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ATTACHMENT BOND INTERFERENCE AND PSYCHOPATHOLOGY: THE SHIELDING EFFECT OF THE EARNED-SECURE ATTACHMENT

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To my parents, my sister,
my grandmothers and my love.

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Abstract

Early-life experiences affecting attachment bond can have long-lasting consequences resulting in negative outcomes, such as depression, in adulthood. However, the formation of an earned-secure attachment with an alternative caregiver can act as a protective factor against attachment disruption with the primary caregiver. The Dopaminergic (DA) system of the Ventral Tegmental Area (VTA) is involved in both attachment bond and depression, and it is particularly sensitive to early-life manipulations. VTA DA neurons are characterized by an inward regulatory mechanism, the hyperpolarized-activated cation current (Ih current) and, interestingly, this mechanism was found altered in animal model of depression. We have previously reported that C57BL/6J female mice exposed to Repeated Cross Fostering (RCF), a procedure affecting attachment bond during a sensitive time window, showed resilience to depression and decreased Ih current in VTA DA neurons. However, females by a different inbred strain, DBA/2J (DBA), exposed to RCF, show increased vulnerability to depressive-like behavior in adulthood. Here, we hypothesized that the opposite phenotype observed in DBA females is related to an opposite alteration of Ih current. Moreover, we also hypothesized that by introducing a stable attachment caregiver during RCF, is sufficient to prevent both behavioral and electrophysiological alterations observed in RCF mice. First, we analyzed the Ih current density in VTA DA neurons of adult RCF and Control females thought patch-clamp recording. Then, we performed RCF procedure introducing a Stable Attachment Figure (SAF) that remains with the pups from PND1 to PND4. After assessing pups' attachment behavior, by analyzing the emitted vocalization while apart from the mother, we tested mice in adulthood to evaluate the rescue effect of SAF on depressive-like phenotype through Forced Swimming Test, Tail Suspension Test and evaluated possible SAF effect on social behavior by Social Interaction Test. Finally, we investigated VTA DA neuron Ih current in RCF+SAF adult females. Our results indicated that depressive-like behavior induced by RCF is linked to increased Ih current density and that rescuing the attachment bond alteration, by providing pups with an alternative stable caregiver is sufficient to prevent these alterations.

1. Introduction

Due to the high plasticity that characterize the early post-natal period, early life experiences play a crucial role in shaping the future brain development and can have long-lasting consequences on adult behavior. Not surprisingly, any environmental perturbation during this sensitive time window may permanently affects the system, with negative consequences in terms of psychopathology. During this period, the primary environment is represented by the interaction between the infant and the caregiver, who takes care of the child providing reassurance and strategies to face adversities. A stable and safe interaction with the caregiver allows the formation of a secure attachment bond, an emotional, reciprocal bond, necessary for the proper brain development. Traumatic experiences or lack of emotional support within the relation with primary caregivers can affect the attachment bond formation, increasing the vulnerability to develop psychopathology in adulthood, such as depression. When the primary caregiver fails to establish a secure attachment with the infant, caregiver other than parents can became alternative attachment figures, by providing stability and emotional support and becoming source of "earned secure attachment". This earned security acts as a protective factor in preventing the negative outcomes induced by the insecure attachment with the primary caregiver. Interestingly, a biological substrate particularly sensitive to early life adversities is the Dopaminergic system arising from the Ventral Tegmental Area (VTA). Interestingly, this brain circuit is involved in both attachment bond formation and depression.

Understanding the physiological alterations induced by early experiences could help to understand the link between VTA, attachment bond and depression.

VTA adaptations have been reported in depressed patients as well as in animal models of both depressive-like behavior and pro-resilient to depression phenotype. The dopaminergic neurons of VTA are characterized by an intrinsic modulatory current, I_h current, that is altered in animal models of depression. A recent study shows that the I_h current of VTA DA neurons is decreased in resilient to depression C57BL/6J female mice early exposed to a manipulation affecting the attachment bond (Repeated Cross Fostering (RCF)). Interestingly, a different inbred strain of mouse exposed to RCF (DBA/2J) shows, in adulthood, increased vulnerability to depressive-like phenotype. In this work we first assess if the opposite behavioral phenotype shown by DBA females, induced by attachment bond interference, is related to an opposite alteration of the I_h current in the VTA DA neurons though an ex-vivo electrophysiological experiment.

Although the earned-secure attachment is believed to act as a protective factor for future behavioral outcomes, the causal and mechanistic pathways linking earned attachment, brain development and

behavioral outcomes have not been established. The current study aims to overcome this gap modeling the earned attachment in mouse.

In our second experiment, we evaluate if by introducing an alternative attachment figure to promote an “earned attachment” during RCF, we prevent the behavioral and neurophysiological outcomes in the adult animals. We used our well-established model of attachment bond interference in mice (RCF) including a non-parental caregiver, a Stable Attachment Figure (SAF), during RCF to mimic the “earned attachment”.

Finally, we start evaluating possible targets that could drive the VTA DA neurons alterations induced by RCF and investigate the preventive effects of SAF.

2. Early life adversities and depression

Early environment, in terms of experiences and relationship occurring during the early stages of development, represents the first environment in which the newborn finds himself interacting with and, therefore, plays a fundamental role. Indeed, attention has been paid to the role of early life experiences in the normal development of the nervous system, in both biological and functional terms. Early experiences could in fact affect emotion, coping strategies to stressful situations and social preferences (Fumagalli et al., 2007; Dion et all., 2022) resulting in pathological outcomes, such as Post-traumatic Stress Disorder (PTSD) and Depression (Klengel and Binder, 2015). The peculiar sensibility of the brain to early environment depends on its high plasticity during development, characterized by critical periods. During these sensitive time windows, experiences and environment translate into potentially irreversible changes that affect brain functions and structures (Burggen and Mueller,2015). When aversive situations are repetitively experienced during this very sensitive period, they can negatively affect the brain development and, consequently, facilitate the expression of negative behavioral outcomes. Not surprisingly, experiencing abuse, maltreatment, impaired relation with caregivers during the early life period is a trigger factor for psychopathology expression such as depression (Kaufman et al., 2000).

Depression is a worldwide disease, an internalizing mood disorder characterized by feelings of sadness and hopelessness, loss of interest and feelings of worthlessness that persist during the days (DSM-V). The wide range of symptoms could occur in comorbidity with other disorders, such as anxiety and addiction, suggesting a possible overlap between neural substrate involved in these diseases (Russo and Nestler, 2013). Clinical and preclinical studies point out an increased vulnerability to develop depressive symptoms following early adverse experiences. The preferential passive coping strategy adopted in dealing with stressful or challenging situations seems to be affected by negative experiences during childhood, together with cognitive and affective deficits (Pechel and Pizzagalli, 2011). Moreover, interacting with unstable and unreliable caregivers could decrease the resilience to coping with stressful situations (Bowlby, 1988). Early experiences could also have a protective role in child future outcome, especially when he experiences a stimulating environment with stable family relationships (Walsh et al., 2019). Differences in resilience or vulnerability to depression are related to individual differences, depending, in turn, by the interaction between genetic background and environment (Fumagalli et al., 2007). Interestingly, epidemiological studies highlight the higher occurrence of almost all the subtypes of depression twice in women than man (ex. Distimic depression or chronic minor depression; Angst and Merikangas, 1997; Ford and

Erlinger, 2004). The increased vulnerability of females could depend on the interaction of several factors: the prevalence of internalizing symptoms of women vs externalizing in men (Rosenfield, 2000); the involvement of hormones dysregulation, mostly related do puberty and menstruation, as a trigger for depression (Albert, 2015); sexually dimorphic brain development of areas involved in depression, such as the Mesocorticolimbic System (Russo and Nestler, 2013). Finally, the prevalence of emotional neglect and adverse experiences during childhood in females than man (Moody et al., 2018) increases the vulnerability to develop depressive symptoms in adolescence ad adulthood.

Whitin the early experiences, the first environment with which the newborn interacts is represented by family, that shapes the developing brain and could thus represent a potential risk factor for mental health. The caregiver, in fact, represents the primary source of care, as well as the tool by which the child acquires and interprets the world around him. Importantly, the caregiver takes care of the children in the most completely way and it is not necessarily represented by the biological parents. In taking care, he/she makes sure that the newborns are safe and healthy, it provides them the resources to grow up, guides them during the development and transmits them values (APA). As a result, the newborn receives protection, nourishment and begins to develop its own personality, benefiting from a stable emotional environment (Bowlby, 1951).
For both humans and other mammals, it is well recognized the important role of the mother in the relationship with the newborn. Besides the pregnancy, the maternal role within the dyad is to provide a secure basis: the child, in order to explore the environment, needs to feel safe in returning and knowing that he will be embraced again and, if necessary, comforted or reassured (Bowlby, 1988) and this increases the possibility of establishing a stable emotional bond. This stability would appear to have protective effects on the child's future behaviors in adverse situations that can normally occurs during lifetime. It has been demonstrated that a responsive caring mother is able to mitigate the cortisol increase during stressful situation, and thus promote a more rapid recovery (Albers et al., 2008). On the other end, experiencing an adverse maternal environment could felicitate development of depression (Rice et al., 2007). However, it should be emphasized that, although the mother may represent the "caregiver par excellence", the safe base role can be assumed by all the parental figures, including the adoptive one.

Adverse experiences related to an inadequate relationship with caregivers, such as an interference of the formation of the attachment bond, are strictly related to development of depression in adulthood.

2.1 Attachment bond: theories and conceptualization

The first authors describing the attachment were Jhon Bowlby and Mary Ainsworth and, since them, the interest in this process and its mechanisms has exponentially grown. The attachment bond is an emotional, reciprocal bond necessary for the establishment of the caregiver-infant's relationship; this bond is universal in mammals (Okabe et a., 2012). Humans have an innate predisposition to develop attachment with caregivers (Sullivan et al., 2011), thus considered an evolutionary tool necessary to satisfy the primary needs of the newborn child, such as eating, feeling safe, clean and protected. Children, in fact, built a psychological construct about the caregivers that allow them to manifest the attachment behavior towards those considered able to face the world adequately (Bowlby, 1969). During the early stage of development children engage pre-programmed behaviors that aim to maintain proximity to the attachment figure (Bowlby, 1988), with the primary function of protection. By attaching to their caregivers, children are more likely to see the world as a welcoming place and, consequently, will be more inclined to exploration, necessary for the proper emotional/social and cognitive development (Winston and Chicot, 2016). The establishment of the attachment bond thus require the internal representation of himself and of the external reality, mediated by the "internal working models" (Bowlby, 1973). Therewith children can internalize the representation of the attachment figures and the relationship that bind them (Bowlby, 1973), allowing them to perceive events, conceive the future and build their plans. Early experiences are important in shaping the formation of the "internal working models", particularly those relating to aversive events; in fact, they can be preserved over time through the internal representations, generating expectations and being used as a guide for future behavior (Baldoni, 2007), acquiring a main role.

There are several mechanisms involved in the formation and maintenance of the attachment bond, starting with the caregiver's recognition until the establishment of a persistent attraction (Numan and Young, 2016). The quality of the interaction plays, of course, a key role by becoming part of the newborn's mental representation (Walsh et al., 2019) and participates in the long-term attraction within the dyad (Numan and Young, 2016).

The formation of the attachment bond starts during the very first sensitive period of life and its perturbation facilitates psychopathological outcomes, such as depression. If good quality interactions with positive early social environment and secure attachment will provide internal resources to face adverse situations (Atwool, 2007), poor quality ones could result in an insecure (anxious, avoidant, disorganized; Grumley et al., 2014) attachment bond. Uncapable of actively face adverse situation,

children with an insecure attachment style are more likely to present depressive symptoms in adulthood (Shaver et al., 2005; Burnette et al., 2009; Jinyao et al., 2012).

The strong relation between attachment bond impairment and depressive symptoms in adulthood was suggested by clinical and preclinical studies (Reite et al., 1978; Bifulco et al., 2006; Spence et al., 2020) mostly in women. Moreover, different patterns of attachment alteration are related with different possible range of pathological outcomes. An anxious attachment style positively correlates with higher risk of anxiety traits in childhood and adolescent (Kerns and Brumariu, 2014), due to the emotional unavailability of the parents, but also with suicidal behavior (Violato and Arato, 2004) and eating disorders (Illing et al., 2010). Disorganized attachment style strongly correlates with personality disorder in adulthood (Westen et al., 2006) such borderline disorder and depression (Brumariu and Kerns, 2010). As a results, the pattern of established attachment will drive the future behavioral strategies in facing experiences in adulthood. When children experiences instability and unreliability, the future behavioral and emotional regulation strategies will be affected (Waters and Waters, 2006).

2.1.2 The protective role of the earned-secure attachment

The separation from the primary source of attachment during early life period is known to promote an initial anger and anxiety phase, followed by feelings of sadness and, if the separation is severe and prolonged, a final response of un-attachment (Bowlby, 1988). What happens if the separation from the primary caregiver is due to its emotional unavailability more than a physical distance? Emotional distance creates confusion in the infant, that will be more likely to develop an insecure attachment pattern (Ainsworth, 1985; Bowlby, 1988) strictly linked to depression in adulthood. The emotional neglect, in fact, is known to alters attachment bond and, consequently, increase the risk of depressive outcomes (Widom et al., 2007; Müller et al., 2019). Indeed, it participate in altering the mental representation of the child, which fails to feel safe and reassured by the primary caregiver (Bowlby, 1988). In order to avoid the alteration of the attachment bond and its negative consequences, another attachment figure can become source of attachment (earned-attachment), providing stability and emotional availability.

People other than parents and close to the family environment, such as a grandparent or an older brother, could impersonating an alternative caregiving figure, especially when the relation with primary caregiver is damaged. The alternative supporting figure can be represented either by someone external to the family, such as a friend's parent, a teacher, a neighbor or, importantly, an adoptive

parent (Saunders et al., 2011). The stronger emotional bond with an alternative caregiving figure experienced during the childhood (Zaccagnino et al., 2014) may help to prevent the negative consequences induced by the negative primary caregiver environment. The alternative caring figure, in fact, could compensate for inadequate parental care and be supportive with the children preventing the risk of future negative outcomes and promoting earned-secure attachment. This classification was identified in adults that did not show impairment in secure attachment's measures in the Adult Attachment Interview (AAI), despite the experience of difficult relationships with their parents during the infancy (Pearson et al., 1994). Due to the negative relation with primary caregivers, people with an earned secure attachment relied on the alternative support figures during the critical period forming a strong emotional bond with them (Zaccagnino et a., 2014). Overall, the earned-security, defined as the processes by which individuals overcome to malevolent parenting experiences (Roisman et a., 2002), act as a protective factor for future behavioral outcomes.

The important role of caregiver during childhood, even different from the parents, is to provide children the suitable instruments to face future adversities and learn to develop trust (Saunders et al., 2011) by forming an adequate attachment bond. If traumatic experiences within the relation with caregivers are able to increase the vulnerability to develop psychopathology in adulthood, environmental protective factors are able to favor a reworking of these traumatic experiences and shielding from aversive consequences.

2.2 Preclinical model of attachment bond alteration: Repeated Cross Fostering

Rodent models emphasize caregiver's role for the offsprings in reducing the perceived of the threat (Opendack and Sullivan, 2017) and point out the first 10 days of life as crucial for attachment bond formation. During this period, rodent pups learn and associate olfactory, visual and auditory stimuli of their caregiver, mainly thought olfactory cues (Opendack and Sullivan, 2017), establishing the attachment bond. In line with clinical studies, pups raised by a mother with poor maternal care during this period show alteration of hypothalamic–pituitary–adrenal (HPA) axis function as well as anxiety and depression (Champagne and Meaney, 2006). Different procedures, as Maternal Separation and Limited Bedding Nesting, interfere with the early environment and induce stress in mice pups with severe consequences in adulthood (Tractenberg et al., 2016; Bath et al., 2016; Rice et al., 2007; Azevedo et al., 2010). These procedures alter the maternal behavior without assessing a possible alteration of the attachment bond between mother and pups.

Recently, it has been characterized a rodent model that aims to interfere with the formation of the attachment bond by creating an unstable environment for the pups. The procedure, known as Repeated Cross Fostering (RCF), consists in fostering pups with four different, adoptive, mothers during the first four days of life (from PND1 to PND4; D'Amato et al., 2011; Ventura et al., 2013; Luchetti et al., 2015, 2016; Di Segni et al., 2016, 2018, 2019; Lo Iacono et al., 2021). The strength of this manipulation is represented by the alteration of the bond between dam and pups, without interfering with the maternal behavior (Di Segni et al., 2018). Cross-fostered pups, in fact, do not differ from control animals for the amount of maternal care received (D'Amato et a., 2011; Luchetti et al., 2015, 2016; Di Segni et a., 2016; Lo Iacono et al., 2021). Additionally, RCF mice pups exhibit a higher number of separation-induced distress ultrasonic vocalization calls (USVs) (Lo Iacono et al., 2021), supporting an increased separation anxiety phenotype induced by the attachment bond interference (D'Amato et a., 2011; Ventura et al., 2013; Lo Iacono et al., 2021). The interference with the formation of a stable and predictable bond with the caregiver induced by RCF (Lo Iacono et al., 2021) has negative consequences in adulthood, depending on genetic background. After RCF exposure, females of C57BL/6J inbred strain of mouse show a pro-resilient behavior to passive coping strategies and depression, while females DBA/2J (DBA) shows an opposite pattern. In fact, RCF DBA female mice show an increased passive coping strategies and depressive-like behavior in adulthood (Lo Iacono et al., 2021; Di Segni et al., D'Addario et al., 2021). Interestingly, RCF was proved to strongly affect females of both strains as supported by the alteration of the transcriptomic pattern in the Ventral Tegmental Area (VTA) observed in both C57 and DBA female mice (Lo Iacono et al., 2021).

The early aversive experiences, as the alteration of the attachment bond, may persistently modify brain development via epigenetic mechanisms, allowing the environment to modulate gene expression and increasing vulnerability to different psychopathologies, such as depression (Burns et al, 2018). Thus, evaluating the neurobiology of attachment could help to understand how its disruption is linked to mood disorders in adulthood.

2.3 Neurobiology of Attachment

Since the attachment bond establishment has an evolutionary purpose, the brain has adapted to favor it, engaging biochemical signaling and neurobiological mechanisms to reinforce and maintain its formation. Preclinical models shed lights on these mechanisms, in order to understand how attachment dysregulation affects brain development resulting in negative pathological outcomes.

Medial Preoptic Area (mPOA) and Bed Nucleus of the Stria Terminalis (BST) regulate the innate, not-learned, motivated behaviors (Numan and Numan, 1996) including the bond pairing. Their role in maternal attachment regulation is related to the involvement of hormones particularly active during mother-pup relation, such as estradiol and prolactin. Their injecting in mPOA during pregnancy and early post-partum period in rodents increases the maternal behavior (Numan and Young, 2016) necessary for offspring attachment. During maternal behavior, MPOA and BST interact with the mesocorticolimbic system, the primary pathway involved in reward learning and evaluation (Numan, 2007; Numan and Stolzenberg, 2008). The maternal behavior is a key element to establish the attachment bond, and the motivation that guide it involves the interaction of Oxytocin (OXT) with the mesocorticolimbic system (Numan and Stolzenberg, 2009). OXT is the primary neuropeptide involved in attachment behavior (Buchheim et al, 2009). It is synthesized in the Paraventricular Nucleus of the Hypotalamus (PVN) and the Supraoptic Nucleus, areas involved in attachment bond, particularly active during lactation (Neuman and Neuman., 1996). OXT release from PVN targets and interacts with the mesocorticolimbic system, in particular the Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAc) (Douglas, 2010). The interaction with the VTA promotes Dopaminergic (DA) firing in the NAc, enhancing the rewarding proprieties of attachment bond establishment (Loth et al., 2021). During pregnancy, the synthesis of OXT receptor increases, together with the synthesis of D2-like DA receptor in NAc (Liu and Wang, 2003), stimulating the formation of pair-bonding. Moreover, increased release of OXT in VTA and NAc strongly enhances caring behavior. Interestingly, this increased activity is evident also in virgin non-lactating females once exposed to pups (Rosenblat, 1967). Consistently with clinical evidence, this supports the point of view that the attachment bond is not strictly related to delivery. The maternal motivation to establish the attachment bond is strong also in virgin females adopting other pups, suggesting the involvement of plasticity mechanisms (Numan and Young, 2016), that seem to be shared with many mammals.

The negative effects of attachment bond disruption could depend in part on the alteration of these complex neurobiological mechanisms. Specifically, the activation of the “attachment circuitry” seems to be related to the dopaminergic mesocorticolimbic system, in particular the VTA, which strengths the motivation driving the maternal behavior and facilitates the attachment bond formation. The finding the this circuitry is found to be also altered in depression strengths the possible relation between the precocious alteration of the bond and the development of depressive symptoms in adulthood.

3. Dopaminergic mesocorticolimbic system: the role of VTA

The VTA is the key area of the dopaminergic mesocorticolimbic system, which extends to striatal and limbic regions, such as Nucleus Accumbens (NAc), Amygdala, Prefrontal Cortex (PFC) and Hippocampus (Olds et al., 1956). The circuitry evolved to pander the normal gratifying essential behaviors, such as eating, drinking, sexual behaviors and, not less important, maternal behaviors and social interactions (Olds et al., 1956; Nestler and Malenka, 2004). The pleasant sensations experienced favor the repetition of the behaviors thus facilitating the consolidation of biologically essential memories. VTA is directly involved in processes related to reward: positive and rewarding stimuli activate it, inducing DA releases in the target areas (Berridge, 2007). Structurally heterogenous, the VTA is mainly constitutes by dopaminergic (DA) neurons (Nair-Roberts et al., 2008) that have different characteristics, in terms of physiological and anatomical criterions (Bjorklund and Dunnett, 2007). VTA projects to different brain areas involved in attachment bond formation and social behaviors, such as PFC and Amygdala c (McCormick et a., 2019; Rincon-Cortes and Grace, 2020). PFC modulates VTA thought a positive feedback loop activated when a cognitive control over reward and social interaction is required (Douma and de Kloet, 2020), while Amygdala inhibits VTA activity in response to emotional aspects of reward (Douma and de Kloet, 2020). Indeed, the activity of the dopaminergic system depends on the valence attributed to the stimuli and when an incorrect activation persists, as in depression, permanent sensibilization-induced impairment in responding to stimuli occurs (Belujon and Grace, 2017). Adult depressed patients show an impairment connectivity between some DAergic system areas probably induced by altered dopaminergic firing from VTA DA neurons (Kumar et al., 2018). These alterations can also depend on negative effects of aversive precocious experiences impacting developing DA system, affecting the ability of the entire circuitry to fulfill its regulation with consequential impairment in reward evaluation, particularly when negative events are experienced during the first two weeks of life (Dinopoulos and Parnavelas, 1991). As a result, adverse experiences occurring during this period could permanently alter the circuitry.

To accomplish its function and regulate DA release, VTA dopaminergic neurons exhibit several intrinsic regulatory mechanisms able to regulate their electrophysiological activity (Marinelli et al., 2006; Kaufling, 2019). Understanding the physiological alterations induced by early experiences in these neurons could help understanding the link between VTA and depression.

3.1 I_h current and depression

In the midbrain system, the dopaminegic (DA) neurons can be divided into several subpopulations, each of which characterized by specific proprieties, including different projection patterns or electrophysiological features (Lammel et al., 2008). A relevant hallmark of the VTA DA neurons is the presence of an intrinsic regulatory mechanism consisting of a balance between excitatory and inhibitory firing, the hyperpolarization-activated cation current (I_h) (Lammel et al., 2008; Shi, 2009). I_h current is a modulatory current activated by membrane hyperpolarization (Neuhoff et al., 2002) and mediated by hyperpolarization-activated cyclic nucleotide-regulated cation channel (HCN; Krashia et al., 2017). Whitin the mesocorticolimbic system, HCN channels are largely expressed and one subunits of these channels, HCN2, is particularly expressed in VTA (Notomi and Shigemoto, 2004). Interestingly, the amplitude of I_h current decreases from lateral to medial regions of the VTA (Krashia et al., 2017), together with membrane proprieties and cell body size change (Krashia et al., 2017), supporting different functions within this area. Conversely, some studies found a large I_h currents in non-DA neurons of the VTA (Margolis et al., 2008; Zhang et al., 2010) suggesting that neurons others than DA can be modulated by this pacemaker activity, or absence of I_h current in DA neurons (Lammel et al., 2008). The heterogeneity of DA neurons within the VTA, strictly connected to its anatomical heterogeneity (Oades and Halliday, 1987), could explain why some of them do not present I_h current. As a meter of fact, ventral subregion of the VTA seems to contain DA neurons with different electrophysiological characteristic (Lammel et al., 2008) although they are poorly investigated, while the medial part meets the Ih current activity (Lammel et al., 2014). Diversity in VTA activity seems to be related to different projection target areas: neurons within the medial VTA project to NAc, while moving to more laterally the projections are more likely to target striatum and mPFC (Ikemoto, 2007).

I_h current has been deeply investigated in animal models, with particular interest in mood disorders. Evidence suggest, in fact, it's alteration induced by stressful experiences (Friedman et al., 2014; Masrouri et al., 2020; D'Addario et al., 2021) strongly connected to passive coping strategies, one of the major characteristic of depressive-like phenotype in rodents. Under stress condition, DA neurons of VTA adapt their activity to better cope with higher activity demand. If a stress is chronically experienced, it could drive to system alterations (Douma and de Kloet, 2020) connected to depression (McGonagle and Kessler, 1990; Hammen, 2005). Interestingly, adult mice exposed to chronic stress in adulthood develop depressive symptoms and I_h current alteration in dopaminergic neurons of VTA (Friedman et al., 2014; Zhong et al., 2018). On the other hand, mice strains that showed resilience to

develop depressive-like phenotype after stress exposure also presented an alteration of the I_h current (Ku et al., 2017, D'Addario et al., 2021). Interestingly, I_h current alteration seems to depend on several factors, like the stress protocol used to evaluate the relation with depressive-like phenotype. As an example, adult mice exposed to a Chronic Social Defeat Stress paradigm, that resulted to be vulnerable to depression, showed an increased I_h current (Friedman et al., 2014) while depressed mice exposed to Chronic Unpredictable Mild Stress showed a reduction of I_h current in VTA DA neurons (Zhong et al., 2018; Lammel et al., 2014). Notably, different patterns of I_h current impairment were found in relation with vulnerability or resilience to depression, strengthening the relationship between the physiological activity of VTA DA neurons (I_h current alteration) and depression.

As previously discussed, one of the major risk factors for developing depression in represented by aversities experienced during the early life period and the formation of the attachment bond is particularly important during this life window. The relation between attachment bond disruption, depression and DA system has been deeply investigated in both clinical and preclinical studies. Nonetheless, the mechanism by which the alteration of the attachment bond interferes with the activity of the DAergic system, increasing vulnerability to subsequent depressive symptoms, is not fully understood.

Specifically, only few studies investigated the relation between early life adversities, depression and I_h current alterations. Recently it was reported an altered I_h current in adult C57 female mice previously exposed to Repeated Cross Fostering (RCF) manipulation (D'Addario et al., 2021), that alters the attachment bond (Lo Iacono et al., 2021). C57 female mice that underwent to RCF, showed a pro-resilient behavior to passive coping strategies as well as reduced density of I_h current in DA neurons of intermediate VTA (iVTA). In addition, control adult females sub-chronically treated with a selective blocker of HCN channels in VTA developed an RCF-like behavior, while RCF females treated with a potentiator of the HCN channels function mimicked the behavioral phenotype of control animals (D'Addario et al., 2021). These results provided a link between I_h current in the VTA DA neurons and resilience to depression induced by early life experiences.

4. Hypothesis

Recently, it has been shown that RCF affects functional proprieties of VTA DA neurons in C57 adult females, and particularly their hyperpolarization-activated cation current (I_h; D'Addario et al., 2021). Notably, in these animals a causative link between I_h current modulation and adult behavior has been demonstrated. Animals exposed to an early life adversity (RCF), that alters the attachment bond, show resilience to passive coping strategies in the adult life (Di Segni et al., 2016; Lo Iacono et al., 2021) and this correlates with the reduction of the I_h current density in DA neurons of the intermediate VTA (D'Addario et al., 2021). In contrast, DBA females exposed to RCF show a depressive-like behavior in adult life (Lo Iacono et al., 2021; *see results section 6.5*). Based on these data, here we hypothesize that this behavioral phenotype is related to increased ih current of VTA DA neurons. To address this point, we first performed voltage- and current-clamp recordings from DA neurons of the intermediate VTA (iVTA) from RCF or Control (Cont) adult DBA female mice.

As well known, the attachment bond perturbation increases vulnerability to psychopathological outcomes expression (Bowlby, 1969). By contrast, a stable environment (e.g., a stable and secure attachment bond during the early life period) acts as protective factor for the future development. Clinical data suggest that caregivers other than parents that can provide emotional and physical support to the children when the early environment is perturbated by instability and insecure attachment; these secondary caregivers can become earned-security figures (Saunders et al., 2011).
The earned-secure attachment acts as an environmental protective factor counteracting the attachment bond disruption consequences. However, the mechanisms underlying these protective effects are not completely known; animal models may help to shed light on these complex processes. For this purpose, we decided to use our well-established model of attachment bond interference in mice (RCF), including a non-parental caregiver, such as a Stable Attachment Figure (SAF) to mimic the earned attachment. This new approach helps us to investigate the potential role of SAF in preventing the behavioral and neurophysiological alterations induced by RCF. Here, we hypothesized that by introducing a stable stimulus able to promote an earned attachment (SAF) during RCF, we can prevent the behavioral and neurophysiological outcomes in the adult animals.

5. Experiment 1: Investigating the RCF effects on electrophysiological proprieties of VTA DA neurons in DBA female mice

5.1 Methods

5.1.1 Animals

DBA/2J (DBA) female mice (Charles River Laboratories, Italy) were housed with water and food available ad libitum, at constant room temperature (21 ± 1 °C) and in a 12:12 h light–dark cycle (lights on at 07:00 a.m.). Adequate measures were taken to minimize pain or discomfort of mice and all experiments were carried out in accordance with Italian national laws (DL 116/92 and DL 26/2014) on the use of animals for research based on the European Communities Council Directives (86/609/EEC and 2010/63/UE). Experimental protocol (no.70/2022) was approved by Italian Ministry of Health. Eight-ten mice of 10-12 weeks old were used for electrophysiological experiments. Six-ten mice for group were used for behavioral experiments during development and in adulthood. RCF and Control (Cont) animals from the same cohort were used for this experiment.

5.1.2. Repeated Cross Fostering

Repeated Cross Fostering (RCF) manipulation was performed as previously described (D'Amato et al., 2011; Di Segni et al. 2016, 2019; Lo Iacono et al., 2021; D'Addario et al., 2021). Pups from the same litter spent the first postnatal day (PND0) with their biological mother; on PND1, litters were randomly assigned to experimental (RCF) or Control (Cont) condition.
RCF pups were fostered by moving the entire litter into the home-cage of a different mother, whose pups had just been removed and moved to another adoptive mother. This procedure was repeated daily, from PND1 to PND4; on PND4, pups were left with the last adoptive mother until weaning (Fig. 1). Cont pups were only picked up daily and reintroduced in their home cage within 30s.
Animals were weaned at PND28, separated by sex and housed in groups of 4 littermates. To avoid litter effects, and to prevent potential female estrous cycle group synchronization, RCF and Cont group were sorted collecting max 2 individual per litter (Di Segni et al., 2016;2018;2019).

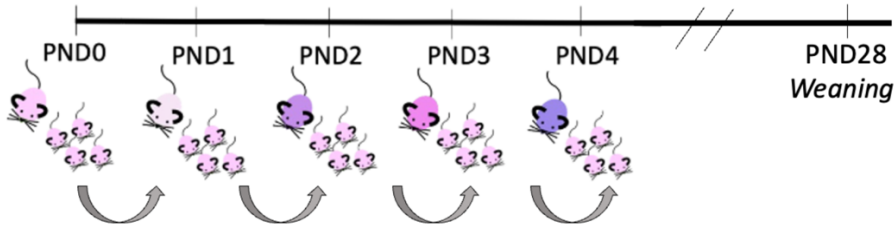

Fig. 1 Schematic representation of Repeated Cross Fostering procedure from PND1 to PND4.

5.1.3 Electrophysiology

5.1.3.1 Midbrain slice preparation

Adult DBA female mice, previously exposed to RCF or CTRL manipulation, underwent no-return deep anaesthesia with halothane (i.p. and gas) to allow their trans-cardiac perfusion with cold (0.5–4 °C), 95%O_2–5%CO_2 – saturated 'slicing' solution (recipe below). This procedure is routinely adopted to minimize the damage inevitably suffered from adult (or aged) brain tissues during the dissection – slicing protocol. The trans-cardiac perfusion is considered satisfactorily completed when internal organs (lungs; liver) appear cleared from blood (whitened) After trans-cardiac perfusion, mice were decapitated; brains were quickly removed from the skull and a tissue block containing the midbrain was isolated and placed in chilled bubbled (95% O_2–5% CO_2) low-sodium N-methyl-D-glucamine (NMDG)-based 'slicing' solution at 0.5–4 °C containing, in mM: 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, and 10 $MgSO_4$ (pH to 7.3–7.4 with 18% hydrochloric acid). As recently published, (D'Addario et al., 2021) horizontal midbrain slices containing the Ventral Tegmental Area (VTA; 250 μm-thick) were prepared using a vibratome (VT1200S, Leica Biosystems), according to Ting et al., 2018.

Thus, once prepared slices were maintained in a becker containing 150mL of NMDG-based, bubbled aCSF solution at 20pproac. 34 °C for 5 min, before adding progressively increasing volumes of the so called 'sodium-spike solution' (2M NaCl in NMDG-based aCSF). For long-term storage, slices were transferred into a HEPES-based aCSF containing: 92mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4, 30mM $NaHCO_3$, 20mM HEPES, 25mM glucose, 2mM thiourea, 5mM Na-ascorbate, 3mM Na-pyruvate, 2mM $CaCl_2$, and 2mM $MgSO_4$ (95%O_2–5%CO_2; pH 7.3–7.4). Slices were let recovering for 1 hour at least before being moved in the recording chamber for the electrophysiological recordings, during which they were perfused continuously (2.5–4 mL/min) with aCSF solution (20pproac. 32 – 34 °C), containing: 126mM NaCl, 24mM $NaHCO_3$, 10mM glucose,

2.5mM KCl, 2.4mM $CaCl_2$, 1.2mM NaH_2PO_4 and 1.2mM $MgCl_2$, saturated with 95%O_2–5%CO_2 (pH 7.4; ~290 mOsm).

5.1.3.2. Electrophysiological recordings

For electrophysiological recordings, single slices were moved to the recording chamber of an upright microscope (Leica) and continuously perfused (3–4 ml/min) with saturated aCSF solution (95% O_2, 5% CO_2). Pipettes were pulled from thick-walled capillaries to a final tip resistance of 5–6 MΩ when filled with an 'intracellular' solution containing: 125mM K-gluconate, 10mM KCl, 10mM HEPES, 2mM $MgCl_2$, 4mM ATP-Mg_2, 0.3mM GTP-Na_3, 0.75mM EGTA, 0.1mM $CaCl_2$, 10mM Phosphocreatine-Na_2 (pH 7.2, ~280 mOsm). Cell-attached and whole-cell patch-clamp recordings were performed from visually identified (40X) VTA DA neurons (MultiClamp 700B and Digidata 1322A; Molecular Devices). Neurons were selected for patching using the following criteria: location in the intermediate region of the VTA (iVTA), according to the map recently described by Krashia et al., (2017); presence of low-frequency, spontaneous regular firing of action potentials (Aps) in cell-attached and whole-cell configuration; presence of I_h current (Krashia et al., 2017). The criteria were validated by post-hoc Thyrosine Hydroxylase (TH) immunostaining on recorded neurons (data not shown; Zhong et al., 2018). Only neurons that met that criteria were include in the study. All recordings were performed at 32–34 °C. Eight-ten animals were used for this experiment and 1-2 slices per mice were analyzed.

Membrane Resistance (R_m), Membrane Time Constant (τ) and Membrane Capacitance (C_m) were measured within 2 min after membrane rupture using the in-built Clampex 9 'membrane test', voltage-clamp protocol, consisting of a 30 ms-long, − 5 or +20 mV step (33.3 Hz) from the Holding membrane Voltage (V_H; − 60 mV). The neuron approximate Resting Potential (RP) was also estimated using the amplifier inbuilt voltmeter immediately after accessing the cell interior.

The hyperpolarization-activated inward current I_h was recorded in whole-cell configuration in response to hyperpolarizing voltage steps (1 sec-long; from −60 to – 120 mV, 20 mV increment, V_H – 60 mV; f_c 2 kHz; sampling 10 kHz).

To investigate the neuronal evoked excitability, different current-clamp protocols were adopted. Firstly, the rheobase (that is, the amplitude of the injected current necessary to induce the first AP) was estimated (Clampfit) from the voltage response to a series of 5 pA-incremental consecutive steps of depolarizing injection current (I_{inj}; 50 ms-long steps; amplitude range: 0 – 0.2 nA).

The under- and supra-threshold properties of iVTA DA neurons were studied using a second I_{inj} step protocol (1 s-long, consecutive current injections; amplitude range: − 0.2 – 0.4 nA). This approach allowed describing the relationship between depolarizing Injection currents and evoked AP Frequency ('*f–I* curve'), used to investigate in details the evoked excitability of iVTA DA neurons in the experimental groups adopted.

All current-clamp recordings were performed at f_c 10 kHz and sampling 50 kHz, with no drugs added to the aCSF and while maintaining their membrane potential at V_H = − 60 mV via the injection of the required steady current.

5.1.3.3 Data analysis

Analysis of current-clamp and voltage-clamp recordings was performed using Igor. Pro 6.32A (WaveMetrics Inc.) with NeuroMatic 2.8 (Rothman and Silver, 2018). The amplitude of I_h currents was measured at the current steady-state and after nulling the current baseline at the interception between the offset of the capacitive peak and the onset of the hyperpolarization-activated inward current, for each step response (Krashia et al., 2017). These amplitude values were used to describe the 'I–V curve', the relationship between the voltage command and the elicited current. In current-clamp experiments, curves for the *f* – I relationships were built after counting the AP elicited per each I_{inj} step using a threshold crossing method.

5.1.4 Statistics

Statistical analysis of patch-clamp data was run in Prism 6 (GraphPad) using Mann–Whitney *U* test, unpaired Student's *t* -tests (with Welch's correction), one-way or two-way repeated measures ANOVA with Bonferroni's test for *post-hoc* analysis, as required. In details, Mann-Whitney test was performed for the analysis of Resting Potential (RP), Action potential (AP, cell-attached configuration), Rheobases and the average of Ih current density parameters. Membrane Time Constant (tau. memb), membrane capacity (C_m) and membrane input resistance (R_m) were analyzed with Whelch's t-test, together with AP in whole-cell configuration. 2-way ANOVA for repeated measure analysis was performed for both *f–I* curves and I-V curves (Condition, 2 levels: RCF and Cont). Normality of data sets was valuated using either Shapiro–Wilk test or D'Agostino & Pearson omnibus normality test. $P < 0.05$ was considered significant. Data are expressed as average values ± SEM

5.2 Results

5.2.1 RCF doesn't affect intrinsic proprieties of VTA DAergic neurons of DBA females

First, we investigated whether RCF induces permanent alterations of the basic, intrinsic properties of iVTA DA neurons, thus interfering with their physiological condition. The exposure to the RCF protocol in early life did not alter the membrane 'resting' potential (RP), membrane time constant (tau memb.), membrane capacity (C_m) or membrane input resistance (R_m) in these neurons (Fig. 2). From these results we could exclude a permanent, general interference by RCF on the physiological conditions of the neurons under investigation.

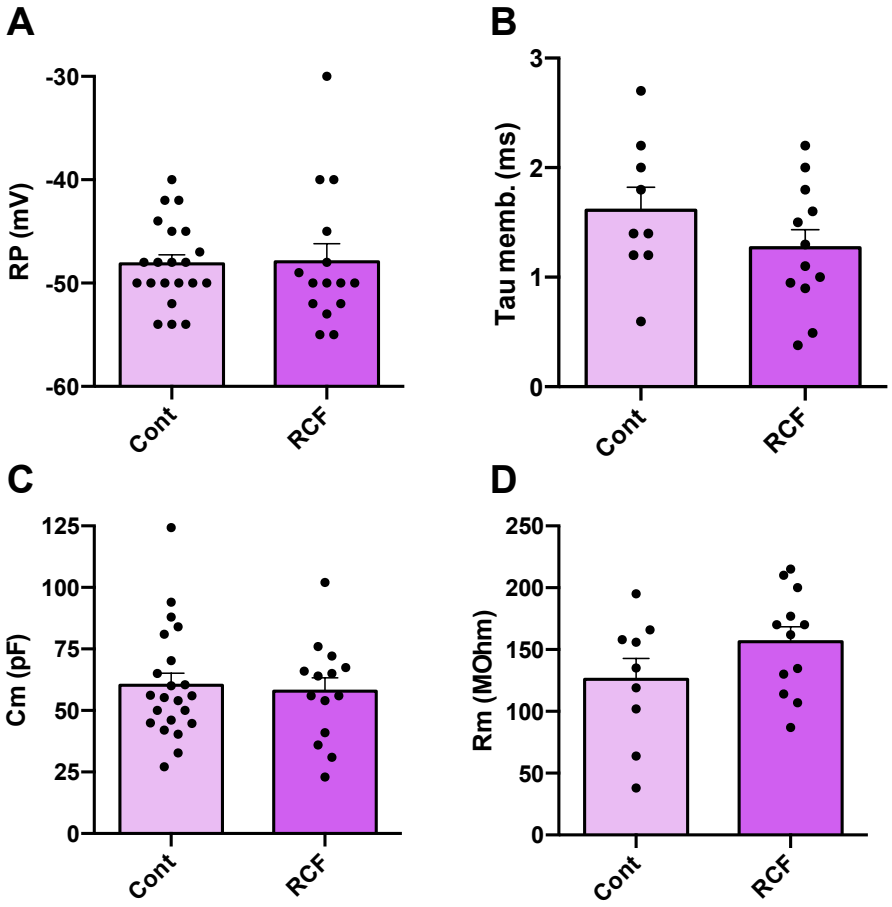

Fig.2 RCF procedure does not alter the intrinsic proprieties of dopaminergic neurons of the intermediate VTA of DBA/2J females. No significant differences between RCF and control (Cont) mice for each of the four intrinsic neuronal proprieties investigated: the membrane 'resting' potential during neuron's pacemaking activity (panel A; Resting Potential, RP, mV; Cont – 48.1 ± 0.9 mV, RCF – 48 ± 2 mV; *p=ns*, 21 and 15 neurons, respectively); the membrane time constant (panel B; tau memb., ms; Cont 1.6 ± 0.2 ms, RCF 1.3 ± 0.2 ms; p= ns t = 1.282, df = 16.36, 9 and 12 neurons, respectively); the membrane capacity (panel C; C_m, pF; Cont 60 ± 5 pF, RCF 58 ± 5 pF; p = ns, t = 0.3391, df = 29.83,

22 and 14 neurons, respectively); and the membrane resistance (panel D; R_m, Mohm; Cont 126 ± 17 Mohm, RCF 156 ± 12 Mohm; p= ns, t = 1.469, df = 15.34, 9 and 12 neurons). (*The electrophysiological characterization was carried out by M Renzi; E Spoleti: G Chilà*).

5.2.2 RCF does not alters spontaneous or evoked firing of DAergic neurons of VTA

Among other proprieties, the spontaneous firing of action potentials typical of the VTA DA neurons (pacemaking activity) is involved in dopamine release in projection areas, such as Nucleus Accumbens (Nac) and Prefrontal Cortex (PFC). Here, we investigated the spontaneous excitability of DA neurons in the iVTA both in cell-attached and in whole-cell configuration.

The action potentials frequency observed in both configurations was unaltered in DA neurons of iVTA from RCF mice compared to controls: in cell-attached, p= ns (Fig.3A). In whole-cell, p= ns, t = 1.182, df = 17.90 (Fig.3B).

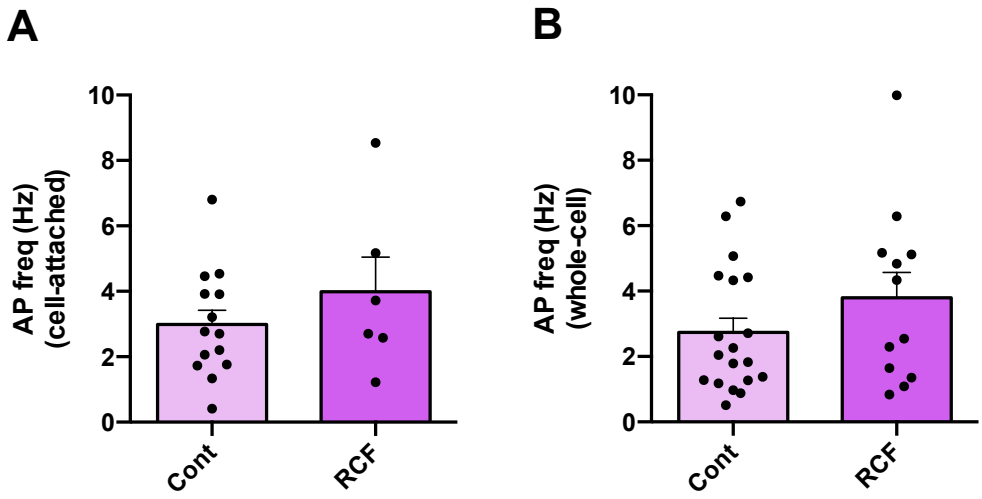

Fig.3 RCF exposure does not alter spontaneous excitability of dopaminergic neurons of the intermediate VTA of DBA/2J females. Scatter-plots depicting the frequency (Hz) of spontaneous Aps recorded from intact DA neurons in cell-attached configuration (A; Cont 3.0 ± 0.4 Hz and RCF 4 ± 1 Hz, 14 and 6 neurons, respectively) or from patched neurons in whole-cell configuration (B, Cont 2.7 ± 0.4 Hz and RCF 3.8 ± 0.8 Hz, 19 and 12 neurons, respectively). No difference was found between RCF and Cont mice. (*The electrophysiological characterization was carried out by M Renzi; E Spoleti: G Chilà*).

To further investigate cell excitability in our model, we evaluated the evoked firing and firstly investigated the neuronal rheobase; similar to spontaneous activity, also rheobase appeared unaffected by the RCF procedure (p= ns; Fig. 4A).

To a deeper level, the evoked excitability of iVTA DA neurons was investigated by building their *f–I* curves (curves describing the relationship between the frequency of evoked firing and the depolarizing injected current). The 2-way ANOVA for repeated measure analysis of the *f–I* curves showed no differences between RCF and Cont ($F(1,35) = 0.4597$, p= ns; Cont n= 22, RCF n= 15), with no significant interaction between "RCF treatment" and "injected current" variables ($F(7, 245) = 0.4480$, p= ns), beside the expected significance of the "injected current" variable ($F(7, 245) = 61.95$, $p < 0.0001$; Fig. 4B). Last, analogous results were obtained by the quantification of the maximum frequency of evoked firing, as predictable (Fig. 4C).

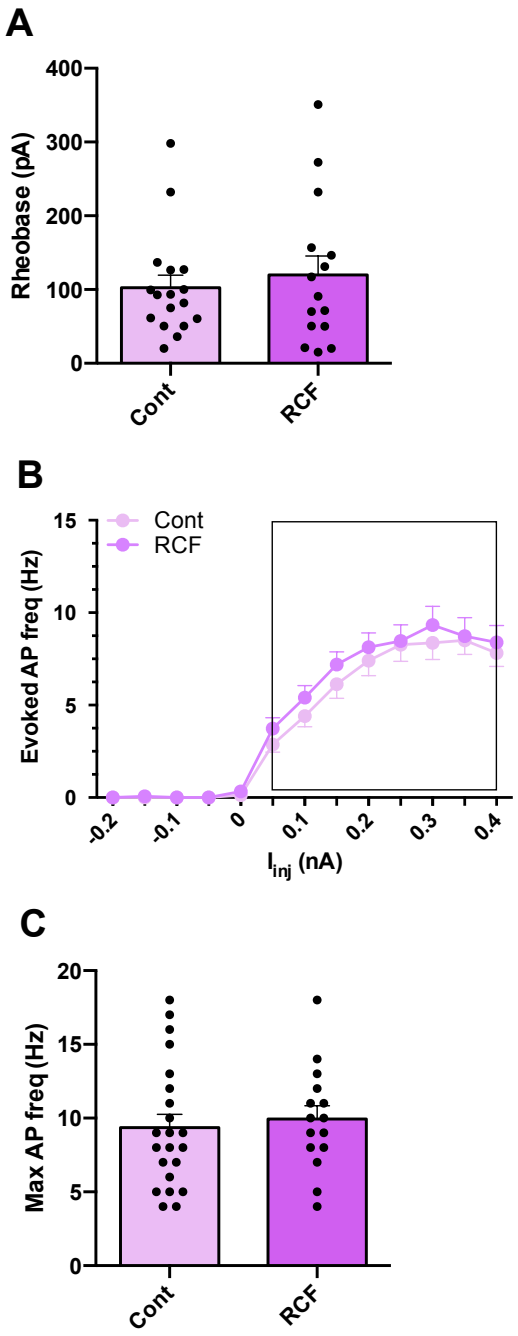

Fig. 4 RCF exposure does not alter the evoked excitability of dopaminergic neurons of the intermediate VTA of DBA/2J females. A) Scatter-plot of rheobase values (pA). No significant differences between RCF and Control (Cont) were found in DA neurons of the iVTA (Cont 103 ± 17 pA; RCF 120 ± 26 pA, 17 and 15 neurons). **B)** *f – I* average curves for Cont and RCF DA neurons of the iVTA. No significant differences were found within the analysis area (squared). **C)** Scatter-plot depicting values for the maximum frequency of AP firing evoked in iVTA DA neurons of DBA RCF *vs* Cont female mice (Cont 9.4 ± 0.9 Hz and RCF 9.9 ± 0.9 Hz, 22 and 15 neurons, respectively; p= ns, t = 0.4442, df = 33.42) (*The electrophysiological characterization was carried out by M Renzi; E Spoleti: G Chilà*).

5.2.3. RCF exposure alters the I_h current density of the intermediate VTA DA neurons

Here, we wanted to investigate if the vulnerability to anhedonic behavioral phenotype shown by DBA female mice was linked to increased I_h current density in the DA neurons of the VTA. To test this hypothesis, I_h current was activated in voltage-clamp experiments applying four steps of hyper-polarizing potential (see Methods 5.1.3.2.) long enough to reach the stationary state of the current itself. The density of the I_h current (in response to the larger hyperpolarizing voltage steps; –120 mV) as well as the current-voltage relationship (I–V curve, showing how a voltage-dependent current changes in response to different command potentials) were thus quantified.

In line with our hypothesis for a correlation between RCF-dependent adult behavior and I_h current modulations in DBA animals, our results showed a significant increase of I_h current in the dopaminergic neurons of intermediate VTA of these females. In details, the average I_h current density in TH^+ neurons of DBA Cont mice was – 1.0 ± 0.1 pA/pS (21 neurons) and – 2.0 ± 0.3 pA/pS (14 neurons; $p<0.005$) in RCF DBA females (V_{cmd} – 120 mV; Fig. 5).

By evaluating the I–V curve populations for our experimental groups using a 2-Way ANOVA test for repeated measure, we found a significant potentiation of the current along the different V_{cmd} for the "RCF treatment" variable (RCF vs Cont; $F(1,33)= 11.02$, $p<0.005$; Cont n = 21, RCF n = 14); a significant interaction between "RCF treatment" and 'V_{cmd}' ($F(3,99)= 8.699$, $p<0.0001$), in addition to the predictable difference found for the 'V_{cmd}' variable ($F(3,99)= 95.95$, $p<0.0001$; Fig. 5C). Bonferroni's post-hoc analysis showed a significant difference of the I_h current density obtained for the most hyperpolarizing V_{cmd}: for V_{cmd} – 100 mV, Cont – 0.53 ± 0.08 pA/pS *vs* RCF – 0.2 ± 1.2 pA; and for V_{cmd} – 120 mV, Cont – 1.0 ± 0.1 pA *vs* RCF – 2.0 ± 0.3 pA/pS.

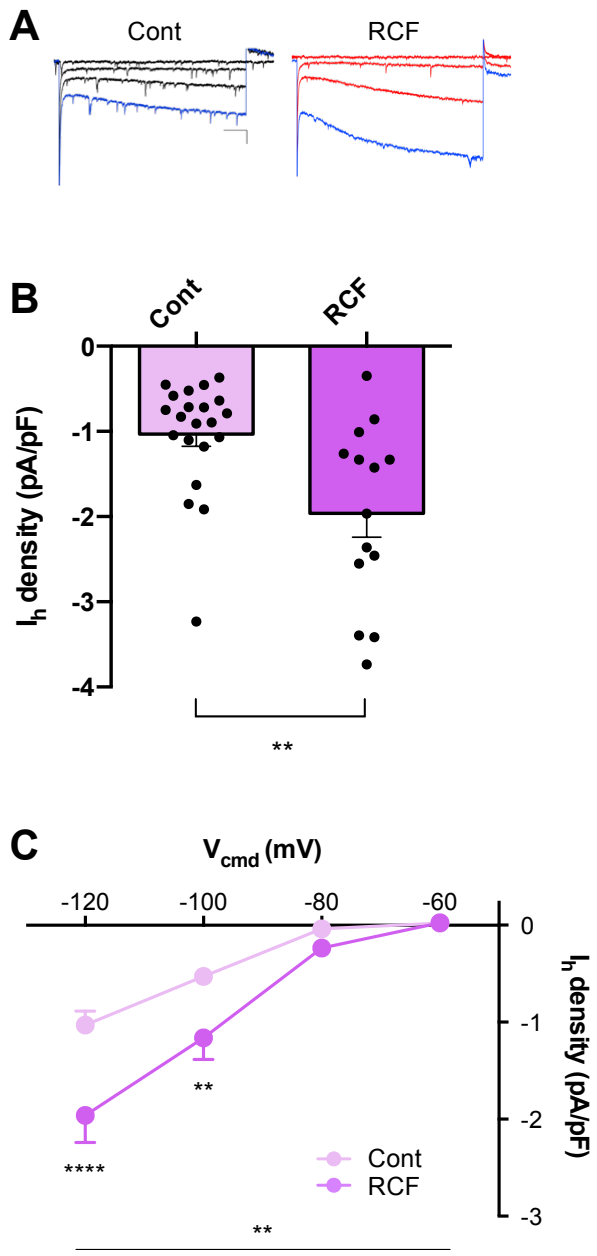

Fig.5 RCF exposure alters the I_h current density in dopaminergic neurons of the intermediate VTA of DBA/2J females A), Typical traces of I_h currents recorded from iVTA neurons activated by steps of V_{cmd} – 120 mV (not shown). The average amplitude of the current at the steady-state of the response was estimated for statistical analysis. In blue, the current response to V_{cmd} – 120 mV used for the statistical analysis shown in B. B) Scatter-plot of the I_h current density (pA/pF) in iVTA DA neurons at the steady-state of the response (V_{cmd} – 120 mV) showing a significant increase of I_h current density in neurons from RCF mice. C) Current density (pA/pF) – Voltage (mV) (I – V) relationship for the neurons investigated (V_{cmd} range: – 60 ~ –120 mV). The analysis showed a significant increase of I_h current density in TH^+ neurons from RCF mice vs Cont. (*The electrophysiological characterization was carried out by M Renzi; E Spoleti: G Chilà*).

5.2.3.1 Significant results summary

As emerged from the electrophysiological analysis, the only significant effect induced by the early life adversities (RCF) concerns the I_h current. RCF manipulation did not affect the intrinsic proprieties, neither the evoked or spontaneous firing of DA neurons. In accord with our hypothesis, RCF females showed an increased I_h current density compared to Cont mice, strengthening the relation with the depressive-like behavior.

6. Experiment 2: Modeling the "earned-secure attachment" as environmental rescue in DBA female mice

Here we decided to evaluate if a Stable Attachment Figure was able to promote an earned attachment (SAF) during RCF and to prevent its the psychopathological and neurophysiological outcomes in adult animals. We propose a rescue model by pairing each litter with a stable virgin, non-lactating dam, that stayed with the pups while they were changing mothers (from PND1 to PND4) (Fig. 6). This female would represent the alternative caregiving figure, acting as a source of alternative attachment (earned attachment), able to elide the negative effects induced by attachment bond interference.

6.1 Methods

6.1.1 Animals

DBA 2/J (DBA) female mice were used for this study (see Methods session 5.1.1 for details). RCF+SAF and Control (Cont+SAF) from two different cohorts were used for behavioral and electrophysiological experiments; however, the same cohort was always used to directly compare the treatment effect within each experiment.

6.1.2 Repeated Cross Fostering with Stable Attachment Figure

The classic version of Repeated Cross Fostering (RCF) was performed as previously described (see Experiment 1, Methods 5.1.2).

The RCF with the Stable Attachment Figure (RCF+SAF) was conducted as follows: DBA pregnant females were individually housed with water and food ad libitum, and nesting material; one week before delivery, a virgin non-lactating dam (SAF) was introduced into the cage with the pregnant dam. Behavior was monitored daily to assess absence of discomfort.

Mouse pups spent the first postnatal day (PND0) with the biological mother and the SAF assigned. From PND1, the litters were randomly assigned to RCF+SAF or Cont+SAF condition. RCF+SAF were fostered by gently moving the entire litter and the assigned SAF into the homecage of a different mother, whose pups and SAF had just been removed and moved to another adoptive mother (Fig. 6) As RCF, this procedure was repeated daily from PND1 to PND4; on PND4, the SAF was removed and placed into another cage while pups were left with the last adoptive mother until weaning. Cont+SAF, that experienced SAF from PND1 to PND4, were only picked up daily and reintroduced in their home cage within 30s.

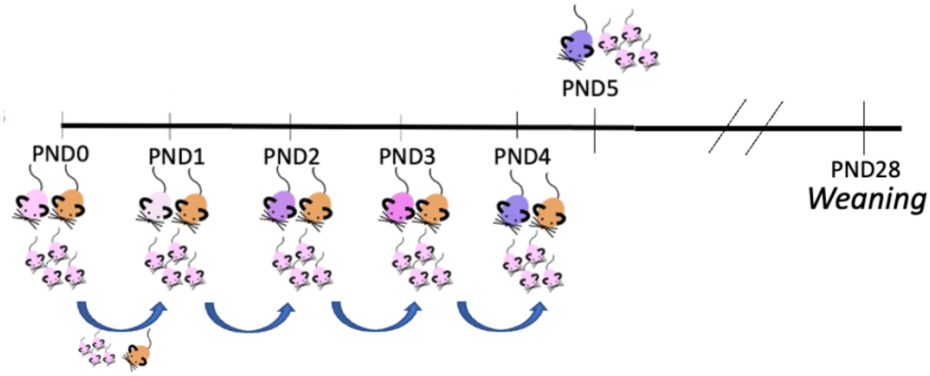

Fig. 6 Schematic representation of Repeated Cross Fostering with SAF procedure

6.1.3 Maternal Behavior Observation

Maternal Behavior Observation (MBO) was conducted as previously described (D'Amato et al., 2011). MBO took place daily, from PND1 to PND7, twice a day (from 12 to 12:30 and from 16 to 16:30), with an instantaneous sampling (2 minutes of sampling/rate) for 16 observation/sampling in each session. From PND1 to PND4, the first session (S1) took place one hour after the RCF procedure. The MBO analysis took in account, for both adoptive mother and the SAF, the following maternal behaviors (MB): Nursing (N), Liking/Grooming (L/G) and the Nesting (B). The non-maternal behaviors (defined as Other Behavior, OB), include Self-Grooming (S) and all the behaviors outside the nest, such as eating, drinking, or not having contacts with the pups (Shoji and Kato, 2006).

6.1.4 Ultrasonic Vocalization Calls

Pup's attachment behavior was measured by Ultrasonic Vocalization Calls (USV's) on PND8, during separation of the mother, as previously described (Cinque et al., 2021; Lo Iacono et al., 2021).
Each pup was individually placed into a sterilized beaker containing Homecage bedding material (Homecage) or clean bedding material (Clean); for RCF+SAF and Cont+SAF groups, mice were also

exposed to SAF bedding material (SAF). The vocalizations were recorded with UltraSoundGate microphone (CM16, AvisoftBioacoustics, Berlin, Germany) placed above 1 cm of the beaker, for 5 minutes, and analyzed with a dedicated software (Avisoft Bioacoustics, Berlin, Germany). The microphone was sensitive to 15-180 kHz frequencies, with a flat response (±6 dB) between 25-140 kHz. USV spectrogram was produced at 488 Hz frequency resolution and 0,512 ms temporal resolution after transferring the recorded files in SasLabPro (AvisoftBioacustic) and converted. To detect USVs a threshold-based automatic algorithm was used. Signal below 20 kHz was deleted to decrease the white noise to 0. No more than 4 pups x litter were tested; the sample size for each group was 6-8 pups, and pups of each group (RCF, Cont, RCF+SAF, Cont+SAF) belonged to 4-5 different litters.

6.1.5 Adult behavioral assay

On PND90, female DBA mice were tested to assess the behavioral phenotype. Animals from different cohorts were used for behavioral tests.

6.1.5.1 Open Field (OF)

Mice were individually placed in the central part of the apparatus and left to explore for 5 minutes. The apparatus consists of a circular open field with 60 cm of diameter and 20 cm of height (Di Segni et al., 2019; Lo Iacono et al., 2021). Distance moved (cm) was recorded and analyzed with the fully automated tracking video system “EthoVision” (Noldus, The Netherlands)

6.1.5.2 Forced Swimming Test (FST)

Forced swimming test (FST) was conducted as previously described (Di Segni et al., 2019; Lo Iacono et al., 2021). Mice were individually introduced into a glass cylinder of 18cm diameter, filled with 20 cm of 28± 2 °C temperature water. The behavior was video-recorded with a digital camera in front of the apparatus for 10 minutes. Mice were then dried and returned into the homecage. The duration of immobility (seconds) was manually scored with Boris by a blind trained observer (Friar and Gamba, 2016).

6.1.5.3 Tail Suspension Test (TST)

Mice were individually suspended by the tail 60 cm above the floor in a neutral plastic chamber using an adhesive tape placed at 1cm from the top of the tail (Yan et al., 2015). Behavior was video-recorded for 10 minutes with a digital camera posed in front of the apparatus and the duration of immobility (total absence of movement, seconds) was manually scored with Boris by a blind trained observer (Lo Iacono et al., 2021).

6.1.5.4 Social Interaction Test (SIT)

Social Interaction Test (SIT) was conducted in a gray rectangular box (60 × 40 × 24 cm) in plexiglass, consisting of a neutral, starting chamber in the center, connected with two "stimulus chamber" containing two identical clear cylinders (plexiglass, 8cm diameters) with multiple small holes (Fiori et al., 2015).

In the habituation session (10 min), mice were placed into the starting chamber and left free to explore the apparatus. During the test session (10 min), an age and sex matched mouse was introduced into one cylinder (pseudo-randomly choses) as a social stimulus, while a neutral object was introduced into the other one. The position of stimuli within cylinders were alternated to avoid potential confounding effects. The sessions were recorded and analyzed by a video-tracking system (EthoVision) to evaluate the time spent in each chamber. The time spent sniffing each cylinder (i.e contacts) during the test was manually scored by a blind trained observer (Lo Iacono et al., 2021) by Boris (Friar and Gamba 2016 Methods in Ecology and Evolution,7,1325–1330).

6.1.6 Ex-vivo electrophysiology

For brain slice patch-clamp experiments, Midbrain slice preparation, Electrophysiological recordings, Data analysis and Statistics were performed as described above (section 5.1.3 to 5.1.4).

6.1.7 Statistics

For behavioral analysis, two-way ANOVA (condition, 2 levels: RCF+SAF, Cont+SAF; bedding, 3 levels: Clean, Homecage, SAF) was used to investigate the pups's vocalizations (USV's). One-way ANOVA (condition, 4 levels: Cont, RCF, Cont+SAF, RCF+SAF) was performed to analyze the MB

from PND1 to PND4, while One-way ANOVA for repeated measure was used to investigate MB during the entire week. One-way ANOVA (condition, 4 levels: Cont, RCF, Cont+SAF, RCF+SAF) was performed to analyze the time spent in immobility during the FST and TST and the percentage of the time spent in the external part of the OF apparatus. Two-way ANOVA for repeated measure was used to investigate the mice behavior during the SIT (RCF, Cont, RCF+SAF, Cont+SAF). Individual between-groups comparisons were performed, when appropriate, by post hoc test (Bonferroni).

6.2 Results

6.2.1 SAF presence rescues pup's attachment behavior and does not interfere with the maternal behavior

To test if the SAF presence during the RCF procedure rescued the formation of the attachment bond, we evaluated the emission of USVs during the separation from the mother in both RCF+SAF and Cont+SAF female pups. In addition, to deeply understand the role of SAF during this very sensitive period, we exposed pups also to the SAF bedding material.

The ANOVA showed a significant bedding effect for both RCF+SAF and Cont+SAF ($F(2,19)= 38.223$; $p<0.0005$). Both groups vocalized less in home-cage bedding material than in the odorless, clean one (Fig.7A *left:* RCF+SAF, $p<0.0005$; *right:* Cont+SAF $p<0.0005$) confirming their ability to be reassured by the presence of the mother odor. This result confirms the rescue of the attachment behavior in RCF+SAF group. Interestingly, when exposed to SAF bedding material, pups from RCF+SAF and Cont+SAF groups vocalized more than in the Homecage, mother's scent (RCF+SAF $p<0.005$; Cont+SAF $p<0.005$) but less than in the clean, odorless bedding (Fig.7 *left:* RCF+SAF, $p<0.05$; *right:* Cont+SAF, $p<0.05$) suggesting a potential role of the SAF for pups.
No significant differences ($F(2,20)=3.313$; p= ns) were evident when analyzing the mean duration of USV across bedding and treatment (Fig.7C RCF+SAF; Fig.7D Cont+SAF).

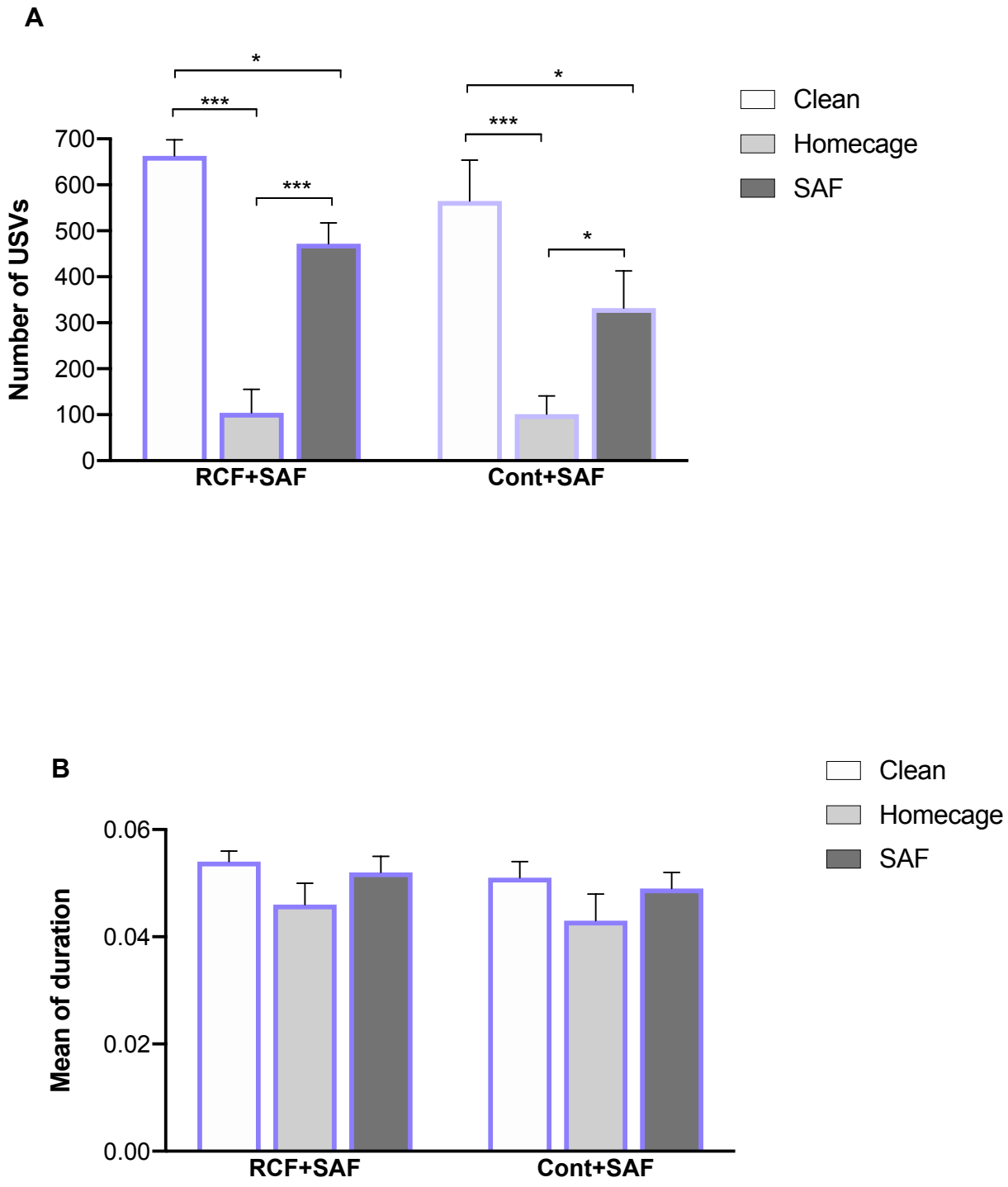

Fig.7 SAF presence during RCF rescued pups' attachment behavior. Statistical analysis showed an increased number of Ultrasonic Vocalization Calls (USV) in clean, odorless, bedding material compared to the homecage one for both RCF+SAF (panel A, *left*) and Cont+SAF (panel A, *right*) mice. When exposed to SAF bedding material, mice showed an increased number of USV compared to homecage condition (panel A, *left* RCF+SAF; panel A, *right* Cont+SAF) but significantly less than the clean bedding material (panel A, *left* RCF+SAF; panel A, *right* Cont+SAF). B) No differences for the mean of USV duration emitted in the three conditions (Clean, Homecage, SAF) for RCF+SAF mice pups (Panel A, *left*) neither for Cont+SAF mice pups (panel B, *right*).

Differently by other early adversities models affecting the maternal behavior, RCF does not impact maternal care (D'Amato et al., 2011). In order to assess if the maternal behavior of adoptive mothers was affected by SAF presence, especially during the four days of co-caring, we observed and analyzed the maternal behavior (Nursing, Grooming/Licking, MB) of both adoptive mother (AM; or the biological mother for Control pups) and SAF.

From PND1 to PND4, both the AM and the SAF took care of pups in the control as well as in RCF condition. There is no difference in the mean of total maternal behavior (MB) from PND1 to PND4 between AM and SAF (Fig. 8A; F(1,26)=0,008; p=ns). Upon days, consistently with literature, the maternal behavior varied but the analysis didn't show any significant interaction between condition and the days for MB of the AM (Fig.8B; F(1,11)=1,865; p= ns).

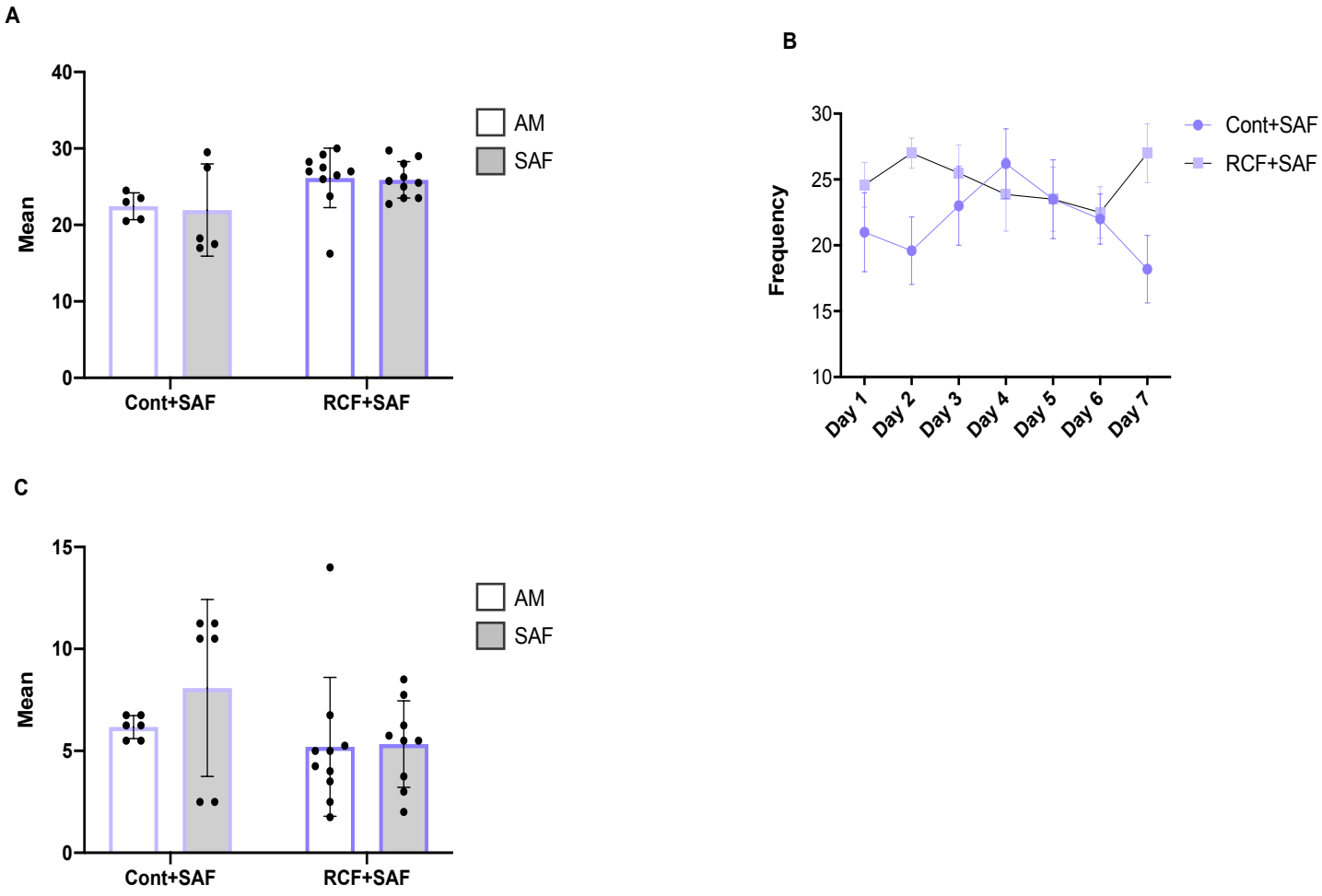

Fig.8 SAF presence during RCF did not alter the maternal behavior. A) Mean of maternal behavior from PND1 to PND4 shown by adoptive mother (AM) compared with the Stable Attachment Figure (SAF) in both Cont and RCF

condition. No difference was evident between the two conditions. B) Frequency of AM maternal behavior during the week of observation. No differences in the amount of maternal was evident between RCF+SAF and Cont+SAF groups. C) Mean of other behaviors (not directed to the pups) from PND1 to PND4 of AM and SAF in Cont and RCF conditions. The analysis showed no differences between the Cont+SAF and RCF+SAF.

No significant difference was evident also in the Other behaviors (behaviors not directed to the pups and outside the nest, OB) between AM and SAF (Fig.8C; F(1,22)=0,377; p=ns).

Analyzing the maternal care from the litter' point of view, we observed a different amount of maternal behavior (MB) in both Cont+SAF and RCF+SAF conditions in comparison with preliminary data on RCF and Cont mice (Fig. 9). The pups that experienced the SAF from PND1 to PND4, received a significantly higher amount of care compared to RCF and Cont litters (F(3,21)= 77,613; p<0.0005). Interestingly and unexpected, post-hoc analysis showed an increase of care received in RCF+SAF compared to Cont+SAF litters (p<0.005) while, consistently with previously published data (D'Amato et al., 2011) we didn't observe any difference between RCF and Cont (p= ns; *preliminary data*).

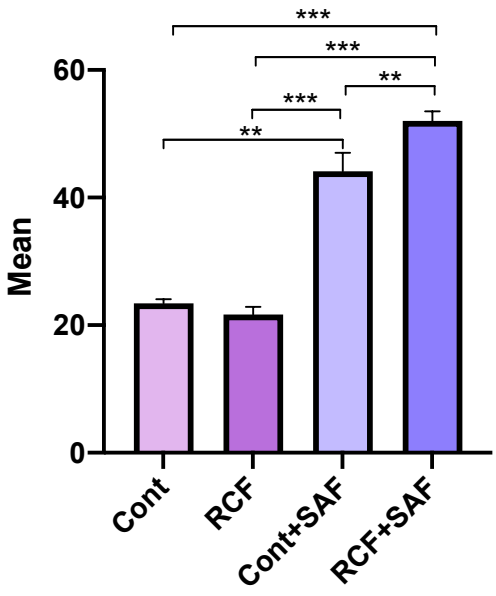

Fig.9 SAF experience during RCF increased the maternal care received. Mean of the total maternal behavior (Nursing/Grooming/Licking, MB) received from PND1 to PND4 by pups in the different experimental conditions (Cont, RCF, Cont+SAF, RCF+SAF). The analysis showed a significant increase of the MB received from pups of SAF condition (Cont+SAF and RCF+SAF) compared to Cont and RCF. In particular, RCF+SAF group received more care compared to the other conditions.

6.2.2 SAF presence rescues adult behavioral phenotype and increases pro-social behavior

DBA female mice of each group were tested in adulthood to assess if the SAF presence during RCF procedure was able to rescue the behavioral alterations induced by attachment bond interference.

One-way ANOVA used for the Open Field (OF) test didn't' show any difference in the time (%) spent exploring the external part of the apparatus in any groups (FIG.10A; F(3,24)= 1,414; p= ns) and in the moved distance (an index of locomotor activity) (Fig.10B; F(3,24= 0,637; p= ns). SAF presence did not impact anxiety-like behavior.

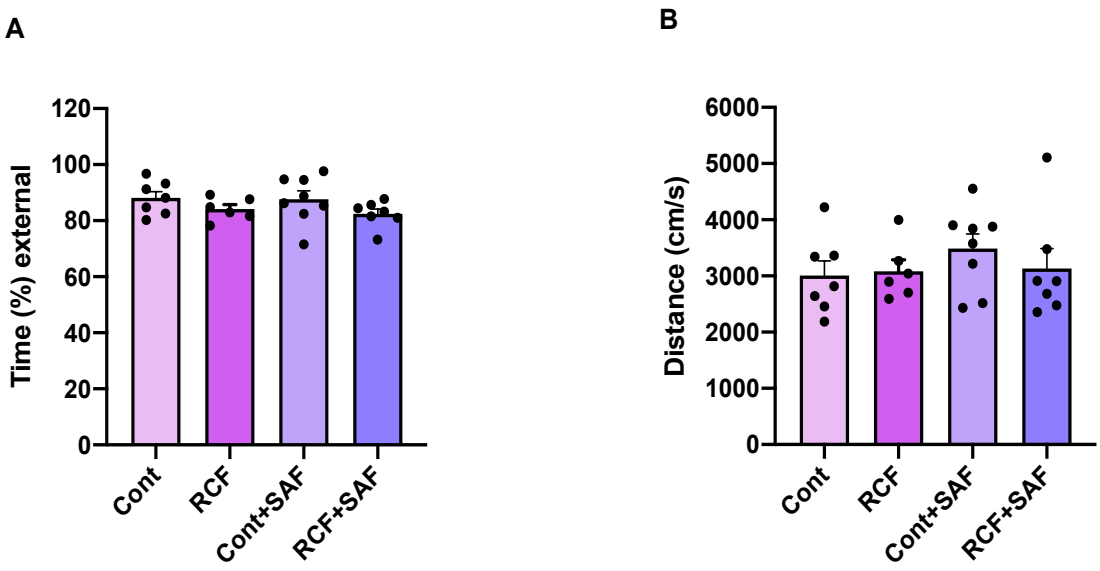

Fig.10 SAF presence during RCF did not affect anxiety-like behavior and locomotor activity in adulthood. A) Scatter-plot of the percentage of time spent exploring the external part of the Open Field (OF) apparatus showing any differences between Cont, RCF, Cont+SAF and RCF+SAF. B) Moved distance (cm/s) in the OF apparatus during the first five minutes of test. The analysis showed no significant differences between the experimental conditions (Cont, RCF, Cont+SAF, RCF+SAF).

The passive coping strategies were measured by Forced Swimming Test (FST) and Tail Suspension Test (TST).

One-way ANOVA was used to analyze the total immobility time in FST, showing a significant treatment difference (Fig.11A; F(3,28)=5.069; p<0.01). As previously published (lo Iacono et al., 2021), RCF mice showed increased time spent in immobility compared to their control (p<0.005),

confirming increased passive coping strategies induced by manipulation. Interestingly, when exposed to SAF during RCF, DBA mice decreased the time spent in immobility. RCF+SAF, in fact, they showed a significantly lower passive coping than RCF ($p<0.005$) group reaching the Cont group levels (p=ns). Cont+SAF mice also showed less immobility than RCF group ($p<0.05$) and didn't differ from both Cont and RCF+SAF (p=ns).

In TST, one-way ANOVA showed a significant treatment effect (Fig.11B; $F(3,21)=5.512$; $p<0.01$). In particular, RCF mice showed the increased passive coping, an index of depressive-like behavior, spending significantly more time in immobility compared to Cont ($p<0,005$). Consistently, RCF+SAF mice decreased the time spent in immobility compared to RCF ($p<0,01$), showing more active coping strategies, and didn't differ from control mice (p=ns). Finally, Cont+SAF group didn't show any difference compared to other groups (p=ns).

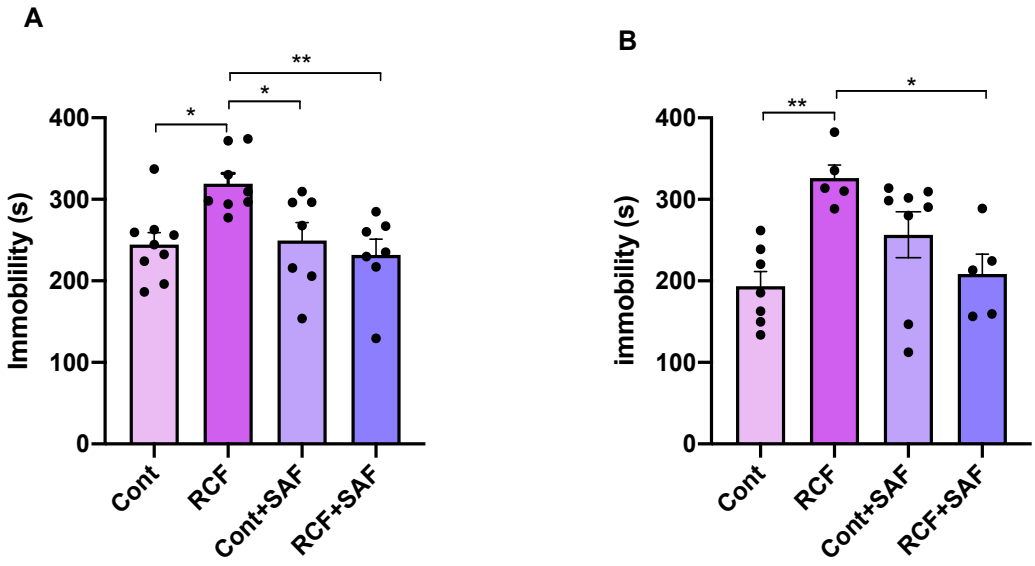

Fig.11 SAF presence during RCF rescued adult behavior reducing passive coping strategies. Scatter-plots showing the time spent in immobility in Forced Swimming Test (panel A) and Tail Suspension Test (panel B) showing the significant reduction of immobility in RCF+SAF in both tests. A) Significant increase of the immobility in RCF mice compared to Cont, Cont+SAF and RCF+SAF. No significant difference was evident between RCF+SAF, Cont+SAF and Cont mice. B) The analysis showed a significant increase of immobility in RCF mice compared with Cont and RCF+SAF mice, while no differences were evident in Cont+SAF compared to other groups.

Finally, animals were tested in Social Interaction Test (SIT) to evaluate the possible effects of SAF experience on social behavior. Previously published data didn't show negative effect of RCF on social behavior (Lo Iacono et al., 2021). However, our interest was to evaluate if the introduction of another source of care, which was proved to actively taking care of the pups, could implement the prosocial behavior of mice, regardless of Cont and RCF condition.

One-way ANOVA for repeated measure showed a significant effect for the time spent exploring the chamber containing the social stimulus (co-specific animal) than the chamber containing the object (Fig. 12A; $F(3,24)=25.616$; $p<0.0005$) for all the groups. Analyzing the quality of the interaction (time of contacts), we found an increased number of interactions with the social stimulus in all the groups (Fig.12B; $F(3,25)=7,253$; $p<0.005$) with a significant interaction condition x pairing ($F(3,25)=3.372$; $p<0.05$). Post-hoc for the time spent interacting with the social stimulus showed a significant SAF effect ($F(3,26)=5.917$; $p<0.005$). RCF+SAF group showed increased number of contacts compared to RCF ($p< 0.01$) and Cont ($p<0.0005$) and, interestingly, Cont+SAF mice also showed increased contacts with the social stimulus compared to their control group (Cont, $p<0.01$). These data suggest increased prosocial behavior induced by SAF, regardless of condition (Cont, RCF; Fig.12C).

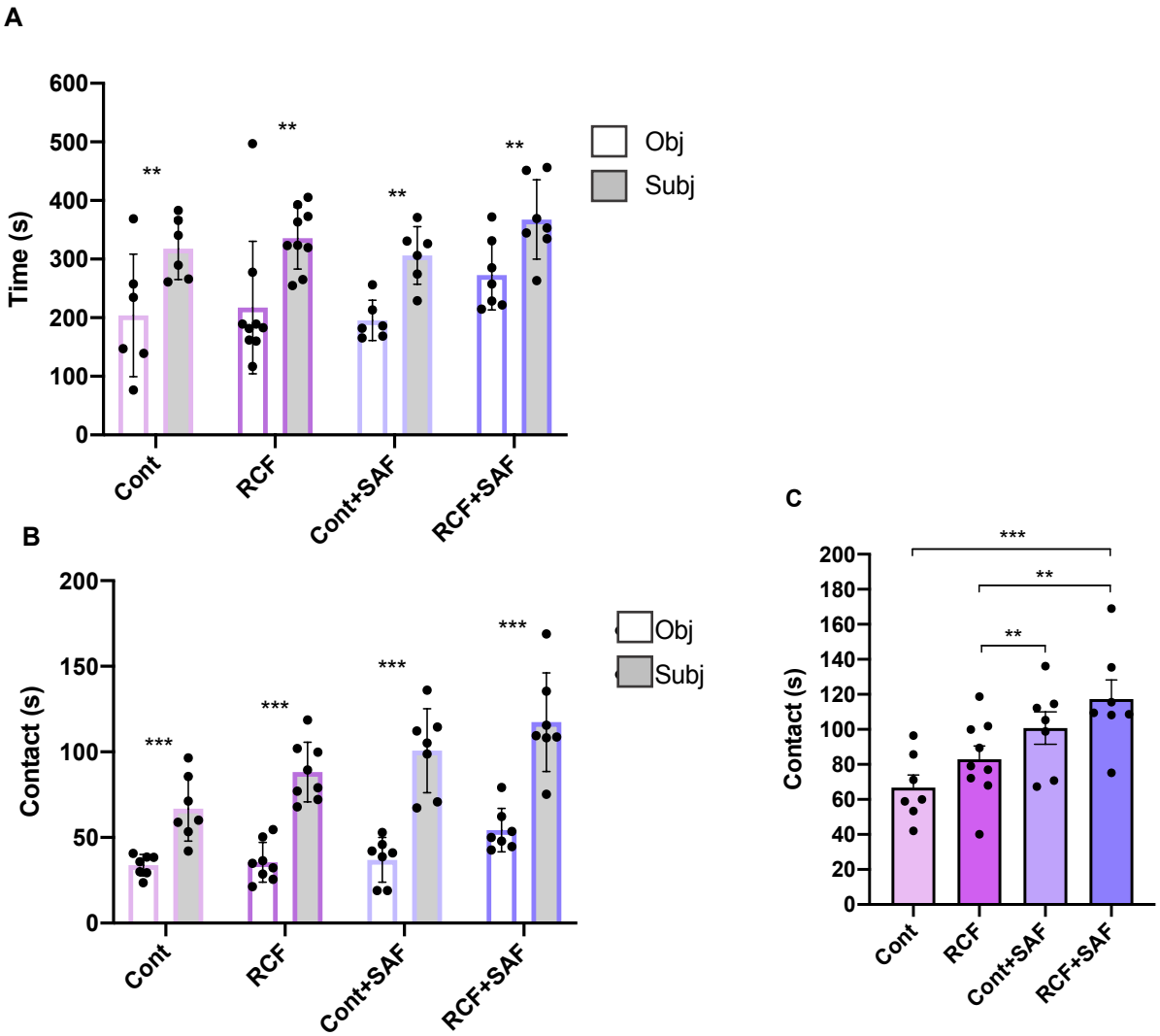

Fig.12 SAF presence during RCF increased prosocial behavior in adulthood. A) Significant difference in the time spent exploring the chamber containing the social stimulus (co-specific mice; Subj) compared with the neutral object (Obj) were evident for all the groups (Cont, RCF, Cont+SAF, RCF+SAF) with no significant differences between them. B) Time of direct interaction with Subj or Obj (contact). The analysis showed a significant increase in the time spent interacting with the Subj compared to Obj for all the groups (Cont, RCF, Cont+SAF, RCF+SAF) with a significant differences between them showed in panel C. C) Post-hoc analysis of the time spent interacting with the Subj between the groups showed a significant increase of contact in both RCF+SAF and Cont+SAF mice compared to Cont and RCF. No differences were evident between Cont+SAF and RCF+SAF.

6.2.3 SAF presence rescues the I_h current density of VTA DAergic neurons

From an electrophysiological point of view, in the previous section, we have demonstrated that in DBA adult females exposed to an early life manipulation altering attachment bond (RCF), DA (TH^+) neurons of the intermediate VTA show a selective, significant potentiation of the I_h current density. This observation fits well with our working hypothesis for a correlation between early aversive life events (RCF), long-term alteration of I_h current of iVTA DA neurons and modulation of the depressive-like behavior in adulthood. Importantly, our new data on DBA mice adds up with our published data on C57 mice (D'Addario et al 2021) indicating a role for the individual genetic background on the RCF protocol outcome on both adult behavior and relevant neuronal functional modulation (I_h).

To pin down the involvement of the maternal bonding within the latter observations, here we attempted to positively interfere with the RCF effects by introducing a Stable Attachment Figure assisting the RCF litters. According to our working hypothesis, we expected the presence of the SAF to elide the RCF effect on the neurophysiological parameters investigated insofar. Thus, we performed voltage-clamp (and current-clamp) characterizations of the DA neurons of the iVTA from RCF+SAF in full analogy with what described above for RCF *vs* Cont groups (Fig. 2 – 5, Chapter 5).

Similarly to what previously described, the introduction of the SAF did not alter the intrinsic properties of these neurons, confirming that brain slice patch-clamp experiments were performed on cells in physiological conditions. In details, for the membrane 'resting' potential we found: Cont+SAF – 56 ± 2 mV, RCF+SAF – 53 ± 2 mV; p= ns, t = 1.053, df = 23.49 (21 and 15 neurons, respectively). For the membrane time constant: Cont+SAF 1.7 ± 0.2 ms, RCF+SAF 2.2 ± 0.2 ms; p= 0.1929, t = 1.340, df = 23.89 (12 and 14 neurons, respectively). For the membrane capacity: Cont+SAF 56 ± 4 pF, RCF+SAF 61 ± 6 pF; p= ns (13 and 14 neurons, respectively). And for the

membrane resistance: Cont+SAF 210 ± 22 MOhm, RCF+SAF 232 ± 24 MOhm; p= ns, t = 0.6716, df = 23.83 (both 13 neurons). Mann-Whitney test was performed for C_m analysis; Welch's t-test was performed for all other parameters investigated.

As per neuronal excitability, similarly to what observed for purely-RCF animals, in RCF+SAF we found no alterations compared to the control group. Thus, spontaneous firing was present, as expected, in TH^+ DA iVTA neurons and appeared unaltered by SAF. In details, in whole-cell the average frequency of spontaneous AP firing was: Cont+SAF 1.2 ± 0.3 Hz and RCF+SAF 1.3 ± 0.4 Hz (9 and 8 neurons, respectively), Student's *t* -test (Welch): p= ns, t = 0.2277, df = 12.97. In line with what found for the spontaneous activity, the evoked excitability also appeared unaltered. Thus, for the rheobase: Cont+SAF 115 ± 31 pA; RCF 104 ± 27 pA (12 and 14 neurons); Mann-Whitney test: p= ns. Likewise, from the 2way-RM ANOVA analysis of the f – I curve populations, we found no differences between RCF+SAF and Cont+SAF (F (1,22) = 0.3966, p= ns; Cont+SAF, 14 neurons, RCF+SAF 10 neurons), with significant interaction between "RCF+SAF treatment" and "injected current" variables (F (7, 154) = 3.163, p<0.005), beside the expected significance of the "injected current" variable (F (7, 154) = 13.04, p < 0.0001). Last, also for the quantification of the maximum frequency of evoked firing, as predictable we found no differences between the groups: Cont+SAF 9 ± 1 Hz and RCF 7.1 ± 0.7 Hz (12 and 11 neurons, respectively), Student's *t* -test (Welch): p= ns, t = 1.229, df = 19.01.

Here, we wanted to verify whether the presence of a SAF could delete such RCF effect, thus returning the I_h current density to control levels. When studying the I_h current profile using voltage-clamp experiments, we found that in presence of the SAF the observed potentiation of the I_h was completely obliterated. This was clear both from the analysis of the RCF+SAF steady-state current elicited by the maximum V_{cmd} adopted (– 120 mV) and from the analysis of the RCF+SAF I – V relationships of the voltage-activated current, which appeared undistinguishable from controls (Fig. 12). The average I_h current density recorded at V_{cmd} – 120 mV from Cont+SAF neurons was – 0.7 ± 0.2 pA/pS and – 1.0 ± 0.3 pA/pS from RCF+SAF cells (both, 13 neurons; Mann-Whitney test: p= ns; Fig. 13A). Similarly, the 2-Way RM ANOVA analysis of the I–V curve populations for the experimental groups under study showed lack of statistical difference, both for the "RCF+SAF treatment" variable (F (1,22)= 0.4767, p= ns; Cont+SAF, n = 11, RCF+SAF, n = 13) and for the interaction between "RCF+SAF treatment" and 'V_{cmd}' (F (3,66) = 0.6330, p= ns), in addition to the predictable difference found for the 'V_{cmd}' variable (F (3,66) = 25.05, p< 0.0001; Fig.13B).

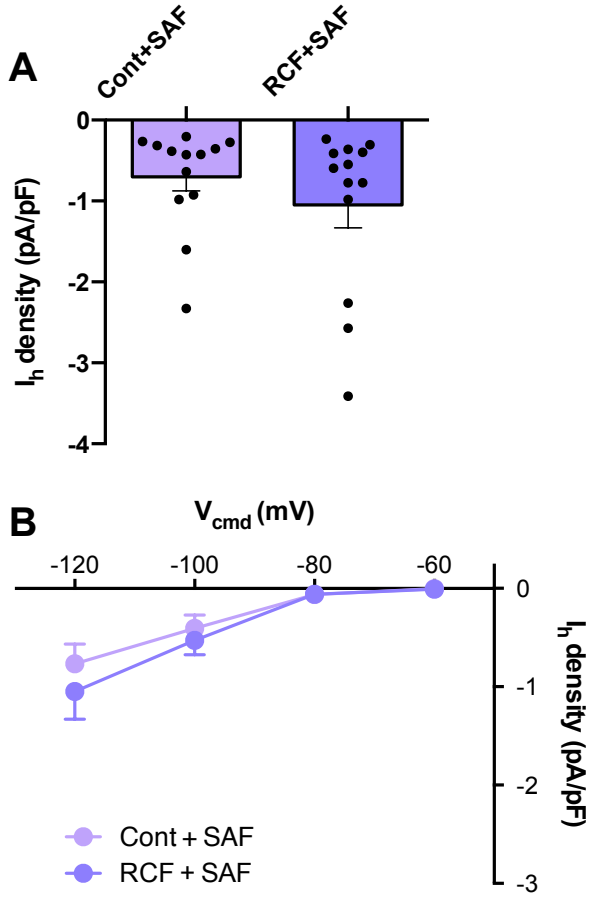

Fig.13 Lack of potentiation of the I_h current in iVTA DA neurons from DBA females in presence of a SAF during RCF. A) Scatter-plot of the I_h current density (pA/pF) in iVTA DA neurons estimated at the steady-state of the response (V_{cmd} – 120 mV) showing no difference across the experimental groups. B) Current density (pA/pF) – Command Voltage (V_{cmd}, mV), 'I – V' average relationships for the neurons investigated. The statistical analysis showed no differences between Cont+SAF and RCF+SAF groups (*The electrophysiological characterization was carried out by M Renzi; E Spoleti: G Chilà*).

Finally, comparing I_h current densities across experiments we could confirm that the presence of the SAF significantly reduced the I_h current density increase originally induced by the RCF protocol: at V_{cmd} – 120 mV, average I_h current density for RCF+SAF neurons was – 1.0 ± 0.3 pA/pS, whilst for RCF neurons it was found to be – 2.0 ± 0.3 pA/pS ($p < 0.01$, Mann Whitney test; respectively, 13 and 14 neurons; Fig.14). In accord with our hypothesis, RCF+SAF didn't differ from Cont group (p = ns, Mann Whitney test; 13 and 21 neurons; Fig. 14) or Cont+SAF (p= ns, Mann Withney Test; 13 neurons both), while Cont+SAF differ from Cont ($p<0.05$, Mann Withney Test; 13 and 21 neurons).

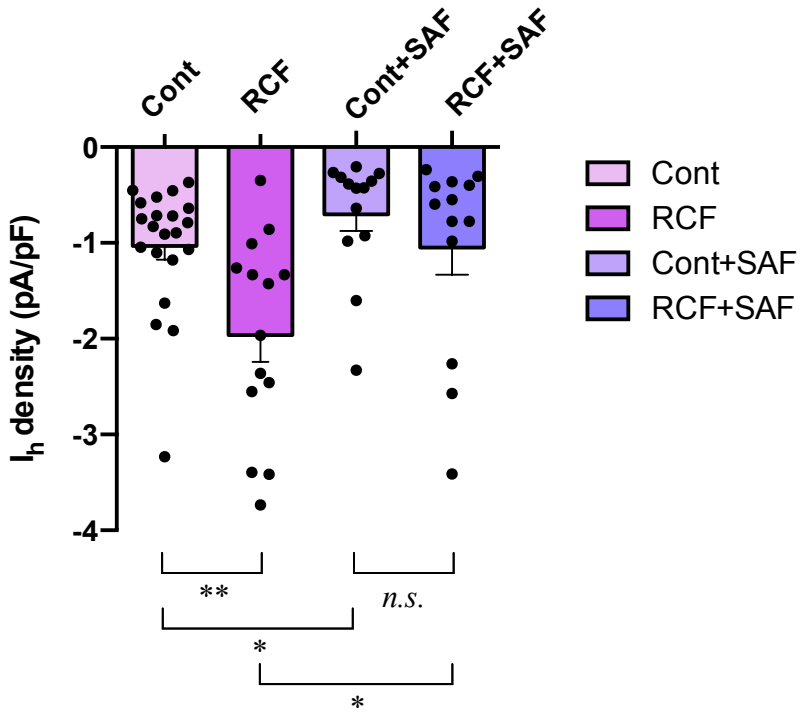

Fig.14 SAF presence during RCF restored the I_h current density of dopaminergic neurons of the intermediate VTA. A) Scatter-plot of the I_h current density (pA/pF) in iVTA DA neurons estimated at the steady-state of the response (V_{cmd} – 120 mV) showing the significant decrease of Ih current density in RCF+SAF group compared to RCF. No differences were evident between RCF+SAF and Cont or Cont+SAF, while the analysis showed a significant reduction of Cont+SAF compared to Cont mice.

7. Preliminary data and future directions

Overall, our results confirmed the role of the SAF who, by promoting an earned-secure attachment prevented the increased I_h current in iVTA DA neurons, paralleling what observed at the behavioral level. Despite our data confirm the initial working hypothesis, our results open more questions.

The first one is to verify whether the rescue effects observed in adulthood in RCF+SAF were driven by the I_h current within VTA DA neurons. In order to establish a causal link between I_h current modulation induced by SAF and its protective effects on behavior, we are planning to perform an intra-VTA treatment with 6-(2,3-Dichlorophenyl)-1,2,4-triazine-3,5-diamine (an I_h current potentiator, Lamotrigine; LTG) in adult RCF+SAF mice. Based on a previously published work (D'Addario et al., 2021), after five days of intra VTA treatment with LTG, we expect to revert the positive effects induced by SAF mimicking the RCF effects, by enhancing the I_h current. To test this hypothesis, mice will be tested in both TST and FST to evaluate the depressive-like behavior and I_h current in VTA DA neurons will be evaluated.

Our second question focus on understanding how RCF manipulation alters the physiology of VTA DAergic neurons, inducing severe consequences on behavior. Since we are altering the formation of the attachment bond during a sensitive time window, when the brain is characterized by high plasticity and sensitive to environmental perturbation, we expect that epigenetic mechanisms play an important role in the RCF effects.

We have previously demonstrated, thought a RNA sequencing experiment, that DBA RCF females showed a tremendously higher number of differently expressed genes (DEGs) in VTA, compared to RCF males of the same strain (Lo Iacono et al., 2021). In particular, RCF females presented 1009 DEGs compared to their control, while males only 2 DEGs. These results suggested that interfering with attachment bond during an early life period affects VTA transcriptomic pattern, and its related functions, in a sex-specific manner (Lo Iacono et al.,2021). This huge difference in DEGs between females and males could depend on presence of a sub-set of genes in females that are more prompt to respond to early environmental manipulation, such as RCF. We interpreted these genes as "plasticity genes" following Belsky suggestion (Belsky et al., 2009). These genes are altered by RCF manipulation and probably involved in adult behavior vulnerability to depressive-like behavior (Lo Iacono et al., 2021) shown by females. In agreement with this conclusion, current results strengthen the involvement of VTA in early adversities induced-alterations (depression), pointing it out it as one of the most important neurobiological substrate candidates. To better understand this point, here are starting analyzing the DEGs by classifying them into functional pathways that could help to understand the mechanisms underlying the relation between RCF, Depression and VTA.

Using QIAGEN Ingenuity Pathway Analysis (IPA), we found several important pathways involved and some of them, in line with our results, are connected to calcium activity or dopamine signaling (Tab.1). These pathways could be related to the physiological properties of VTA DA neurons, that are altered by RCF and rescued by SAF experience. Among the significant pathways showed in the Tab.1, we found the estrogen signaling pathway to be altered by RCF in DBA females.

As well know, estrogens are deeply expressed in the mesocorticolimbic system and are involved in DA regulation, together with androgens (Abuele and Kritzer, 2012; Eck and Bangasser 2020; Brown et al., 2015). Indeed, androgens end estrogens are strictly related and work together in regulating brain development during early stages. Estrogens and androgens are essential during development: perturbations that alters them could affect their organizational effects with long-lasting consequences on brain functions (Eck and Bangasser 2020). Not surprisingly, aversive events could impact gonadal

hormones and their receptors, altering DA system consequently, due to their involvement in programming brain development during the sensitive period (Eck and Bangasser 2020).

Estradiol role, in fact, starts very early in development, during the embryonic phase, driving DA neurons differentiation and continues during early stages of life by modulating DA synthesis (Kipp et al., 2006; Varshney and Nalvarte, 2017). Moreover, estrogens are involved in DA neurons activity regulation, modulating the firing rate (Di Paolo, 1994; Dazzi et al,, 2007). Similarly, androgens are involved in brain regulation, especially influencing DA neurotransmission and firing (Di Paolo, 1994; Sotomayor-Zárate et al., 2014) within the mesocorticolimbic system. Interestingly, both preclinical and clinical studies suggest the involvement of gonadal hormones impairment as a potential cause for depression (Shors and Leuner, 2003; Albert, 2015).

Canonical pathways	p value
Oxidative Phosphorylation	9,17E-13
Mitochondrial Dysfunction	1,86041E-10
GP6 Signaling Pathway	1,07004E-07
Dopamine-DARPP32 Feedback in cAMP Signaling	5,38477E-05
Sirtuin Signaling Pathway	0,000191818
Protein Kinase A Signaling	0,000410147
Estrogen Receptor Signaling	0,000494549
Axonal Guidance Signaling	0,000621871
Phosphatidylglycerol Biosynthesis II (Non-plastidic)	0,001216856
D-myo-inositol-5-phosphate Metabolism	0,001518423
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	0,002965676
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	0,002965676
Semaphorin Signaling in Neurons	0,003026182
Opioid Signaling Pathway	0,003507288
Netrin Signaling	0,003769578
RhoA Signaling	0,003786104
Endocannabinoid Neuronal Synapse Pathway	0,00418353
Regulation of Actin-based Motility by Rho	0,004245744
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0,004450473
CDK5 Signaling	0,004595671
CDP-diacylglycerol Biosynthesis I	0,004886066
3-phosphoinositide Degradation	0,006157631
Sphingosine and Sphingosine-1-phosphate Metabolism	0,006590121
Sucrose Degradation V (Mammalian)	0,006590121
Aspartate Biosynthesis	0,00794607
Glutamate Degradation II	0,00794607
Semaphorin Neuronal Repulsive Signaling Pathway	0,008626247
nNOS Signaling in Skeletal Muscle Cells	0,010687254
Calcium Signaling	0,011569551
3-phosphoinositide Biosynthesis	0,012939674
Gap Junction Signaling	0,012939674
Superpathway of Inositol Phosphate Compounds	0,01305662
Cardiac β-adrenergic Signaling	0,013109804
RhoGDI Signaling	0,013350918
Senescence Pathway	0,014133656
Dopamine Receptor Signaling	0,014968619
L-cysteine Degradation I	0,01534038
Synaptic Long Term Potentiation	0,015874154
Corticotropin Releasing Hormone Signaling	0,020135776
Synaptic Long Term Depression	0,020280202
Integrin Signaling	0,021588173
cAMP-mediated signaling	0,021797727
Salvage Pathways of Pyrimidine Ribonucleotides	0,022114209
RAR Activation	0,023259037

Tab.1 List of the pathways clustering the DEGs altered by RCF in DBA/2J female mice obtained using QIAGEN Ingenuity Pathway Analysis (IPA). The table shows only the significant pathway in order of p-value.

Finally, strong evidence supports the relation between gonadal hormones and Oxytocin (OXT) (Insel et al., 1993; Tokui et al., 2021), the principal neuropeptide involved in the attachment bond formation. OXT plays an important role in hormones regulation and as previously described, is involved in DA system regulation (Baskerville and Douglas, 2010). OXT is able to excites VTA DA neurons increasing firing and DA release in the target areas (Xiao et al., 2018). Not less important, it was suggested a possible role for OXT in modulating Ih current (Tang et a., 2014; Liu et al., 2022),

although the mechanism is not yet completely known. Due to these evidences, we decided to conduct a preliminary experiment to investigate a possible relation between attachment bond alteration induced by RCF, estrogens, androgens and OXT receptors in order to clarify the potential mechanisms altered by RCF responsible for adult psychopathological outcomes.

Through rt-qPCR we analyzed gonadal hormones and OXT receptors mRNA levels on PND5, immediately after RCF manipulation, and in adulthood in both RCF and Cont DBA mice. Moreover, we performed the same analysis in adult RCF+SAF mice, in order to investigate whether the presence of a stable caregiver during RCF was able to prevent the detrimental effects induced by this manipulation.

7.1 Methods

7.1.1 Animals

DBA/2J female mice of 5 days old were used for young receptor assessment, 10–12 weeks old were used for adult one.

7.1.2 RCF

Both Repeated Cross Fostering (RCF) and Repeated Cross Fostering with Stable Attachment Figure (RCF+SAF) were performed as previously described (see Experiment 1, Methods 5.2 and experiment 2, Methods 6.2).

7.1.3 Tissue Isolation and RNA preparation

For mice pups analysis, RCF and Cont brain were collected at PND5 and half brains were stored at -80°C. 10–12 weeks old RCF, Cont, RCF+SAF and Cont+SAF female DBA mice were scarified by cervical dislocation and dissected brains were stored at -80 °C. Bilateral punches of VTA were obtained from coronal slices, following Franklin and Paxinos atlas coordinates, no thicker than 300 um using stainless-steel tube (0,5 mm diameter) and stored at -80 °C before the assay. RNA from punches or from half-brain were isolated using Total RNA purification Plus Kit (Norgen Biotek, Thorold, Canada) and RNA quantity was determined by absorbance at 260 nm with NanoDrop UV-VIS spectrophotometer.

7.1.4 rt-qPCR

Random complementary DNA sequences were obtained using Hight Capacity Reverse Transcriptin Kit (Applied Biosystem, Branchburg, NJ, USA) for the reverse transcription of mRNAs: Estrogen alfa (ERa), beta (ERb), androgen (AR) and oxytocin (OXT). Around 10 ng of cDNA templates were amplified by qPCR with Taqman tenchology, using 7900HT thermal cycler apparatus equipped with the SDS software version 2.3 (Applied Biosystems) for data collection. Cd values were normalized to averaged measures of Tata Binding Protein (TBP, Taqman assay ID Mm00446973_m1) for Era (ID Mm00433149_m1), ERb (ID Mm00599821_m1), AR (ID Mm00442688_m1) and OXTr (ID Mm01182684_m1). Data were run in triplicate and were expressed as Relative Expression, according to $\Delta C(t)$ method.

7.1.5 Statistics

One-way ANOVA (treatment, 4 levels: Cont, RCF, Cont+SAF, RCF+SAF for adult mice; treatment, 2 levels: Cont, RCF for pups) was used to analyze the relative mRNA levels of AR, ER and OXT receptors.

7.2 Results

7.2.1 **RCF manipulation affects androgens and oxytocin receptors mRNA levels in mice pups**

Preliminary results from half-brain receptors mRNA levels in RCF and Cont female mice pups are showed in Fig.15. One-way ANOVA showed a significant reduction of AR mRNA levels in RCF female pups compared to control (Fig. 15A; $F(1,7)= 6,64$; $p<0.05$). Decreased mRNA levels were evident also for OXT receptor (Fig.15B; $F(1,7)=12,279$;$p<0.01$). No significant effect was detected

for both Era and ERb levels (Fig.15C-D; ERa: F(1,8)=3,744; p=ns; ERb: F(1,9)=0,096; p=ns).

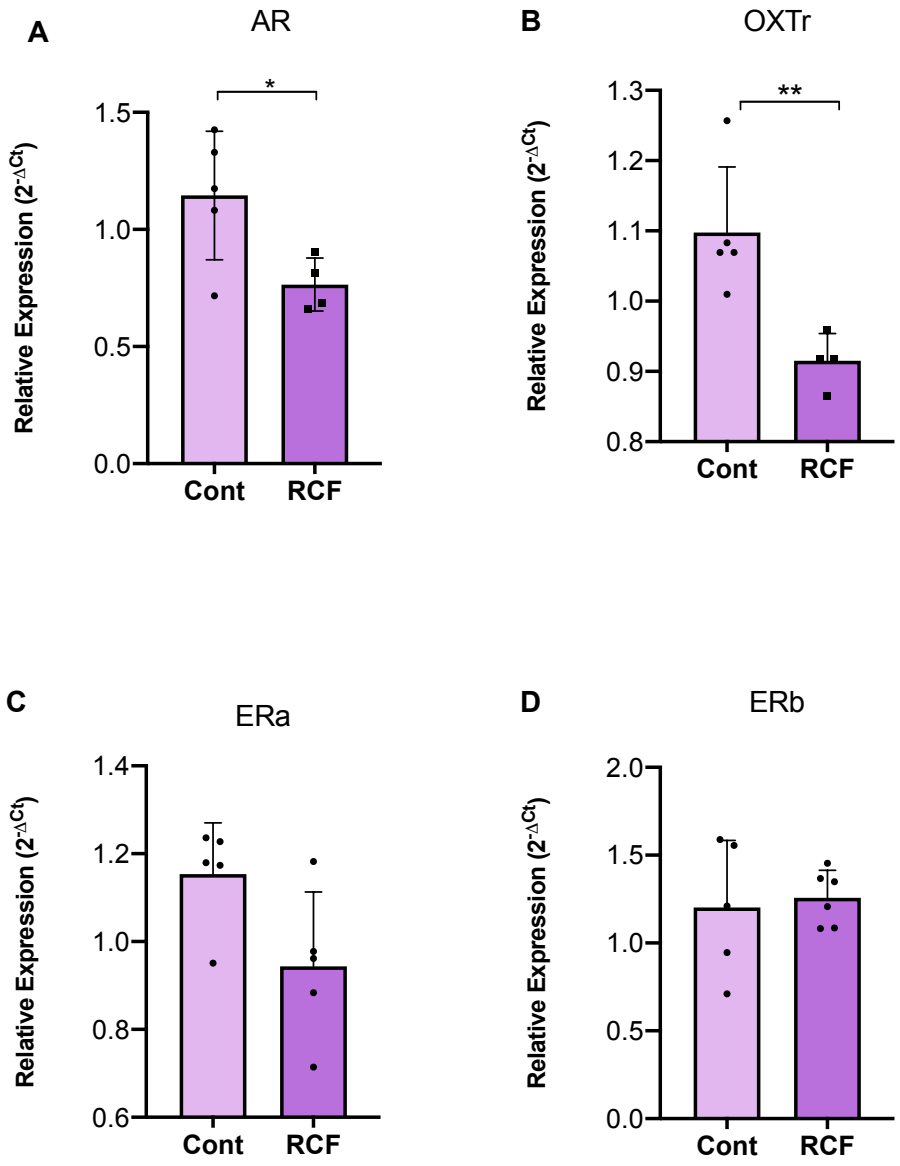

Fig.15 RCF affects mRNA levels of Androgen and Oxytocin receptor in mouse pups. Scatter-plot showing the mRNA levels ($\Delta C(t)$) of half-brain of RCF and Cont mice at PND5. The nalysis showed significant decrease in RCF mice vs Cont for Androgen (panel A) and Oxytocin (panel B) receptor mRNA levels. No difference was evident for Estroge alfa (panel C) and beta (D) between RCF and Cont.

7.2.1 SAF rescues androgens receptor mRNA levels in Ventral Tegmental Area of adult mice

Based on the preliminary results obtained on PND5, we decided to analyze the targets we found altered (AR and OXTr) in the VTA of adult RCF and Cont female mice through rt-qPCR. The analysis was performed also in RCF+SAF and Cont+SAF, to evaluate if experiencing SAF during RCF have prevented possible alterations.

One-way ANOVA showed a significant treatment effect (Fig.16; $F(3,14)= 3,413$; $p<0.05$). In particular, RCF mice showed increased mRNA levels of AR compared to Cont ($p<0.05$), Cont+SAF ($p<0.05$) and RCF+SAF ($p<0.05$). Importantly, experiencing SAF during RCF was able to prevent this increase. No difference, in fact, was evident between Cont, RCF+SAF and Cont+SAF (p=ns)

The analysis showed a significant treatment effect for OXTr mRNA levels ($F(3,23)= 4,916$; $p<0.01$). No significant difference was evident between RCF and Cont DBA female mice (p=ns). One-way ANOVA showed a significant increase of OXTr mRNA levels in both RCF+SAF and Cont+SAF compared to both Cont and RCF (RCF+SAF vs Cont $p<0.05$; RCF+SAF vs RCF $p<0.01$; Cont+SAF vs Cont $p<0.05$; Cont+SAF vs RCF $p<0.01$), suggesting a general effect of the SAF.

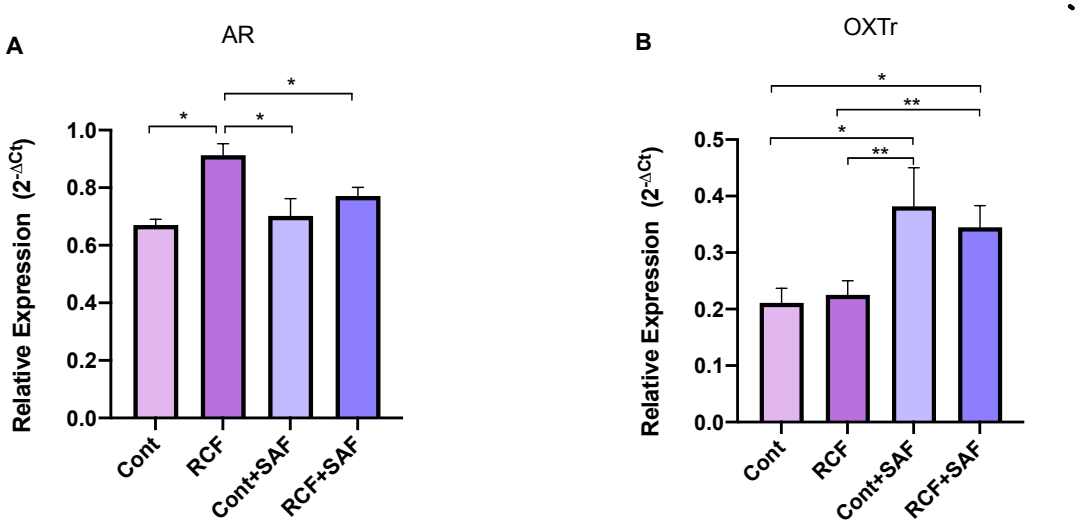

Fig.16 SAF presence rescued AR mRNA levels and increased OXTr mRNA levels in VTA of adult mice. A) Significant difference of mRNA levels of Androgen receptor (AR) of RCF females compared to Cont, Cont+SAF and RCF+SAF. B) No significant difference in mRNA levels of Oxytocin receptor (OXTr) between Cont and RCF. Both Cont+SAF and RCF+SAF showed a significant increase compared to Cont and RCF.

These preliminary results indicate alterations of AR mRNA levels in VTA induced by RCF manipulation, that seem to be prevented by SAF experience. A "SAF effect" was evident for OXT receptor. Further studies will evaluate more deeply these alterations also investigating possible receptors alterations in other aeras of the mesocorticolimbic pathway involved in pair bonding, such as NAc, mPFC and Amy.

8. Discussion

The early postnatal period represents a sensitive time window particularly vulnerable to environmental factor (Burggen and Mueller, 2015) that can lead to negative outcomes later in life, such as depression. Indeed, experiencing negative events and instability during this sensitive developmental period represents one of the major risk factors for developing depression in adulthood (Kauffman et al., 2000; Burns et al, 2018). In all mammalian species, the first environment is represented by caregivers (in general, parents). Beside taking care of the infant, caregivers also provide emotional support allowing the offspring to establish a secure attachment bond (Bowlby, 1988; Atwool, 2007). Upon early development, the attachment bond plays a critical role for the offspring's future development (Rice et al., 2008; Azevedo et al., 2010) and any perturbation of it may have severe long-lasting consequences (Tractenberg et al., 2016), including depression. In order to understand how interfering with attachment bond formation could lead to maladaptive behavior, to investigate the underlying mechanisms involved is mandatory. Previous studies showed a link between attachment bond and development of the dopaminergic (DA) systems (Rincon-Cortes and Grace, 2020; He et al., 2021) which is particularly vulnerable to early life experiences (Dinopoulos and Parnavelas, 1991). Interestingly, it was also demonstrated an alteration of the DA system in early life adversities-induced depression (Belujon and Grace, 2017; Kumar et al., 2018). The mesocorticolimbic pathway plays has an important role in mediating reward processing and is involved in stress response (Neslter and Malenka, 2003; Berridge, 2007). This system extends from the Ventral Tegmental Area (VTA) to Nucleus Accumbens (NAc), Prefrontal Cortex (PFC) and Amygdala. The VTA represents the key area of the DAergic system and, interestingly, its projections to brain regions such as Amygdala, NAc and lateral septum have been implicated in the formation of attachment bond (McCormick et a., 2019; Rincon-Cortes and Grace, 2020). The DA neurons of VTA are regulated by an excitatory/inhibitory synaptic balance and by electrophysiological characteristics, such as hyperpolarization-activated cation current (Lammel et al., 2008; Shi, 2009). The I_h current represents a hallmark of DA neurons and is mediated by hyperpolarization-activated cyclic nucleotide gated (HCN) channels (Krashia et al., 2017), widely expressed in the mesocorticolimbic system

(Notomi and Shigemoto, 2004). As matter of fact, I_h current has been proved to be involved in depression (Friedman et al., 2014; Masrouri et al., 2020; D'Addario et al., 2021). Rodent models found an alteration of this current in mice showing a depressive-like phenotype after stress exposure (Zhong et al., 2018), as well as in resilient to depression mice (D'Addario et al., 2021). Not less important, I_h current is also affected by early life adversities exposure (Masrouri et al., 2020; D'Addario et al., 2021). A recent work unrevealed a possible link between attachment bond interference and depression, by using the Repeated Cross Fostering (RCF) manipulation. RCF manipulation affects the formation of attachment bond between dam and the offspring by inducing an unstable environment (D'Amato et al., 2011; Di Segni et al., 2016; Lo Iacono et al., 2021). This procedure is known to have negative consequences in adulthood depending on the genetic background. C57BL/6J (C57) female mice exposed to Repeated Cross Fostering (RCF) during the first days of life, showed a pro-resilient to depression behavior, as well as reduced density of I_h current in neurons of the intermediate VTA (D'Addario et al., 2021). In the same work, control adult female mice repeatedly treated with a potentiator of I_h current intra-VTA developed a RCF-like behavior, providing a link between I_h current in the VTA and depression-like phenotype. We have previously demonstrated that RCF is able to induce an opposite adult phenotype in DBA/2J (DBA) female mice: RCF DBA females showed increased passive coping strategies and depressive-like behavior in adulthood (Lo Iacono et al., 2021). In order to better understand the link between I_h current and depression, here we decided to evaluate if DBA female mice that underwent to RCF manipulation showed also an opposite alteration of I_h current. We first evaluated the intrinsic proprieties of VTA DA neurons of the iVTA in RCF animals, and we did not find any differences induced by RCF when compared to controls. These results are consistent with the lack of alteration of the spontaneous activity of DA neurons from RCF C57 female mice reported in D'Addario *et al*., 2021. We then evaluated the spontaneous and the evoked firing of the VTA DA neurons, which is involved in DA release in the projection areas, in both cell-attached and in whole-cell configuration.

Overall, differently from what observed in C57 mice, in DBA adult females the attachment bond interference modelled by the RCF did not induce a persistent alteration of cell excitability in DA neurons of the iVTA. Finally, we quantified I_h current density and the current-voltage relationship (I-V curve) in DBA females mice exposed to RCF compared to their controls. In line with our hypothesis, we found a significant increase of I_h current density in DBA RCF female mice, confirming a correlation between RCF-dependent adult behavior and I_h current modulation. As described above, there is an apparent causal link between early aversive life events (RCF), long-term alteration of I_h current in iVTA DA neurons and modulation of the mood behavior in the individuals' adult life. Such

link appears to be of some relevance, as it is preserved across animal strains: when comparing the behavioral and electrophysiological outcome of RCF in C57 and DBA adult females (D'Addario et al., 2021; present data), we found opposite results. Importantly, our new data on DBA mice adds up with previously published data on C57 mice (D'Addario et al., 2021) indicating a role for the individual genetic background on the RCF outcome on both adult behavior and relevant neuronal functional modulation (I_h). So far, our results point to a pivotal role of the correct formation of the attachment bond during early life period in preventing long-lasting impairment on behavioral and electrophysiological outcomes.

To allow a secure attachment with the offspring, caregiver has to embody a secure basis (Bowlby, 1969). When the principal caregiver, like the parents, fails to become it, caregivers other than parents could provide the necessary support (Saunders et al., 2011) for a proper brain development. Earned-attachment figures embodying alternative caregiving figures, that allow the correct formation of the attachment bond and act as protective factors. Alternative caregivers could, in fact, prevent the negative consequences induced by an incorrect attachment bond formation. Who could represent an alternative caregiver for a child? Someone close to the family, such as a grandparent, a big brother or a step-parent; either someone external to the family, such as friend's parent, a teacher, a neighbor or a social assistant (Saunders et al., 2011). Although clinical studies reported a significant positive effect of the alternative caregiver in reorganizing the previous relational experiencing and in mediating future ones (Saunders et al., 2011; Cook et al., 2006), no pre-clinical model has, to date, been proposed. Consistently, there are no evidence for possible mechanisms underlying the protective effects of the earned-attachment. To fill this gap, we propose a model of alternative caregiver figure exploiting our well-established model of attachment bond interference (RCF) to try to prevent its negative consequences. During the RCF manipulation, we add a non-lactating virgin female that remained with the pups while exchanging adoptive mothers acting as a Stable Attachment Figure (SAF). We hypothesized that SAF, embodying the earned attachment, could prevent the alterations induced by RCF during a very sensitive time window. Fist, we assessed if SAF presence was able to restore the attachment in female mice pups recording and analyzing the distress Calls (USV's). USV calls are emitted with different purposes in different circumstances. For example, pups are usually calmed by the odor of the mother and when exposed to stranger's odors, like adult males, pups drastically decrease the calls and stop them (Sungur et al., 2016). Evolutionary, this allow them to not get caught when a situation is experienced as dangerous. On the other hand, when exposed to an odorless, potentially dangerous environment, pups increase their calls to reach their mother. Indeed,

in accord with previously published data (Lo Iacono et al., 2021), Cont mice emitted a higher number of USVs when exposed to a clean bedding material compared to the home-cage one, that had calming effects on them. On the other hand, RCF mice showed higher number of USVs regardless of the condition: RCF are, in fact, unable to benefit of the presence of the mother cues (Lo Iacono et al., 2021; current data). According to our hypothesis, mice pups that experienced SAF during RCF showed an opposite pattern in comparison with RCF mice. SAF presence positively affected pups that emitted a lower number of vocalizations in the home-cage that in the clean, potentially dangerous, one. Moreover, when tested in SAF bedding material mouse pups emitted an higher number of USVs compared to the home-cage condition, but less than the clean one, indicating that SAF was recognized by pups. The rescue effects of SAF could be related to the increased levels of care that pups received during the first four days of life. In fact, if we compare the amount of care that pups in SAF condition (both Cont+SAF and RCF+SAF) received with RCF and Cont mice, we noticed an increased amount of care due to the presence of both adoptive mother and SAF. This result was consistent with other studies in literature using another source of care as environmental enrichment (Garbugino et al., 2016; Middei et al., 2022). Importantly, observing the maternal behavior we didn't note any difference in care between RCF+SAF and Cont+SAF, indicating that SAF presence didn't alter the adoptive mother behavior (reffered as the biological for the Cont+SAF confition): both took care of the pups independently of the condition (RCF or Cont). Therefore, an additional, stable, source of care during the first four days of life positively affected mouse pups contrasting the RCF effects and, in turn, rescuing the adult behavioral alterations. Adult RCF+SAF female mice showed less passive coping strategies compared to RCF mice, confirming the positive effect of the earned-secure attachment in preventing the long-lasting negative consequences. Since we were adding a social stimulus during a sensitive time window, we decided to also test the animals in Social Interaction Test to evaluate if the additional source of care could have impacted the sociability. Although RCF did not affect social behavior in DBA female mice (Lo Iacono et al, 2021; present data), RCF+SAF showed an increased pro-social behavior compared to the other group and, not surprisingly, the pro-social behavior of Cont+SAF increased too. While for RCF+SAF the additional care compensated for maternal instability, Cont+SAF benefited from double source of care in absence of any attachment bond interference. We could interpret the SAF as an environmental enrichment, that, providing additional care, was able to increase the sociability.

Finally, we asked which could be the mechanisms involved. Since RCF induced a long-term VTA DA neurons alteration, strongly related to the behavioral phenotype, we decided to evaluate whether SAF prevented this alteration. As for RCF and Cont, SAF presence did not induce modifications with

respect to cell excitability in VTA DA neurons and RCF+SAF did not differ from Cont+SAF group. The relevance of the maternal bond in these long-term, neuronal alterations is demonstrated by the observation that the introduction of the SAF during the development of RCF DBA mice rescued the RCF-induced alterations. In according to our behavioral results showing a rescue, RCF+SAF mice showed a decreased I_h current density compared to RCF mice, reaching the Cont level. Unexpectedly, Cont+SAF female mice showed a reduction of I_h current even more marked compared to their control mice. Again, as for the social behavior, we interpreted this data as a result of the increased maternal care received during early life period that may be related to the prosocial behavior. Further studies are needed to investigate this point and to evaluate the role of the additional source of care during the first four days of life.

But what causes the I_h current alteration observed in iVTA DA neurons of RCF females? One possible explanation for the I_h current density increase could be the enhanced expression of the HCN channels mediating this current. However, already in D'Addario et al 2021 (where a decrease of the current was observed in C57 RCF adult females, as discussed) no alteration in the total protein for the most abundant channel subunit expressed (HCN2) was found in the VTA. Although we cannot rule out this possibility, results from our previous work prompted us to hypothesize different, not related to HCN expression, mechanisms underlying the I_h current potentiation observed in DBA mice, such as different inter-neuronal and intracellular signaling pathways. Further experiments will be performed to investigate this possibility. To establish a direct cause-effect relationship between I_h current and behavior in our animal model, we also plan to carry out an experiment aims to increase (by repeated infusion of a I_h current potentiator, Lamotrigine) the I_h current in VTA DA neurons of RCF+SAF animals at the levels observed in RCF mice. If our hypothesis is correct, this treatment should be reverting the behavioral and electrophysiological positive effects induced by SAF, providing a causal link between attachment bond alteration, earned attachment, I_h current and depression.

Lastly, we still have one last question left to answer: how does RCF alter VTA DA neurons physiology? Several studies reported the role for gonadal hormones in modulating DA transmission (Eck and Bangasser, 2020; Roberts et al., 2022; Brown et al., 2015) supporting the idea that both androgens and estrogens modulate DA function in the mesocorticolimbic system, thus affecting the motivational processes altered in depression. Whitin the circuitry, in fact, estrogens receptor alfa (Era), beta (ERb) and androgen receptor (AR) are widely expressed (Aubele and Kritzer, 2012; Eck and Bangasser, 2020). Both gonadal hormones influence the developing brain by regulating several functions. As an example, evidence suggest a role for estradiol in guiding DA midbrain differentiation during embryonic phase and DA synthesis during early post-natal period (Kipp et al., 2006; Varshney

et al., 2017). On the other hand, androgens influence dopaminergic neurotransmission and directly interact with estrogens in regulating motivating behavior (Sotomayor-Zárate et al., 2014; Aubele and Kritzer, 2012). Interestingly, gonadal hormones seems to strictly interact with Oxytocin (OXT; Insel et al., 1993; Tokui et al., 2021; Baskerville and Douglas, 2010), the primary neuropeptide involved in attachment bond formation and maintenance (Buchheim et al, 2009). OXT is synthesized within the pyramidal neurons of the paraventricular nucleus of the hypothalamus (PVN), that interact with the mesocorticolimbic system. OXT release from PVN targets VTA and NAc, with a consequent modulation on DA system (Baskerville and Douglas, 2010), particularly increasing DA release by exciting VTA DA neurons (Xiao et al., 2018). Few recent studies also suggest a possible role of OXT in regulating Ih current within the VTA (Tang et a., 2014; Liu et al., 2022), although the mechanism is not yet completely known.

Interestingly, we have recently reported a strong alteration in gene expression within the VTA of adult DBA mice induced by RCF during the early life period (Lo Iacono et al., 2021). Through the VTA RNA-sequencing, we were able to detect more than 1000 Differently Expressed genes (DEGs) altered by RCF manipulation, that could possibly explain the physiological changes observed in adulthood. Here we clustered the DEGs into functional pathways by using QIAGEN Ingenuity Pathway Analysis (IPA) and, among several interesting pathways (involved in calcium activity and DA signaling, see Tab.1), we found the estrogen receptor signaling pathway altered by RCF.

Since gonadal hormones and their receptors can be impacted by postnatal aversive experiences and these changes may alter VTA DA system (Eck and Bangasser 2020), here we performed a preliminary experiment to investigate a potential involvement of estrogen, androgens and oxytocin receptors in mediating the RCF-induced psychopathological outcomes. We first decided to evaluate through rt-qPCR mRNA levels of ERs, AR and OXTr on half-brain of DBA mouse pups on PND5, to assess a direct effect induced by RCF. The preliminary results obtained showed a reduction in RCF compared to Cont pups only for AR and OXTr. Conversely, we didn't observe any difference in Era and ERb mRNA levels induced by the early life manipulation. To deeply understand the effects of attachment bond alteration, we decided to start by evaluating the two targets altered by RCF in adulthood, directly targeting the VTA. In addition, we also tested mice that experienced SAF from PND1 to PND4 to evaluate a possible protective effect of the alternative caregiving figure. Opposite to what we observed in pups, we found increased AR mRNA levels into the VTA of RCF compared to Cont and, interestingly, it SAF experience was able to prevent this alteration. Regarding the OXTr mRNA levels analysis, we did not observe any difference induced by RCF, but we observe a general effect induced by SAF. Both Cont+SAF and RCF+SAF, in fact, showed an increased mRNA levels of OXTr compared to other groups.

During development, gonadal hormones exert organizational effects leading to permanent consequences on brain development and function. Therefore, we hypothesized that the different effect found in adulthood could depend on the fact that both ERs and AR act as transcription factors and regulates specific gene transcription (Cornil et al., 2015; Heinlein and Chang, 2002). Moreover Oxytocin, particularly sensitive to early life manipulation, appears to be regulated by both of them (Hiroi et al., 2013). Based on their peculiar role during development, gonadal hormones and oxytocin's mRNA receptor evaluation needs to be performed also on young mice that experienced the SAF, to evaluate the short-term effect of the manipulation. For this purpose, we planned to evaluate the receptors' mRNA levels of RCF+SAF and Cont+SAF also on PND5, immediately after the early life manipulation. Unveiling their potential change during the life-span, as well as the effects induced by early manipulations on their expression, may shield light on their role in mediating the alteration observed in adulthood. Early life experiences can, indeed, impact gonadal hormones, Oxytocin and their receptors, with long lasting consequences on DA signaling (Eck and Bangasser, 2020). Further studies are needed to deeply investigate this possibility.

9. Conclusion

In line with clinical studies, our results show that interfering with the formation of the attachment bond during a critical time window produces long-lasting negative consequences. Indeed, using our animal model of attachment bond interference, the Repeated Cross Fostering (RCF), we confirmed the link between this alteration and depressive outcomes in adulthood, also pointing out the alteration of the physiology of dopaminergic neurons of VTA. When we analyzed one of the main characteristics of DA neurons, the I_h current, we found an increase induced by the early life manipulation. Clinical studies highlight the protective role of a caregiver other than parents that provide an earned secure attachment when the primary caregiver fail to become a secure basis. Unfortunately, there are no animal models that evaluated how an alternative caregiving figure may prevent the negative outcomes and which mechanisms are involved. Here, by exploiting our well-established model of attachment bond interference, we proposed a new model that aim to mimic the earned attachment through a stable alternative caregiving figure (Stable Attachment Figure, SAF). When the unstable attachment pattern is rescued by the presence of the SAF, promoting the earned secure attachment, both depressive-like behavior and physiological alterations of VTA DA neurons are prevented. We point out a pivotal role for I_h current and hormones in mediating the behavioral alterations induced by RCF, prevented by the earned attachment. Indeed, providing a stable early environment during the developmental period had a protective role on the negative consequences

induced by attachment bond interference. Overall, our results provide interesting new insight on the neurobiological mechanisms of earned attachment.

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