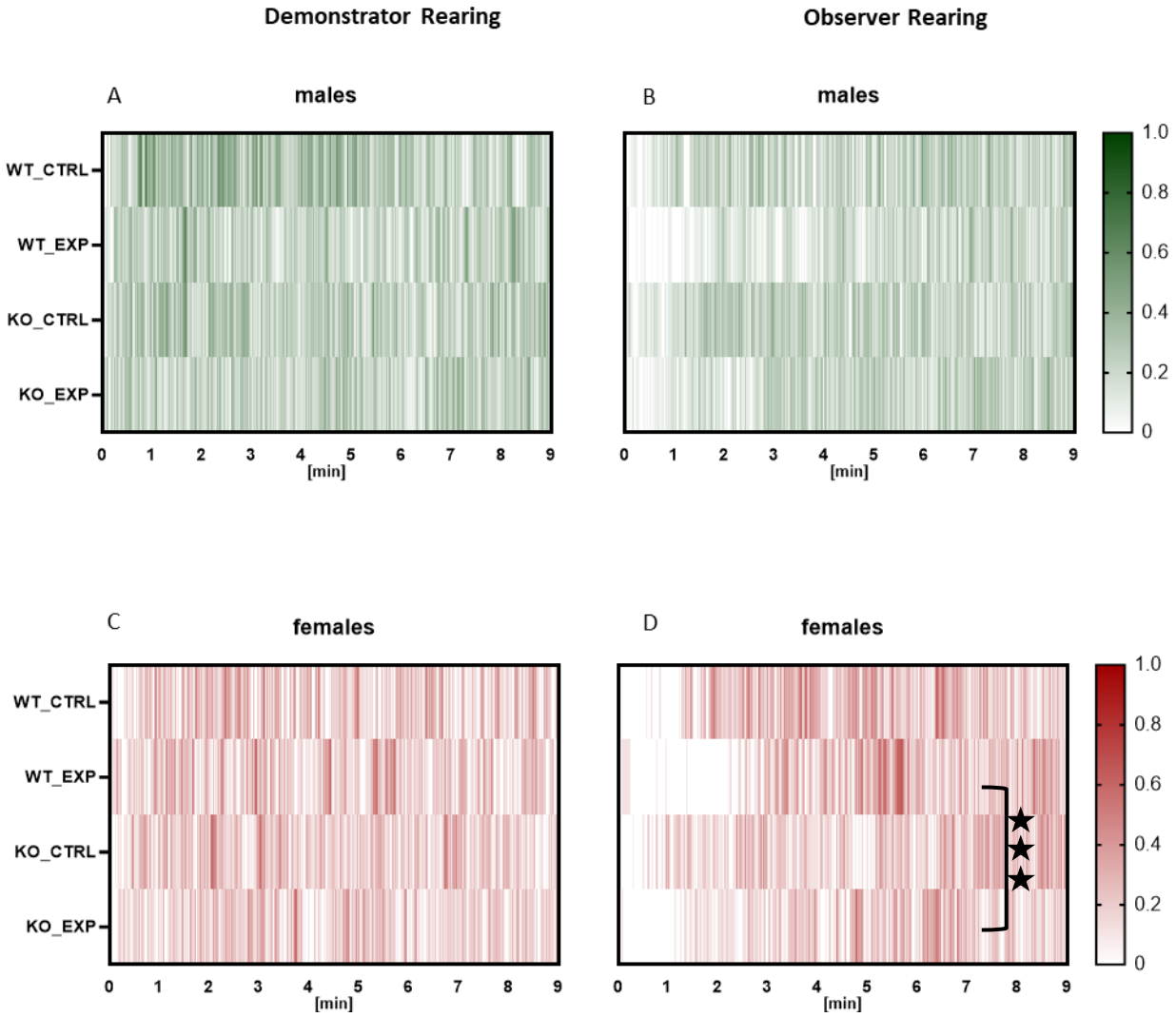

Figure 14. Rearing behavior dynamics. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Rearing behavior provided on graphs represents Rearing of Observers and Demonstrators over nine minutes of recorded interactions. Each vertical line represents one second, while color intensity codes duration of measured behavior in a given second. Three stars corresponds to $p<0.0005$ between WT_EXP and KO_EXP group.

4.2.3. Stereotypic behavior

Autism Spectrum Disorder is often characterized with the occurrence of stereotypies. Fragile X patients often exhibit stereotypic and self-injurious behaviors, starting at the age of 12-15 months (Hessl et al., 2008; Neri, 2017; D. Zhang et al., 2018). Here I assessed digging in the bedding performed by both animals as a potentially stereotypic coping response. On **Figure 15** I show that there were no significant differences between groups. What is worth noticing is that there is a tendency for lower amounts of digging in the EXP group in male Demonstrators (**A, C**) and female Observers from both genotypes (**B, D**). Analysis was done with the use of non-parametric Kruskal - Wallis test with post-hoc Dunn's multiple comparison test.

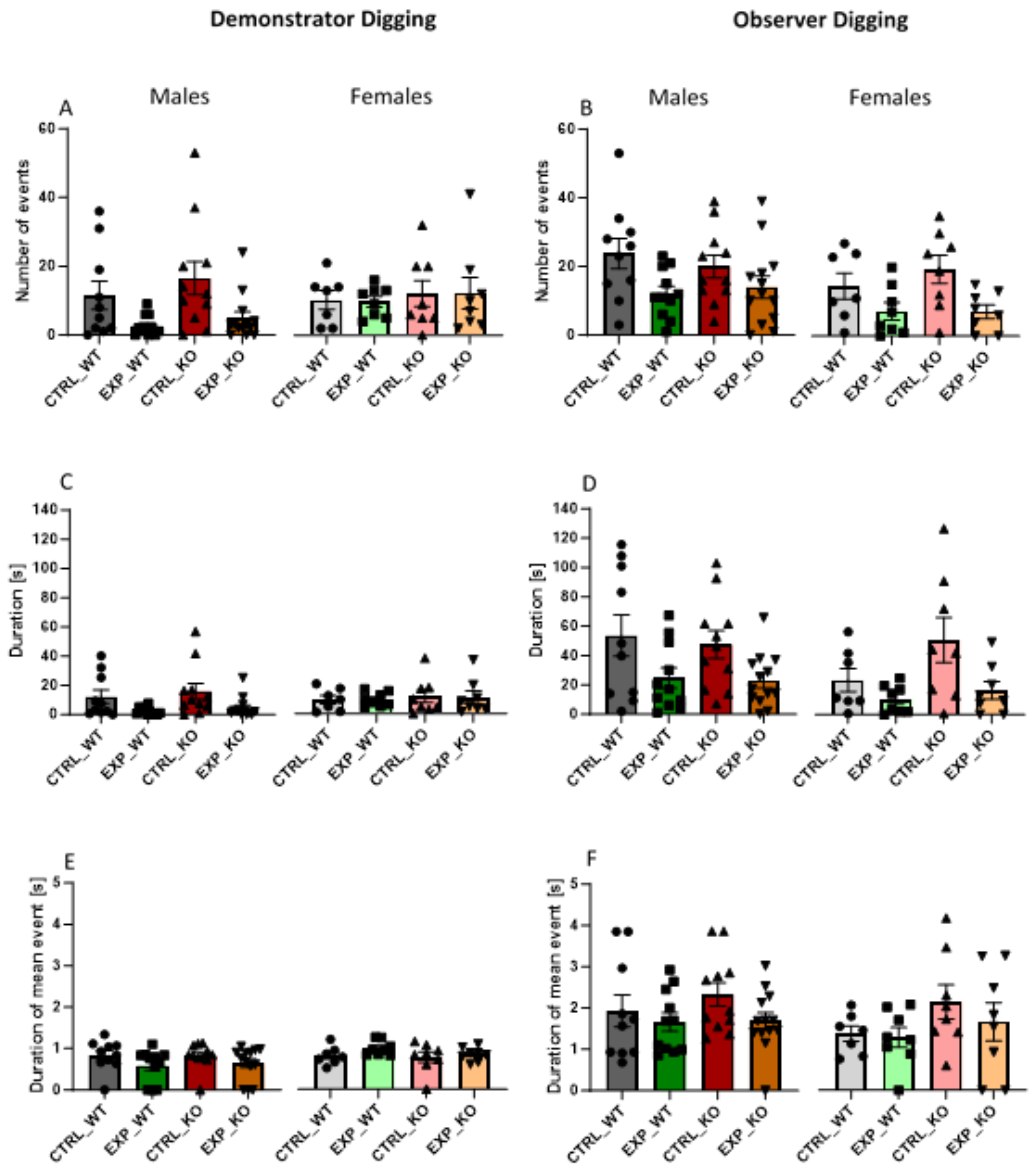

Figure 15. Stereotypic behavior. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Digging behavior provided on graphs represents digging of Observer and Demonstrator. All data are shown as mean ± s.e.m, dots represent each individual.

Another example of a stereotypic behavior which I assessed is selfgrooming of both Demonstrators and Observers. **Figure 16** shows self-grooming data and in the case of Demonstrators there were no significant differences noticed in the number of events (**A**), duration of behavior (**C**) and mean duration of an event (**E**). There was also no distinction in the number of events in male and female Observers from either group (**B)** but we could see that

female Observers from experimental KO group (**D**) self-groomed more than their peers from the experimental WT group. Analysis was done with the use of non-parametric Kruskal - Wallis test with post-hoc Dunn's multiple comparison test, * corresponds to $p<0.05$.

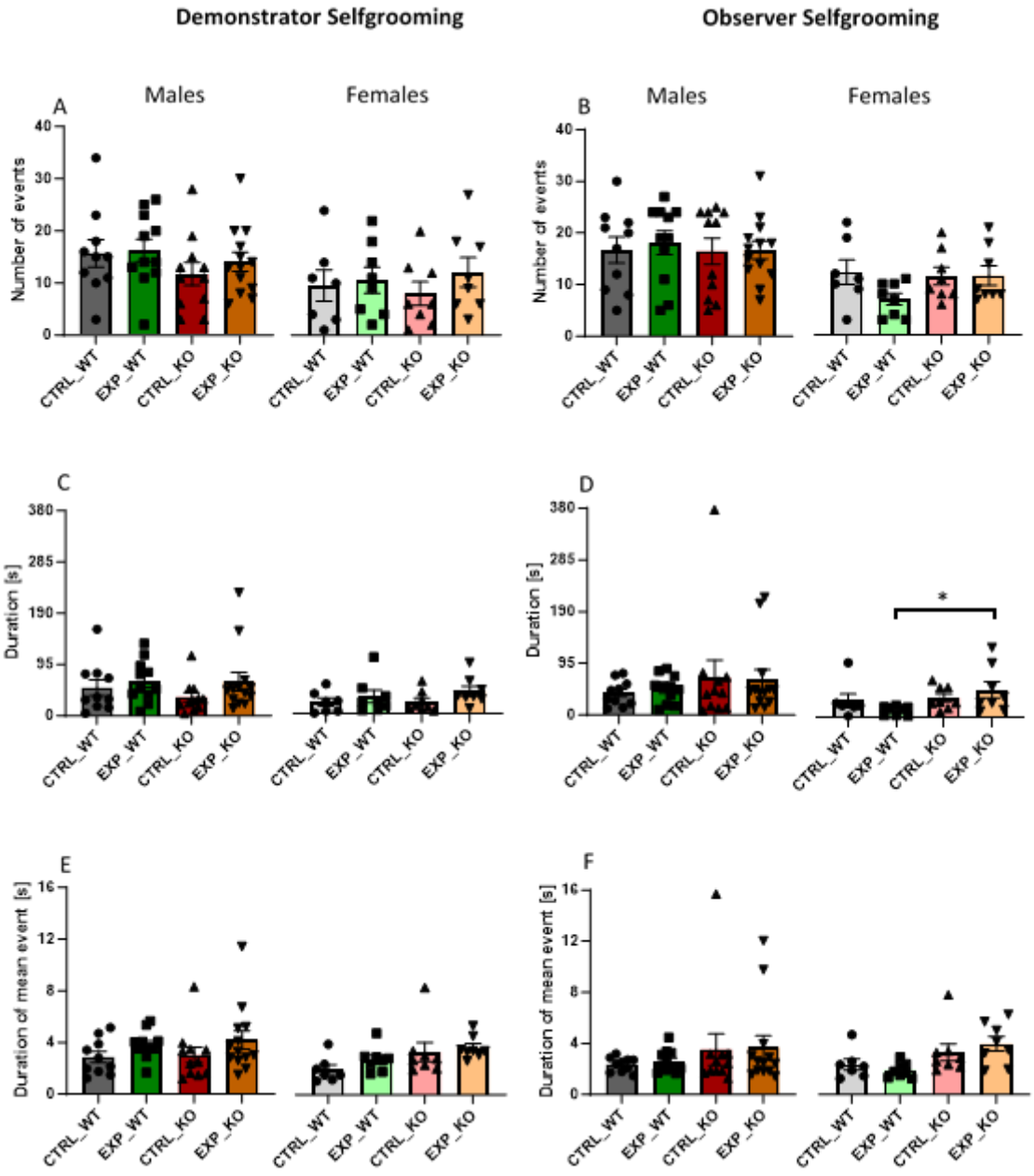

Figure 16. Stereotypic behavior. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Self-grooming behavior provided on graphs represents selfgrooming of Observers and Demonstrators. All data are shown as mean ± s.e.m, dots represent each individual.

4.2.3.1. The dynamics of stereotypic behaviors

The dynamics of stereotypic behaviors clearly shows that they occur after the initial bout of social investigation (especially sniffing of the anogenital regions). **Figure 17** shows distribution of very sparse episodes of digging behavior performed by male Demonstrators (**A)** and female Demonstrators (**C**). In the case of Observers we can see that KO females from the control group display more profound digging behavior (**D**) compared to other female groups. Observer males had a similar digging pattern across all groups (**B**). We can also see an overall pattern that animals from EXP groups dig less.

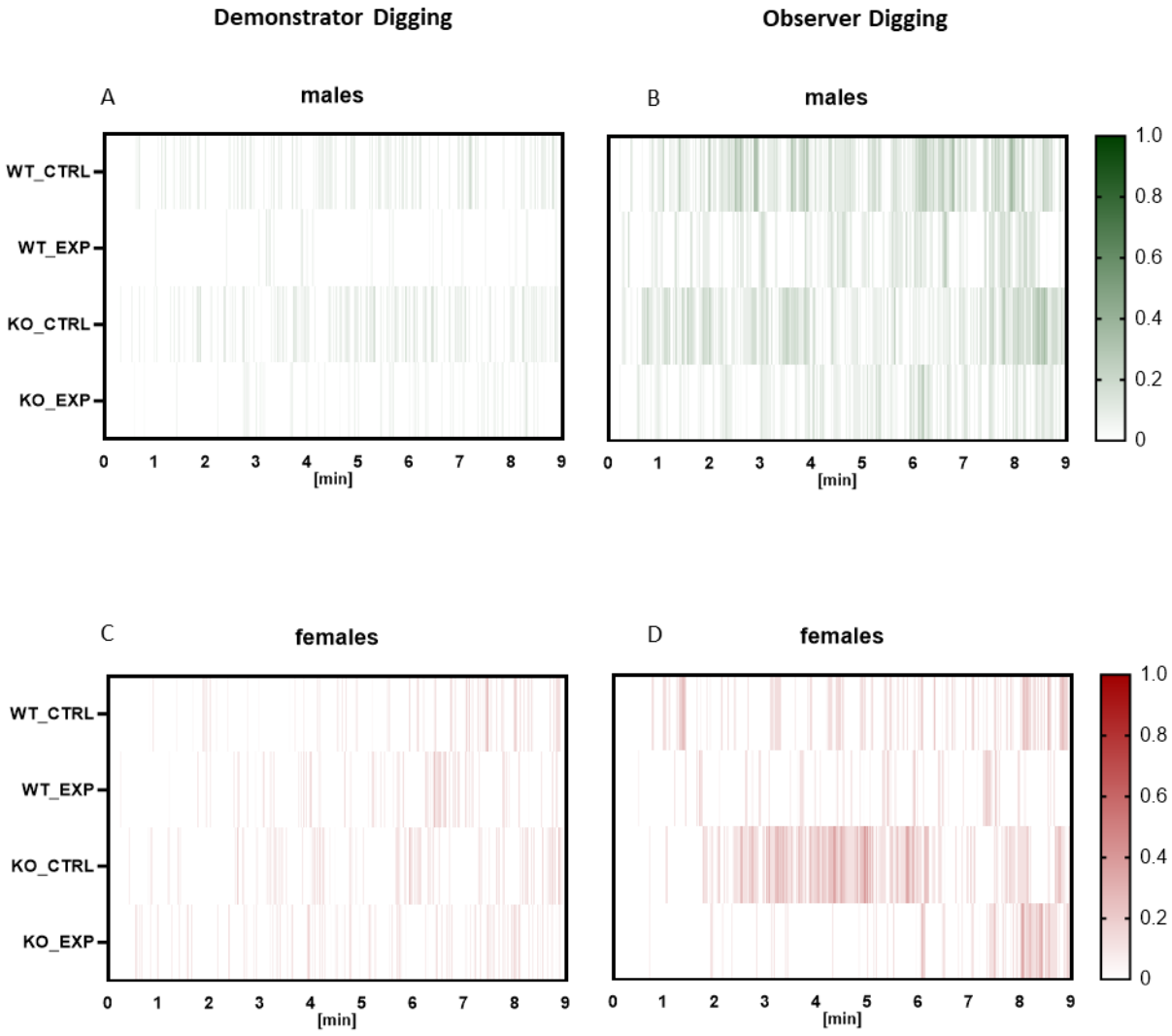

Figure 17. Digging behavior dynamics. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Digging behavior provided on graphs represents Digging of Observers and Demonstrators over nine minutes of recorded interactions. Each vertical line represents one second, while color intensity codes duration of measured behavior in a given second.

The dynamics of self-grooming reveal a less pronounced pattern. Male (**Figure 18 A**) and female (**C**) Demonstrators self-groom in a similar way in control and experimental groups. In the case of male Observers (**B**) we also can see a similar distribution of behavior during 9 minutes of recording, while in female Observers, I noticed that after the initial part (when mice focus primarily on sniffing behavior) (**Figure 16 D**) there was an increase in self grooming in Observers from the KO experimental group, which was not observed in WT experimental group (**D**).

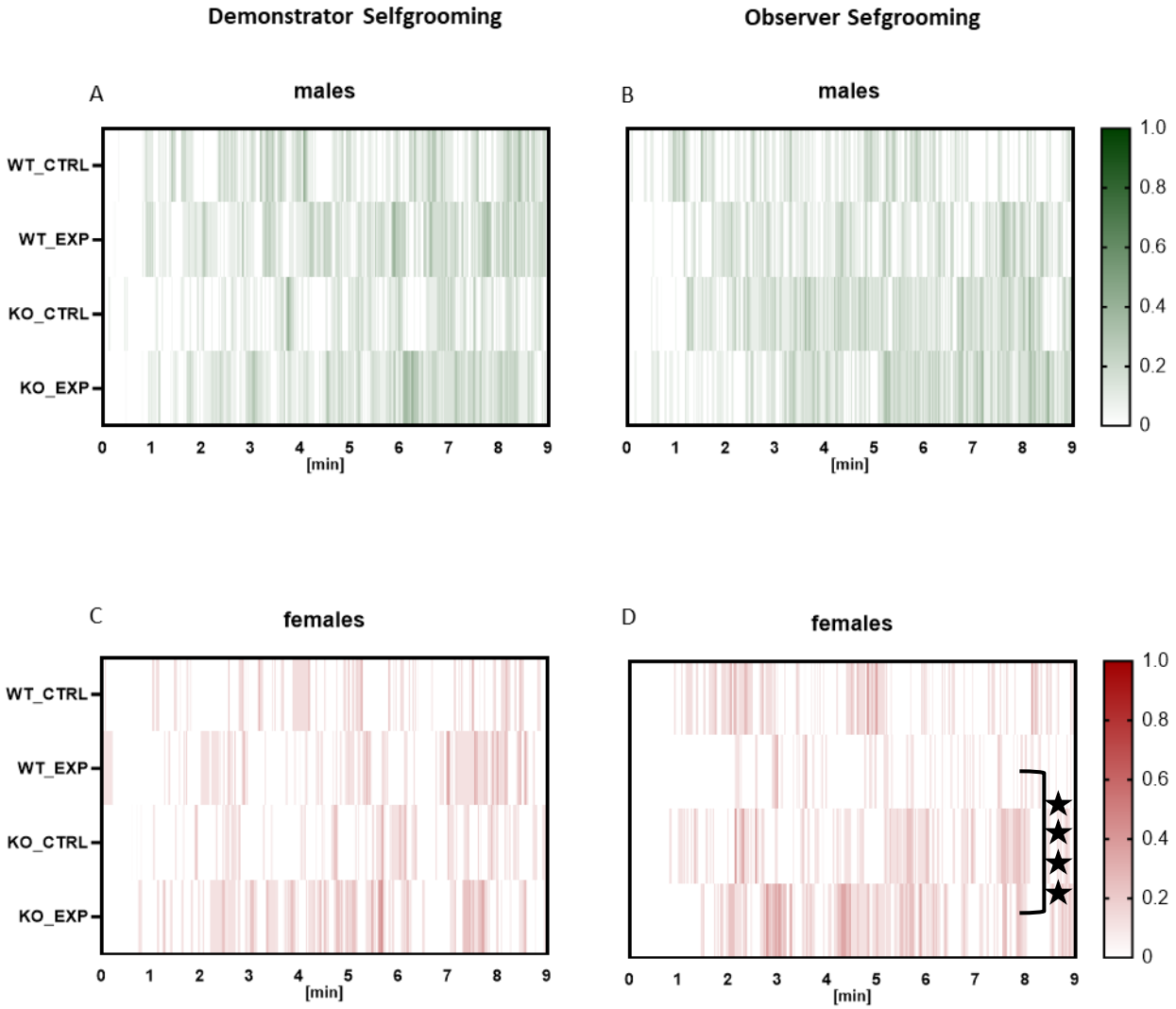

Figure 18. The dynamics of selfgrooming behavior. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Graphs represents Selfgrooming of Observers and Demonstrators over nine minutes of recorded interactions. Each vertical line represents one second, while color intensity codes duration of measured behavior in a given second. Four stars correspond to $p<0.0001$ between WT_EXP and KO_EXP group.

I also wanted to assess if there was any synchronization of behaviors between the Demonstrator and the Observer and to do that, I measured correlation between total time spent on a given behavior within each pair of animals. I calculated Pearson coefficient and plotted them in heatmaps in which the value is color coded and, * corresponds to $p<0.05$, ** corresponds to $p<0.01$. First I checked the exploratory behavior - rearing (**Figure 19**) and the only significant strong positive correlation (0.89) was found between Demonstrator and Observers in males from the WT Experimental group. No correlation in rearing behavior was found in females or other groups of males.

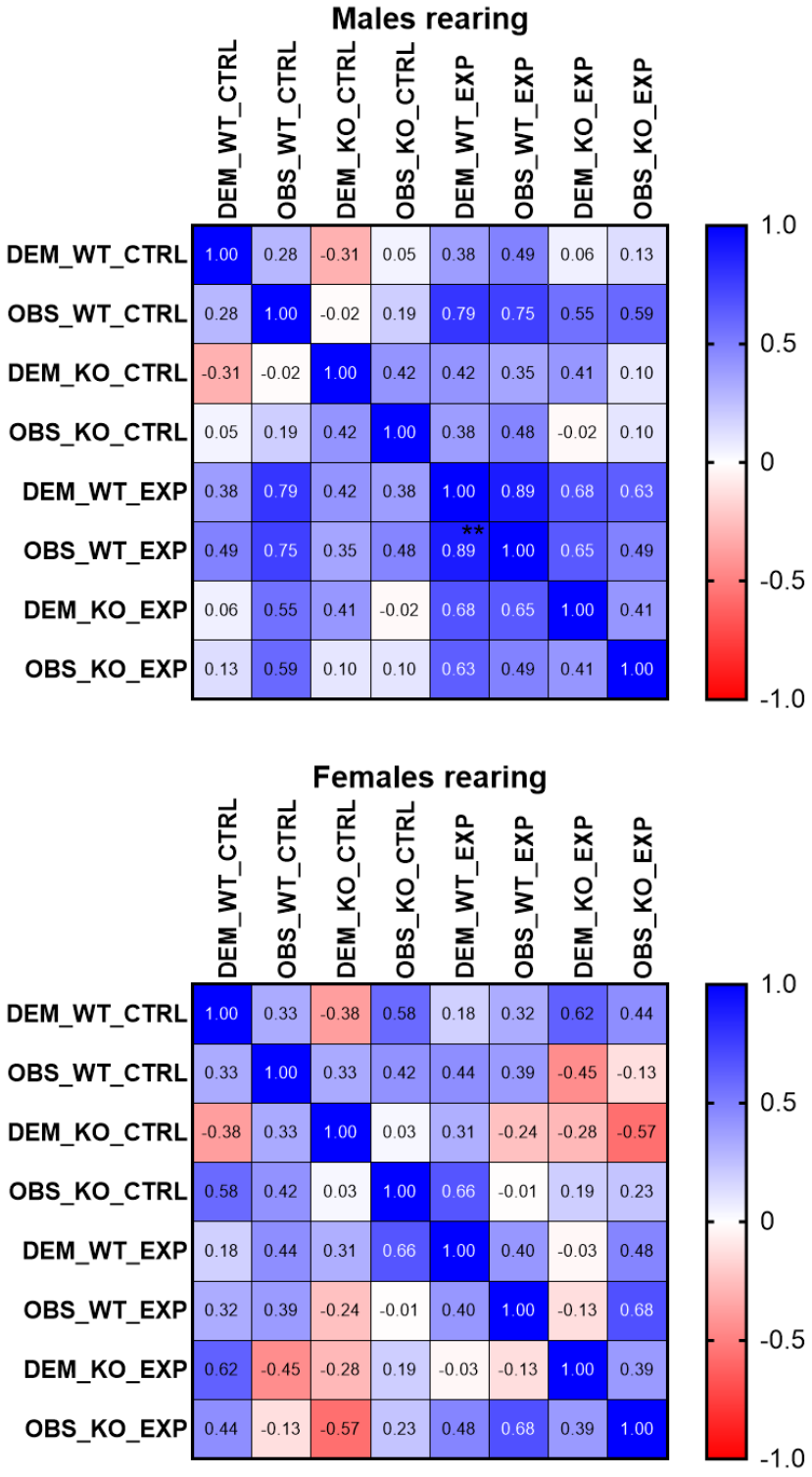

Figure 19. Correlation of rearing behavior between the Demonstrator and the Observer. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Two stars corresponds to $p < 0.01$.

Then I measured the correlation of the Demonstrator and the Observer stereotypic behavior - digging in bedding (**Figure 20**). Significant positive correlation was found only in KO males in the experimental group and no other examples of behavior synchrony was observed in other groups.

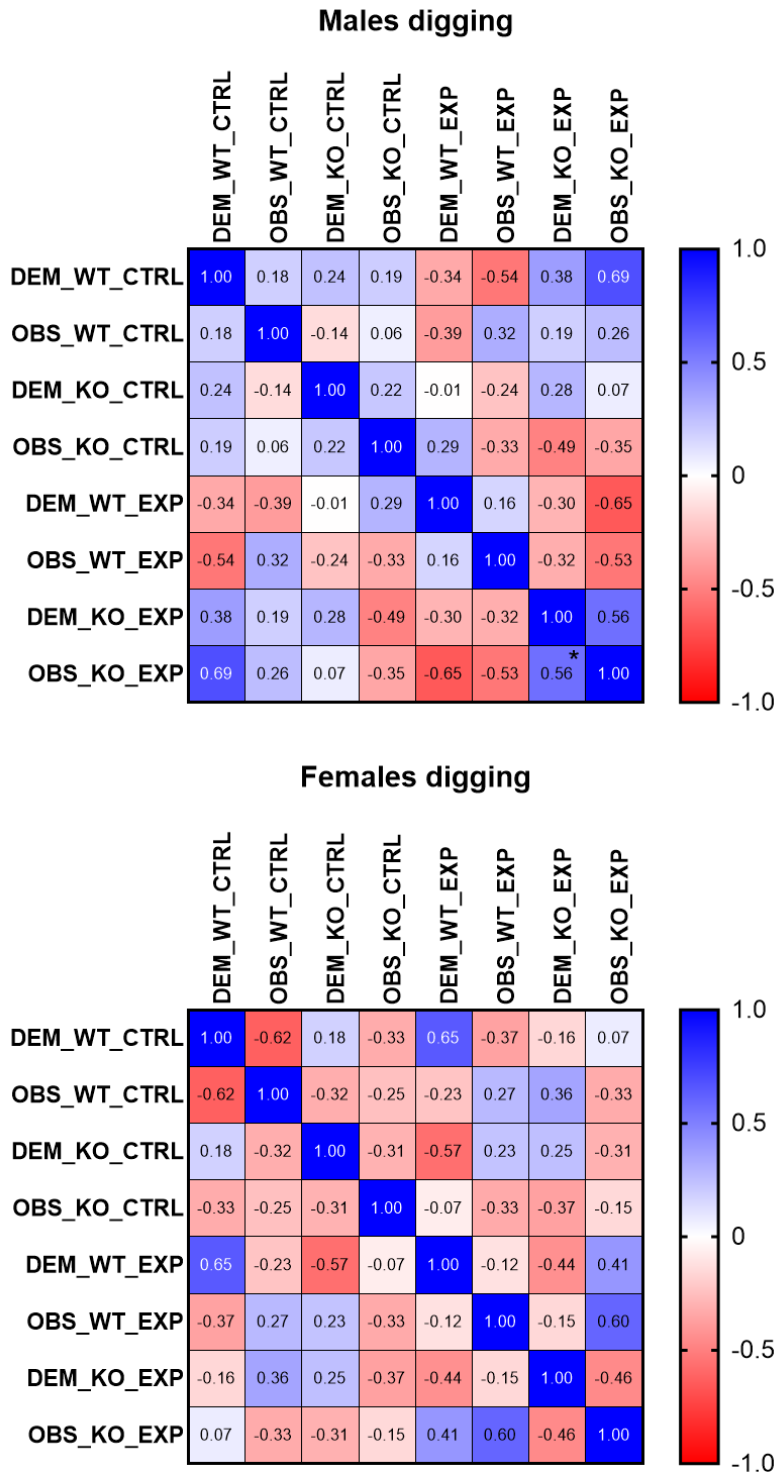

Figure 20. Correlation of digging behavior between the Demonstrator and the Observer. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. One star corresponds to $p < 0.05$.

Another stereotypic behavior which I assessed was the selfgrooming behavior (**Figure 21**). There was a positive correlation between Demonstrator and Observer in both males and females from KO strain in the experimental group. This effect was not observed in control groups or in WT strain regardless of sex.

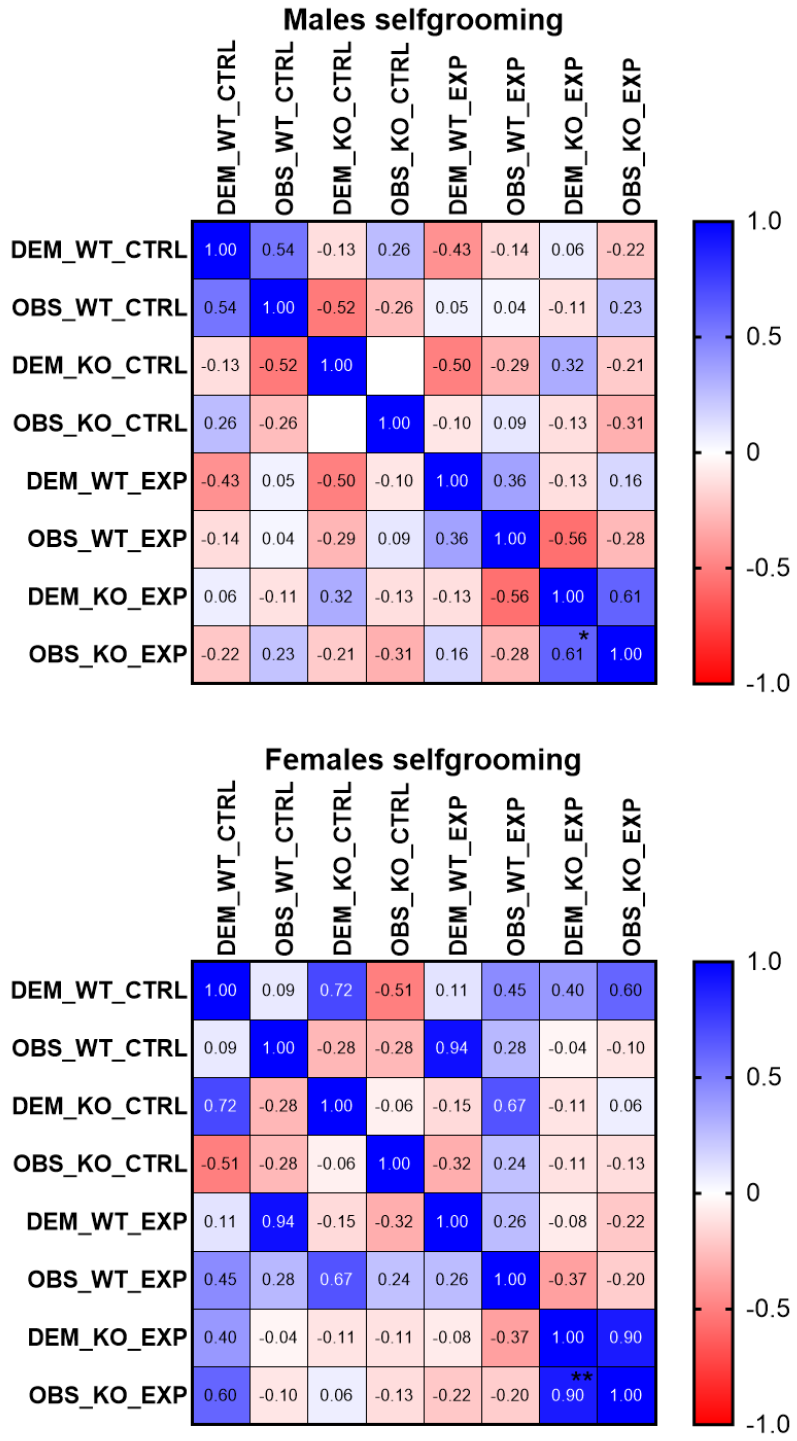

Figure 21. Correlation of selfgrooing behavior between the Demonstrator and the Observer. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Two starts corresponds to p <0.01.

4.2.4. Locomotor activity

Another important aspect of mouse behavior which may influence the total amount of specific, directed behaviors is the activity of the animals. Here I checked if there were any differences in overall locomotor activity measured by Total distance (**A, B**), Mean Velocity of

animal (**C, D**) and Mean Distance between the Demonstrator and the Observer (**E, F**) between control and experimental group and I did not see any differences. Analysis was done with the use of non-parametric Kruskal - Wallis test with post-hoc Dunn's multiple comparison test.

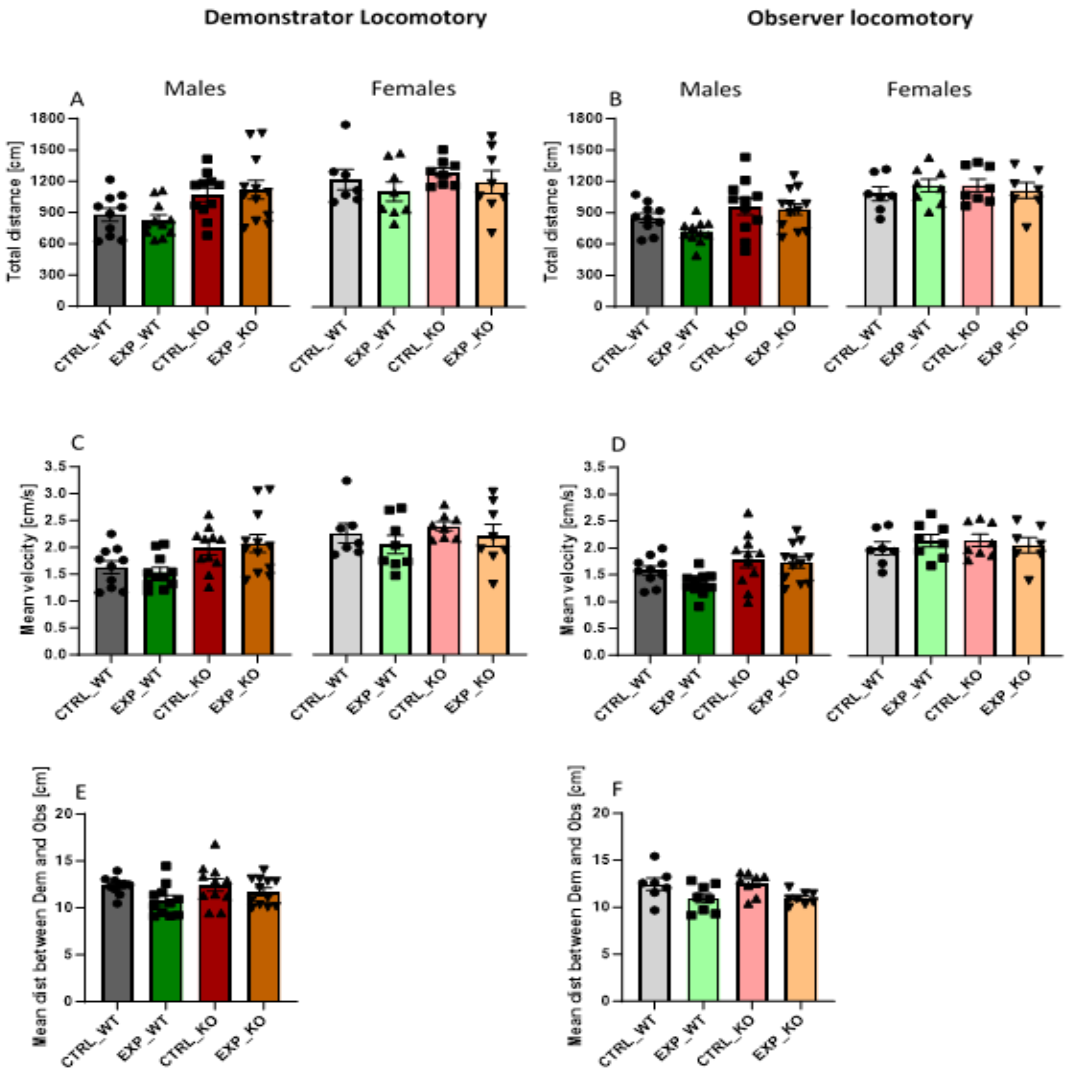

Figure 22. Locomotor activity. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Rearing behavior provided on graphs represents rearing of Observer and Demonstrator. All data are shown as mean ± s.e.m, dots represent each individual.

4.3. Neuronal activation pattern (c-Fos expression)

4.3.1. The Amygdala

To assess which brain structures may constitute parts of the neural circuitry involved in controlling emotional contagion occurring during the Remote Fear Transfer the number of c-Fos positive nuclei was counted in the amygdalar complex and the prefrontal cortex. These brain areas were chosen as being active during Remote Transfer of Fear in our previous study done with a normo-social strain of mice - c57BL/6J mice (Meyza et al., 2015). For the analysis of the activation patterns in the amygdalar complex I considered separately: the basolateral, the central (divided into centromedial and centrolateral), and the medial nuclei. For the medial prefrontal region, I calculated the numbers of c-Fos positive nuclei separately for the prelimbic and the infralimbic cortices.

In line with our expectations, I observed increased c-Fos activity in the medial nuclei of the amygdala in Demonstrators exposed to footshocks (directly experiencing the aversive stimulation, **Figure 23 C**). To our surprise there was no such increase in the basolateral nuclei of the amygdala except in female Demonstrators from KO genotype (**A**). I did not, however, observe an increase in c-Fos labelling in Observers interacting with stressed Demonstrators in either the basolateral or medial part of the amygdala (**B, D**).

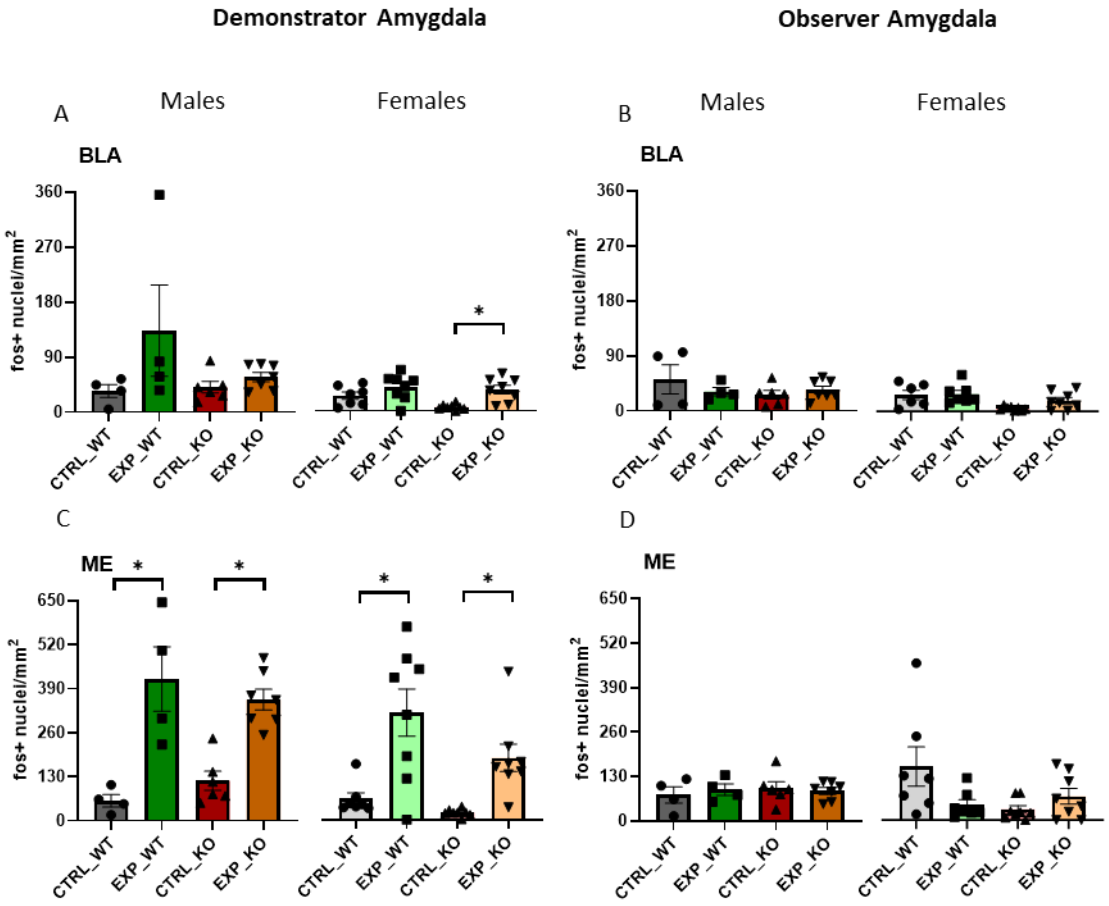

Figure 23. c-Fos levels in the amygdalar nuclei. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Results are presented as density of c-Fos positive nuclei per mm^2. All data are shown as mean ± s.e.m, dots represent each individual.

Next, the activity of the centromedial and the centrolateral nuclei was investigated. **Figure 24** shows a rise in c-Fos positive nuclei density in the centromedial nuclei of the KO Demonstrator females from the experimental group compared to the control group. Such effect is not observed in WT females or in males (**A**). Interestingly, there was an increased (as compared to control) activation of the centromedial nuclei of the amygdala in WT Demonstrator males, an effect which was not present in KO males or in females (**C**). In Observer brains I did not observe changed patterns of c-Fos expression regardless of sex and genotype. For statistical analysis Mixed-effects with post-hoc Tukey's multiple comparison test was used, * corresponds to $p<0.05$.

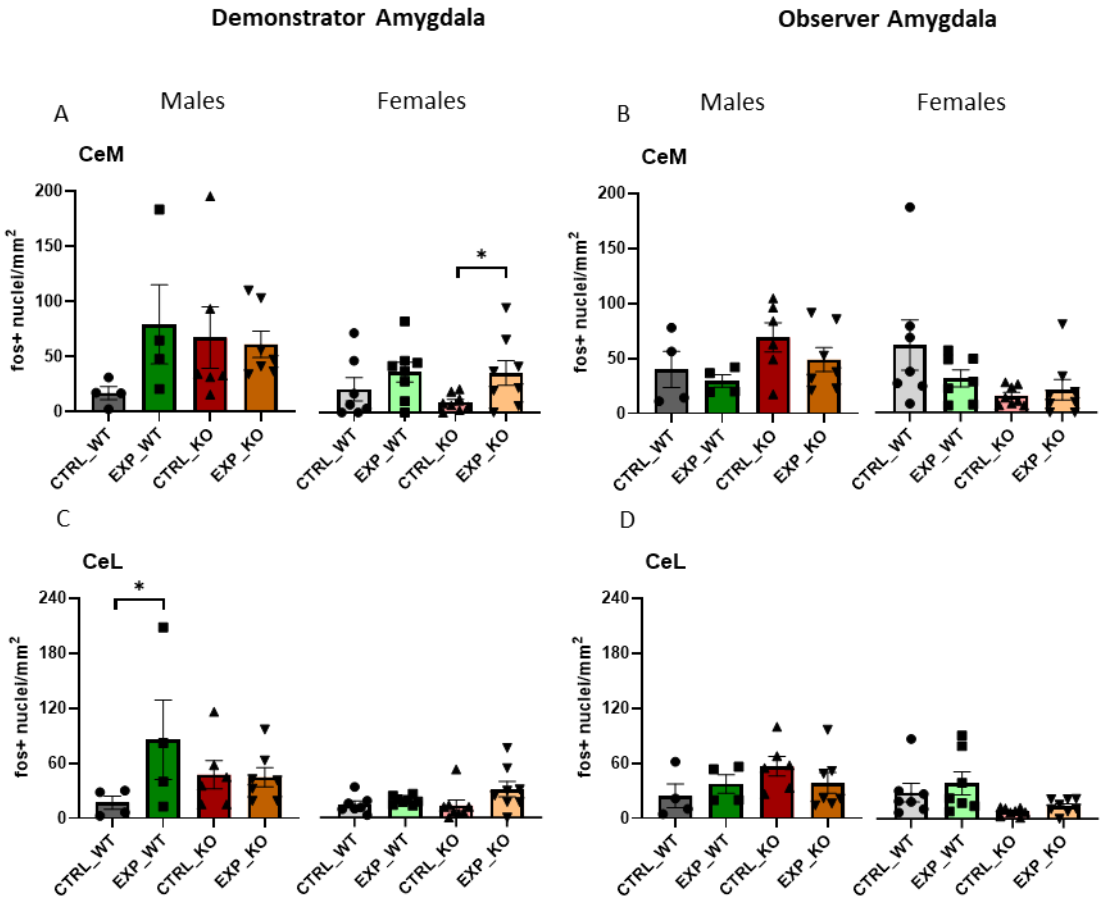

Figure 24. Number of c-Fos positive nuclei in the central amygdala. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Results are presented as density of c-Fos positive nuclei per mm^2. All data are shown as mean ± s.e.m, dots represent each individual.

4.3.2. The Prefrontal Cortex

Activity of the prefrontal cortex was scored separately for the prelimbic and infralimbic parts. On **Figure 25** we can see that footshock experienced by female Demonstrators from both genotypes induced an increase in c-Fos levels in both parts of the prefrontal cortex (**A, B**) while in males the effect was not present in the infralimbic cortex of WT Demonstrators and both cortices in KO Demonstrators (**A**). In the case of male Observers there was no increase in neuronal activation of either of the assessed areas (**B, D**), but what is worth noticing is that there

was a significant increase in the number of c-Fos positive nuclei within the infralimbic part of the prefrontal cortex in female Observers from the Experimental group in both genotypes. Seeing as this parallels the effect in Demonstrators, it may indicate that interaction with a stressed cagemate was enough to trigger c-Fos response (**D**). For statistical analysis Mixed-effects with post-hoc Tukey's multiple comparison test was used, * corresponds to $p<0.05$, ** corresponds to $p<0.001$.

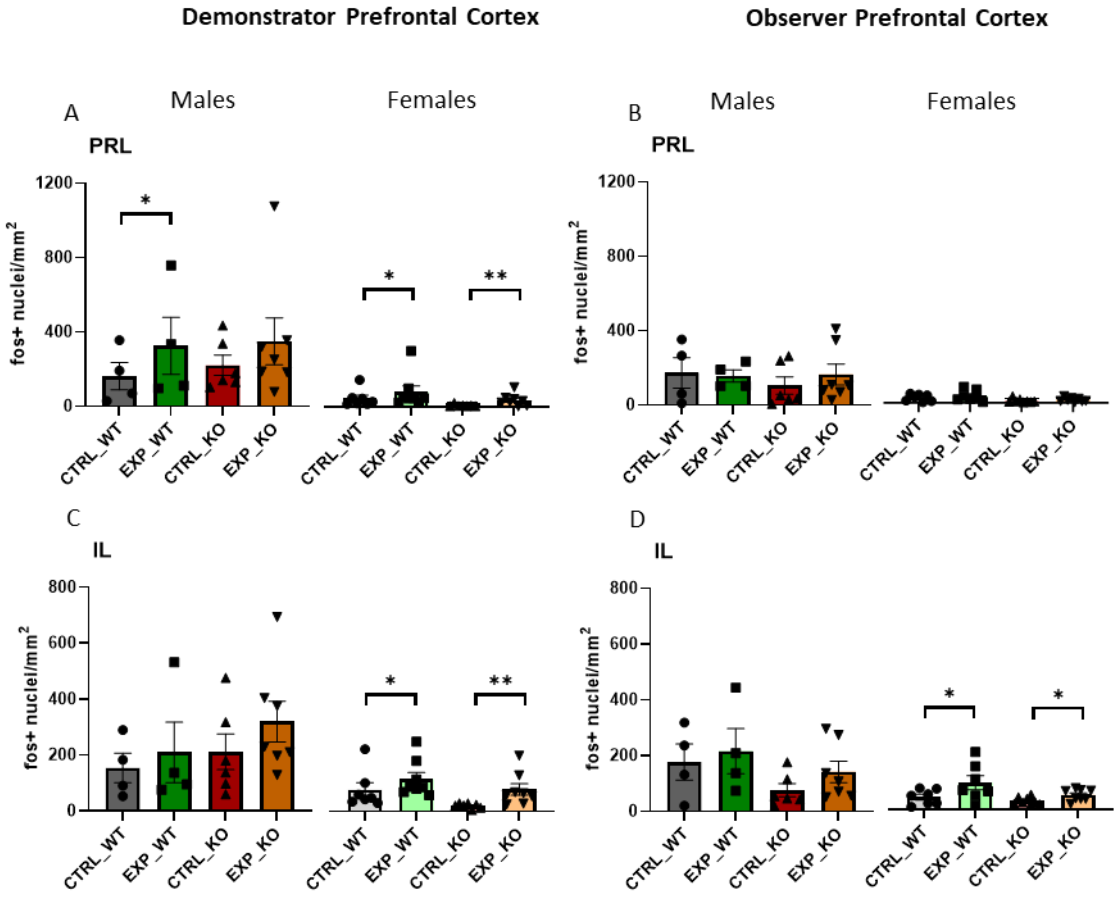

Figure 25. c-Fos levels in the prefrontal cortex. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Results are presented as density of c-Fos positive nuclei per mm^2. All data are shown as mean ± s.e.m, dots represent each individual.

5. Discussion

Emotional contagion is the necessary basis for empathy and thus for high quality social interactions (which can be impaired in people with Autism Spectrum Disorder). Studying this phenomenon gives us an insight into the neuronal mechanism responsible for deficiencies in emotional processing and can help us develop new treatments for Autism Spectrum Disorder. The aim of this study was to assess emotional contagion abilities of mice lacking a functional *FMRP1* gene responsible for controlling synaptic functionality and plasticity (Hou et al., 2006; Huber et al., 2002).

The results obtained here show that male WT Observers were able to detect the change in Demonstrators' emotional state and showed increased sniffing behavior towards it as compared to the Observers from the control group (paired with non-shocked Demonstrators). This confirms the occurrence of emotional contagion in these mice, contrary to their KO peers. KO Observers from the Experimental group did not show an increase in interest in the stressed cage mate. This result is consistent with literature. Moy et al (Moy et al., 2009) showed that animals lacking the *Fmr*1 gene have less preference towards stranger mice in comparison to controls with a functioning gene even though their sociability was not affected.

It is worth mentioning that the team led by Moy in their studies used mice lacking FMR1 protein in two background strains: C57BL/6J and FVB/129. And only the knockout mice on FVB/129 background (also used here) had shown deficits in social preference. Keum et al. (Keum et al., 2016) assessed empathic fear response, by employing Observation Fear Learning paradigm among 11 strains of mice. In this test the Observer through direct witnessing of the Demonstrator being shocked acquires a context-dependent fear response. Observational Fear Learning relies on the assumption that, for Observers to acquire fear response without experiencing a direct foot shock transfer of emotional arousal from Demonstrator has to occur. The degree of such emotional contagion can be measured by measuring freezing response of the Observer (Jeon et al., 2010; Jeon & Shin, 2011). Interestingly, familiarity with the Demonstrator may enhance such empathic response. One of the strains which exhibited high empathic response was C57BL/6J while FVB strain was characterized with low empathic response, which in turn may explain differences between background strain found by Moy group and moderate behavioral effects observed in my study. Empathy differences, assessed by physiological and behavioral responses dependent on strains were also reported by Chen et al

(Chen et al., 2009). In this study C57BL/6J mice were able to acquire Pavlovian association of Conditional Stimuli (tone) to Unconditional Stimuli (foot shock) by only observing object mice being subjected to aversive stimuli while mice from BALB/cJ strain did not develop that association.

As previously mentioned, familiarity may enhance empathic response. Study led by Gonzalez-Liencres (Gonzalez-Liencres et al., 2014) reported that when the Observer is witnessing a cage mate being foot shocked it is more likely to show a freezing response compared to observing a non-familiar mouse. They also reported a correlation in the number of fecal droppings between a familiar Demonstrator and an Observer (as an indication of stress, in comparison to non-cagemate pairs). In my experiments, mice were paired for at least three weeks prior to the onset of behavioral testing to enhance the probability of emotional contagion. The effect of that was clearly seen in females tested in my paradigm. Contrary to males, female Observers from both genotypes detected the change in Demonstrators' emotional state. Observer mice, regardless of genotype, sniffed the body of their stressed cage mate more than the Observers paired with a non-stressed Demonstrator. It is worth noticing that like males, most of the sniffing was performed right after the Demonstrator was put back into the safe environment of the home cage. This was to be expected since mice, even after quite short separation, consider the returning Demonstrator as something novel. In control groups regardless of sex and genotype initial sniffing does not develop into longer bouts of interest in the partner. In the Observers from the Experimental group these were intensified, which suggests that they had to notice the altered emotional state of their partner and continued sniffing to gather more information.

There was a subtle peak of rearing activity in the middle of interaction after the initial sniffing period ends. It is found only in female WT Observers from the Experimental group. One could speculate that this was driven by increased motivation to survey the environment upon receiving information from the stressed partner. No such increase in rearing activity was observed in KO female Observers from the Experimental group. Mice from this group displayed increased stereotypic behavior, namely they self-groomed more. In rodents, self-grooming is usually executed through a steady patterned sequence starting from the nose to the face, later moving to the head and finishing on the body and tail (Kalueff et al., 2016) and its prolonged or distorted occurrence is often linked with repetitive behaviors found in neurodevelopmental disorders such as Autism Spectrum Disorder (Li et al., 2024). Furthermore, grooming behavior is often associated with stress coping (Kalueff & Tuohimaa, 2004, 2005; Spruijt et al., 1992).

It was shown to be elevated after or during exposure to stressful conditions (Spruijt et al., 1992; van Erp et al., 1994). Interestingly, in my study the dynamics histogram of self-grooming behavior showed that it happened after the initial sniffing phase, so after interactions with the stressed Demonstrator. KO females displayed more grooming behavior than their WT counterparts, which could indicate that they responded to detection of the distress of the cage mate with this stress (coping) response. This would be in line with clinical reports showing higher cortisol levels associated with stereotypy in people diagnosed with Autism Spectrum Disorder (De Vaan et al., 2020; Yang et al., 2015). Although it is worth noticing that others have reported that elevated stereotypies are linked to lower levels of cortisol in saline (Gabriels et al., 2013).

Increased grooming behavior may be one of the reasons for higher prefrontal cortex activation since the prefrontal cortex is one of the many brain structures involved in controlling grooming behaviors (Ahmari et al., 2013; Burguière et al., 2013; Pinhal et al., 2018). Since altered levels of grooming behavior may be used as a marker of stress it is not only the amount of grooming but specific changes in grooming sequence (incorrect transitions between body parts) that can be used as a more sophisticated tool to assess stress response in rodents (Kalueff & Tuohimaa, 2004). Thanks to the rapid development of machine learning based tools, it is now possible to assess small changes in behavior on a better time scale and with less effort and more validity. It would be beneficial to check if FMR1 KO mice can be characterized with incorrect sequence of self-grooming. Subtle differences in the dynamics of this behavior suggest further analysis could be informative (e.g. for rearing in WT females from Experimental group). The in-depth insight into the behavioral sequence with the resolution of one second (obtained here thanks to the use of machine learning algorithms for pose assessment and behavior recognition) allowed us to also see that the onset of self-grooming only happens after the initial bout of sniffing and that there is a sequence pattern in mouse behavior worth investigating. Unfortunately, due to the nature of recording (top view of the cage) I was not able to obtain videos from which I would be able to characterize selfgrooming behavior with more detail. To asseas subtle changes of grooming behavior sequence side view of the cage and high resolutions recording would be necessary. A similar approach to interpret behavior by taking into consideration the whole behavior dynamics rather than only the total amount of measured behavior was proposed by Wiltschko (Wiltschko et al., 2015). They suggested that all behavioral bouts happen in sequences and deciphering them could be interpreted as mouse body language.

Emotional contagion is often described as an automatic process dependent upon mimicking behavior of a Demonstrator by the Observer (Iacoboni, 2009; Prochazkova & Kret, 2017). To explore that approach I measured the strength of correlation between total duration of several behaviors within pairs to answer the question whether the display of a certain behavior is manifested by the Demonstrator the Observer would follow. In the case of rearing behavior, I noticed strong positive correlation in males from the experimental WT group, which was not found in control settings. It may suggest the mimickry-like nature of emotional contagion since overall rearing levels did not change between control and experimental group but rearing behavior became more synchronized when the Demonstrator was subjected to footshocks. Since no such increase in sniffing behavior was seen in males from the KO group, we can assume that emotional contagion did not occur, and the lack of synchronization could be expected. But the same was not true when I looked at stereotypic behavior - digging in bedding. I noticed that digging of the Observer from the KO group is correlated with digging of the stressed Demonstrator. That in turn may hint that KO Observers were, to some degree, able to notice the altered emotional state of the Demonstrator. Interestingly in females no correlation in rearing or digging in any of the group was found which is in line with sex differences reported previously for Remote Fear Transfer paradigm (Mikosz et al., 2015).

Next stereotypic behavior which I assessed was the grooming behavior and here I found that there was a strong positive correlation between Demonstrators and Observers from the experimental KO group in both males and females which is not present in control groups or the experimental WT group. Looking at these results from the strain perspective it could be interpreted as an altered response to stressful stimuli. Compared to the WT strain, where the Observer after interactions with a stressed Demonstrator exhibited signs of exploratory behavior (rearing), the KO strain expressed their distress by excessive grooming and/or digging behavior.

Many scientists study emotional contagion with the use of the Observational Fear Learning paradigm. Its primary parameter used to quantify levels of emotional contagion is the freezing response of the Observer during and after witnessing the Demonstrator being subjected to aversive stimuli (foot shocks). The Remote Transfer of Fear paradigm used here does not induce such behavior. Our previous experiments (Meyza et al., 2015) and the current one showed that no freezing is displayed by either Demonstrators or Observers regardless of strain/genotype and sex. In Remote Transfer of Fear paradigm, social interactions are recorded in the safe environment of the home cage after the Demonstrator mouse receives footshocks in

an adjacent room (the Observer is not able to see, hear or detect the smell of the Demonstrator while it is being stressed). Since the danger in our protocol is not imminent, freezing behavior is not necessary and is replaced by more active coping strategies. The question concerning social interactions and their deficits, however, remains the same. Do the roots for it stem from improper display of changed emotional state or from an inability to receive signals about it.

Sex differences in empathic abilities have been well documented - for review see (Rochat, 2023). Empathy index was assessed in children and adolescents by questionnaires in which you had to mark on a scale if you agree or not with statements such as: 'It makes me sad to see a girl who can't find anyone to play with' or 'Boys who cry because (they) are happy are silly'. Results showed that empathy was highest by girls towards other girls and lesser towards boys, but still higher than empathic responses displayed by boys towards girls. The least empathic response was recorded when it concerned affection shown by boys towards other boys (Bryant, 1982). A similar study done on Norwegian students aged 13-16 has shown that empathic responsiveness is the highest when displayed by girls towards other girls and that boys exhibit less concerns towards a distressed male peer (Endresen, 2001). Research performed by Schulthe-Rüther (Schulte-Rüther et al., 2008) tested how human subjects will react to emotion expressing faces. One assessment focused on the subjects own emotional response after seeing an emotional face while the second task was to evaluate others face displaying various emotions. In both assignments (self and other) women ranked level of emotional response higher than men and brain activation measured by functional magnetic resonance imaging also showed an increase of activity in structures controlling emotional perspective taking (part of mentalizing network), such as the prefrontal cortex and the anterior cingulate cortex. Study performed on baboons showed that females had increased emotional contagion, as compared to males. It was measured by contagious yawning. The authors explained this effect through stronger relationships formed by females through infant care and the occurrence of alloparental care (Palagi et al., 2009). Interestingly, when studying contagious yawning, if the model exhibiting a yawn is a female the yawn contagion is the strongest (Demuru & Palagi, 2012). Pain response of female mice observing a conspecific in pain was also higher than that of male Observers (Langford et al., 2006). Female mice also approached other mice in pain more often than males did (Langford et al., 2010). Research group led by Ben-Ami Bartal et al (Bartal et al., 2011) showed that female rats were more eager to release constrained conspecifics than males.

In the case of consolation, more advanced level of empathy response, it was noticed that there is a higher chance to exhibit consolation behavior towards a stressed individual by female bystanders than by male ones (Romero et al., 2010). A similar effect was seen in lowland gorillas (Cordoni et al., 2006). In the 2006 study (Singer, 2006) volunteers had to play an economic game, where opponents were told to play fairly or unfairly. Next, volunteers observed their opponents receiving pain and their empathic response was assessed with fMRI. Interestingly both sexes showed activation in the fronto-insular and anterior cingulate cortex towards fair players, while only women exhibited empathic responses towards players who played unfairly. Moore (Christov-Moore et al., 2014) suggests that sex differences are both caused by phylogenetic and ontogenetics roots and are strengthened even more by social expectations about gender roles.

When measuring changes in behavior it is of most importance to also quantify locomotor characteristics of animals to check if differences in the behavior do not stem from altered activity. For this reason, I measured the total distance run by both Demonstrators and Observers, mean velocity and the mean distance between Observer and Demonstrator. There were no differences within the same genotype and sex between control and experimental group, which clearly indicates that changes in the behavior levels were not due to the altered activity levels of mice.

When assessing neural activity via c-Fos pattern expressions at first we looked at the brains of the Demonstrators to see which areas will become more activated in response to the foot shock. All of the demonstrators from both sexes regardless of the genotype reacted to aversive stimulation with an increase in the number of c-Fos positive cells in the amygdala and the prefrontal cortex. As anticipated, the most activated amygdala part was the medial nucleus. In our previous experiments conducted on normo-social C57BL/6 mice the medial nucleus of the amygdala was also highly activated in Demonstrators in the same behavioral paradigm (Meyza et al., 2015). Medial amygdalar nucleus is considered a main hub for social information incoming via olfactory cues - the principal sense used by mice (Li et al., 2017). Medial amygdalar nucleus is also responsible for processing predator odor and is a relay from lateral amygdalar nucleus further through the hypothalamus to the PAG (responsible among others for behavioral response to threat) for predator cues obtained by other senses (Gross & Canteras, 2012). It is also worth noticing that beside higher c-Fos amount in CeL amygdala of male WT Demonstrators, BLA and CeM in KO female Demonstrators there was not robust increase in c-Fos in the basolateral or central nuclei of the amygdala. According to the previous research,

activation of these structures is usually accompanied by freezing behavior elicited by fear conditioning in single animals (Goosens & Maren, 2001; Herry & Johansen, 2014; LeDoux, 2000).

One of the reasons for lower activation of the amygdala may be that, immediately after footshocks the Demonstrator was taken back to the safe environment of the home cage which could alleviate fear. Furthermore, social interactions with a cage mate could also have an effect on c-Fos activity through a phenomenon called the 'Social Buffering'. We must remember that information within a pair of mice does not go only in one way from Demonstrator to Observer. Observers' behavior also influences the way the Demonstrator behaves, especially when the Observer notices the altered emotional state of the Demonstrator and directs its undivided attention to it. Evidence from human studies suggests that presence of someone familiar can lower everyday stress and helps in faster recovery from trauma (Bowen et al., 2014). Similar results were obtained from studies on non-human primates (Gunnar et al., 2015; Sanchez et al., 2015). Social buffering was also observed in prairie voles (Burkett et al., 2016). Presence of a rat partner decreases freezing in previously fear conditioned rats during fear extinction (Gorkiewicz et al., 2023) or when exposed to the conditioning context (Kiyokawa et al., 2004). (Hennessy et al., 2009; Kikusui et al., 2006) showed that the effect size of 'Social Buffering' is influenced by familiarity and whether or not the partner animal is naive to the fear conditioning. Experiments performed on mice have shown that exposure to non-fearful mice before fear conditioning may reduce long-term contextual fear memory (Guzmán et al., 2009). Interestingly, an increase in Demonstrator's amygdala activity, other than in the medial nucleus, was found in KO strain in Experimental groups (BLA and CeM in females) which may indicate that social interactions could not alleviate these Demonstrators' fear to the same extent as in WT mice.

The c-Fos activation pattern in the amygdala of the Observers did not seem to change regardless of group or sex. This result is somewhat unexpected, although in our previous study using another ASD mouse model, the BTBR T+ tj/J mice, there was also no c-Fos increase in Observers paired with stressed Demonstrators, an effect which we associated with decreased social interaction leading to lack of emotion transfer. In the normo-social strain, c57BL/6 mice, the basolateral nucleus of the amygdala of Observers paired with stressed Demonstrators was activated (Meyza et al., 2015). The same behavioral paradigm conducted on rats showed that in Observers both the basolateral and central amygdala were activated (Knapska et al., 2006) which suggests that emotional contagion is both species and strain dependent. This is in line

with the Observational Fear Learning study results showing that FVB strain (the background strain for our Fmr1 KO) is less social and less prone to emotional contagion than c57BL/6 mice (Keum et al., 2016).

In our previous study (Meyza et al., 2015) c57BL/6 male Demonstrators and Observers responded to the behavioral challenge with an increase in neuronal activation in both the prelimbic and infralimbic parts of the prefrontal cortex. Here we saw a similar increase in WT Demonstrators but in the prelimbic cortex only. We did not see a similar activation in either KO Demonstrators or the Observers of either genotype. While the lack of an increase in the number of c-Fos positive cells in KO Observers could be related to diminished transfer of emotions due to low amounts of sniffing in these pairs of mice, the lack of activation in WT Observers seems to have a different background. Contrary to males, the exposure to a stressed cage mate evoked a significant activation of the infralimbic and prelimbic prefrontal cortex in female Demonstrators regardless of genotype. Interestingly, it coincided with a period of increased body sniffing which enabled emotional contagion. Seeing as this increase was found only in the experimental group it suggests that the interaction itself with a fear conditioned partner is sufficient to trigger a c-Fos response in the prefrontal cortex of the female Observers. A study done on rats showed that an interaction with a recently stressed Demonstrator triggers c-Fos increase in the prefrontal cortex of male but not female Observers (Mikosz et al., 2015). This further confirms differences between rats and mice and that emotional contagion in rodents, especially in the context of sex is still not well understood. Stronger activation of the prefrontal cortex may also explain the lower activity of the amygdala seen in the current study as the role of the prefrontal cortex in controlling and attenuating fear responses is well known (LeDoux, 2000; Maren, 2011). Our results are also compatible with the hypothesis proposed by Pare and Quirk (Paré & Quirk, 2017), saying that prefrontal neurons through reciprocal connection between mPFC and basal nuclei of the amygdala modulate behavioral responses. In this hypothesis it is proposed that there is a subset of neurons in the basolateral amygdala that control different behaviors linked to fear response. To elicit one of such behaviors the responsible group of neurons has to be activated while the other subset of cells has to be inhibited through connections from mPFC.

On **Figure 26** I summarized the c-Fos data showing differences between control and experimental group in Demonstrators and Observers, while on **Figure 27** summarized differences between WT and KO strains are shown.

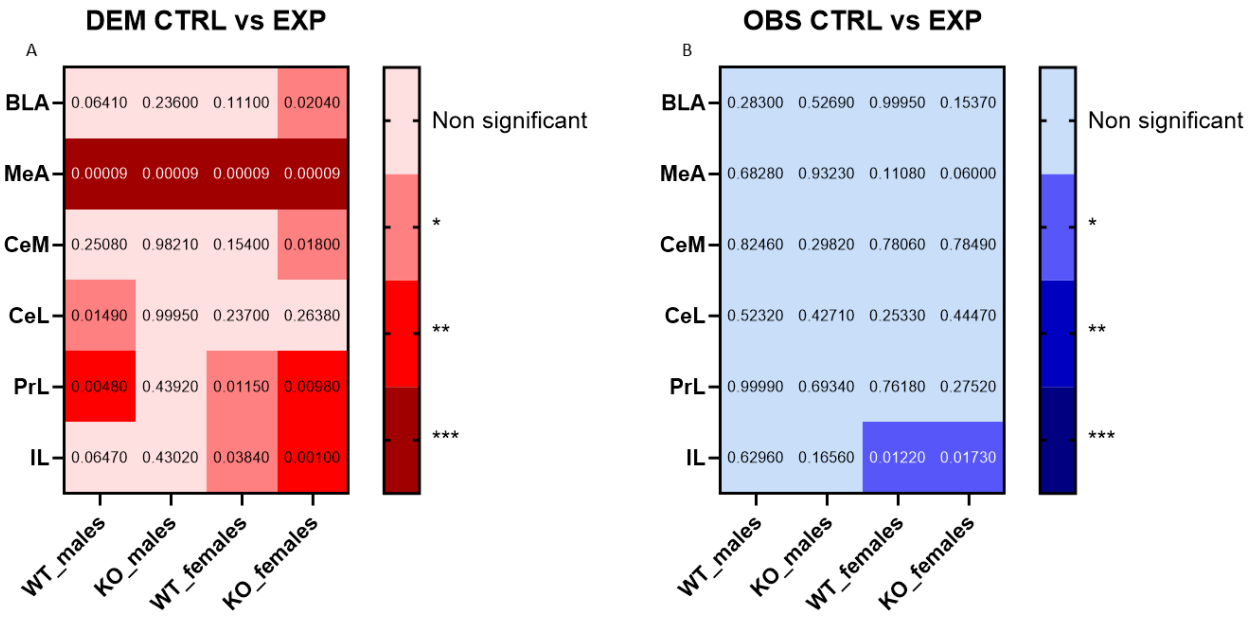

Figure 26. Summarized c-Fos data compared between control and experimental group. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Significance levels are color coded.

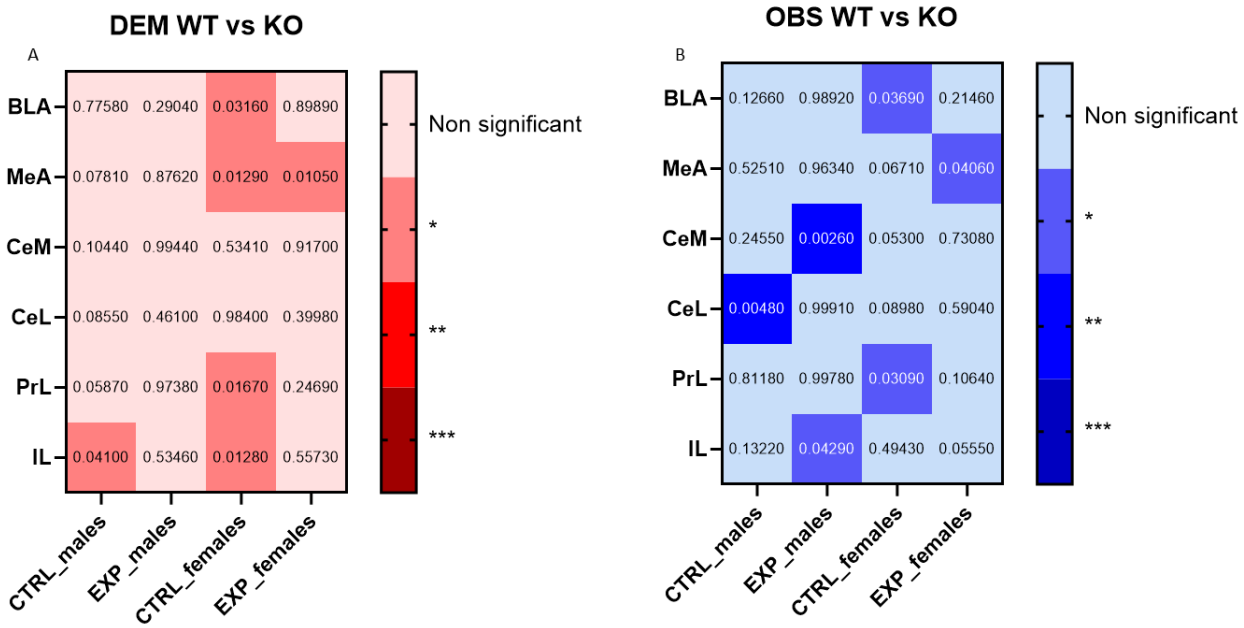

Figure 27. Summarized c-Fos data compared between WT and KO strains. WT - Wild Type genotype, KO - Knockout genotype, CTRL - Control group, EXP - Experimental group. Significance levels are color coded.

When analyzing c-Fos expression pattern it is important to remember about the limitations of this method: its low temporal resolution and that it might not show every activated cell (Hudson, 2018). Further studies employing other techniques would also be necessary to

characterize the activated population (as e.g., excitatory or inhibitory neurons as well as neurons co-expressing other markers).

One of the target genes of the AP-1 transcriptional factor, which c-Fos is a part of, is matrix metalloproteinase-9 (MMP-9) (Okulski et al., 2007; Rivera et al., 2010). As previously mentioned Fragile X Syndrome is characterized with an unusual phenotype of dendritic spines. Mice without FMR1 (FX KO) have less developed dendritic spines (characterized with immature phenotype: more thin and long spines and less mushroom shaped ones), which reflects neuronal morphology found in people diagnosed with Fragile X Syndrome (Comery et al., 1997; Irwin et al., 2002; McKinney et al., 2005).

This phenotype is directly linked to the lack of FMR1 protein, a crucial inhibitory factor for local translation of many genes tightly connected with synaptic function. One of its target genes is MMP-9, a protein whose main function is to re-organize the extracellular matrix, necessary for dendritic spines to be able to change their shape in response to stimulation. Higher levels of MMP-9 found in FMR1 KO mice are thought to be responsible for impaired dendritic spine morphology (Phillips & Pozzo-Miller, 2015). Cross breeding of FMR1 KO mice with mice lacking MMP-9 resulted in normalizing their asocial phenotype, moreover rescuing the morphology of dendritic spines (Sidhu et al., 2014). Lowering levels of MMP-9 with minocycline treatment also results in normalization of spine morphology in FX KO mice, simultaneously improving their social skills (Dziembowska et al., 2013).

Research conducted on rodent models of ASD point to imbalance between excitatory and inhibitory activity in the brain as one of the reasons behind impairment of social interactions. In FMR1 KO mice a distorted excitation/inhibition balance resulting with hyper-excitability of neocortical circuits was found (Gibson et al., 2008), alongside alterations in neurotransmitter levels (Contractor et al., 2015) and enhanced neocortical excitability due to the elevated neuroplasticity (Gonçalves et al., 2013; Zhang et al., 2014).

Altered activity of the prefrontal cortex may also be related to changed sensory functions in people with ASD who have difficulties with reading emotional faces (Marco et al., 2011). BTBR mice, an idiopathic mouse model of ASD, also display gaze avoidance (Blanchard et al., 2012) which could explain their difficulties at reading emotional information from their conspecifics. Thanks to the robust development of machine learning tools combined with high-quality video capture scientists are now able to study mouse facial expressions at previously unavailable levels of detail. It is worth pursuing to find out if mice can get information from

faces even though vision is not their main sense. Another way of communication between rodents is through ultrasonic vocalizations. In rats the two main types of vocalizations fall in 22kHz and 50kHz bands. The first one is associated with an aversive state while the second one with appetitive responses (Brudzynski, 2021). Mice vocalize less, mainly during pup separation, territorial fights and courting assays (Yao et al., 2023). We recorded and examined USVs during the Remote Transfer of Fear but there were too few episodes of it and the distribution was not representative of any group or genotype to warrant an in-depth analysis of this mode of communication.

Mice use olfactory cues as a main method to gather information about the environment. In trained tasks mice quickly learned the location of a reward following odors while visual cues were eliminated. On the other hand, when olfactory cues were not present, animals were not able to perform the task (Gire et al., 2016). (Girard et al., 2016) showed that FVB mice are capable of learning odor-reward associations in olfactory tubing maze. Mice use urinary scent marking behavior towards other males in novel environments as a way to assert dominance (Arakawa et al., 2007) and it was shown that mice can distinguish between specific odor cues (Hurst, 1989). It was reported that mice are able to discriminate between two mice of the same species as well as between animals from different species solely based on olfactory cues (Bowers & Alexander, 1967). Individual mice are equipped with original scent due to the existence of polymorphic genes named odortypes which are mainly linked to major histocompatibility complex genes (Yamazaki et al., 1999). Due to the high sensitivity of a mouse olfactory system, mice are able to detect and avoid infected conspecific (Beauchamp & Yamazaki, 2003). In a paradigm chosen in this thesis I could not focus on examining olfactory cues since testing was performed in a safe environment of the home cage so the used bedding was not a good place to extract any smells from.

As always when conducting experiments in a highly controlled, laboratory environment it is worth asking about the ethological relevance of results obtained that way. Especially when studying sociability of mice, one has to remember that mice living in nature are particularly territorial and their social interactions are limited to mating, nursing offspring and defending its territory (Chambers et al., 2000; Crowcroft, 1955). Males principally are fighting for resources with other males, while females have more chances to interact with conspecifics. Even though one might argue that learning about social interactions in artificial settings may be pointless, studies suggests that when space is limited males aggression decreases which may facilitate social interactions between them (Berdoy & Drickamer, 2007; Brown, 1953; Davis, 1958).

Nevertheless, it is crucial that scientists should conduct experiments in more natural settings and utilize both females and males in studying social interactions to get a broader understanding of mechanisms responsible for emotional responses. Especially in the light of the sex-specific responses observed in this study.

6. Summary

The aim of this study was to expand our knowledge about the emotional contagion abilities of mice with a full knockout of the FMRP protein (a monogenetic model of ASD). Results obtained in this study point to differences in responses to a stressful peer depending on the sex and the genotype of the animal. Males from FMR1 KO strain showed deficits in social behavior compared to their WT counterparts (e.g lower body sniffing during exposure to a stressed cagemate). After social interactions with a stressed Demonstrator male WT Observers synchronized their rearing behavior while KO Observers synchronized selfgrooming which could be interpreted as a stereotypic response typical of ASD. Similar effect was observed in females. Observers from both genotypes showed interest in stressed Demonstrators, but their reactions were different. Females from the WT group responded with more exploratory behavior (rearing), while mice from the KO group committed to selfgrooming. On the neuronal level I could not see an increase in neuronal activation patterns (c-Fos expression) in males. In females on the other hand there was a significant increase in c-Fos expression in the prefrontal cortex of Observers. This pattern shows resemblance to the activity pattern of c-Fos found in Demonstrators. Behavior analysis was conducted with the use of a novel approach utilizing machine learning tools. This approach enabled investigation of subtle changes in behavior (otherwise difficult to achieve with the use of standard manual tools). Moreover, the same model can be used in other projects after brief re-training.

7. Conclusions

Results obtained in this study validate *Fmr1* knockout strain as a mouse model for studying emotional contagion deficits and point to the importance of further research on both sexes since clear sex differences in both the extent and the neuronal background of emotional contagion were observed. The behavioral analysis performed here proves the feasibility of the machine-learning approach in dissecting autism-like phenotype in mice.

8. References

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9. Publication record of the PhD Candidate

1. Andraka, K., Kondrakiewicz, K., Rojek-Sito, K., Ziegart-Sadowska, K., Meyza, K., **Nikolaev, T.**, Hamed, A., Kursa, M., Wójcik, M., Danielewski, K., Wiatrowska, M., Kublik, E., Bekisz, M., Lebitko, T., Duque, D., Jaworski, T., Madej, H., Konopka, W., Boguszewski, P. M., & Knapska, E. (2021). Distinct circuits in rat central amygdala for defensive behaviors evoked by socially signaled imminent versus remote danger. *Current Biology : CB*, *31*(11), 2347-2358.e6.

2. de Hoz, L., Gierej, D., Lioudyno, V., Jaworski, J., Blazejczyk, M., Cruces-Solís, H., Beroun, A., Lebitko, T., **Nikolaev, T**., Knapska, E., Nelken, I., & Kaczmarek, L. (2017). Blocking c-Fos Expression Reveals the Role of Auditory Cortex Plasticity in Sound Frequency Discrimination Learning. *Cerebral Cortex*, 1–11.
3. Kaźmierowska, A. M., Kostecki, M., Szczepanik, M., **Nikolaev, T**., Hamed, A., Michałowski, J. M., Wypych, M., Marchewka, A., & Knapska, E. (2023). Rats respond to aversive emotional arousal of human handlers with the activation of the basolateral and central amygdala. *Proceedings of the National Academy of Sciences of the United States of America*, *120*(46), e2302655120.
4. Kondrakiewicz, K., Rokosz-Andraka, K., **Nikolaev, T**., Górkiewicz, T., Danielewski, K., Gruszczyńska, A., Meyza, K., & Knapska, E. (2019). Social Transfer of Fear in Rodents. *Current Protocols in Neuroscience*, *90*(1), e85.
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8. Puścian, A., Winiarski, M., Łęski, S., Charzewski, Ł., **Nikolaev, T**., Borowska, J., Dzik, J. M., Bijata, M., Lipp, H.-P., Dziembowska, M., & Knapska, E. (2021). Chronic fluoxetine treatment impairs motivation and reward learning by affecting neuronal plasticity in the central amygdala. *British Journal of Pharmacology*, *178*(3), 672–688.