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EFFECTS OF EARLY LIFE STRESS ON MESOCORTICOLIMBIC DOPAMINE SYSTEM FUNCTION AND RELATED DISORDERS IN PRE-ADOLESCENT HYPOMORPHIC MAOA MICE

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1. Introduction

Aggression represents an innate and essential instinct for species survival. However, during childhood and adolescence it may deteriorate into a pathological condition characterized by repetitive and persistent behavioral and emotional impairments, which are cardinal symptoms of conduct disorders (CD) (Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5)). A high comorbidity between psychiatric disorders and aggressiveness during childhood and adolescence has been shown, thus suggesting that human aggression might represent an endophenotype of mental disorders during this neurodevelopmental window (Loeber *et al.*, 2000). One of the most common psychiatric comorbidities observed in aggressive individuals is substance use disorder.

Aggression is a multi-factorial disorder depending on the interaction between genetic and environmental (G×E) factors (Caspi *et al.*, 2002; Viding, 2006). Particularly, the interaction between a genetic predisposition to a low activity of monoamine oxidase A (MAOA) enzyme and the exposure to early life traumas, such as child neglect and/or abuse, significantly increases the probability to manifest aggression during adolescence and adulthood. Low MAOA activity results in high monoamine brain levels, such as serotonin (5-HT) and dopamine (DA), which are considered a hallmark of aggressiveness in humans. Similarly, in rodents, perturbations of monoamine signaling during specific developmental windows are also associated with development of aggressive behavior (Yu *et al.*, 2014).

Considering the devastating socio-economic impact of aggressiveness and psychiatric comorbidity on affected patients, one of the major goals of research is to identify therapeutic strategies that can be used to control aggression and violence. Hence, it is paramount to deepen our understanding on circuit and neuronal mechanisms underlying pathophysiology of aggressive behavior. In this regard, the development of animal models mimicking aggressive disorders helps to define mechanistic insights underlying aggression and, therefore, to design potential treatment options. For the purpose of my thesis project, we have utilized a transgenic hypomorphic (MAOA^{Neo}) mouse model of aggressive behavior with a high translational validity as it recapitulates G×E interaction (Godar *et al.*, 2019), which helps elucidating neurobiological bases of aggression and to identify novel targets for its prevention.

1.1. Aggressive behavior

Aggressiveness is an adaptative behavior developed for species survival with primary functions such as securing access to food and a safe sleeping place. Within a reproductive context, this behavior is essential for obtaining resources aimed at successfully transmitting genetic information into the next generation (Silk *et al.*, 2003). In rodents, aggressiveness is usually related to territorial nature of the individual, as rivals and foreign intruders are excluded from one's territory rather than being tolerated. Typically, males engage in aggressive behavior to reach dominance in a social context, to defend territories and to secure resources. On the other hand, females usually engage in aggressive behavior during postpartum period to protect and defend the progeny (Brain, 1981; Huntingford and Turner, 1988; de Almeida *et al.*, 2005). According to ethological and sociobiological perspective, aggressiveness is typically displayed by animal species living in socially organized and cohesive groups, such as primates and rodents (Barnett, 1975, Gendreau and Archer, 2005). Considering the social nature of these species, aggression is a mechanism used to establish and maintain a social hierarchy.

In 1943, the psychologist Abraham Maslow conceptualized his theory of a hierarchy of needs, postulating his "Theory of Human Motivation" in which human needs are hierarchically organized in a pyramid. Specifically, at the bottom of the pyramid there are basic and physiological needs, such as obtaining food, water, shelter, warmth, and mate. Since the human nature is driven by essential instinct for survival, these primary needs must be addressed before humans move on to the higher level of needs. After these needs, the other one is represented by safety, including the protection from threat and violence, to provide emotional and financial stability as well as health (Maslow, 1943). Once these basic needs have been fulfilled, the third level of Maslow's pyramid includes social needs, such as love, acceptance, and belonging, that are important for interpersonal relationships. The fourth degree is represented by the self-worth, accomplishment, and respect of others, playing a central role in motivating behavior. At the last and highest level of Maslow's hierarchy, there are self-actualization needs referring to the personal realization, self-fulfillment and seeking personal growth (Maslow, 1970).

According to this theory, aggressiveness is carried out to fulfill primary needs. Particularly, the ability to be aggressive toward others represents an innate tendency aimed at maintaining and enhancing one's own health and will, to gain access to valuable resources, as well as to prevent threats from others (Buss and Duntley, 2006). Thus, aggression can be defined as a

behavior with the intent to inflict physical damage on another individual whenever the interests of two or more conflict. Although aggressiveness represents a competitive advantage for survival, it is time and effort consuming and it can often be dangerous to life and health. Thus, when aggressive behavior is exaggerated, persistent and manifested out of context, it can be considered pathological (Nelson and Treinor, 2007). In particular, when pathological aggression is manifested during adolescence as being disproportionate to any provocation and causing physical harm to others, it is regarded a key symptom of CD.

Therefore, while physiological aggression regards an adaptive mechanism, pathological aggression represents an inappropriate or disproportionate response to social norms and cultural practices, typically resulting in acts of violence and delinquency (Kolla and Bortolato, 2020). CD is a psychiatric disorder characterized by repetitive and persistent behaviors that violate the rights of others, such as physical aggression towards people or animals, theft, property damage and rule violations (DSM-5). CD and the associated antisocial behavior are the most common mental and behavioral problems manifesting during middle childhood or early adolescence (Cohen *et al.*, 1993; Polanczyk *et al.*, 2015), with a prevalence rate of about 3% in school-aged children (Erskine *et al.*, 2014). CD is diagnosed by the presence of at least 3 out 15 criteria or symptoms that characterize this disorder, which are grouped in main categories, including aggression acts to people and animals, destruction of property, deceitfulness or theft, serious violations of rules (DMS-5). It is a heterogeneous disease often associated with other mental conditions or disabilities, such as autism spectrum disorders (ASD), attention- deficit/hyperactivity disorder (ADHD), oppositional defiant disorder (ODD), substance misuse in adolescence, depression, and anxiety (Angold *et al.*, 1999; Thapar *et al.*, 2001; Moffitt and Scott, 2008). CD cannot be considered an episodic disease, with defined onset and offset phases, as affected individuals manifest chronic symptoms, and they often develop personality disorders. Moreover, they can manifest criminal behavior in adulthood and, according to major categories of psychopathological conditions, it has a constellation of symptoms and behaviors that can vary in terms of presence/absence and severity (Kim-Cohen *et al.*, 2003). As abovementioned, aggression is one of the main behaviors associated with CD as well as the most predictive factor for later negative outcomes related to CD (Hyde *et al.*, 2015). In this framework, aggression is categorized in two subtypes, proactive and reactive, which are largely supported by psychological, social, and biological studies (Wrangham *et al.*, 2018).

Proactive aggressive behavior, also termed instrumental aggression, is a goal-directed behavior. It is acquired during development to obtain a reward or positive outcome, such as attention, money, political power. This form of aggression is also known as cognitive or premeditated because the attack is planned to obtain a positive outcome that is perceived as appropriate only with a low effort (Dodge *et al.*, 1997; Brendgen *et al.*, 2011). The attention is directed toward a target and is often accompanied by a lack of emotional arousal, empathy, and remorse (Frick *et al.*, 2003). Examples of instrumental aggression are bullying, stalking and premeditated crimes.

Reactive aggressive behavior (hereinafter AB), often termed impulsive or emotional aggression, occurs as a defensive response to a real or perceived threat or provocation (Dodge and Coie, 1987; Brendgen *et al.*, 2015). The aim is to remove the provoking stimulus. This hostile nature is typically associated with anger and impairments in social information processing, emotional regulation, verbal intelligence, impulse control, and executive functioning (Dodge *et al.*, 1997; Lemerise and Arsenio, 2000; Connor *et al.*, 2003; Dodge and Pettit, 2003; Marsee and Frick, 2007; Arsenio *et al.*, 2009). Of note, this form of aggression has genetic and environmental bases. Among genetic factors, low activity of MAOA enzyme plays an important role, while among environmental factors, early life child abuse and maltreatment represent vulnerability factors (Dodge *et al.*, 1997; Connor *et al.*, 2004).

1.2. The etiopathogenesis of aggressive behavior

AB is a multifactorial disorder influenced by genetic, neurobiological, and psychosocial factors. It is related to impulsivity, violence, irritability, and hostility. It exhibits a high comorbidity with a range of psychiatric conditions, including schizophrenia, bipolar disorder, dementia, personality disorders (*i.e.*, borderline, and antisocial disorders), post-traumatic stress disorders, traumatic brain injury, addiction, and pervasive developmental disorders (Comai *et al.*, 2012). Impairment of several neurotransmitter system, such as 5-HT, DA, norepinephrine (NE), nitric oxide, and neuroactive steroids, alterations in synaptic plasticity and receptor subunits, including 5-HT1B, γ-aminobutyric acid A (GABAA), GABAB, and glutamate (N-methyl D-aspartate [NMDA]) receptors, are implicated in the etiology of aggression and in the manifestation of antisocial behavior (Nelson and Trainor, 2007; Comai *et al.*, 2012; Waltes *et al.*, 2016). Notwithstanding, AB pathogenesis depends

on the effects of different environmental stimuli, including child neglect, maltreatment, early traumas, stress, depression, substance abuse in specific genetic and neurobiological contexts (Nelson and Trainor, 2007).

1.2.1. Genetic factors

Dysfunction of 5-HT system plays a role in AB (Comai *et al.*, 2012; Godar *et al.*, 2019). In humans, the presence of polymorphisms onto genes involved in 5-HT synthesis and transmission are associated with aggression (Craig and Halton, 2009; Zalsman *et al.*, 2011), as well as with other psychological disorders, such as depression (Zill *et al.*, 2004; Levinson, 2006; Lohoff, 2010) and anxiety (Hovatta and Barlow, 2008). Patients affected by AB, such as aggression, violence and impulsivity show differences in the levels of 5-HT or its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the cerebrospinal fluid (Brown and Linnoila, 1990; Stanley *et al.*, 2000; Placidi *et al.*, 2001). In particular, low levels of 5-HT are associated with AB and represent a trait for engaging in AB aimed at dominance and reproductive success (Seo and Patrick, 2008). In males, a positive correlation has been found between low 5-HT brain levels and the manifestation of high trait aggression (Linnoila *et al.*, 1983; da Cunha-Bang *et al.*, 2016). Preclinical evidence has confirmed the role of two 5-HT receptor subtypes in mediation of AB, *i.e.*, 5-HT1 and 5-HT2. Specifically, increasing 5-HT activity by administering 5-HT precursors, 5-HT- reuptake inhibitors or 5-HT1A- and 5-HT1B-receptor agonists has been shown to blunt aggression in different species, including humans (Unis *et al.*, 1997; Chiavegatto and Nelson, 2003). Lesions of the serotoninergic nucleus dorsal raphe by administration of 5,7-dihydroxytriptamine (5,7-DHT) resulted in a reduction of offensive behavior in the resident-intruder test (De Almeida and Lucion, 1997). In mice, 5-HT1A agonists prevent AB induced by social isolation (Sánchez *et al.*, 1993) and 5-HT1B receptor agonists inhibit several types of AB (Fish *et al.*, 1999). Particularly, 5-HT1B receptor activation in prefrontal cortical areas, specifically the orbitofrontal cortex (OFC), suppresses AB, thus suggesting an important role of this area in the inhibitory control of aggression when expressed as impulsive behavior (Blair, 2004; De Almeida *et al*., 2006). Accordingly, the interaction between GxE factors led to an up-regulation of 5-HT2A receptors in the prefrontal cortex (PFC) during the first postnatal week resulting in the manifestation of antisocial behaviors later in life, as indexed by an increased AB and a reduction in social interaction (Godar *et al.*, 2019).

Multiple studies aimed at investigating the heritability of AB have identified stressful environmental stimuli and *Maoa* gene as two key players for the development of

pathological aggression (Raine, 2002). Particularly, the interaction between early life adversities and *Maoa* gene represents the best-characterized model implicated in the etiopathology of AB (Caspi *et al.*, 2002; Bortolato *et al.*, 2008, 2013). Specifically, Caspi and colleagues (2002) demonstrated that deficient MAOA activity increased the risk of developing CD and antisocial behavior in boys that had experienced abuse and maltreatment. Genetic studies and meta-analyses have also confirmed the relevance of such a G×E interaction in the manifestation of AB (Kim-Cohen *et al.*, 2006; Meyer-Lindenberg *et al.*, 2006; Guo *et al.*, 2008; Fergusson, 2010). Among animal studies, Godar and colleagues (2019) were the first to develop a mouse model recapitulating this G×E interaction to be able to study its resulting molecular alterations. In particular, Godar and colleagues (2019) took advantage of a transgenic *Maoa* mouse mutant, displaying a low MAOA enzymatic activity but no spontaneous aggression (Bortolato *et al.*, 2011). In order to mimic child neglect and abuse, mice were exposed to early-life stress (ES) during the first postnatal week. The relevance of this G×E interaction is then revealed by the manifestation of AB during adolescence and adulthood only in transgenic mice exposed to ES, which also show deficits in social approaches. This evidence supports the validity of this mouse model, the first one to be recapitulating AB pathognomonic clinical phenotypes (Godar *et al.*, 2019).

Clinical and preclinical studies have demonstrated that, among monoamines, the activation of DA system also plays a critical role in modulation of AB. For example, an antagonist of DA D2 receptor (DAD2R), haloperidol, has been used for decades to treat AB in psychotic patients (Glazer and Dickson, 1998; Fitzgerald *et al*., 1999). This drug also reduces violent outbursts in individuals affected by dementia and borderline personality disorder, as well as in children and adolescents affected by CD (Comings *et al*., 1994; Pies and Popli, 1995; Masi, 2004; Tyrer *et al.*, 2008; Beauchaine, 2012). Accordingly, several studies show the involvement of mesocorticolimbic DA system in the preparation, execution, and consequences of AB (van Erp and Miczek, 2000; Ferrari *et al.*, 2003; de Almeida *et al.*, 2005). Thus, not only an increase in DA levels or its metabolites in PFC or nucleus accumbens (NAc) is implicated in the initiation and maintenance of attacks towards a threat, but also in a defensive response to attacks. In fact, DA levels are enhanced in NAc core of resident rats 60 minutes after the exposure to an intruder (van Erp *et al.*, 2000). Interestingly, an increase in tyrosine hydroxylase (TH)**,** the rate-limiting enzyme of catecholamines biosynthesis, and in DA transporter (DAT) mRNA levels has been found in the ventral tegmental area (VTA) of aggressive male mice after a repeated confrontation with

conspecifics (Filipenko *et al.*, 2001). Moreover, significant differences in DA levels and expression of DAD1Rs and DAD2Rs were detected in the mesocorticolimbic DA system of two strains of mice, A/J and BALB/cJ, which are docile and aggressive, respectively. (Couppis *et al.*, 2008). Accordingly, *in vivo* studies in freely moving rats demonstrated an increased DA activity during aversive condition such as defeat stress (Anstrom *et al.*, 2009). Furthermore, a correlation between increased mesocorticolimbic DA levels and fighting behavior has been found in socially isolated mice (Hadfield, 1983; Tidey and Miczek, 1996; Ferrari *et al.*, 2003).

Genetic studies assessing the role of enzymes involved in DA metabolism, such as MAO and catechol-O-methyltransferase (COMT), or DAT, confirmed DA central role in modulating AB. Among these genes, *Maoa* results largely linked to AB, the reason for which it is termed the ‘‘warrior gene’’ (McDermott *et al.*, 2009). The first evidence for a key role of this gene in AB stems from a study carried out in patients of a Dutch family affected by Brunner syndrome: this is a X-linked recessive disorder characterized by a point nonsense mutation in exon 8 of *Maoa* gene and associated with a reduced enzyme activity (Brunner *et al.*, 1993a, 1993b). Affected males of this family showed abnormal levels of disruptive and violent behaviors in response to frustration, anger, provocation, and maladaptive environmental triggers. Moreover, they displayed other symptoms, including borderline intellectual disability, sleep disturbance and stereotyped hand movements (Brunner *et al.*, 1993a, 1993b, 1996). Recently, two clinical studies documented other cases of Brunner syndrome and the role of *Maoa* gene in the ontogeny of this syndrome (Piton *et al.*, 2014; Palmer *et al.*, 2016). Similar to the patients described by Brunner, these individuals reported attention deficit and intellectual disability, which were often accompanied by other autistic–like traits as well as impulsive and explosive behaviors. In both reports, affected males showed MAOA deficits and the same biochemical abnormalities described in the Brunner syndrome (Bortolato *et al.*, 2018).

Genetic studies on polymorphic variants of *Maoa* gene revealed a functional link between low activity of MAOA and AB (Zhang-James *et al.*, 2019). This variable-number tandem repeat polymorphism (uVNTR) in *Maoa* gene on chromosome Xp11.23 is characterized by alleles with 30 pb repeats located 1.2kb upstream of the transcription initiate site (Sabol *et al.*, 1998). Specifically, the presence of 3 or 5 repeats produces a genotype with low activity allele (MAOA-L), in contrast to high activity allele (MAOA-H) found when 3.5 or 4 repeats are present (Sabol *et al.*, 1998; Huang *et al.*, 2004; Manca *et al.*, 2018; Kolla and Bortolato,

2020). In humans, imaging studies demonstrated that MAOA-L male exhibited a lower amygdala activity with a resulting increased activation of ventromedial PFC (vmPFC), a brain region strongly implicated in socio-affective and socio-cognitive abilities, two essential processes for social interaction (Buckholtz *et al*., 2008). Moreover, patients affected by Brunner syndrome exhibit an increased neuronal network activity in cortical areas associated with a higher expression of NMDA receptor subunits GluN2A and GluN2B (Shi *et al*., 2019). Furthermore, patients carrying MAOA-L genotype showed alterations in the event-related potential (ERP) during an emotion-processing task in the right medial frontal and premotor areas, right superior temporo-occipital, left inferior and superior parietal regions, all brain areas implicated in face and emotion perception. Thus, neuronal alterations showed by MAOA-L males may be associated with anger processing, in agreement with antisocial traits (Williams *at al*., 2009). Finally, violent crime episodes were more frequent in incarcerated male offenders carrying MAOA-L mutation (Stetler *et al.*, 2014).

In rodents, Cases and colleagues (1995) developed the first transgenic line of mice deficient *maoa* (MAOA knockout (KO) mice) characterized by a phenotype akin the Brunner syndrome, which show increased brain levels of monoamines during adolescence. Other studies investigated the relationship between MAOA deficits and antisocial behavior. Specifically, MAOA KO mice displayed anxiety-like behaviors, defensive and neophobic responses to minor or innocuous stimuli, such as unfamiliar objects in their home cage (Kim *et al.*, 1997; Godar *et al.*, 2011), as well as a lower response to stressful and threatening contingencies, including predator cues, physical restraint, and cold temperature (Popova, 2006; Godar *et al.*, 2011, 2014). Furthermore, MAOA KO mice showed a marked reduction in social and environmental exploration (Bortolato *et al.*, 2011; Godar *et al.*, 2011). Interestingly, behavioral phenotypes of MAOA KO mice reproduced all core symptoms observed in autism spectrum disorder patients, confirming the antisocial nature of this disorder (Bortolato *et al.*, 2013). MAOA KO mice also show morphological and cytoarchitectonic alterations in brain cortical areas, including reduced callosal thickness in the rostral regions, increased apical arborization of pyramidal cells in orbitofrontal cortex, and dysmorphism of barrel fields in layer IV of the somatosensory cortex (Cases *et al.*, 1995; Bortolato *et al.*, 2013). These anomalies are associated with social and emotional impairments, as well as with the manifestation of repetitive behaviors. The severity of these traits is inversely correlated with MAOA enzyme catalytic activity representing a biomarker

for antisocial disorder (Alia-Klein *et al.*, 2008; Bortolato *et al.*, 2013). AB observed in MAOA KO mice is also associated with deficits in information processing encoded by the PFC, a brain region that also plays a key role in modulating aggression. Particularly, these deficits depend on alterations in NMDA receptors subunit composition. Specifically, MAOA KO mouse exhibits a higher expression of GluN2A and GluN2B subunits in PFC, which is associated with alterations in PFC connectivity and AB (Bortolato *et al.*, 2012). Of note, NMDA receptors play a central role in the regulation of information processing, implicated in aggressive and defensive responses (Gould and Cameron, 1997; Carobrez *et al.*, 2001). Accordingly, the function and subunit composition of these receptors is influenced by monoamine neurotransmitters (Masuko *et al.*, 2004; Yuen *et al.*, 2005; Bortolato *et al.*, 2012). Collectively, these findings show that low activity of MAOA enzyme represents a vulnerable genetic factor for aggressive and antisocial behavior. Of note, in humans, early life adversities impact on MAOA-L genotype by increasing the predisposition to engage in criminal and violent acts at adulthood.

1.2.2. The interplay between gene and environment

Several environmental factors can interact with genetic background and predispose to AB: these are uninvolved and/or permissive parenting style, exposure to early traumas and/or specific forms of maltreatment and abuse, and also to substances of abuse. Several independent studies have also shown that carriers of MAOA-L variants with a history of maltreatment during childhood display a significantly higher risk of developing AB (Caspi *et al.*, 2002; Kim-Cohen *et al.*, 2006; Williams *et al.*, 2009; Fergusson *et al.*, 2011).

Different experimental protocols can be used in rodents to study the effect of distinct environmental factors mimicking early life adversity, for instance maternal separation and social isolation. Maternal care and the relationship between mother and infant are very important for social animals. Maternal separation during early life leads to long-lasting alterations in behavioral (*e.g.*, AB), emotional, and cognitive functions in the offspring that can manifest during adolescence and adulthood (Veenema, 2009). Similarly, the stress related to post-weaning social isolation is one of the most frequently used social stressor paradigms for studying the impact of adversities during adolescence: at weaning, the animal is singly housed and, therefore, deprived from social interactions with conspecifics. Post-weaning is usually the time when play behavior begins and is enhanced in order to prepare the individuals for adult social behaviors, including defense and copulation. As a result, social deprivation during such an important developmental window results in changes in

neurodevelopment that may enhance vulnerability to psychiatric disorders (Walker *et al.*, 2019). Among the most commonly used paradigms to study AB in rodents, the resident–intruder test provides an excellent predictive validity toward human aggression (Umukoro *et al.*, 2013). This paradigm studies the resident animal's response to a conspecific intruder: a rodent is housed (singly or with a female) in a situation mimicking a natural environment during which the animals can establish territoriality and dominance. When a resident rodent meets an unfamiliar conspecific intruder in its territory (cage), a fight may occur during which different types of behaviors (*e.g.*, number or duration of approach, investigation, fighting, chasing, posture) may be related to discrete neuronal substrates involved (Koolhaas *et al.*, 1980). Finally, the interactions between the resident and the intruder depend on several factors, such as age, hormonal status, exposure to drugs or environmental factors.

Neurobiological and epidemiological evidence suggests that exposure to early life adversities can cause enduring brain abnormalities that may affect the quality of life throughout the lifespan (Anda *et al.*, 2005), particularly when interact with genetic risk factors (Foley *et al.*, 2004; Meyer-Lindenberg *et al.*, 2006; McDermott *et al.*, 2009; McDermott *et al.*, 2013; Godar *et al.*, 2016). For example, it has been shown that MAOA-L males with history of child abuse or neglect had a higher prevalence of antisocial behaviors than males with only one of the two risk factors (Caspi *et al.*, 2002; Kim-Cohen *et al.*, 2006). Abused children with low-activity of MAOA developed CD and hostility during the peri-adolescence period (Ferguson *et al.*, 2011). Of note, low activity of MAOA has also been shown to interact with maternal stress and to influence negative emotionality in infants (Hill *et al.*, 2013). Relationship between low activity of *MAOA* and early life traumas or stress also increased the vulnerability to develop antisocial behaviors during adolescence (Shih *et al.*, 1999; Nelson and Trainor, 2007; Godar *et al.*, 2016). A reduced MAOA activity might be crucial during early postnatal life, since it reaches its peak concentrations between birth and postnatal day 10 (PND10) in rodent midbrain (Vitalis *et al.*, 2002; Tong *et al.*, 2013). In mice, the first and second postnatal weeks of life represent temporal windows during which MAOA plays a key role in brain development (Bortolato *et al.*, 2013). Perseverative and aggressive behaviors showed by adult MAOA KO mice are associated with high levels of 5-HT during the first and second postnatal week of life (Bortolato *et al.*, 2012, 2013): pharmacological manipulation during the first postnatal week with the inhibitor of 5-HT synthesis, parachlorophenylalanine (PCPA), reduced 5-HT forebrain levels in in 8-day old and adult mice, but did not prevent aggression in adult mice (Bortolato *et al.*, 2013). These

observations are suggestive of additional MAOA substrates (*e.g.*, DA and NE) other than 5-HT to be involved in the manifestation of aggressive traits. Accordingly, Yu and coworkers (2014) showed that when DA signaling was pharmacologically enhanced during peri-adolescence, adult mice displayed increased aggression. They also show a causal relationship between optogenetic stimulation of VTA DA neuron activity and the manifestation of AB (Yu *et al.*, 2014). Collectively, these findings support the role of different monoamines during two discrete developmental windows in the manifestation of AB at adulthood. Specifically, during early postnatal period an increased 5-HT activity results in anxiety/depression-related behaviors, whereas at peri-adolescence an enhanced DA function is causally tied to the manifestation of AB in adulthood. Remarkably, these two stages of life are key periods for brain structures maturation and functions, as well as to the development of anxiety-, fear-, and play-behavior (Walker *et al.*, 2019). Thus, the exposure to stressful events (*e.g.*, child neglect or isolation) may disrupt these developmental windows resulting in long-term consequences in brain function and plasticity that can persist later in life and influence behavioral responses (Chen and Baram, 2016; Walker *et al.*, 2019).

1.2.3. An animal model of interaction between low activity MAOA and early life stress

In 2011, Bortolato and collaborators developed the first *Maoa* hypomorphic mouse line, generated through the insertion of a neomycin resistance cassette (Neo) into the intron 8 of the *Maoa* gene. This mouse line, called Maoa^{Neo}, is characterized by reduced levels and activity of MAOA enzyme. The diminished MAOA activity in the brain resulted in a significant increase of monoamine levels, in particular 5-HT and NE, mimicking MAOA KO mouse. These molecular alterations were accompanied by morphological and behavioral changes. In particular, Maoa^{Neo} mice showed differences in the pattern of dendritic arborization in pyramidal neurons, which were characterized by an increased number and length of apical branches in the OFC. Among behavioral features, Maoa^{Neo} mice showed impairments in social behavior, compulsive behaviors in response to neutral environmental stimuli or mild stressors, but a lack of spontaneous AB (Bortolato *et al.*, 2011). Indeed, this Maoa^{Neo} mouse line is not aggressive unless exposed to an early stress regimen, which consists of maternal separation and daily saline intraperitoneal injections (during the first post-natal week), followed by five days of social isolation at weaning (Godar *et al.*, 2019). This represents the first mouse model of GxE interaction implicated in the pathogenesis of aggression and antisocial behaviors.

1.3. Mesocorticolimbic dopamine system and aggressive behavior

Aggression and antisocial behaviors reflect a dysregulation in DA mesocorticolimbic circuits (Godar *et al.*, 2016). DA among conventional neurotransmitters is one of the most studied since its discovery (Carlsson *et al.*, 1957). DA plays a key role in many brain functions, such as reward-based and associative learning, motivation, memory, cognitive control in decision-making**,** and motor function **(**Cools, 2008; Salamone and Correa, 2012; Schultz, 2013; Berridge and Kringelbach, 2013; Gillies *et al.*, 2014; Klein *et al.*, 2019). Two main brain areas within the midbrain show high expression of DA neurons: the VTA and the substantia nigra pars compacta (SNc). The VTA and SNc are separate brain areas that play important roles in the neuromodulation of DA-related behavior via two distinct DA pathways, *i.e.*, nigrostriatal and mesocorticolimbic one (Root *et al.*, 2016; Morales and Margolis, 2017). The nigrostriatal pathway consists of DA projections arising from the SNc to the dorsal striatum (Haber and Fudge, 1997) and controls movement. Alterations in the nigrostriatal pathway have been linked to deficits in locomotor activity, as those displayed in Parkinson's disease. The mesocorticolimbic pathway consists of DA projections from the VTA to the PFC (Ikemoto, 2007; Morales and Margolis, 2017) and the NAc (Edwards *et al.*, 2017; Yang *et al.*, 2018) and encodes motivation, reward, and cognition (**Fig. 1**). Dysfunctions of this pathway are associated with psychiatric disorders, including schizophrenia, ADHD (Edwards *et al.*, 2017; Hauser *et al.*, 2017; Yang *et al.*, 2018), and substance use disorders (Wise and Robble, 2020).

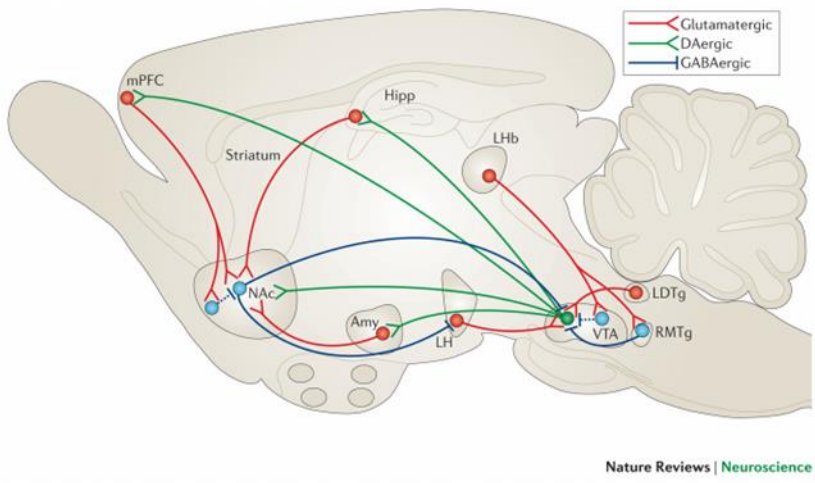

Figure 1. Mesocorticolimbic system. Schematic of main DA, GABA, and glutamate inputs and outputs of VTA and NAc (DA=green; GABA=blue; glutamate=red), in rodent brain. The NAc receives major glutamate inputs from medial PFC (mPFC), hippocampus (Hipp), and amygdala (Amy). On the other hand, the VTA

receives inputs from: laterodorsal tegmental nucleus (LDTg), lateral habenula (LHb), and lateral hypothalamus (LH; from Russo and Nestler, 2013).

Mesocorticolimbic DA neurons represent in the VTA the most prevalent cell type (~65%); the remaining cells are GABA (~30%) and glutamate neurons (~2-3%) (Margolis *et al.*, 2006; Morales and Margolis, 2017). DA cells are identified by the presence of *i*) TH, the main enzyme involved in catecholamine synthesis (Daubner *et al.*, 2011); *ii*) the Pitx3, an homeobox transcription factor expressed by DA neurons and important for the development and maintenance of DA neuron phenotype (Zhao *et al.*, 2004; Smidt *et al.*, 2012); *iii*) the DAT, an integral membrane protein that removes DA from the synaptic cleft into the cytosol of DA neurons (Lammel *et al.*, 2015; Stuber *et al.*, 2015). VTA DA neurons are a heterogeneous population characterized by well-defined electrophysiological properties and behavioral actions (Lammel *et al.*, 2014; Morales and Margolis, 2017). VTA DA system consists of several DA neuron subpopulations with different properties and projection targets (Lammel *et al.*, 2008). These neurons mainly project to NAc and other cortical and subcortical brain regions, such as PFC, amygdala, hippocampus (HP), and olfactory tubercule (Björklund and Dunnett, 2007), with a defined topography. In particular, anterior-lateral VTA DA neurons project to the core of the NAc, whereas DA neurons of the medial posterior VTA mainly project to the core and medial shell of the NAc as well as the medial PFC (mPFC) and basolateral amygdala (BLA) (Lammel *et al.*, 2008; Ikemoto, 2010). *Ex vivo* electrophysiological studies show that DA neurons can be identified by specific electrophysiological and molecular properties. The lateral VTA DA neurons are characterized by *i*) the presence of large hyperpolarization activated currents (I_h), which are mediated by hyperpolarization-activated cyclic nucleotide–gated (HCN) inwardly rectifying non-specific cation channels (Margolis *et al.*, 2006); *ii*) a slower spontaneous firing rate; *iii*) a larger cell body size compared to surrounding putative GABA neurons; *iv*) a pharmacological inhibition of their pacemaker activity induced by DAD2R agonists; *v*) higher DAT, VMAT expression (Ford *et al.*, 2006; Margolis *et al.*, 2012). Medial posterior VTA DA cells do not present I_h (sag) current, but exhibit faster firing frequency, small neuron cell body, low DAT, VMAT expression and respond to opioid receptor activation (Ford *et al.*, 2006). Moreover, they lack somatodendritic inhibition mediated by DAD2R autoreceptors (Lammel *et al.*, 2008). VTA DA neuron subpopulations, including those projecting to the HP and ventral pallidum (VP) have to be electrophysiologically

characterized yet. The diversity of VTA DA neuron function also depends on the wide variety of local and afferent inputs (Watabe-Uchida *et al.*, 2012; Faget *et al.*, 2016).

In rodents, the manifestation of AB is associated with NAc DA levels as the block of DA transmission in NAc attenuates aggressive responses (Couppis and Kennedy, 2008). Accordingly, in DAT KO mice, the increased striatal DA levels induce AB (Rodriguiz *et al.*, 2004). Additionally, increased striatal DA and DOPAC levels during peri-adolescence are associated with manifestation of AB in adulthood, and opto-stimulation of VTA DA neurons enhanced the aggression during the resident intruder test (Yu *et al.*, 2014). To the best of our knowledge this is the first and only evidence of a causative role of DA in the regulation of AB.

1.4. Endocannabinoid modulation of mesocorticolimbic system

VTA DA neurons represent a key node in the mesocorticolimbic reward system (Volkow *et al.*, 2019). Their activity is influenced by many neurotransmitter systems, including acetylcholine, glutamate, and GABA, opioids, and peptides. The endocannabinoid (eCB) system influences excitatory and inhibitory signaling onto the VTA, thus contributing to the reinforcing effects of drugs through modulation of hedonic responses or inhibition of negative affective states (Volkow *et al.*, 2019). This neurotransmitter system is implicated in regulating several physiological and pathological conditions. Specifically, eCB signaling modulates motivation for pleasurable natural stimuli, such as social interaction, sexual activity, and food, but also influences the rewarding effects of addictive drugs (Trezza *et al.*, 2010; Klein *et al.*, 2012; Silvestri and Di Marzo, 2013; Zlebnik and Cheer, 2016). Moreover, alterations in eCB system have been observed in many disease states, such as anxiety depression, pain, inflammation, and neurodegenerative disorders (Wei *et al.*, 2017).

The eCB system comprises a family of lipid ligands and G protein-coupled receptors, as well as the enzymatic machinery involved in the biosynthesis and degradation of the ligands. Two types of cannabinoid receptors exist: type 1 cannabinoid receptors (CB1Rs; encoded by *Cnr1* gene) and type 2 (CB2Rs; encoded by *Cnr2*). Both receptors are coupled to similar transduction systems. Particularly, CBR activation inhibits cAMP formation through its coupling with $G_{i/o}$ proteins, thus resulting in a decreased protein kinase A-dependent (PKA) phosphorylation, whose outcome is a presynaptic inhibition of neurotransmitter release (Melis and Pistis, 2007). CB1Rs are only expressed in the brain, and they are particularly

abundant in different brains areas (*e.g.*, amygdala, cingulate cortex, PFC, VP, caudate, putamen, NAc, substantia nigra pars reticulata (SNr), lateral hypothalamus, and HP) that are involved in the regulation of reward, drug addiction and cognitive function (Busquets-Garcia *et al.*, 2018). CB2Rs are mainly expressed by immune cells, although evidence suggests their presence in neurons and microglia of several brain regions, including striatum, amygdala, HP and VTA (Van Sickle *et al.*, 2005; Manzanares *et al.*, 2018).

The best-characterized eCBs are N-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG). According to their canonical mode of actions, eCBs are synthesized and released *on demand* by postsynaptic neurons and travel retrogradely across the synaptic cleft to bind to and activate CB1Rs located on presynaptic terminals, resulting in a decreased neurotransmitter release. They are synthesized by cleavage of membrane phospholipid precursors, N-arachidonoyl-phosphatidylethanolamine (NAPE) and 1,2-diacylglycerol (DAG), for AEA and 2-AG respectively, then they are immediately released mostly through Ca^{2+}-dependent mechanisms. AEA synthesis derives from the metabolism of its precursor via the N acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), whereas 2-AG from the hydrolytic metabolism of its precursor the sn-1-selective DAG lipases (DAGLs), DAGLα and DAGLβ. AEA is primarily catabolized by fatty acid amide hydrolase 1 (FAAH1), whereas 2-AG by monoacylglycerol lipase (MAGL) and, to a lesser extent, by α,β- hydrolase 6 (ABHD6), cyclooxygenase 2 (COX2) and FAAH1. eCB enzymatic machinery has distinct cellular and anatomical locations, with MAGL predominantly localized in presynaptic terminals and FAAH1 enzyme in neuronal postsynaptic domain. AEA and 2-AG exert agonist activity at both CB1Rs and CB2Rs. Particularly, AEA binds with a slightly higher affinity to CB1R than to CB2R, while 2-AG binds with equal affinity at CB1Rs and CB2Rs. Interestingly, AEA and 2-AG exhibit agonist properties on several other receptors, including peroxisome proliferator activated receptors (PPARs), G protein-coupled receptor 55 (GPR55) and GPR119. Moreover, AEA exerts potent agonist effects at transient receptor potential (TRP) vanilloid channels, including TRPV1 (Parsons and Hurd, 2015).

eCB system plays an important role in encoding natural and drug rewards through the interaction with mesolimbic DA system (Sagheddu *et al.*, 2015; Manzanares *et al.*, 2018). Specifically, eCBs increase both tonic and phasic DA release by means of a CB1Rs-dependent mechanism into VTA (Melis and Pistis, 2012; Spanagel, 2020; Peters *et al.*, 2021b). eCB regulates the DA system through a fine tuning of the balance between inhibitory

and excitatory transmission (Covey *et al.*, 2017). Particularly, DA neurons are inhibited by GABA inputs arising from VP, rostromedial tegmental nucleus (RMTg), and VTA interneurons resulting in inhibition of spontaneous firing and/or bursting activity of VTA DA neurons. Among eCBs, the 2-AG is synthetized and released by DA neurons after activation of group I mGluR and plays a role in reducing GABA transmission on VTA DA neurons. Indeed, pharmacological block of synthesis and/or release of 2-AG restores the decreased response of DA cells to GABA inputs (Pan *et al.*, 2008a). Given that GABA inputs on VTA are crucial to modulate the activity of DA neurons, the transient reduction of this inhibitory transmission may lead to a phasic excitation of DA neurons. Similarly, eCBs acting through CB1R decrease glutamate transmission on VTA DA neurons (Melis *et al.*, 2004a). The excitatory afferents on midbrain arising from cortical and rostral regions result in a fine modulation of DA neuron activity through CB1Rs (Kortleven *et al.*, 2011; Melis and Pistis, 2012; Morales and Jagerovic, 2020; Peters *et al*., 2021a).

Since eCB signaling modulates both excitatory and inhibitory transmission on DA neuron, it represents a key player of the mechanisms underlying plasticity of DA system. Particularly, the 2-AG, released *on demand* by DA neurons, mediates both short- and long-term form of synaptic plasticity. Among the short-term forms of plasticity, 2-AG is a key player in the expression of a depolarization-induced suppression of inhibition (DSI) or excitation (DSE), two forms of short-term plasticity that are necessary to reduce GABA or glutamate transmission, respectively (Melis and Pistis, 2007). The synthesis and release of 2-AG by DA neurons results from a rise of intracellular Ca^{2+} levels in response to activation of different types of receptors on DA membrane surface, such as group I metabotropic receptors (mGluRs), voltage-gated calcium channels (VGCC) and muscarinic acetylcholine receptors, which mobilize stored Ca^{2+} via phospholipase C (PLC) (Verma *et al*., 2001; Maejima *et al.*, 2001; Hashimotodani *et al.*, 2005; Kreitzer and Regehr, 2002; Kim *et al.*, 2002; Castillo *et al*., 2012; Kano, 2014).

Ultimately, eCBs modulate both tonic and phasic DA neuronal activity (Melis *et al.*, 2004b; Xu and Chen, 2015). Moreover, eCBs are involved in the *in vivo* modulation of VTA DA neurons synaptic strength, firing rate, and pattern of discharge (Melis *et al.*, 2004b; Chen *et al.*, 2007). Among the long-term forms of synaptic plasticity, 2-AG mediates the expression of long-term depression (LTD) and the inhibition of long-term potentiation (LTP) of VTA DA neurons, which play a role in memory consolidation and behavioral learning. Particularly, 2-AG mediates low frequency stimulation (LFS)-induced LTD. Moreover, the

release of 2-AG prevents the spike time-dependent LTP induction, but not its expression (Kortleven *et al.*, 2011). In a similar fashion, cocaine-induced potentiation of excitatory plasticity is influenced by 2-AG involved in LTD while impairing LTP to limit the effect of increased excitatory transmission and protect DA cells from excitotoxicity (Pan *et al.*, 2008a).

Midbrain DA cells are sensitive to the exposure of exogenous cannabinoid agonists, such as Δ^9-tetrahydrocannabinol (THC) and WIN55,212-2. Specifically, both CB1R agonists increased DA neuronal activity (Gessa *et al*., 1998) and DA release in target regions, such as NAc and PFC (Pistis *et al.*, 2002; Cheer *et al.*, 2004).

Since the eCB release is triggered by different patterns of stimulation (*i.e.*, depolarization, stimulation of excitatory efferences, burst firing induction) similarly to those caused by exposure to salient behavioral events, the eCBs may have a critical role in the modulation of signal-to-noise ratio of DA activity, especially during emotional and sensory processing (Melis and Pistis, 2007). Therefore, a disruption in the eCB signaling, including alterations in eCB levels and/or CB1R expression and function, represents a pathophysiological substrate of neuropsychiatric disorders, such as schizophrenia, ASD, Parkinson's disease and drug abuse (Zamberletti *et al.*, 2017, 2021).

The eCB system plays a crucial role also in the neurobiological mechanism underlying drug addiction. Particularly, eCBs and CBRs are widely expressed in areas of mesocorticolimbic system involved in the initiation and maintenance of substance abuse and in the development of drug addiction (Manzanares *et al.*, 2018; Peters *et al.*, 2021b). eCBs modulate the balance between excitatory and inhibitory synaptic activity in the reward pathway. Specifically, the eCB action on excitatory transmission correlates with drug seeking/taking behavior, whereas the modulation on inhibitory inputs is particularly involved in withdrawal and drug craving behaviors. Therefore, eCB participates to this behavior through the regulation of DA activity. Accordingly, the evidence that activation of CB1R through agonists (*i.e.*, WIN55,212-2) and the increase of 2-AG levels in VTA are associated with the expression of LTD and depression of inhibitory transmission, along with an increase in the motivational and reinforcing effects of several substance of abuse, such as alcohol, opiates, and psychostimulant (*i.e.*, cocaine,), support the role of eCB in the cycle of drug addiction (Melis *et al.*, 2009; Lecca *et al.*, 2012; Tong *et al.*, 2017; Engi *et al.*, 2021). On the other hand, the pharmacological and genetic impairments of eCB signaling reduces the motivational and rewarding effects of these drugs through a modulation of DA activity (Manzanares *et al.*,

2018; Spanagel, 2020). Indeed, the blockade of CB1R decreases nicotine and ethanol self-administration and NAc DA release in rodents exposed to THC, nicotine, ethanol, or cocaine (Cohen *et al.*, 2002; Hungund *et al.*, 2003; Soria *et al.*, 2006). Accordingly, CB1R antagonist treatment prevents the rewarding effects of THC and WIN, as well as the enhanced striatal DA levels (Solinas and Goldberg, 2005; Wenzel and Cheer, 2014, 2018). Similarly, genetic manipulation of eCB system shows that CB1R KO mice display a reduction of conditioned place preference to ethanol and cocaine, which is associated with a compensatory increase in striatal DAD2R expression (Houchi *et al.*, 2005). This evidence supports the role of eCB system in the reinforcing and motivational properties of different drugs of abuse via the modulation of DA transmission. Particularly, eCB system plays a key role in cocaine reinforcement-related behaviors after subchronic exposure and after withdrawal. In fact, an increase in 2-AG levels and a decrease of AEA levels in PFC, NAc, dorsal striatum and HP are observed after repeated cocaine self-administration and passive exposure. Conversely, reduced levels of these eCBs result after 10 days of abstinence (Bystrowska *et al.*, 2014; 2018), along with an up-regulation of CB1R in cortical and subcortical brain areas (Adamczyk *et al.*, 2012). Similarly, subchronic cocaine exposure during early adolescence increases CB1R density and decreases CB2R expression in PFC, an effect that subsides at adulthood (García-Cabrerizo and García-Fuster, 2016). This latter finding confirms how critical are developmental periods, which are characterized by a higher vulnerability to cocaine effects.

1.5. Aggressive behavior and drug addiction

Drug addiction is a chronic relapsing brain disorder due to repetitive use of substances that alters reward circuit functions and related behaviors thereby leading to drug-induced plasticity changes. Drug addiction is characterized by three stages: 1) drug-compulsive seeking and taking (binge/intoxication); 2) loss of control in limiting intake (preoccupation/anticipation); 3) emergence of a negative emotional state (*e.g.*, dysphoria, anxiety, irritability) that reflects a motivational withdrawal syndrome (withdrawal/negative effect) (Koob and Volkow, 2010, 2016; DMS-5). Various neuronal circuits are involved during these stages. Specifically, in the first stage, the reinforcing effects of drugs of abuse depend on stimulation of mesolimbic DA transmission (Di Chiara and Imperato, 1988; McGregor and Roberts, 1993). The second phase involves areas such as BLA, insula, HP

and PFC to control executive functions, whereas the third stage engages the extended amygdala to promote the negative reinforcement and the development of addiction (Koob and Volkow, 2010).

The transition from controlled to compulsive drug-seeking and drug-taking behavior is influenced by numerous factors. Drug exposure induces lasting neuroadaptations in motivational systems that drive drug-seeking and drug-taking behaviors. Although the first stage of drug use is motivated by hedonic processes, prolonged drug exposure progressively blunts reward system function, thereby leading to escalated frequency and amount of drug consumption, resulting in a dependent state characterized by negative affective symptoms (*e.g.*, dysphoria, anxiety, and irritability) that emerge during abstinence. These negative emotional states arise from the recruitment of stress signaling systems (involving corticotropin releasing factor and dynorphin) and dysregulation of mechanisms that constrain these responses (such as neuropeptide Y and nociception). Prolonged and compulsive drug consumption can alleviate negative affective states through negative reinforcement (Koob and Volkow, 2010; 2016). Moreover, clinical studies reveal that the interaction between MAOA-L alleles and child traumas plays an important role in the development of substance abuse later in life (Fite *et al*., 2018; 2020).

Notably, a high comorbidity exists between AB and substance abuse (Seo *et al.*, 2008) and this depends on common biological predisposing factors, including imbalance in neurotransmitters and executive brain functions. Among these, alterations in the 5-HT and DA systems (Soderstrom *et al.*, 2003) and prefrontal function (Goldstein *et al.*, 2005) potentially represent the link between AB and its comorbidity disorders, such as drug abuse. On one hand, low DA levels contribute to drug- and alcohol-seeking behavior, increased DA signaling is observed in limbic brain regions (*e.g.*, NAc) in cocaine-treated rats (Hurd and Ungerstedt, 1989), in type II alcohol users (Tiihonen *et al.*, 1995), and aggressive rats and humans (Miczek *et al.*, 2002). On the other hand, dysfunction in PFC, particularly in orbitomedial areas, is a common condition in AB and drug abuse. Accordingly, PFC plays a key role in the modulation of self-control and in processing salience attribution to social-affective and environmental stimuli, so that its disruption is associated with an increased propensity to impulsive and compulsive behavior, including AB and drug abuse (Davidson, 2002; Korponay *et al.*, 2017). Moreover, mesocorticolimbic system plays a critical role in processing reward-related stimuli, including those of drugs of abuse (Wise, 2008; Volkow and Morales, 2015). Accordingly, brain imaging studies demonstrate that PFC circuit is

disrupted in substance abusers, and it is associated with an inability to control AB (Davidson, 2002, Ochsner and Gross, 2005), as well as a compulsive drug intake (Goldstein and Volkow, 2011). An increased DA activity has been observed in limbic regions, such as NAc, in different population of substance abusers, as well as in aggressive individuals (Hurd and Ungerstedt, 1989; Miczek *et al.*, 2002). This evidence suggests that the dysregulation of mesocorticolimbic DA system may contribute to the etiology of both AB and substance abuse, thus representing a common biological substrate for the comorbidity between these disorders.

2. Aims of the study

We took advantage of the hypomorphic ($MAOA^{Neo}$) mouse model, which recapitulates the interactions between low activity of MAOA variants and child neglect (G×E) (Godar *et al.*, 2019), predisposing to AB to examine, by means of behavioral and *ex vivo* electrophysiological analyses, whether:

1) synaptic alterations of mesocorticolimbic DA system are necessary and sufficient for the genesis of AB during pre-adolescence;

2) this GxE interaction enhances the vulnerability to psychostimulant effects of cocaine.

3. Material and methods

3.1. Animals

All experimental procedures were in accordance with the National Institute of Health guidelines and approved by the Animal Use Committees of the Universities of Cagliari, Utah, and Kansas (USA). In Italy, all procedures were performed in accordance with the European legislation (EU Directive, 2010/63) and were approved by the Animal Ethics Committees of the University of Cagliari and by Italian Ministry of Health (auth. n. 659/ 2015-PR and 621/2016-PR). We made all efforts to minimize animal pain and discomfort and to reduce the number of animals used. Male MAOA^{Neo} mice on 129S6 genetic background and their WT counterparts were used in this study.

3.1.1. MAOA^{Neo} mice

The generation of MAOA^{Neo} mice was made at University of Utah and Kansas. Briefly, the MAOA^{Neo} construct was specifically designed to harbor a floxed neomycin selection cassette (*Neo*^{R}) in intron-12 of the *Mao*a gene and a loxP sequence in intron-11. This configuration was devised to generate a hypomorphic variant in which either the *Neo*^{R} cassette or exon-12 (which encodes for the active site of the enzyme) may be removed upon recombination with *Cre* sequences. Shortly, a MAOA^{Neo} targeting vector was generated from the plasmid pPGK1neo, containing a phosphoglycerate kinase-1 (*PGK1*) promoter followed by *Neo*^{R} cassette and a polyadenylated signal flanked by two *lox*P sequences, and a 9-kb *Bam*HI MAOA clone. This MAOA^{Neo} targeting vector was electroporated into embryonic stem cells and incubated in the culture medium with G418 for neomycin- resistance selection. Two independent neomycin-resistant homologous recombinant clones were selected among the embryonic stem cell lines and used to generate two chimeric lines of 129S6 mice. Then, they were crossed with WT 129/SvEvTac6 female mice, to select for germ-line transmission of MAOA^{Neo} genotype among WT littermates. F1 female MAOA^{Neo} mice (HZ) were selected to cross with WT 129/SvEvTac6 male mice to obtain F2 male MAOA^{Neo} mice, which were used to establish a colony (Bortolato *et al.*, 2011). Since *Mao*a is an X-linked gene, male offspring of MAOA^{Neo} mice dams were either MAOA^{Neo} or WT, the presence of *lox*P sequences was then ascertained by genomic DNA PCR.

3.1.3. Animal husbandry

Pregnant MAOA^{Neo} mice were single housed 3 days prior to parturition. Only litters with more 4 pups (and at least 2 males) were used, and all litters with more than 8 pups were culled to 8 at PND1 to assure uniformity of litter size. Litter effects were minimized by using mice from at least 6 different litters for behavioral and electrophysiological experiment. Bedding was changed in all cages at PND7 and PND14, and mice were weaned at PND21. Animals were grouped-housed in cages with food and water available *ab libitium*, except during the periods of social isolation, in which they were isolated in their home cage with *ab libitium* access to food and water. The room was maintained at 22°C, on a 12 h/12h light/dark cycle from 7 a.m. to 7 p.m. Behavioral and electrophysiological experiments occurred between 10 a.m and 6 p.m. during the light phase of the light/dark cycle.

3.2. Early life stress procedure (ES)

In order to simulate child neglect and maltreatment, male pups were subjected to a daily stress regimen of maternal separation and saline injection respectively, during the first postnatal week (from PND1 to 7), hereafter designated as early-life stress (ES) (**Fig. 2**). During maternal separation, pups were removed from the home cage and placed into a new cage in a separate temperature-controlled (25 °C) room for 2–3 h/day. Manipulations were performed in a pseudorandom fashion and at different times during the light cycle. Physiological saline injections were performed using a micro-injector connected to a Hamilton syringe (10 µL/g body weight). The stressfulness of intraperitoneal injection was ensured by verifying the response from each pup, which typically consisted of rapid limb and head movements after the puncture. At PND22, mice were singly housed for 5 days before performing behavioral and electrophysiological experiments. This regimen was implemented to enhance the translational relevance of the animal model (Godar *et al.*, 2019).

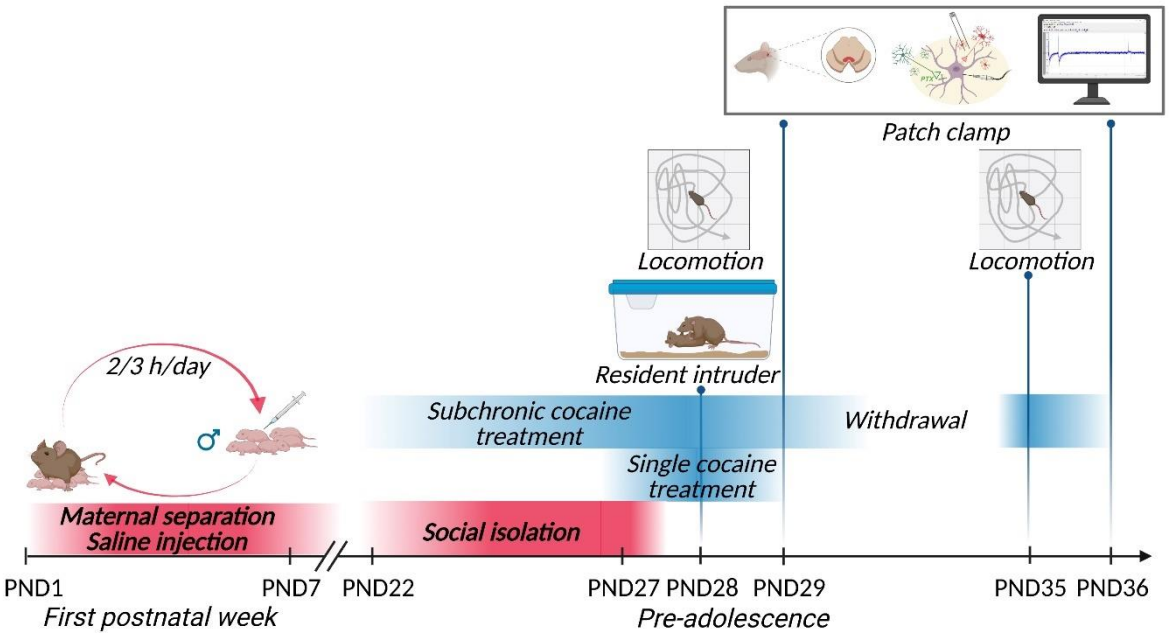

Fig 2. Schematic timeline of the experimental design. Pre-adolescence male pups were subjected to a daily stress regimen of maternal separation and saline injection during the first postnatal week (from postnatal day 1 (PND1) to PND7) followed by social isolation for 5 days at PND22 before performing resident intruder test and electrophysiological experiments. At pre-adolescence mice received single or subchronic daily i.p. injection of cocaine (15 mg/kg) before to test locomotor activity and perform electrophysiological experiments, as well as after one week of withdrawal.

3.3. Resident intruder paradigm

Aggression was tested in the resident intruder task. Male mice were isolated for 5 days (from PND22 to PND27) in their home cages (**Fig. 2**). This duration of isolation was chosen because it has been assessed as sufficient to evoke a robust aggressive response in 129S6 MAOA^{Neo} mice, and optimal to capture marked differences in aggression in comparison to WT conspecifics. During this period, the bedding of the cage was not cleaned to let the resident establish its own territory. On the first day after isolation, resident mice were exposed for 10 min to age- and weight-matched intruder WT males from different litters. The intruder was placed at the opposite side of the resident in a counterbalanced position (right or left). Behavior was video monitored from an adjacent room, recorded, and scored by trained observer blind to both genotype and treatment. Measurements included: (a) number and duration of fighting behavior; (b) latency between the introduction of the intruder and the first attack; (c) social exploration, in terms of frequency and duration of active social approach; (d) latency to first "non fighting" social approach; (e) episodes of tail rattling, a prominent feature of aggressive phenotype in mice; (f) number and duration of chasing behavior of the resident towards the intruder; (g) rearing behavior, as index of general motor activity of the resident. At the end of the test, resident and intruder mice were

returned to their respective home cages. Prior to behavioral testing, animals were acclimated to the room in which the behavioral paradigm took place for a period of 30 minutes. This approach minimized the possible errors associated with environmental visual, odorous, and auditory cues during the testing phase.

3.4. Open field test

Mouse locomotor activity was tested in the open field arena, consisting of a clear Plexiglas square cage (11cm long x 11 cm wide x 30 cm high) covered by clear Plexiglas plate. Each box had a set of photocells located at right angles to each other and projecting horizontal infrared beam.

The day of the test, mice were placed in the center of the arena and their basal locomotor activity was measured as total number of sequential infrared beam breaks in the horizontal sensor, recorded every 5 minutes, beginning after the mice was placed in the cage. To evaluate the effect of cocaine on locomotor behavior, animals were first habituated to the apparatus for 15-30 min to obtain a stable measure of baseline activity, then after cocaine administration the locomotor activity was assessed for 120 min. We tested the locomotor activity of mice in response to three different protocols of cocaine exposure (**Fig. 2**): (a) after a single cocaine administration; (b) the last day of subchronic cocaine exposure (5-7 days); (c) after a challenge with cocaine following one week of withdrawal from cocaine subchronic exposure.

3.5. *Ex vivo* electrophysiological experiments

MAOA^{Neo} and WT mice were anesthetized with isoflurane until loss of righting reflex and decapitated, then horizontal VTA brain slices (250 μm) or coronal PFC slices (300 μm) were cut using a vibrating slicer (Leica). To assess the effect of cocaine after subchronic treatment and after one week of withdrawal, VTA slices were prepared 20-24 h and one week after the last cocaine injection, respectively (**Fig. 2**). For midbrain, slices were prepared in ice cold (4–6°C) low-Ca^{2+} solution containing the following (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 0.625 $CaCl_2$, 18 $NaHCO_3$ and 11 glucose (300-306 mOsm). For PFC, slices were prepared in ice cold (4–6°C) sucrose-based physiological solution containing the following (in mM): 87 NaCl, 75 sucrose, 25 glucose, 5 KCl, 1.25 NaH_2PO_4, 21 $MgCl_2$, 0.5

$CaCl_2$. Immediately after cutting, slices were transferred to a holding chamber with an artificial cerebrospinal fluid (aCSF) at 37°C and saturated with 95% O_2 and 5% CO_2 containing the following (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$ and 11 glucose (300-306 mOsm). For midbrain, slices were allowed to recover for at least 1 h before being placed, as hemislices, in the recording chamber and superfused with aCSF (36–37 °C) saturated with 95% O_2 and 5% CO_2. For PFC, slices were recovered in aCSF at 37°C for 30 min and then were stored (>1 h) at room temperature until recording. Cells were visualized with an upright microscope with infrared illumination (Axioskop FS 2 plus; Zeiss), and whole-cell patch-clamp recordings were made by using an Axopatch 200 B amplifier (Molecular Devices).

Voltage-clamp recordings of evoked excitatory post-synaptic currents (EPSCs) were made with electrodes filled with a solution containing the following (in mM): 117 Cs methanesulfonic acid, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 Mg_2ATP, and 0.25 Mg_2GTP, pH 7.2–7.4, 275–285 mOsm. Picrotoxin (100 μm) was added to the ACSF to block GABAA receptor-mediated inhibitory post-synaptic currents (IPSCs). Voltage-clamp recordings of evoked IPSCs were made with electrodes filled with a solution containing the following (in mM): 144 KCl, 10 HEPES, 3.45 BAPTA, 1 $CaCl_2$, 2.5 Mg_2ATP and 0.25 Mg_2GTP, pH 7.2–7.4, 275–285 mOsm. All GABAA IPSCs were recorded in the presence of, 6-cyano-2,3-dihydroxy-7- nitro-quinoxaline (10 μM) and 2-amino-5-phosphonopentanoic acid (AP5; 100 μM) to block AMPA and NMDA -receptors-mediated synaptic currents, respectively. Experiments were begun only after series resistance had stabilized (typically 15–40 MΩ). Series and input resistance were monitored continuously on-line with a 5 mV depolarizing step (25 ms). Data were filtered at 2 kHz, digitized at 10 kHz, and collected on-line with acquisition software (pClamp 10; Molecular Devices).

DA neurons from the lateral portion of the posterior VTA were identified by the following criteria: cell morphology and medial anatomical location to the medial terminal nucleus of the accessory optic tract; the presence of a large hyperpolarization activated current (I_h), assayed immediately after break-in in response to 13 incremental 10 mV hyperpolarizing steps (250 ms) from a holding potential of -70mV; slow pacemaker-like firing rate (<5 Hz); long action potential duration (>2 ms) (Johnson and North, 1992). PFC pyramidal neurons were identified by their peculiar shape, the presence of a prominent apical dendrite, and the distance from the pial surface (layers 5/6).

A bipolar, stainless steel stimulating electrode (FHC) was placed ~100–200 μm rostral to the recording electrode and was used to stimulate at a frequency of 0.1 Hz. Paired stimuli were given with an interstimulus interval of 50 ms, and the ratio between the second and the first postsynaptic currents (PSC2/PSC1) was calculated and averaged for a 5 min baseline (Melis *et al.,* 2002). NMDA EPSCs were evoked while holding cells at +40 mV. The AMPA EPSC was isolated after bath application of the NMDA antagonist D-AP5 (100 μM). The NMDA EPSC was obtained by digital subtraction of the AMPA EPSC from the dual (AMPA + NMDA-mediated) EPSC (Ungless *et al.,* 2001). For evoked field excitatory postsynaptic potential (fEPSP) recordings, extracellular recording electrodes were filled with ACSF. Field potentials were stimulated at 0.1 Hz (100 ms pulse) with the stainless-steel stimulating electrode placed in layer 2/3 as previously described (Lafourcade *et al.,* 2007). The depolarizing pulse used to evoke depolarization-induced suppression of inhibition (DSI; Vincent *et al.,* 1992; Pitler and Alger, 1994) was a 3 s step to +40 mV from holding potential (-70 mV) (Melis *et al.,* 2004a). This protocol was chosen on the evidence of a manifest eCB tone when VTA DA cells are held at +40 mV for 3 s (Melis *et al.,* 2004a, 2009). The magnitude of DSI was measured as a percentage of the mean amplitude of consecutive IPSCs after depolarization (acquired between 5 and 15 s after the end of the pulse) relative to that of 5 IPSCs before the depolarization (Melis *et al.,* 2004a). Bath application of WIN was performed as follows: WIN was applied for 5 min at the lowest concentration, then, another 5 min with the next increasing WIN concentration. The effect of WIN on GABAA IPSCs was taken at the 5^{th} minute of bath application and normalized to the baseline (5 min before drug application). We chose this protocol because it has been shown that at physiological temperatures WIN-induced effects on GABAA IPSCs recorded from VTA DA neurons reached their peak at this time. The effect of JZL (100 nM) on GABAA IPSCs was taken after 5 min bath application (Lecca *et al.,* 2012). Each hemislice received only a single drug exposure. All drugs were dissolved in DMSO when it was needed. The final concentration of DMSO was <0.01 %.

3.6. Drugs

SCH23390 hydrochloride, AM281 and JZL184 were purchased from Tocris Bioscience. Alpha-methyl-p-tyrosine (AMPT) and WIN were purchased from Sigma-Aldrich, cocaine hydrochloride was purchased from Johnson Matthey and sulpiride was purchased from

Teofarma. AMPT (200 mg/kg), SCH23390 hydrochloride (0.1 mg/kg), cocaine hydrochloride (15 mg/kg) and sulpiride (10 mg/kg) were dissolved in 0.9% saline solution, whilst AM281 (2.5 mg/kg) was dissolved in dimethyl sulfoxide (DMSO) 3% and saline 0.9% of final volume. AMPT and SCH23390 were administered 30 min prior to testing. Sulpiride and AM281 (2.5 mg/kg) were administered 20 min before cocaine injection. All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

3.7. Statistical analysis

All the numerical data are given as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism (version 6.01). Kolmogorov-Smirnov and Bartlett's tests were used to verify data normality and homoscedasticity. Data were compared and analyzed utilizing two-way ANOVA for repeated measures (treatment x time), when appropriate, or Student's *t*-test followed by Tukey's and Sidak's *post-hoc* test. Predicted Gaussian curves were built upon nonlinear regression analysis of frequency distribution. Significance threshold was set at 0.05.

4. Results

4.1. Synaptic alterations of the mesocorticolimbic DA system in MAOA^{Neo} mice exposed to early life stress are necessary and sufficient for the genesis of AB during pre-adolescence

4.1.1. The role of DA signaling in the manifestation of AB in MAOA^{Neo} mice subjected to early life stress

To induce AB at preadolescence, MAOA^{Neo} mice were subjected to early life stressful procedures to reproduce physical abuse and neglect during the first postnatal weeks of life, and subsequently, to social isolation at weaning. Accordingly, the first postnatal week of life and pre-adolescence period represent two important developmental windows during which DA plays a critical role in the etiology of AB at adulthood (Yu *et al.*, 2014). We first addressed the role of DA in this mouse model of G×E interaction leading to AB during pre-adolescence.

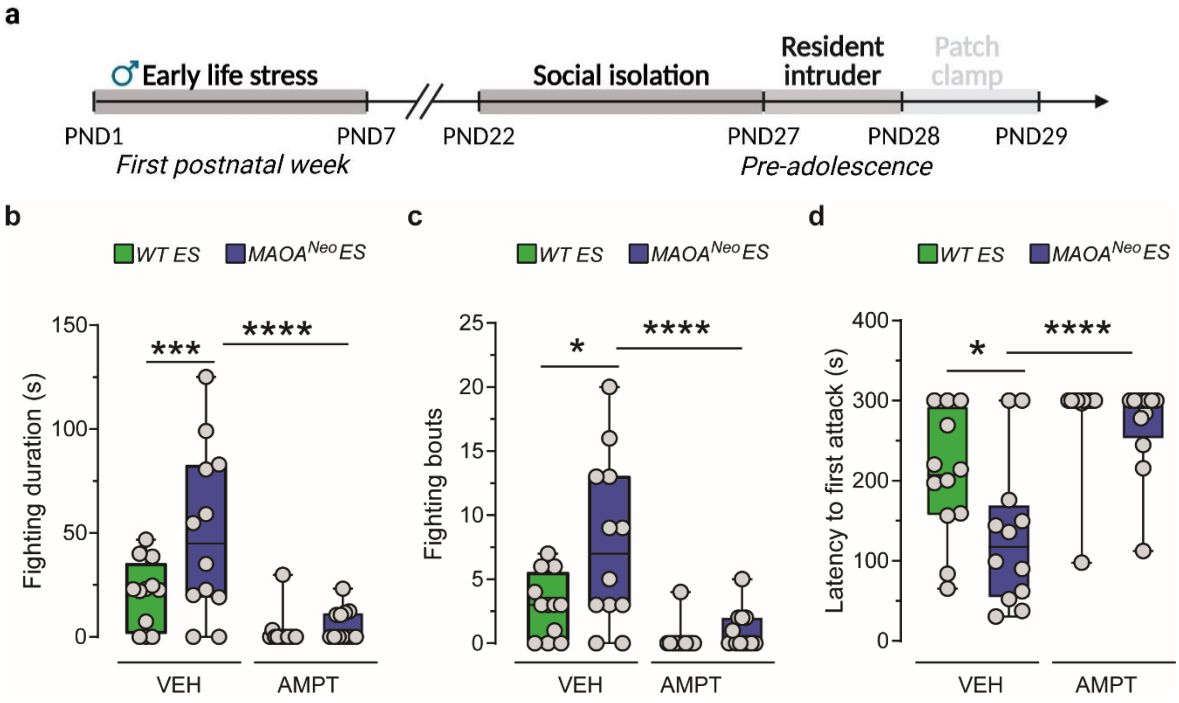

Fig 3. DA synthesis inhibition prevents aggressive behavior in MAOA^{Neo} mice subjected to early stress. (**a**) Schematic timeline of experimental design. Graphs showed the effect of acute administration of the tyrosine hydroxylase enzyme inhibitor alpha-methyl-para-tyrosine (AMPT; 200 mg/kg i.p.) in MAO^{Neo} ES mice on duration (**b**), number of episodes (**c**) of fighting behaviors and in the latency to the first attack (**d**). Graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single animal (n_{mice}=12 per group). Asterisks in the figure refer to main effect of genotype and treatment, revealed by 2-way ANOVA analyses; ****p < 0.0001, ***p < 0.001, *p < 0.05.

We confirmed that AB was displayed in a resident-intruder test at pre-adolescence (PND28-30) by MAOA^{Neo} mice subjected to early life stress (ES; Godar *et al.*, 2019): MAOA^{Neo} ES mice exhibited an increased fighting duration (**Fig. 3b**, 2-way ANOVA, main effect of genotype: $F_{(1, 50)}$=30.57: $p < 0.001$), number of fighting bouts (**Fig. 3c**, 2-way ANOVA, main effect of genotype $F_{(1,44)}$=6.90, $p < 0.05$), and a decreased latency to first attack (**Fig. 3d**, main effect of genotype: $F_{(1,44)}$=6.90, $p < 0.05$) towards a foreign conspecific. We also did not find differences in AB between MAOA^{Neo} and WT mice (data not shown). To assess whether DA was involved, we pharmacologically blocked *in vivo* the synthesis of DA via acute administration of TH enzyme inhibitor alpha-methyl-para-tyrosine (AMPT, 200 mg/kg, i.p.) 30 min before the resident intruder test. AMPT abolished the aggressive phenotypes in MAOA^{Neo} ES mice, as detected from effect of treatment in all parameters (fighting duration**, Fig. 3b**, 2-way ANOVA, main effect of treatment: $F_{(1,44)}$=22.84, $p < 0.0001$; fighting bouts, **Fig. 3c**, 2-way ANOVA, main effect of treatment: $F_{(1,44)}$=20.60, $p < 0.0001$; latency to first attack**, Fig. 3d**, 2-way ANOVA, main effect of treatment $F_{(1,44)}$=20.60, $p < 0.0001$). Significant differences in genotype×treatment interactions were also found for the duration and number of attacks (**Fig. 3b,c**, 2-way ANOVA, duration, $F_{(1,44)=}$4.08, $p < 0.05$, *post-hoc* Tukey's test, MAOA^{Neo} ES VEH vs MAOA^{Neo} ES AMPT, $p < 0.001$; bouts, $F_{(1,44)}$=3.96, $p < 0.05$; , *post-hoc* Tukey's test, MAOA^{Neo} ES VEH vs MAOA^{Neo} ES AMPT, $p < 0.001$), but not for the latency (**Fig. 3d**, 2-way ANOVA, $F_{(1,44)}$=2.08). These data suggest that the interaction between low MAOA activity and ES elicits AB at pre-adolescence that requires DA signaling.

4.1.2. Early life stress alters MAOA^{Neo} mouse post-synaptic responsiveness of VTA DA neurons to excitatory inputs

Next, we investigated whether this GxE interaction could affect synaptic properties of VTA DA neurons during pre-adolescence. To this aim, we obtained whole-cell patch-clamp recordings from putative DA neurons in the lateral portion of the posterior VTA and we electrically evoked excitatory post-synaptic currents (EPSCs) by stimulating rostral afferents. When we examined the current-voltage relationship (I–V) of AMPAR-mediated EPSCs, we found that ES impacted only MAOA^{Neo} mouse DA cells. In particular, MAOA^{Neo} ES mouse current-voltage relationship (I-V) curves are non-linear and exhibit an inward rectification (**Fig. 4b**, 2-way ANOVA, interaction $F_{(1,24)}$= 6.8; $p < 0.01$; genotype $F_{(1,24)}$= 71.2; $p < 0.0001$; ES $F_{(1,24)}$= 20.6; $p = 0.0001$; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT, $p < 0.0001$; MAOA^{Neo} ES vs MAOA^{Neo}, $p < 0.001$; MAOA^{Neo} ES vs WT ES, $p < 0.0001$),

which is an electrophysiological signature of GluA2-lacking AMPARs, which are calcium permeable (Bellone and Lüsher, 2005; Bellone *et al.*, 2011; Hausknecht *et al.*, 2015). However, no differences in synaptic strength elicited by paired stimuli (50 ms interval) as measured as paired-pulse ratio (PPR), were found among genotypes and ES (**Fig. 4c**, 2-way ANOVA, interaction $F_{(1,31)}$= 0,4; p = 0.53).

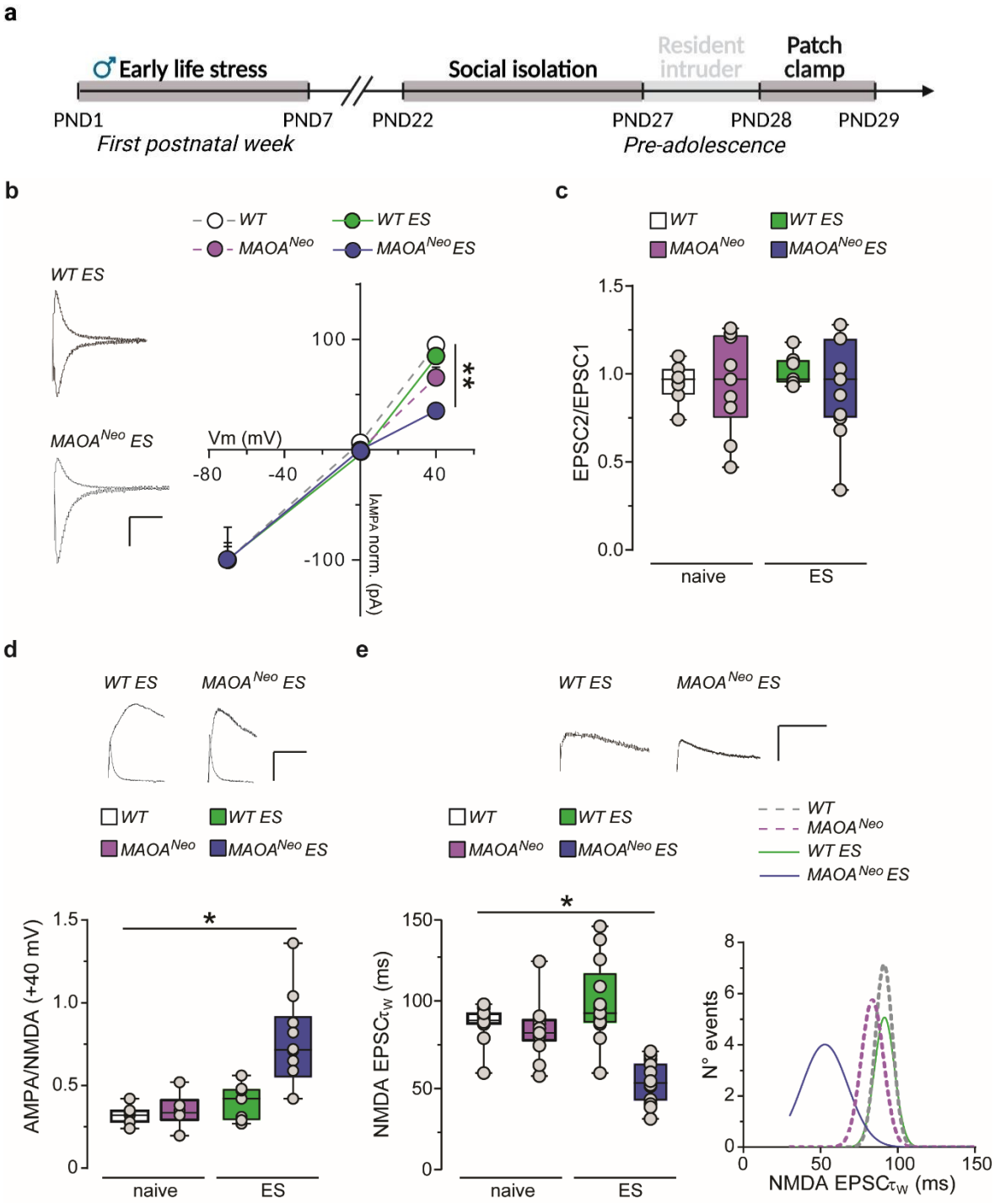

Fig. 4. Early life stress affects excitatory postsynaptic responsiveness of VTA DA neurons in MAOA^{Neo} mice. (**a**) Schematic timeline of experimental design. (**b**) Current-voltage relationship (I-V) curves of AMPA EPSCs recorded from DA neurons of WT (n_{cells}=7, n_{mice}=4), MAOA^{Neo} (n_{cells}=6, n_{mice}=3), WT ES (n_{cells}=6,

n_{mice}=4) and MAOA^{Neo} ES (n_{cells}=10, n_{mice}=8) mice. Insets show representative traces of AMPA EPSCs recorded at -70 and +40 mV from WT ES and MAOA^{Neo} ES. Calibration bar: 10 ms, 50 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**c**) Bar graph summarizing the effect of ES on paired-pulse ratio (EPSC2/EPSC1) of AMPA EPSCs recorded from WT (n_{cells}=5, n_{mice}=3), MAOA^{Neo} (n_{cells}=10, n_{mice}=5), WT ES (n_{cells}=7, n_{mice}=4) and MAOA^{Neo} ES (n_{cells}=11, n_{mice}=8) slices. (**d**) ***Top***, insets show representative traces of AMPA and NMDA EPSCs recorded from DA neurons held at +40 mV in slices from WT ES and MAOA^{Neo} ES mice. Calibration bar: 20 ms, 100 pA. ***Bottom***, quantification of the data summarizing ES effect on AMPA/NMDA ratio recorded from WT (n_{cells}=7, n_{mice}=4), MAOA^{Neo} (n_{cells}=6, n_{mice}=3), WT ES (n_{cells}=7, n_{mice}=4) and MAOA^{Neo} ES (n_{cells}=10, n_{mice}=8) slices. (**e**) ***Top***, insets show representative traces of NMDA EPSCs recorded at +40 mV in slices from WT ES and MAOA^{Neo} ES mice. ***Bottom (right)***, quantification of the data showing NMDA EPSC decay time kinetic (weighted tau, τ) in MAOA^{Neo} ES (n_{cells}=14, n_{mice}=8), WT ES (n_{cells}=13, n_{mice}=7), MAOA^{Neo} (n_{cells}=13, n_{mice}=7) and WT (n_{cells}=11, n_{mice}=5) slices. ***Bottom (left)***, A predicted Gaussian showing distribution of NMDAR EPSCs τ in the different groups of mice. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; $^{**}p < 0.01$, $^{*}p < 0.05$. Representative traces of AMPA and NMDA EPSC of WT and MAOA^{Neo} mice are not indicated, they are not different from those obtained from WT ES mice.

Changes in AMPAR expression and function often increase the ratio between AMPAR- and NMDAR-mediated EPSCs amplitude (Bariselli *et al.*, 2016), a common electrophysiogical assay to evaluate synaptic strength. Therefore, to determine whether excitatory synapses were potentiated or depressed (Ungless *et al.*, 2001), we examined the AMPA/NMDA ratio in our mouse model. The analysis revealed that putative DA neurons from MAOA^{Neo} ES mice exhibited a higher AMPA/NMDA ratio compared to WT ES and unstressed mice (**Fig. 4d**, 2-way ANOVA, interaction $F_{(1,26)}$= 6.1; p = 0.02; genotype $F_{(1,26)}$= 7.9; p = 0.009; ES $F_{(1,26)}$=12.8; p = 0.001; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT, p < 0.001; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.01; MAOA^{Neo} ES vs WT ES, p < 0.01). We also computed NMDAR-mediated EPSC decay time kinetic (expressed as weighted tau (τ) and predicted Gaussian curves) and found that it is faster in DA neurons recorded from MAOA^{Neo} ES mice, as compared to WT ES and unstressed mice (**Fig. 4e**; 2- way ANOVA, interaction $F_{(1,47)}$=20.9; p < 0.0001; genotype $F_{(1,47)}$= 29.2; p < 0.0001; ES $F_{(1,47)}$= 3.5; p = 0.06; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT, p < 0.0001; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.001; MAOA^{Neo} ES vs WT ES, p < 0.0001), suggesting an increase in the ratio between GluN2A and GluN2B subunits in NMDA receptors (Bellone *et al.*, 2011). Taken together, these findings indicate that increased AMPA/NMDA ratio induced by ES in MAOA^{Neo} mice may reflect GluA2-lacking AMPAR insertion in DA neurons, which might be one feature reflecting an enhanced postsynaptic responsiveness to glutamate transmission.

4.1.3. Early life stress affects synaptic properties and plasticity of PFC neurons in MAOA^{Neo} mice

Imbalances in information processing within the PFC largely depends upon changes in monoamine levels (Levy, 2004; Stahl, 2009) and NMDAR function (Homayoun *et al.*, 2005; Jackson *et al.*, 2004), which enable temporal and spatial summation of incoming inputs (Miller and Cohen, 2001; WA *et al.*, 2010). To investigate potential impact of ES on MAOA^{Neo} PFC, we examined excitatory synaptic properties of layer 5/6 pyramidal neurons. We found no effect of either genotype or ES on I-V relationship of AMPAR-mediated EPSCs (**Fig. 5a**, 2-way ANOVA, interaction $F_{(1,24)}$=2.5; p = 0.13). However, ES induced a depression of AMPAR-mediated EPSCs recorded from pyramidal neurons only in MAOA^{Neo} mice (**Fig. 5b**, 2-way ANOVA, interaction $F_{(1,25)}$=17.7; p = 0.0003; genotype $F_{(1,25)}$=5.9; p = 0.02; ES $F_{(1,25)}$=0.1; p = 0.81; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT ES, p < 0.001; WT ES vs WT, p < 0.05; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.05). ES increased AMPA/NMDA ratio in both genotypes (**Fig. 5c**, 2-way ANOVA, interaction $F_{(1,23)}$=2.6; p = 0.11; *post-hoc* Tukey's test: WT ES vs WT, p < 0.0001; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.01; MAOA^{Neo} ES vs WT ES, p < 0.05). Notably, ES also prolonged the decay time of NMDAR-mediated EPSCs exclusively in MAOA^{Neo} mouse pyramidal neurons (**Fig. 5d,** 2-way ANOVA, interaction $F_{(1,41)}$=11,2; p = 0.0018; genotype $F_{(1,41)}$=17,5; p = 0.0001; ES $F_{(1,41)}$=7,6; p = 0.008; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT ES, p < 0.0001; WT ES vs WT, p < 0.0001; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.001). As expected, RO25698 (0.03-1 μM), a GluN2B-specific antagonist, more potently reduced NMDAR-mediated EPSC amplitude in MAOA^{Neo} ES mouse PFC pyramidal neurons when compared to WT ES and naïve mice (**Fig. 5e**, 2-way RM ANOVA, interaction $F_{(9,57)}$=6.4; p < 0.0001; 0.03 μM, WT ES vs MAOA^{Neo} ES: *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT ES, p < 0.01; WT ES vs WT, p < 0.0001; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.0001). Expression of GluN2B-containing NMDARs favors synaptic plasticity induction bi-directionally (Barria and Malinow, 2005). Particularly, in the PFC, the insertion of GluN2B enhances synaptic responses to presynaptic stimulation and facilitates long-term potentiation (LTP) (Cui *et al.*, 2011; Ruan *et al.*, 2014), similarly to the effects of an increased background of DA signal (Matsuda *et al.*, 2006; Ruan *et al.*, 2014) via DAD1R activation (Lewis and O'Donnell, 2000; Ruan *et al.*, 2014). We, therefore, hypothesized that the enhanced DA levels induced by MAOA low activity, and characterizing MAOA^{Neo} mice, might affect PFC output function in response to ES. To test this hypothesis, we recorded field potentials in PFC layer 5/6 evoked by stimulating layer

2/3 and applied a theta burst stimulation protocol (TBS; 50 Hz, 100 pulses 4 times at 0.1 Hz) that induces LTP only when slices are primed with DA (Matsuda *et al.*, 2006). As predicted, LTP could be induced only in MAOA^{Neo} ES mice (**Fig. 5f**, 2-way RM ANOVA, interaction $F_{(72,408)}$=3.4; $p < 0.0001$; genotype x ES $F_{(3,17)}$=13,93; $p < 0.0001$). Together, these results suggest that the exposure to ES alters NMDAR function and enables long-term plasticity in PFC pyramidal cells of MAOA^{Neo} ES mice.

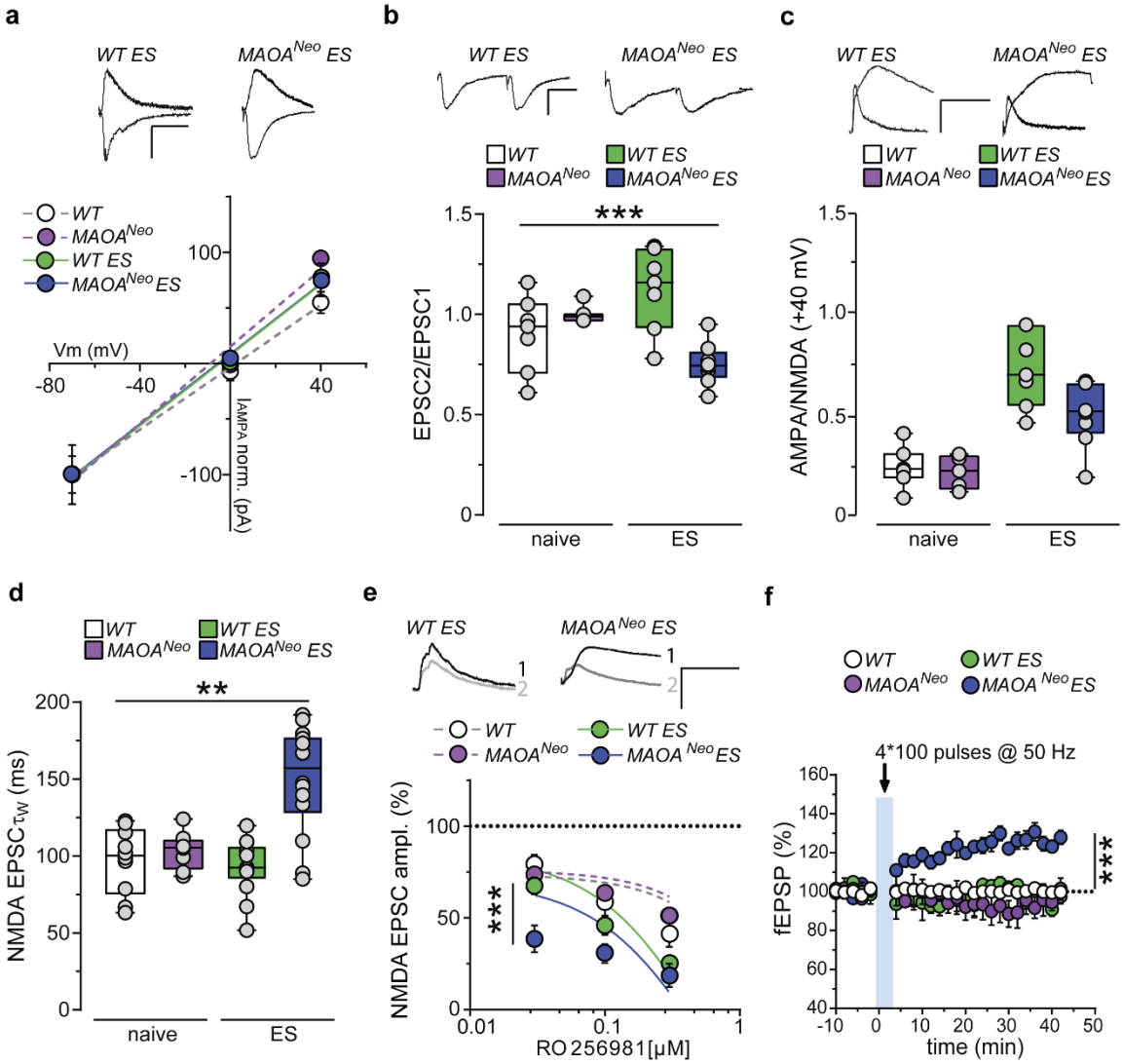

Fig. 5. Early stress alters MAOA^{Neo} mouse PFC function. (**a**) Current-voltage relationship (I–V) curves of AMPA EPSCs recorded from PFC layer 5/6 pyramidal neurons in WT (n_{cells}=7, n_{mice}=3), MAOA^{Neo} (n_{cells}=5, n_{mice}=2), WT ES (n_{cells}=7, n_{mice}=3) and MAOA^{Neo} ES (n_{cells}=7, n_{mice}=4) mice. Insets show representative traces of AMPA EPSCs recorded at -70 and +40 mV from WT ES and MAOA^{Neo} ES mice. Calibration bar: 10 ms, 50 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**b**) Bar graph summarizing the effects of ES on paired-pulse ratio (EPSC2/EPSC1) of AMPA EPSCs recorded from WT (n_{cells}=7, n_{mice}=4), MAOA^{Neo} (n_{cells}=7, n_{mice}=4), WT ES (n_{cells}=7, n_{mice}=5) and MAOA^{Neo} ES (n_{cells}=8, n_{mice}=6) slices. Insets show representative traces of paired AMPA EPSCs. Calibration bar: 20 ms, 100 pA. (**c**) Quantification of the data summarizing ES effect on AMPA/NMDA ratio recorded from WT (n_{cells}=7, n_{mice}=3), MAOA^{Neo} (n_{cells}=5, n_{mice}=2), WT ES (n_{cells}=7, n_{mice}=3) and MAOA^{Neo} ES (n_{cells}=8, n_{mice}=4) slices. Insets show

representative traces of AMPA and NMDA EPSCs recorded from layer 5/6 pyramidal neurons held at +40 mV in slices from WT ES and MAOA^{Neo} ES mice. Calibration bar: 10 ms, 100 pA. (**d**) Quantification of the data showing NMDAR EPSC decay time kinetics (weighted tau, τ) in WT (n_{cells}=10, n_{mice}=8), MAOA^{Neo} (n_{cells}=8, n_{mice}=6), WT ES (n_{cells}=13, n_{mice}=12) and MAOA^{Neo} ES (n_{cells}=14, n_{mice}=13) slices. (**e**) Dose-response curves for percentage inhibition in NMDA EPSC amplitude by the GluN2B selective antagonist RO256981 in WT (n_{cells}=6, n_{pups}=3), MAOA^{Neo} (n_{cells}=6, n_{mice}=3), WT ES (n_{cells}=6, n_{mice}=3) and MAOA^{Neo} ES (n_{cells}=5, n_{mice}=3). Insets show representative traces of NMDA EPSCs, before (black) and after (grey) RO256981 (0.03 μ) bath application. Calibration bar: 100 ms, 200 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**g**) The time course graph shows the mean change in the amplitude of extracellular field potentials (fEPSPs) measured over the indicated theta burst stimulation (TBS: 50 Hz, 100 pulses, four times at 0.1 Hz, at the arrow) from PFC layer 5/6 only in MAOA^{Neo} ES (n_{cells}=5, n_{mice}=4), WT (n_{cells}=5, n_{mice}=3), MAOA^{Neo} (n_{cells}=5, n_{mice}=3) and WT ES (n_{cells}=6, n_{mice}=3) slices. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; ***p < 0.001, **p < 0.01, *p < 0.05. Representative traces of AMPA and NMDA EPSC of WT and MAOA^{Neo} mice are not indicated, they are not different from those obtained from WT ES mice.

4.1.4. Blockade of DAD1R signaling restores PFC function and behavior

In the PFC, phasic DA release following burst firing of VTA DA cells modulates the depolarization of membrane potential of pyramidal neurons via activation of DAD1Rs (Lewis and O'Donnell, 2000). The modulation of excitatory transmission by DAD1R activation in PFC might, therefore, enable synaptic plasticity and facilitate input-target association during reward learning and top-down information processing (Ruan *et al.*, 2014). Thus, we decided to address the role of DAD1R activation in TBS-induced LTP in pyramidal neurons of PFC of MAOA^{Neo} ES mouse. To test this, we pharmacologically blocked DAD1Rs *in vivo* with the DAD1R antagonist SCH23390 (0.1 mg/kg, i.p.) and 30 min after, acute PFC-containing slices were prepared. This manipulation fully blocked TBS-induced LTP in MAOA^{Neo} ES mice without affecting fEPSP in WT ES mice (**Fig. 6b**, 2-way RM ANOVA, interaction $F_{(72,432)}$=2.3; p < 0.0001; SCH23390 on genotype x ES, $F_{(3,18)}$=16.0; p < 0.0001).

Next, to ascertain whether DAD1R blockade could prevent AB in MAOA^{Neo} ES mice, SCH23390 (0.1 mg/kg, i.p.) or vehicle (VEH) was administered 30 minutes before resident-intruder test.

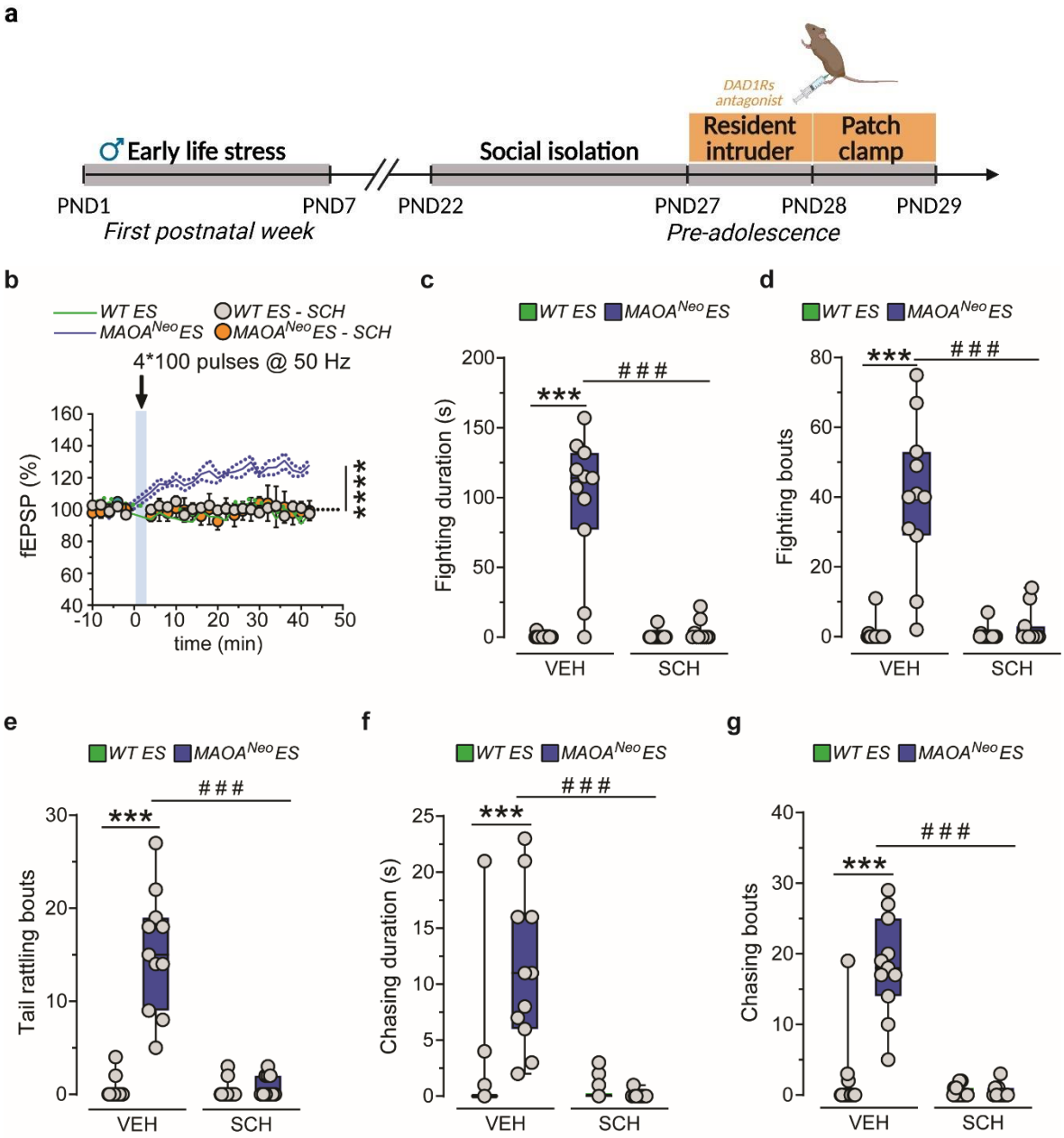

Fig. 6. *In vivo* blockade of DAD1Rs prevents aggressive behavior and restores PFC plasticity in MAOA^{Neo} mice subjected to early life stress. (**a**) Schematic timeline of experimental design. (**b**) *In vivo* administration of the DAD1R antagonist SCH23390 (0.1 mg/kg, i.p., 30 minutes before slice preparation) prevented TBS-induced fEPSPs recorded from PFC layer 5/6 in MAOA^{Neo} ES (n_{cells}=6, n_{mice}=6) slices, whereas it did not affect fEPSP amplitude in WT ES (n_{cells}=6, n_{mice}=6) slices. Green and blue lines represent time course of the effects shown in **Fig. 5f** for WT ES and MAOA^{Neo} ES mice, respectively. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. The aggressive behavior in MAO^{Neo} ES mice was abolished by the selective blockade of DAD1R, SCH23390, expressed as decreased fighting duration (**c**), fighting bouts (**d**), episodes of tail rattling (**e**), duration (**f**) and number of chasing behavior (**g**). Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single animal (n_{mice}=11-18 per group). Main effects are not indicated. 2-way ANOVA; *post-hoc* Tukey's test; ****p < 0.0001, ***/###p < 0.001.

As predicted, pharmacological blockade of DAD1Rs abolished the manifestation of AB at pre-adolescence in MAOA^{Neo} ES mice, as revealed by the reduction in fighting duration and

bouts (**Fig. 6c,** duration, 2-way ANOVA, interaction treatment x genotype $F_{(1,54)}$=67.23 p < 0.0001; *post-hoc* Tukey's test: MAOA^{Neo} ES VEH vs WT ES VEH, p < 0.05; WT ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; MAOA^{Neo} ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; **Fig. 6d,** bouts, 2-way ANOVA, interaction treatment x genotype $F_{(1,54)}$=48.75 p < 0.0001; *post-hoc* Tukey's test: MAOA^{Neo} ES VEH vs WT ES VEH, p < 0.0001; WT ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; MAOA^{Neo} ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001). In addition, to further confirm the increase of AB observed in MAOA^{Neo} ES mice, we analyzed other behavioral phenotypes that may be detected during the resident intruder test, which are strongly correlated with AB (Miczek *et al.*, 2002). We found a marked change in the number of episodes of tail rattling (**Fig. 6e,** 2-way ANOVA, interaction treatment x genotype $F_{(1,54)}$=84.92 p < 0.0001; *post-hoc* Tukey's test: MAOA^{Neo} ES VEH vs WT ES VEH, p < 0.0001; WT ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; MAOA^{Neo} ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001) as well as the duration and frequency of chasing behavior of the resident toward the intruder (**Fig. 6f,** duration, 2-way ANOVA, interaction treatment x genotype $F_{(1,54)}$=20.51, p < 0.0001; *post-hoc* Tukey's test: MAOA^{Neo} ES VEH vs WT ES VEH, p < 0.0001; WT ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; MAOA^{Neo} ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; **Fig. 6g,** frequency, 2-way ANOVA, interaction treatment x genotype $F_{(1,54)}$=60.91, p < 0.0001; *post-hoc* Tukey's test: MAOA^{Neo} ES VEH vs WT ES VEH, p < 0.0001; WT ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001; MAOA^{Neo} ES SCH vs MAOA^{Neo} ES VEH, p < 0.0001).

4.2. MAOA^{Neo} mice exposed to early life stress exhibit an enhanced vulnerability to psychostimulant effects of cocaine at pre-adolescence

4.2.1. Early life stress enhances psychostimulant response to repeated cocaine exposure in MAOA^{Neo} mice

Since AB is one predictive risk factor associated with the development of addictive disorders (Ernst *et al.*, 2006; Jester *et al.*, 2008; Mathias *et al.*, 2015) and, in males, low activity of MAOA uVNTR alleles predisposes to earlier onset of substance abuse (Vanyukov and Tarter, 2000; Kirisci *et al.*, 2004), we hypothesized that our mouse model could be more susceptible to psychostimulant effects of cocaine. To this aim, we examined cocaine effects on their spontaneous locomotor activity in an open file arena. Acute administration of cocaine (15 mg/kg, i.p.) increased locomotor activity in MAOA^{Neo} mice, in terms of

horizontal counts and duration (**Fig. 7b**, 2-way RM ANOVA, interaction genotype x ES $F_{(3,40)}$=31.98; p < 0.0001). The effect of GxE interaction could only be revealed upon one week of daily treatment with cocaine (**Fig. 7c**, 2-way RM ANOVA, interaction genotype x ES interaction $F_{(3,54)}$=29.78; p < 0.0001), an effect that also persisted after one week of withdrawal (**Fig. 7d**, 2-way RM ANOVA, interaction $F_{(3,32)}$=12.01; p < 0.0001).

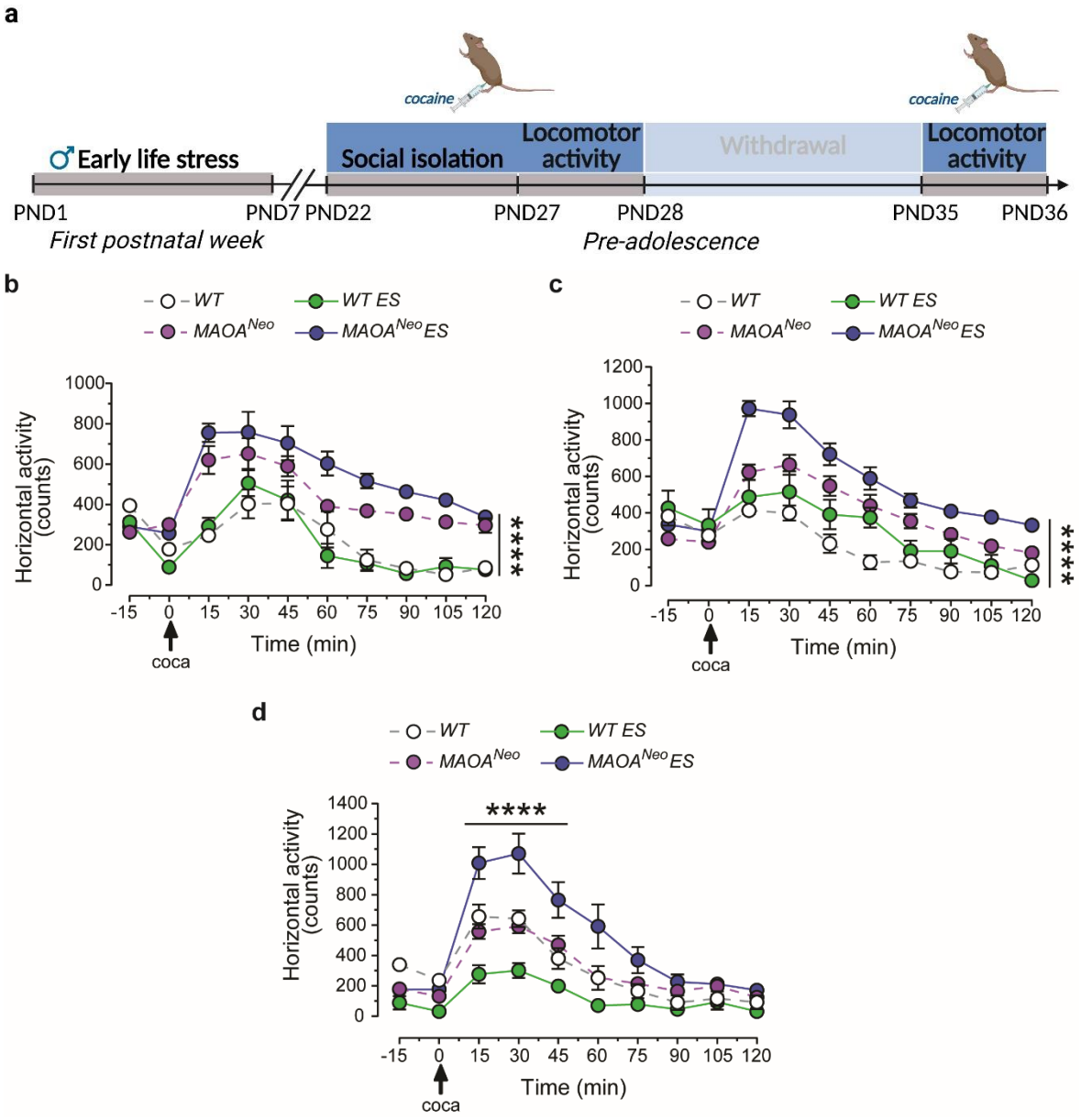

Fig. 7. Psychostimulant effects of cocaine in MAOA^{Neo} mice subjected to early life stress. (**a**) Schematic timeline of experimental design. (**b**) Acute cocaine administration (15 mg/kg, i.p.) increased the locomotor activity of MAOA^{Neo} mice, in terms of horizontal activity and duration. (**c**) Subchronic exposure to cocaine (1 week) enhanced the psychostimulant response to cocaine in MAOA^{Neo} mice exposed to early life stress (ES). (**d**) This enhanced locomotor activity persisted long after one week of withdrawal. Graphs show spontaneous locomotor (horizontal) activity measured as counts (bin=15 min) in a novel open field arena. Each symbol represents the average value (± s.e.m.) (n_{mice}=6-19 per group). Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; ****p < 0.0001.

4.2.2. In vivo subchronic exposure to cocaine abolishes the effects of early life stress on basal synaptic properties of excitatory inputs on VTA DA cells in MAOA^{Neo} mice

Single and repeated *in vivo* exposure to cocaine or other drugs of abuse alters synaptic plasticity in the VTA, which plays an important role in behavioral consequences of cocaine use and abuse (Ungless *et al.*, 2001; Borgland *et al.*, 2004; Yuan *et al.*, 2013). We, therefore, evaluated whether similar adaptations could also occur in MAOA^{Neo} ES mice, which could contribute to its increased psychostimulant effects. To this aim, we subjected the mice to a week of cocaine treatment and 24 hours after the last cocaine administration we prepared the brain slices to measure excitatory synaptic properties of putative DA neurons.

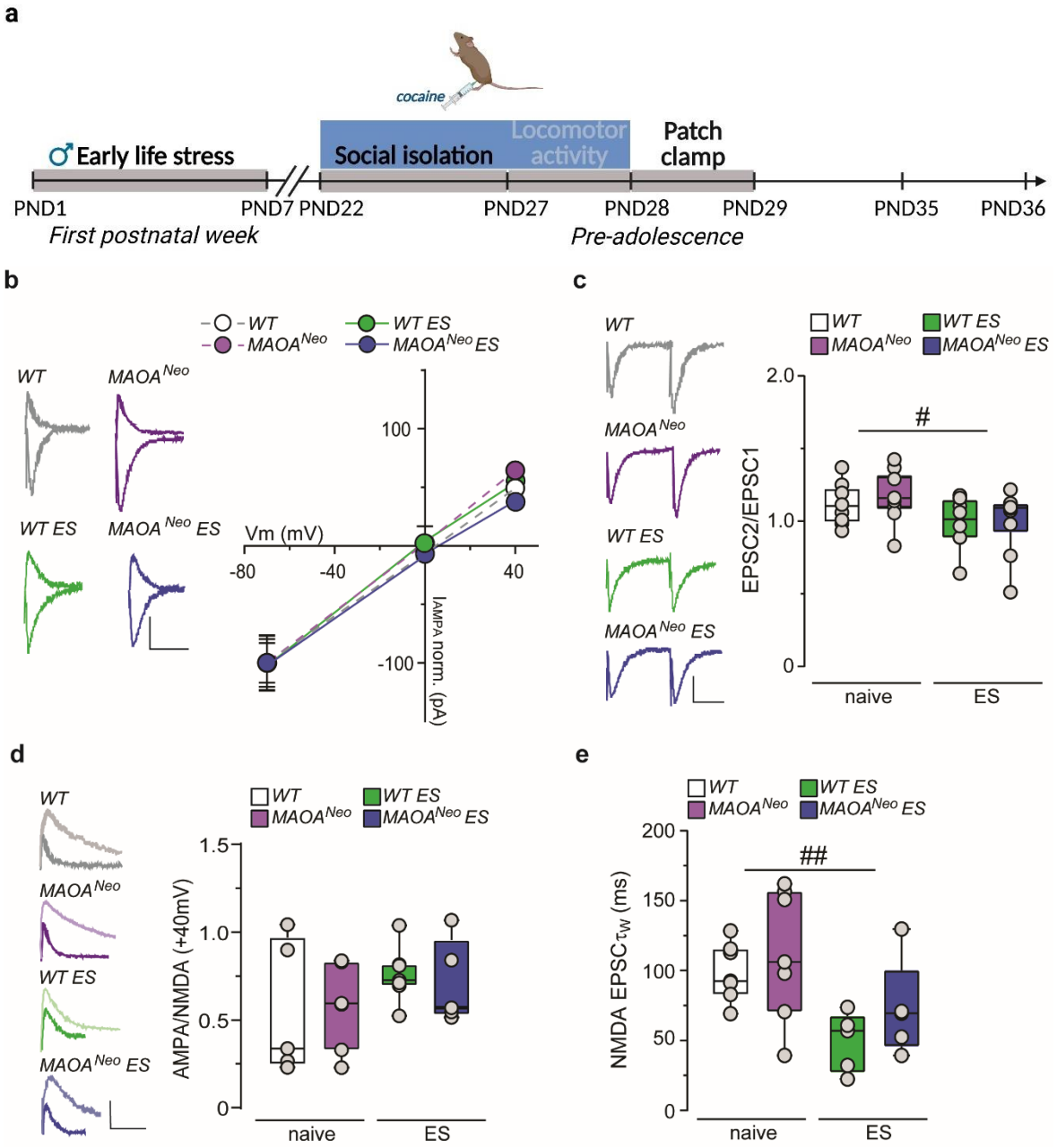

Fig. 8. Excitatory synaptic properties of VTA DA neurons are not changed by subchronic exposure to cocaine in MAOA^{Neo} mice subjected to early life stress. (**a**) Schematic timeline of experimental design. (**b**) Current-voltage relationship (I–V) curves of AMPA EPSCs recorded from DA neurons 24 hours after the last cocaine injection of subchronic treatment in WT (n_{cells}=7, n_{mice}=5), MAOA^{Neo} (n_{cells}=7, n_{mice}=7), WT ES (n_{cells}=6, n_{mice}=7) and MAOA^{Neo} ES (n_{cells}=5, n_{mice}=5) mice. Representative traces of AMPA EPSCs recorded at −70 mV, 0 mV and +40 mV from WT, MAOA^{Neo}, WT ES and MAOA^{Neo} ES mice are shown on the left. Calibration bar: 25 ms, 50 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**c**) Graph panel summarizing the effect of ES on paired-pulse ratio (EPSC2/EPSC1) of AMPA EPSCs recorded from WT (n_{cells}=9, n_{mice}=10), MAOA^{Neo} (n_{cells}=10, n_{mice}=12), WT ES (n_{cells}=8, n_{mice}=8) and MAOA^{Neo} ES (n_{cells}=10, n_{mice}=9) slices 24 hours after the last treatment of subchronic cocaine exposure. (**d**) The AMPA/NMDA ratio was not affected by subchronic exposure to cocaine in MAOA^{Neo} ES mice. Insets show representative traces of AMPA, and NMDA EPSCs traces recorded from DA neurons held at +40mV in slices from WT (n_{cells}=5, n_{mice}=5), MAOA^{Neo} (n_{cells}=7, n_{mice}=7), WT ES (n_{cells}=7, n_{mice}=7) and MAOA^{Neo} ES (n_{cells}=5, n_{mice}=5) mice. Calibration bar: 25 ms, 50 pA. (**e**) Quantification of the data showing NMDAR EPSC decay time kinetics (weighted tau, τ) in WT (n_{cells}=5, n_{mice}=5), MAOA^{Neo} (n_{cells}=7, n_{mice}=7), WT ES (n_{cells}=7, n_{mice}=7) and MAOA^{Neo} ES (n_{cells}=5, n_{mice}=5) slices. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Simbols in the figure refer to the main effect of ES revealed by 2-way ANOVA analyses; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$.

We found no differences on I-V relationship of AMPAR-mediated EPSCs among groups (**Fig. 8b**, 2-way ANOVA, interaction genotype x ES $F_{(3,63)}$=0.41; p = 0.74). Statistical analysis revealed a main effect of ES in the measure of PPR of AMPA-mediated EPSCs in both WT and MAOA^{Neo} mice DA neurons (**Fig. 8c,** 2-way ANOVA, interaction $F_{(1,33)}$=0.15; p = 0.70; genotype $F_{(1,33)}$=0.59, p = 0.44; ES $F_{(1,33)}$=6.56, p = 0.02). Also, no differences were observed in excitatory synaptic strength, expressed as AMPA/NMDA ratio, among groups (**Fig. 8d,** 2-way ANOVA, interaction genotype x ES $F_{(1,20)}$=0.11; p = 0.74). However, a main effect of ES was revealed in the decay time of NMDAR-mediated EPSCs in both WT and MAOA^{Neo} mouse DA neurons (**Fig. 8e,** 2-way ANOVA, interaction $F_{(1,20)}$=0.13; p = 0.72; genotype $F_{(1,20)}$=1.69, p = 0.20; ES: $F_{(1,20)}$=10.39, p = 0.004). These findings suggest that subchronic exposure to cocaine, by affecting excitatory synapses of the other experimental groups, abolishes the effects of ES on basal synaptic properties of excitatory inputs on DA cells in MAOA^{Neo} mice (Frau *et al.*, 2019).

4.2.3. In vivo subchronic cocaine exposure reduces synaptic efficacy of inhibitory inputs on VTA DA neurons in MAOA^{Neo} mice exposed to early life stress

Since subchronic cocaine reduces GABA inhibition in rat DA neurons (Liu *et al.*, 2005; Pan *et al.*, 2008a), we next examined the properties of GABA inputs on VTA DA neurons. We first examined synaptic efficacy of evoked GABAA-mediated inhibitory postsynaptic currents (IPSCs) by building input-output (I-O) curves. 24 hours after the last cocaine injection, we observed a decreased synaptic inhibition of DA cells in MAOA^{Neo} ES mouse DA neurons (**Fig. 9a**, 2-way RM ANOVA, interaction $F_{(3,30)}$=2.87, p =0.05; *post-hoc*

Tukey's test: 75 µA, 100 µA, MAOA^{Neo} ES vs WT, $p < 0.05$; MAOA^{Neo} ES vs MAOA^{Neo}, $p < 0.01$; MAOA^{Neo} ES vs WT ES, $p < 0.05$). This was associated to a paired-pulse facilitation of GABAA IPSCs at higher stimulus intensities (**Fig. 9b**, 2-way ANOVA, interaction $F_{(1,47)}=4.98$, $p = 0.03$, genotype $F_{(1,47)}=6.97$, $p = 0.011$, ES $F_{(1,47)}=8.43$, $p = 0.005$; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT, $p < 0.001$; MAOA^{Neo} ES vs MAOA^{Neo}, $p < 0.01$; MAOA^{Neo} ES vs WT ES, $p < 0.01$) and to a decreased CV (expressed as $1/CV^2$) of IPSCs, an index of changes in presynaptic function (Faber and Korn, 1991) (**Fig. 9c**, 2-way ANOVA, interaction $F_{(1,40)}=6.59$, $p=0.01$; genotype $F_{(1,40)}=5.77$, $p = 0.02$, ES $F_{(1,40)}=15.90$, $p < 0.001$; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT, $p < 0.001$; MAOA^{Neo} ES vs MAOA^{Neo}, $p < 0.001$; MAOA^{Neo} ES vs WT ES, $p < 0.01$).

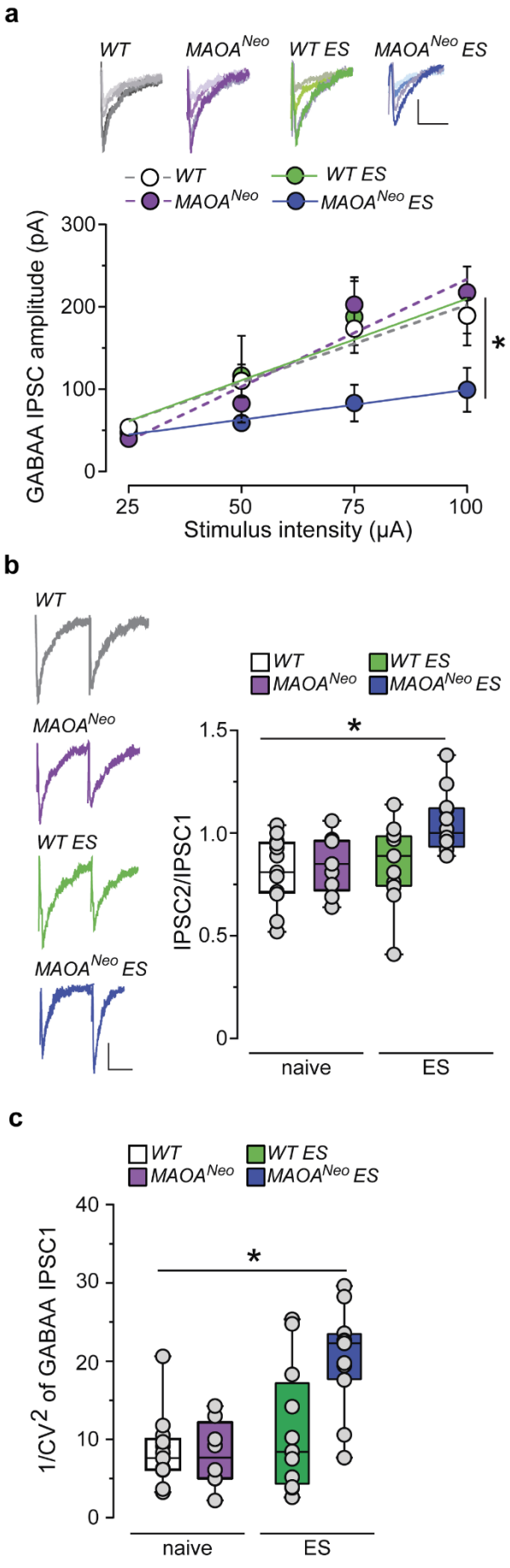

Fig. 9. Subchronic cocaine exposure reduces synaptic inhibition onto DA neurons in MAOA^{Neo} mice subjected to early life stress. (**a**) Input–output relationships of GABAA-mediated IPSCs in WT (n_{cells}=8, n_{mice}=8), MAOA^{Neo} (n_{cells}=7, n_{mice}=7), WT ES (n_{cells}=8, n_{mice}=8) and MAOA^{Neo} ES (n_{cells}=11, n_{mice}=6) mice 24 hours after the last treatment of subchronic cocaine exposure. Insets show representative traces of GABAA IPSC recorded at each stimulus intensity. Calibration bar: 25 ms, 50 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**b**) Graph panel summarizing the averaged paired-pulse ratio (IPSC2/IPSC1) of GABAA IPSCs for all cells recorded 24 hours after the last treatment of subchronic exposure to cocaine in WT (n_{cells}=13, n_{mice}=10), MAOA^{Neo} (n_{cells}=9, n_{mice}=10), WT ES (n_{cells}=13, n_{mice}=12) and MAOA^{Neo} ES (n_{cells}=12, n_{mice}=12) mice. Inset shows representative traces of paired GABAA IPSCs recorded from VTA putative DA neurons. Calibration bar: 25 ms, 50 pA. (**c**) Effect of *in vivo* subchronic exposure to cocaine on coefficient of variation ($1/CV^2$) of GABAA IPSC1 from WT (n_{cells}=13, n_{mice}=10), MAOA^{Neo} (n_{cells}=9, n_{mice}=10), WT ES (n_{cells}=13, n_{mice}=12) and MAOA^{Neo} ES (n_{cells}=12, n_{mice}=12). (**d**) Dose–response curves for percentage inhibition in amplitude of GABAA IPSC by the cocaine bath-application displaying a larger effect in MAOA^{Neo} ES (n_{cells}=5, n_{mice}=5) than in WT (n_{cells}=7, n_{mice}=7), MAOA^{Neo} (n_{cells}=5, n_{mice}=5) and WT ES (n_{cells}=7, n_{mice}=6) cells recorded 24 hours after the last injection of subchronic cocaine exposure. Insets show IPSC traces recorded after 1 µM (green and blue) and 3 µM (grey) cocaine application. Calibration bar, 50 ms, 100 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; **p < 0.01, *p < 0.05.

Taken together, these results suggest that MAOA^{Neo} ES DA cells after repeated cocaine exposure exhibit a reduced synaptic efficacy of GABA afferents that could be ascribed to a decreased probability of neurotransmitter release.

Reduced efficacy of synaptic inhibition induced by acute cocaine is a reversible effect, concentration-dependent and DAD2R-mediated (Pan *et al.*, 2008a). To determine whether MAOA^{Neo} ES DA cells were more sensitive to cocaine acute effects, we compared the effects of increasing cocaine concentrations on GABAA IPSCs.

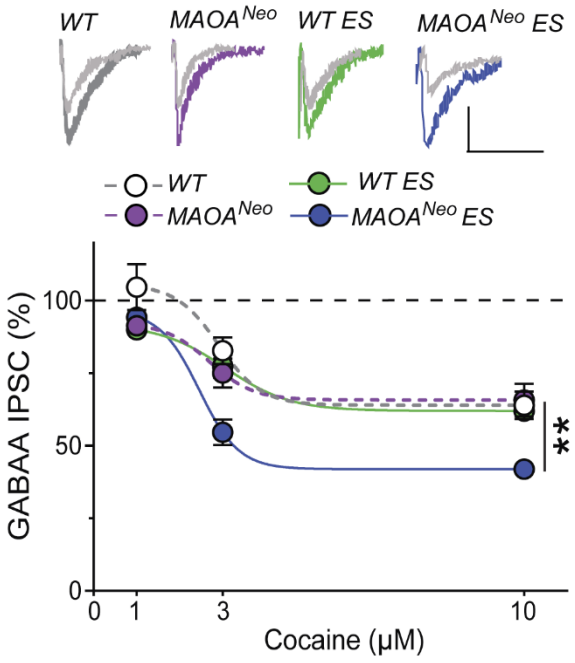

Fig. 10. Subchronic cocaine exposure induces acute depression of evoked IPSC onto DA neurons in MAOA^{Neo} mice subjected to early life stress. Dose–response curves for percentage inhibition in amplitude of GABAA IPSC by the cocaine bath-application displaying a larger effect in MAOA^{Neo} ES (n_{cells}=5, n_{mice}=5) than in WT (n_{cells}=7, n_{mice}=7), MAOA^{Neo} (n_{cells}=5, n_{mice}=5) and WT ES (n_{cells}=7, n_{mice}=6) cells recorded 24 hours after the last injection of subchronic cocaine exposure. Insets show IPSC traces recorded after 1 µM (green and blue) and 3 µM (grey) cocaine application. Calibration bar, 50 ms, 100 pA. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; **p < 0.01

Cocaine is more potent in reducing GABAA IPSCs on VTA DA neurons recorded from MAOA^{Neo} ES mice in comparison to the other groups (**Fig. 10**, 2-way RM ANOVA, interaction $F_{(6,40)}$=2.53, p = 0.04, genotype x ES $F_{(3,20)}$=6.96, p = 0.002, concentration $F_{(2,40)}$=71.07, p < 0.0001; *post-hoc* Tukey's test: 3µM: MAOA^{Neo} ES vs WT, p < 0.001; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.05; MAOA^{Neo} ES vs WT ES, p < 0.01; 10µM: MAOA^{Neo} ES vs WT, p < 0.01; MAOA^{Neo} ES vs MAOA^{Neo}, p < 0.01; MAOA^{Neo} ES vs WT ES, p < 0.05).

Since MAOA^{Neo} ES mice displayed a behavioral sensitivity to cocaine after one week of withdrawal (**Fig. 7d**), we evaluated whether this was associated with long lasting alterations of inhibitory synaptic strength on VTA DA neurons. MAOA^{Neo} ES DA cells still exhibited a reduced efficacy of GABAA IPSC as revealed by I-O curves (**Fig. 11b**, 2-way RM ANOVA, interaction $F_{(4,35)}$=3.97, p = 0.01; *post-hoc* Tukey's test: 75 µA, MAOA^{Neo} ES after withdrawal vs WT, p < 0.05; MAOA^{Neo} ES after withdrawal vs MAOA^{Neo}, p < 0.01; MAOA^{Neo} ES after withdrawal vs WT ES, p < 0.05; 100 µA, MAOA^{Neo} ES after withdrawal vs WT, p < 0.05; MAOA^{Neo} ES after withdrawal vs MAOA^{Neo}, p < 0.05p; MAOA^{Neo} ES after withdrawal vs WT ES, p < 0.05) along with the paired-pulse facilitation (IPSC2/IPSC2) of IPSCs at higher stimulus intensities (**Fig. 11c**, 2-way ANOVA, interaction $F_{(2,57)}$=2.62, p

= 0.08; genotype $F_{(2,57)}$=5.02, p < 0.01; ES $F_{(1,57)}$=4.98, p < 0.03; *post-hoc* Tukey's test: $MAOA^{Neo}$ ES after withdrawal vs WT, p < 0.01; $MAOA^{Neo}$ ES after withdrawal vs $MAOA^{Neo}$, p < 0.05; $MAOA^{Neo}$ ES after withdrawal vs WT ES, p < 0.01; $MAOA^{Neo}$ ES vs WT, p < 0.001; $MAOA^{Neo}$ ES vs $MAOA^{Neo}$, p < 0.01; $MAOA^{Neo}$ ES vs WT ES, p < 0.01), an effect that is not accompanied by differences in $1/CV^2$ (**Fig. 11d**, 2-way ANOVA, interaction $F_{(2,50)}$=5.53, p < 0.01; genotype $F_{(2,57)}$=4.62, p = 0.01; ES $F_{(1,50)}$=34.76, p < 0.0001; *post-hoc* Tukey's test: $MAOA^{Neo}$ ES after withdrawal vs $MAOA^{Neo}$, p = 0.2; $MAOA^{Neo}$ ES after withdrawal vs WT ES, p = 0.8, $MAOA^{Neo}$ ES after withdrawal vs $MAOA^{Neo}$ ES, p = 0.7; $MAOA^{Neo}$ ES vs WT, p < 0.001; $MAOA^{Neo}$ ES vs $MAOA^{Neo}$, p < 0.001; $MAOA^{Neo}$ ES vs WT ES, p < 0.001).

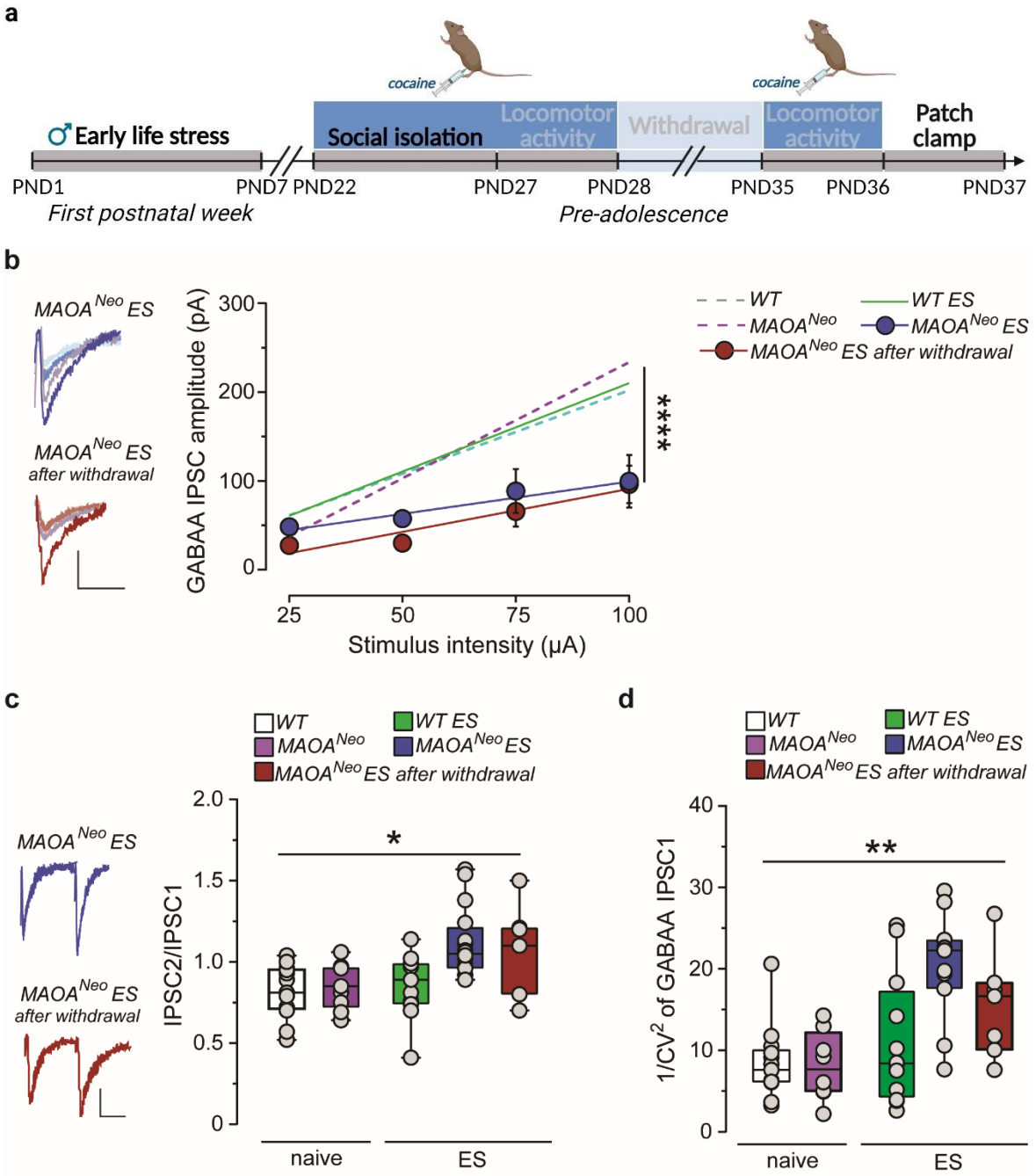

Fig. 11. Subchronic cocaine effects on synaptic properties of inhibitory inputs lasts for one week in VTA DA neurons of MAOA^{Neo} mice subjected to early life stress. (**a**) Schematic timeline of experimental design. (**b**) Input–output relationships of GABAA-mediated IPSCs showing that subchronic cocaine effect in reducing synaptic efficacy of these synapses persists after one week of withdrawal in MAOA^{Neo} ES mice (n_{cells}=6, n_{mice}=3) in comparison to other groups recorded after 7 days of cocaine injection, WT (n_{cells}=8, n_{mice}=8), MAOA^{Neo} (n_{cells}=7, n_{mice}=7), WT ES (n_{cells}=8, n_{mice}=8) MAOA^{Neo} ES (n_{cells}=11, n_{mice}=6). Each symbol represents the averaged value (± s.e.m.) obtained from different cells. Left-hand panel shows representative traces of IPSC recorded at each stimulus intensity for MAOA^{Neo} ES after subchronic exposure and after one week of withdrawal. Calibration bar: 25 ms, 50 pA. (**c**) Graph panel displays that the facilitation in the averaged paired-pulse ratio (IPSC2/IPSC1) of GABAA IPSCs lasts for one week in MAOA^{Neo} ES (n_{cells}=6, n_{mice} =3) mice in comparison to PPR of WT (n_{cells}=13, n_{mice}=10), MAOA^{Neo} (n_{cells}=9, n_{mice}=9), WT ES (n_{cells}=13, n_{mice}=12) and MAOA^{Neo} ES (n_{cells}=12, n_{mice}=12) recorded 24 after the end of subchronic treatment. Insets show representative traces of paired GABAA IPSCs recorded from VTA putative DA neurons for MAOA^{Neo} ES after subchronic exposure and after one week of withdrawal Calibration bar: 25 ms, 50 pA. (**d**) Graph panel showing the effect of *in vivo* subchronic exposure to cocaine on 1/CV^2 of GABAA IPSC1 persists after one

week of withdrawal in MAOA^{Neo} ES (n_{cells}=6, n_{mice} =3) mice in comparison to WT (n_{cells}=13, n_{mice}=10), MAOA^{Neo} (n_{cells}=9, n_{mice}=9), WT ES (n_{cells}=13, n_{mice}=12) and MAOA^{Neo} ES (n_{cells}=12, n_{mice}=12). Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; ****p < 0.0001, **p < 0.01, *p < 0.05.

4.2.4. Subchronic cocaine exposure enables DSI in VTA DA neurons of MAOA^{Neo} mice subjected to early life stress

Since the reduction of inhibitory transmission on rat midbrain DA neurons after repeated cocaine exposure depends on activation of CB1 and DAD2 receptors (Pan *et al.*, 2008a), we sought to determine the involvement of eCB signaling in the effects observed in MAOA^{Neo} ES mice. To this aim, we phasically activated eCB signaling by depolarizing DA cells (from -70 to +40 mV) for a subthreshold duration (*i.e.*, 3 s; Melis *et al.*, 2009, 2013) that does not induce DA cells to express a short-term form of synaptic plasticity termed depolarization-induced suppression of inhibition (DSI) (Pitler and Alger, 1994). Remarkably, DSI could only be expressed by MAOA^{Neo} ES mouse DA neurons (**Fig. 12a,** 2-way RM ANOVA, interaction genotype x ES $F_{(3,462)}$=9.44, p<0.0001; *post-hoc* Tukey's test: 10 s, MAOA^{Neo} ES vs WT, p = 0.03; MAOA^{Neo} ES vs MAOA^{Neo}, p = 0.03; MAOA^{Neo} ES vs WT ES, p = 0.02; 20 s, MAOA^{Neo} ES vs MAOA^{Neo}, p = 0.04; 30 s, MAOA^{Neo} ES vs WT, p = 0.01; 40 s, MAOA^{Neo} ES vs WT ES, p = 0.013; **Fig. 12b,** 2-way ANOVA, interaction $F_{(1,22)}$=10.25, p=0.004, genotype $F_{(1,22)}$=7.4, p=0.01, ES $F_{(1,22)}$=11.21, p=0.003; *post-hoc* Tukey's test: MAOA^{Neo} ES vs WT, p = 0.001; MAOA^{Neo} ES vs MAOA^{Neo}, p = 0.003; MAOA^{Neo} ES vs WT ES, p = 0.0008). This phenomenon was accompanied by an increased PPR (**Fig. 12c,** paired *t* test, p = 0.005), suggestive of a decreased probability of GABA release (Melis *et al.*, 2002 and references therein).

The observations that DSI is not expressed when mice are not exposed to cocaine (data not shown) as well as in the other experimental groups exposed to cocaine are suggestive of a specific effect due to GxE interaction. As such, subchronic cocaine exposure could functionally activate eCB system only in MAOA^{Neo} ES mice by inducing metaplastic changes in the molecular architecture of this signaling pathway (Melis *et al.*, 2014). To test this hypothesis, we first assessed whether differences in CB1R function/number might occur at GABA synapses arising from rostral afferents onto VTA DA neurons by building a concentration-response relationship for the CB1R/CB2R agonist WIN55,212-2 (WIN; 0.01-3 µM). No differences were observed among groups (**Fig. 12d**, 2-way RM ANOVA, interaction $F_{(15,87)}$=1.0, p = 0.45, genotype x ES $F_{(3,18)}$=1.18, p = 0.36, concentration

$F_{(5,87)}$=34.58, p <0.0001). Next, we examined the possibility of an increased 2-AG tone, which could explain the larger DSI, by blocking the activity of its principal degrading enzyme, *i.e.*, monoacylglycerol lipase (MAGL; Blankman *et al.*, 2007). When we bath applied the potent MAGL inhibitor JZL184 (Long *et al.*, 2009; 100 nM ,10 min of pre-incubation), differences in DSI and paired-pulse facilitation were no longer observed among experimental groups (**Fig. 12e**, left panel (time course of DSI), 2-way RM ANOVA, interaction genotype x ES $F_{(3,16)}$=2.43, p = 0.10; right panel (IPSC2/IPSC2), 2-way ANOVA, interaction $F_{(1,16)}$=0.13, p = 0.73, genotype $F_{(1,16)}$=0.03, p = 0.87, concentration $F_{(1,16)}$=0.16, p = 0.69). Since JZL184 reduced GABAA IPSC amplitude to a similar extent among groups (data not shown), we can reasonably rule out that MAOA^{Neo} ES mice have an increased 2-AG tone but suggest that the rate of its degradation differs in MAOA^{Neo} ES mice.

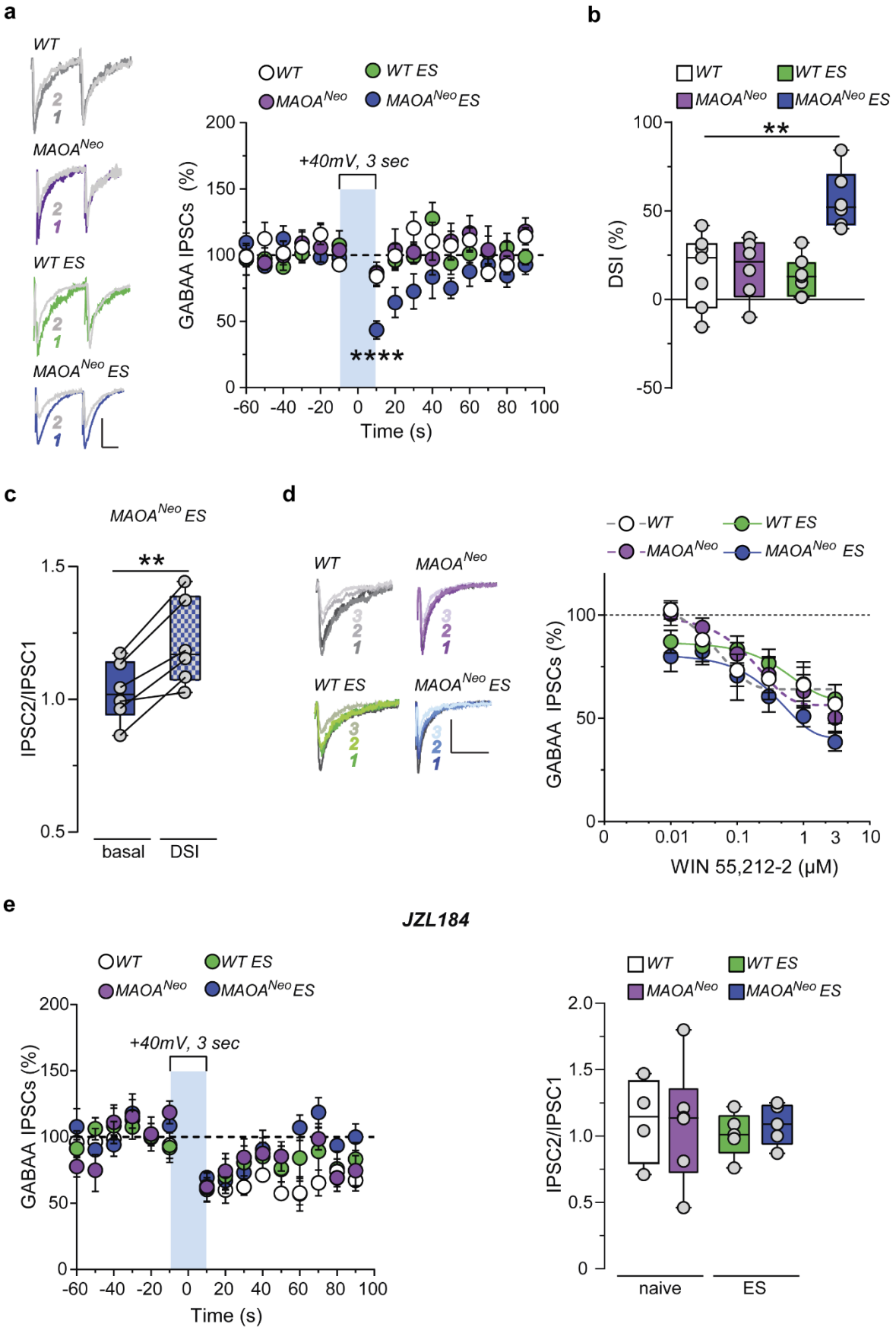

Fig. 12. Endocannabinoid modulation of inhibitory transmission arising from rostral afferents is shown by MAOA^{Neo} mice subjected to early life stress after subchronic cocaine exposure. (**a**) Graph panel

showing the time course of DSI. Application of a depolarizing pulse from -70 to +40 mV for 3 sec on VTA DA neurons induced reduction of GABAA IPSCs evoked by stimulating rostral afferents in MAOA^{Neo} ES mice recorded 24 hours after the end of subchronic treatment. Note that in MAOA^{Neo} ES DSI decay time is prolonged. GABAA IPSCs amplitude was normalized to the averaged value (dotted line) before depolarization. Each symbol represents the averaged value (± s.e.m.) obtained from different cells, WT (n_{cells}=7, n_{mice}=7), MAOA^{Neo} (n_{cells}=6, n_{mice}=7), WT ES (n_{cells}=7, n_{mice}=7) and MAOA^{Neo} ES (n_{cells}=6, n_{mice}=7). Left panel shows representative traces of IPSC recorded before and after DSI. Grey traces represent the GABAA IPSCs after the depolarizing step. Calibration bar, 25 ms, 50 pA. (**b**) Averaged data for DSI induced by depolarizing pulse with a duration 3 sec are plotted. (**c**) Bar graph summarizing the averaged paired-pulse ratio (IPSC2/IPSC1) of rostral GABAA IPSCs for all cells recorded in MAOA^{Neo} ES mice before (basal) and after (DSI) depolarization pulse. (**d**) Dose–response curves for percentage inhibition in amplitude of GABAA IPSCs by the CB1 receptor agonist WIN55,212–2 as recorded from VTA DA cells and evoked by stimulating rostral afferents in WT (n_{cells}=5, n_{mice}=4), MAOA^{Neo} (n_{cells}=4, n_{mice}=2), WT ES (n_{cells}=7, n_{mice}=6) and MAOA^{Neo} ES (n_{cells}=5, n_{mice}=5). Each symbol represents the averaged value (± s.e.m.) obtained from different cells. The inset shows IPSC before (grey), and during 0,01μM (1), 0,1 μM (2), 3 μM (3) perfusion of WIN. Calibration bar, 25 ms, 100 pA. (**e**) The left graph displays that MAGL inactivation produced no differences in paired-pulse ratio (IPSC2/IPSC1) of rostral GABAA IPSCs for all cells recorded in WT, MAOA^{Neo}, WT ES and MAOA^{Neo} ES. The right panel shows the time course of DSI in the presence of JZL184 in WT (n_{cells}=4, n_{mice}=4), MAOA^{Neo} (n_{cells}=5, n_{mice}=4), WT ES (n_{cells}=6, n_{mice}=3) and MAOA^{Neo} ES (n_{cells}=5, n_{mice}=3). JZL184 induced similar DSI among groups. GABAA IPSCs amplitude was normalized to the averaged value (dotted line) before depolarization. Each symbol represents the averaged value (± s.e.m.) obtained from different cells. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses and two-tailed unpaired t-test; ****$p < 0.0001$, **$p < 0.01$.

Of note, the prolonged DSI was still expressed by DA cells in MAOA^{Neo} ES mice after one week of withdrawal (**Fig. 13a,** 2-way RM ANOVA, interaction genotype x ES $F_{(4,26)}$=8.07, $p < 0.001$; *post-hoc* Tukey's test: 10 s, MAOA^{Neo} ES after withdrawal vs WT, $p = 0.03$; MAOA^{Neo} ES after withdrawal vs MAOA^{Neo}, $p < 0.05$; MAOA^{Neo} ES after withdrawal vs WT ES, $p < 0.05$; MAOA^{Neo} ES vs WT, $p < 0.05$; MAOA^{Neo} ES vs MAOA^{Neo}, $p < 0.05$; MAOA^{Neo} ES vs WT ES, $p < 0.01$; **Fig. 13b,** 2-way ANOVA, interaction $F_{(2,32)}$=13.70, $p < 0.0001$, genotype $F_{(2,32)}$=6.9, $p = 0.003$, ES $F_{(1,32)}$=39.13, $p < 0.0001$; *post-hoc* Tukey's test: MAOA^{Neo} ES after withdrawal vs WT, $p < 0.001$; MAOA^{Neo} ES after withdrawal vs MAOA^{Neo}, $p < 0.001$; MAOA^{Neo} ES after withdrawal vs WT ES, $p < 0.001$; MAOA^{Neo} ES after withdrawal vs WT ES, $p < 0.05$; MAOA^{Neo} ES vs WT, $p < 0.001$; MAOA^{Neo} ES vs MAOA^{Neo}, $p < 0.01$; MAOA^{Neo} ES vs WT ES, $p < 0.001$), an effect still accompanied by an increased PPR (**Fig. 13c**, two-tailed paired *t* test, $p = 0.027$). Altogether, these data indicate that repeated exposure to cocaine *in vivo* increases phasic eCB signaling on GABA inputs and that this effect is long lasting along with their behavioral sensitivity (**Fig. 7d**).

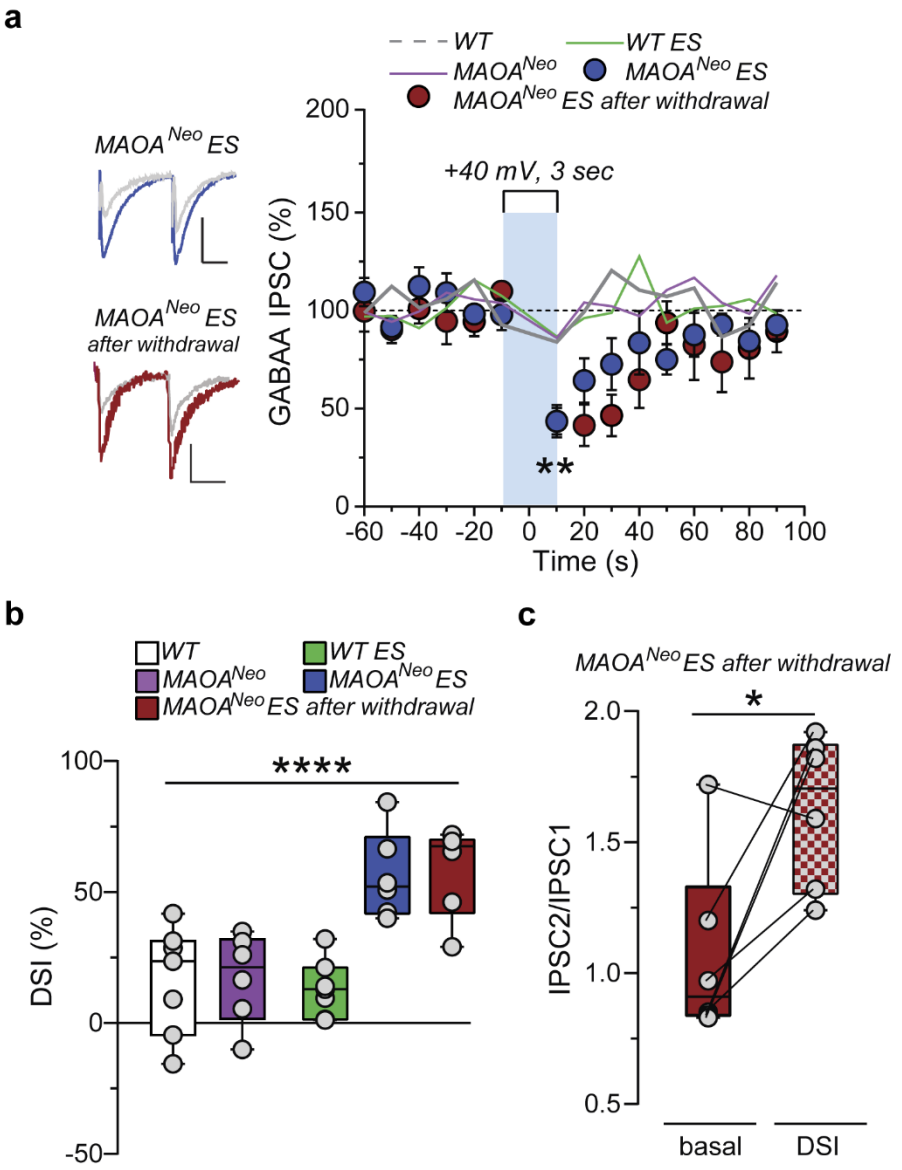

Fig. 13. Subchronic cocaine effects on DSI lasts for one week in VTA DA neurons of MAOA^{Neo} mice subjected to early life stress. (**a**) Graph panel showing the time course of DSI. Application of a depolarizing pulse from -70 to +40 mV for 3 sec on VTA DA neurons enables DSI of GABAA IPSCs evoked by stimulating rostral afferents in MAOA^{Neo} ES after withdrawal (n_{cells}=5, n_{mice}=3) mice similarly to MAOA^{Neo} ES after subchronic exposure. GABAA IPSCs amplitude was normalized to the averaged value (dotted line) before depolarization. Each symbol represents the averaged value (± s.e.m.) obtained from different cells WT (n_{cells}=7, n_{mice}=7), MAOA^{Neo} (n_{cells}=6, n_{mice}=6), WT ES (n_{cells}=7, n_{mice}=6) and MAOA^{Neo} ES (n_{cells}=6, n_{mice}=6). Left-hand panel shows representative traces of IPSC recorded before and after DSI. Grey traces represent the GABAA IPSCs after the depolarizing step. Calibration bar, 25 ms, 50 pA. (**b**) Averaged data for DSI induced by depolarizing pulse with a duration 3 sec are plotted. (**c**) Bar graph summarizing the averaged paired-pulse ratio (IPSC2/IPSC1) of rostral GABAA IPSCs for all cells recorded in MAOA^{Neo} ES mice after one week of withdrawal before (basal) and after (DSI) depolarization pulse. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; ****p < 0.0001, **p < 0.01, *p < 0.05.

4.2.5. Role of DAD2 and CB1 receptors in cocaine-induced decrease of GABA efficacy on VTA DA neurons

In the VTA, GABA release is regulated by eCBs (Riegel and Lupica, 2004) via inhibition of a cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase activity (PKA) pathway downstream of CB1R activation (Vogel *et al.*, 1993; Howlett *et al.*, 2002; Chevaleyre *et al.*, 2007). Cocaine mobilizes 2-AG from DA neurons and inhibits GABA

release by activating CB1Rs (Pan *et al.*, 2008a; Wang *et al.*, 2015). Cocaine-induced activation of DAD2R by DA also inhibits GABA release via a cAMP-PKA pathway (Pan *et al.*, 2008a). Thus, we examined whether this common pathway downstream of DAD2Rs and CB1Rs could account for our effects in pre-adolescent MAOA^{Neo} ES mice. First, we pharmacologically blocked *in vivo* DAD2R by administering sulpiride (10 mg/kg, i.p, 20 min before cocaine). Differences in GABAA IPSCs amplitude (**Fig. 14b**, 2-way RM ANOVA, interaction $F_{(4,33)}$=1.65, p = 0.018; *post hoc* Tukey's test: 100 µA, MAOA^{Neo} ES sulp + coca vs MAOA^{Neo} ES coca, p < 0.001; MAOA^{Neo} sulp + coca vs MAOA^{Neo} ES coca, p < 0.5), in PPR (**Fig. 14c**, 2-way ANOVA, interaction $F_{(1,41)}$=7.04, p = 0.01, genotype x ES $F_{(1,41)}$=2.47, p = 0.12, sulp+coca x coca $F_{(1,41)}$=1.52, p = 0.22; *post-hoc* Sidak's test: MAOA^{Neo} ES sulp + coca vs MAOA^{Neo} ES coca, p = 0.04) and DSI (**Fig. 14d,** left panel, 2-way RM ANOVA, interaction genotype x ES $F_{(4,27)}$=0.65, p=0.63; *post-hoc* Tukey's test: 10 s, MAOA^{Neo} ES sulp + coca vs MAOA^{Neo} ES coca, p = 0.005; WT ES sulp + coca vs MAOA^{Neo} ES coca p = 0.004; right panel, 2-way ANOVA, interaction $F_{(3,44)}$=9.24, p < 0.0001, genotype x ES $F_{(3,44)}$=2.80, p = 0.05, sulp+coca vs coca $F_{(1,44)}$=2.80 p = 0.007; *post-hoc* Tukey's test: MAOA^{Neo} ES sulp + coca vs MAOA^{Neo} ES coca, p < 0.0001; WT ES sulp vs MAOA^{Neo} ES coca, p = 0.0001; MAOA^{Neo} sulp + coca vs MAOA^{Neo} ES coca, p < 0.05; WT sulp + coca vs MAOA^{Neo} ES coca, p = 0.01) were no longer observed in DA cells among groups.

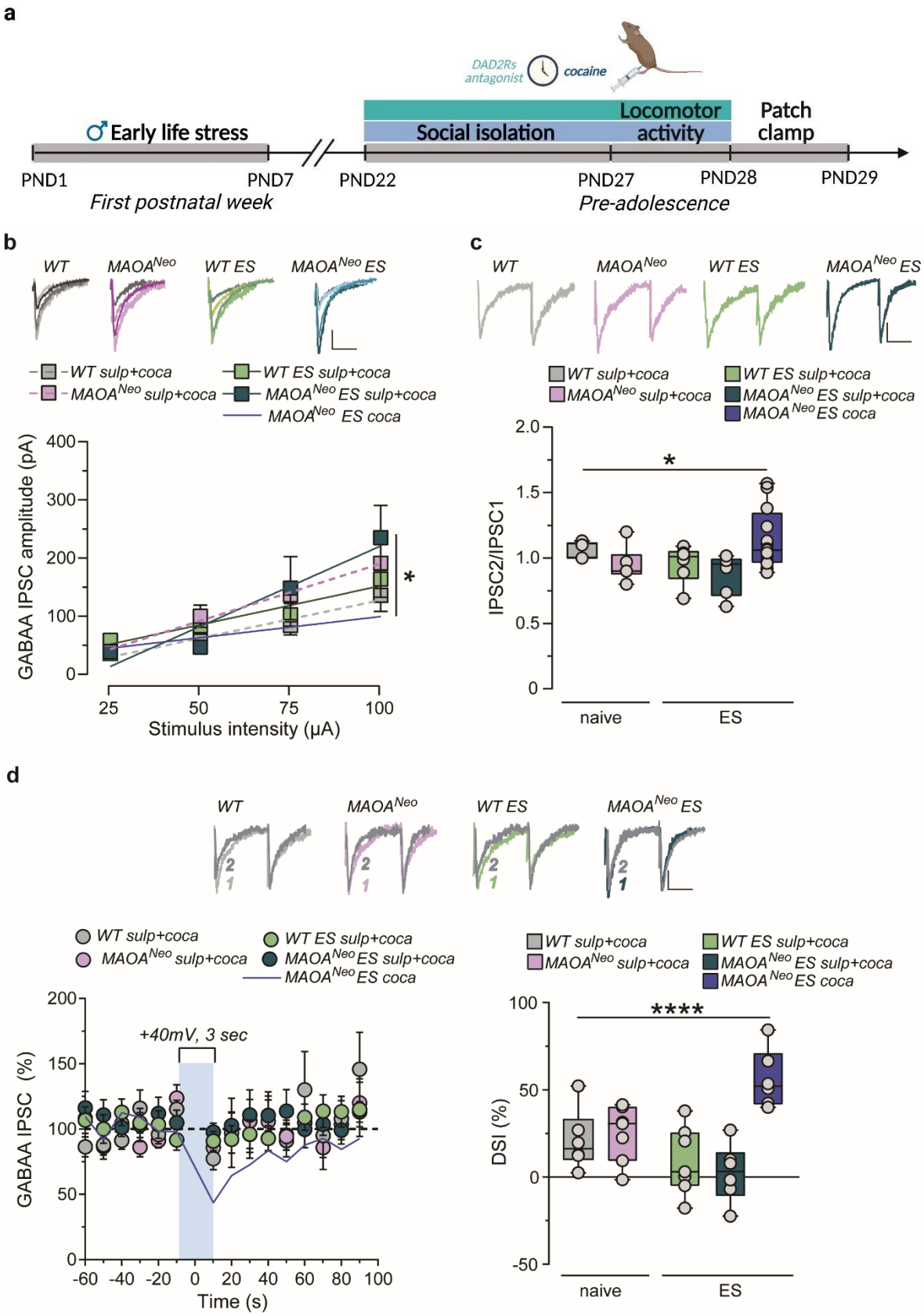

Fig. 14. ***In vivo*** **pretreatment with DAD2R antagonist sulpiride abolishes differences in synaptic properties of inhibitory inputs and blocks DSI in VTA DA neurons.** (**a**) Schematic timeline of experimental design. (**b**) ***Top***, representative traces of IPSC recorded in response of incremental stimulus intensities. Calibration bar: 25 ms, 50 pA ***Bottom***, the graph of input–output relationships of GABAA-mediated IPSCs

shows the effect of pretreatment with DAD2R antagonist sulpiride (sulp, 10 mg/kg, i.p) 20 minutes before each intraperitoneal cocaine injection (coca, 15 mg/kg) for 7 days. In midbrain slice prepared 24h after the last cocaine injection, sulpiride blocks cocaine-induced reduction in the amplitude of GABAA IPSC of MAOA^{Neo} ES (n_{cells}=6, n_{mice}=4) in comparison to MAOA^{Neo} ES coca (n_{cells}=11, n_{mice}=7) and abolishing differences with WT sulp+coca (n_{cells}=8, n_{mice}=4), MAOA^{Neo} sulp+coca (n_{cells}=7, n_{mice}=4) and WT ES sulp+coca (n_{cells}=7, n_{pups}=4). Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**c**) Graph panel summarizing the effect of sulpiride pretreatment on averaged paired-pulse ratio (IPSC2/IPSC1) of GABAA IPSCs for DA cells recorded in WT sulp+coca (n_{cells}=8, n_{mice}=4), MAOA^{Neo} sulp+coca (n_{cells}=7, n_{mice}=4), WT ES sulp+coca (n_{cells}=7, n_{mice}=4) and MAOA^{Neo} ES sulp+coca (n_{cells}=6, n_{mice}=4) in comparison with MAOA^{Neo} ES coca (n_{cells}=11, n_{mice}=7) mice. (**d**) ***Left panel,*** graph showing the effect of pretreatment with sulpiride on time course of DSI. Application of a depolarizing pulse from -70 to +40 mV for 3 sec on VTA DA neurons blocks DSI of GABAA IPSCs evoked by stimulating rostral afferents in MAOA^{Neo} ES mice. GABAA IPSCs amplitude was normalized to the averaged value (dotted line) before depolarization. Each symbol represents the averaged value (± s.e.m.) obtained from different cells, WT sulp+coca (n_{cells}=6, n_{mice}=4), MAOA^{Neo} sulp+coca (n_{cells}=7, n_{mice}=4), WT ES sulp+coca (n_{cells}=7, n_{mice}=4) and MAOA^{Neo} ES sulp+coca (n_{cells}=6, n_{mice}=4) in comparison to MAOA^{Neo} ES coca (n_{cells}=6, n_{mice}=6) mice. ***Right panel,*** averaged data for DSI induced by depolarizing pulse with a duration 3 sec are plotted. ***Top***, representative traces of IPSC recorded before and after DSI. Grey traces represent the GABAA IPSCs after the depolarizing step. Calibration bar, 25 ms, 50 pA. Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; ****p < 0.0001, **p < 0.01, *p < 0.05.

Next, we examined the role of CB1Rs by blocking them *in vivo* with the antagonist AM281 (2.5 mg/kg, i.p. 7 days, 20 min before cocaine injection). AM281-treated mice show a normal inhibitory synaptic transmission (**Fig. 15b**, 2-way RM ANOVA, interaction $F_{(1,59)}$=12.37, p < 0.001; *post-hoc* Tukey's test: 75 µA, MAOA^{Neo} ES AM281+coca vs MAOA^{Neo} ES coca, p = 0.03; 100 µA, MAOA^{Neo} ES AM281+coca vs MAOA^{Neo} ES coca, p = 0.02), along with the PPR (**Fig. 15c**, 2-tailed unpaired *t*-test, p = 0.25). Also, AM281-treated mouse DA cells did not express DSI (**Fig. 15d,** left panel, 2-way RM ANOVA, interaction genotype x ES x pretreatment $F_{(1,193)}$=13.35, p < 0.001; right panel**,** 2-tailed npaired *t*-test, p = 0.01).

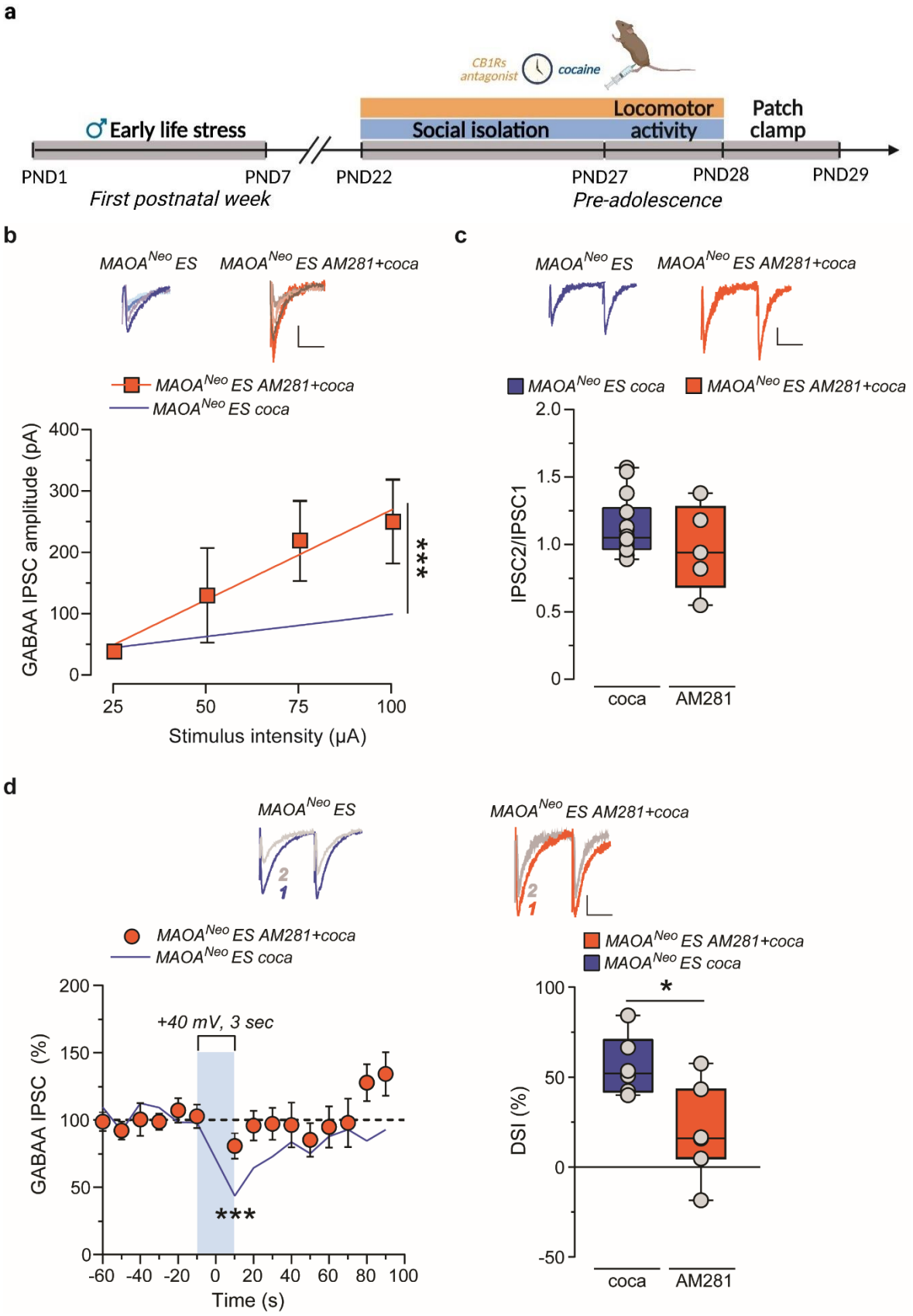

Fig. 15. *In vivo* pretreatment with CB1R antagonist AM281 abolishes differences in synaptic properties of inhibitory inputs and blocks DSI in VTA DA neurons in MAOA^{Neo} mice subjected to early life stress.

(**a**) Schematic timeline of experimental design. (**b**) ***Top***, insets show representative traces of IPSC recorded in response of incremental stimulus intensities. Calibration bar: 25 ms, 50 pA ***Bottom***, the graph of input–output relationships of GABAA-mediated IPSCs shows the effect of pretreatment with CB1R antagonist AM281 (2.5 mg/kg, i.p) 20 minutes before each intraperitoneal cocaine injection (coca, 15 mg/kg) for 7 days on midbrain slice prepared 24h after the last cocaine injection. AM281 abolishes cocaine-induced reduction in the amplitude of GABAA IPSC in MAOA^{Neo} ES mice (n_{cells}=6, n_{mice}=3) in comparison to MAOA^{Neo} ES coca (n_{cells}=11, n_{mice}=8). Each symbol represents the averaged value (± s.e.m.) obtained from different cells. (**c**) Graph panels summarizing the effect of pretreatment with AM281 on averaged paired-pulse ratio (IPSC2/IPSC1) for DA cells recorded in MAOA^{Neo} ES AM281+coca (n_{cells}=6, n_{mice}=3) and MAOA^{Neo} ES coca (n_{cells}=11, n_{mice} =8) mice. (**d**) ***Left panel***, graph panel showing the effect of pretreatment of AM281 on time course of DSI. Application of a depolarizing pulse from -70 to +40 mV for 3 sec on VTA DA neurons prevents DSI of GABAA IPSCs evoked by stimulating rostral afferents in MAOA^{Neo} ES mice. GABAA IPSCs amplitude was normalized to the averaged value (dotted line) before depolarization. Each symbol represents the averaged value (± s.e.m.) obtained from different cells in MAOA^{Neo} AM281+coca (n_{cells}=6, n_{mice} =3) and MAOA^{Neo} ES coca (n_{cells}=6, n_{mice}=7) mice. ***Right panel***, Averaged data for DSI induced by depolarizing pulse with a duration 3 sec are plotted. ***Top***, representative traces of IPSC recorded before and after DSI. Grey traces represent the GABAA IPSCs after the depolarizing step. Calibration bar, 25 ms, 50 pA. (**e**) Unless otherwise indicated, graphs show box-and-whisker plots (including minima, maxima and median values, and lower and upper quartiles) with each circle representing a single cell recorded. Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; **p < 0.01, *p < 0.05.

Lastly, we examined whether either drug affected cocaine-induced hyper-locomotion. Pretreatment with either DAD2 and CB1R antagonist prevented cocaine-induced hyperlocomotion in MAOA^{Neo} ES mice (**Fig.16a**, 2-way RM ANOVA, interaction genotype x ES x pretreatment $F_{(7,73)}$=12.33; p < 0.0001; **Fig.16b**, 2-way RM ANOVA, interaction genotype x ES x pretreatment $F_{(1,22)}$=4.05; p = 0.056). Collectively, these results are suggestive of a role for both DAD2 and CB1 receptors in the reduced GABA inhibition on VTA DA neurons and the enhanced sensitivity to cocaine stimulant effects.

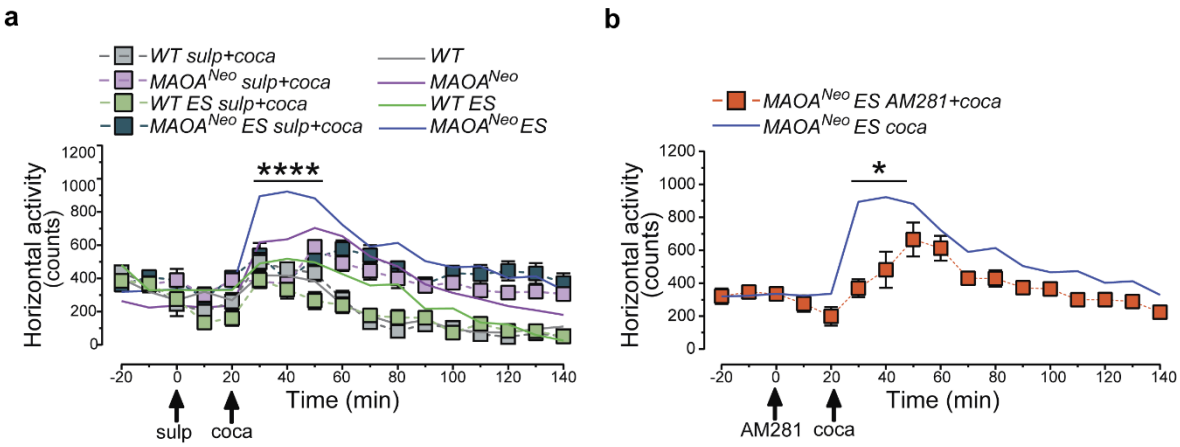

Fig. 16. *In vivo* pretreatment with antagonists to D2 and CB1 receptors reduces cocaine-induced hyperlocomotion in MAOA^{Neo} mice subjected to early life stress. (**a**) Pretreatment of mice with DAD2R antagonist sulpiride (sulp, 10 mg/kg, i.p) 20 min before each intraperitoneal cocaine injection (coca, 15 mg/kg) for 7 days abolishes the hyperlocomotion in MAOA^{Neo} mice exposed to early life stress in comparison to other groups. (**b**) Pretreatment of mice with CB1R antagonist AM281 (2.5 10 mg/kg, i.p) 20 min before each intraperitoneal cocaine injection (coca, 15 mg/kg) for 7 days abolishes the cocaine-induced hyperlocomotion

in MAOA^{Neo} mice exposed to early life stress. Graphs show spontaneous locomotor activity measured as counts of 10 minutes before and after drugs injection at the last day of treatment in a novel open field arena. Each symbol represents the average value (± s.e.m.) (n_{mice}= 5-18 per group). Asterisks in the figure refer to the interaction revealed by 2-way ANOVA analyses; ****$p < 0.0001$, *$p < 0.05$.

5. Discussion

The main finding of the present thesis is that dysfunctions of mesocortical DA system, which are secondary to the interaction between ES and low activity of the MAOA enzyme (*i.e.*, MAOA^{Neo} mouse), confer vulnerability traits to individuals at pre-adolescence. This susceptibility is so far revealed by an AB and an increased sensitivity to psychostimulant effects of cocaine. Both these traits are associated to alterations of synaptic properties of DA neurons and changes in behavioral responses.

The finding that MAOA^{Neo} ES mice display an AB during the resident intruder test supports clinical and preclinical evidence of an interplay between functional polymorphism of *Maoa* gene and ES, including social isolation, which acts as a predictive risk factor for the emergence of AB at pre-adolescence (Caspi *et al.*, 2002; Viding and Frith, 2006; Godar *et al.*, 2019). Of note, this is associated with a dysfunctional mesocortical DA system at pre-adolescence. Accordingly, perturbations in monoamine signaling occurring during development contribute to the ontogeny of AB (Comai *et al.*, 2012; Yu *et al.*, 2014; Godar *et al.*, 2019). In particular, the causal link between AB and a hyperdopaminergia during peri-adolescence is substantiated by optogenetic stimulation of VTA DA neurons, which is tied to AB (Yu *et al.*, 2014). In agreement with Yu et al. (2014), we observed that pharmacological blockade of DA signaling, either by inhibiting DA synthesis or by blocking D1 receptors with AMPT and SCH23390 respectively, prevented AB manifestation in MAOA^{Neo} ES mice. The observation that putative DA neurons of the VTA show an increased post-synaptic responsiveness to excitatory inputs, without any change in the properties of inhibitory afferents, at pre-adolescence may alter the balance between inhibition and excitation by favoring this latter. This is consistent with the role of midbrain DA neurons in coding and modulating emotional and adaptative behaviors (Pignatelli and Bonci, 2015). Our working hypothesis is that an enhanced DA cell function might decrease background and evoked activity of PFC pyramidal neurons, thereby affecting the global PFC network. This would support that the reduced signal-to-noise ratio within the PFC results in the decreased activity of pyramidal cells associated with inadequate behavioral responses (Kroener *et al.*, 2009). Since the PFC plays a key role in modulating AB and in initiating appropriate behavioral response (Davidson, 2002; Lösel *et al.*, 2007; Pardini *et al.*, 2011), the dysregulation in PFC functions that we observed might be crucial for the etiopathogenesis of AB (Bortolato *et al.*, 2012). Therefore, a rise in DA background within

the PFC, along with an enhanced probability of glutamate release and an increased ratio of GluN2B/GluN2A subunits in NMDA receptors on pyramidal cells, might account for the expression of long-term potentiation (LTP) that we observe selectively in our mouse model. Since within the PFC NMDA receptors are involved in information processing as well as in the spatial and temporal summation of converging inputs (Bortolato *et al.*, 2021), such alterations might contribute to impairments in social responses and in the manifestation of AB displayed by MAOA^{Neo} ES mice. Thus, given that PFC integrates multiple synaptic signals arising from different brain areas and projects to several subcortical-limbic regions regulating emotional processing of social and environmental stimuli (Bortolato *et al.*, 2012), it is plausible that the imbalance between VTA and PFC function might contribute to an impairment of executive function in MAOA^{Neo} ES mice, which attribute a hostile bias to social stimuli thereby resulting in a marked AB. Accordingly, in humans, the exposure to early life adversities produces long-lasting changes in structure and function of DA systems and in an enhanced connectivity among the PFC, cingulate and striatal networks (Pizzagalli, 2014; Ironside *et al.*, 2018). Alterations in cortical networks following stress may disrupt the top-down control of response inhibition after deviations in social cue coding and increase the sensitivity to negative valence (Grandjean *et al.*, 2016). Moreover, deficits in executive functions are correlated with the manifestation of reactive aggression, a specific form of AB (Crick and Dodge, 1996; McAuliffe *et al.*, 2006). Of note, abnormalities in cortical and limbic network are also involved in other psychiatric conditions such as ADHD, bipolar disorder, as well as in the phobia characterizing anxiety disorders (Burke *et al.*, 2002; Lösel *et al.*, 2007; Lewis *et al.*, 2008; Stahl, 2009a, 2009b).

The present findings also highlight that genetic background makes the difference, which lead to the manifestation of AB, when an individual is exposed to early life adversities. Indeed, both adolescent and adult C57BL/6J male mice exposed to ES during the first postnatal week and to social isolation after weaning do not develop AB (Veenema *et al.*, 2007; Tsuda *et al.*, 2011), whereas in pre-adolescent MAOA^{Neo} mice these are necessary and sufficient to reliably produce an AB (Godar *et al.*, 2019; present data). Of note, by leveraging on the mesocortical DA system alterations occurring exclusively in pre-adolescent MAOA^{Neo} mice, we were able to design an effective pharmacological intervention that restored PFC functions and prevented AB. We, therefore, speculate that DAD1Rs might be involved in the proper interpretation of social cues and suggest this as a potential molecular target for the treatment

of AB. Noteworthy, age-specific medications that prevent and treat different subtypes of AB still represent an unmet clinical need (Zuddas, 2014).

Our data support the diathesis stress model, where individuals carrying genetic predispositions and exposed to stressful life experience are more vulnerable to exhibit antisocial behaviors and to abuse drugs (Greven *et al.*, 2019). With regard to drug abuse, animal studies suggest that early life traumas (*e.g.*, maternal isolation during the first post-natal week of life) increase the susceptibility to develop substance abuse later in life, as well as to enhance acquisition of cocaine self-administration during adulthood (Kosten *et al.*, 2000). Of note, this enhanced susceptibility increases in presence of genetic predispositions associated with polymorphisms in proximity of genes involved in the synthesis or metabolism of monoamines (*i.e.*, DA beta hydroxylase, tryptophan hydroxylase 2), as well as of genes associated with eCB system (CB1R; Pierce *et al.*, 2018). Accordingly, the enhanced sensitivity to cocaine in this GxE mouse model persisted after one week of withdrawal. This is particularly relevant when one takes into account the genetic background of these mice that is the 129S6 mouse strain. In fact, 129S6 mice are characterized by a lower or null locomotor response to increasing doses of cocaine, especially when compared to other mouse strains or Sprague Dawley rats (Schlussman *et al.*, 1998; Thomsen and Caine, 2011).

Mechanistic insights of this behavioral sensitivity to subchronic cocaine effect reveal marked effects at synapses on DA cells. On one hand, we found no differences at excitatory synapses among experimental groups. This apparent "no result", instead, is in line with the evidence that cocaine potentiates excitatory synaptic transmission in the VTA after both single and repeated exposure (Borgland *et al.*, 2004; Mameli *et al.*, 2011; Yuan *et al.* 2013). Cocaine, by potentiating excitatory transmission on DA cells, abolished the selective changes at these synapses that are associated with this GxE interaction (Frau *et al.*, 2019). On the other hand, inhibitory transmission on DA neurons was selectively reduced in MAOA^{Neo} ES mice. Of note, such an effect on inhibition was unveiled only upon subchronic cocaine exposure and persisted after one week of withdrawal. This is consistent with both a decreased inhibition and the expression of inhibitory-LTD (I-LTD) in VTA DA neurons following a subchronic *in vivo* cocaine exposure in Sprague Dawley rats (Pan *et al.*, 2008a,b) and might contribute to the enhanced sensitivity to psychostimulant effects of cocaine. Subchronic cocaine also reveals the role of a phasic activation of eCB system in this GxE interaction, yet another mechanism that could underpin the sensitivity to cocaine psychostimulant effects. One could

plausibly speculate that cross-sensitization might result from a facilitation of LTP induction upon the release of eCBs that transiently suppress and filter out inhibitory inputs on DA cells. Although the expression of a DSI, persisting even after one week of withdrawal, suggests an involvement of eCB phasic signaling in the regulation of both GABA release and behavioral readout, at this stage we cannot rule out other metaplastic changes of eCB system. As a matter of fact, functional activation states of eCB signaling have been proposed to contribute to metaplastic control of synaptic and behavioral functions (Melis *et al.*, 2014). At this stage, we can only infer that homeostatic adaptations of eCB signaling following *in vivo* experience (*e.g.*, ES, cocaine exposure) do not affect CB1R expression/function by triggering CB1R downregulation or desensitization. In fact, dose-relationship curves for the effects of a CB1R/CB2R agonist (WIN) on GABA postsynaptic current amplitude do not show any difference among the experimental groups. We can also reasonably rule out that alterations in endogenous tone of 2-AG occur at these synapses, since these latter would produce adaptive changes in CB1R number and/or function. In addition, prolonged tonic 2-AG signaling would lead to downregulation of CB1Rs (Chanda *et al.*, 2010) that we did not have measured *in vivo* or *ex vivo*. Consistently, pharmacological blockade of MAGL does not reveal an endogenous tone of 2-AG at these synapses in MAOA^{Neo} ES mice, although it enables phasic activation of 2-AG signaling and the resulting expression of DSI in all experimental groups. Further studies are warranted to verify if changes in sensitivity and/or activity of MAGL occur in MAOA^{Neo} ES mice. Additionally, since the functional role of 2-AG on expression of DSI in VTA also depends on DAGL expression and function (Chevaleyre and Castillo, 2003), we cannot exclude its involvement in the expression of this form of eCB-mediated short-term synaptic plasticity in this mouse model of GxE interaction exposed to cocaine.

Presynaptic CB1R activation is necessary for the expression of I-LTD and DSI in the VTA through the recruitment of presynaptic and postsynaptic mechanisms. Our observations that *in vivo* pharmacological blockade of CB1Rs prevents cocaine-induced hyperlocomotion in MAOA^{Neo} ES mice, DSI expression, and restores synaptic efficacy of GABA transmission supports that eCB signaling via activation of CB1Rs are necessary for these phenomena. This is in line with the evidence that CB1Rs activation plays a role in the induction of short- and long-term synaptic plasticity in the VTA (Pan *et al.*, 2008a, Melis *et al.*, 2009, 2013, 2014). Among the mechanisms involved in the regulation of GABA release and, therefore, in the expression of eCB-mediated I-LTD in the VTA, cAMP/PKA signaling pathway plays

an important role (Bonci and Williams, 1996; Melis *et al.*, 2002; Pan *et al.*, 2008b). Particularly, GABA release is inhibited by eCBs via CB1R activation (Riegel and Lupica, 2004; Melis *et al.*, 2009, 2013, 2014) thereby inhibiting cAMP/PKA pathway (Chevaleyre *et al.*, 2007; Pan *et al.*, 2008b). Moreover, cocaine mobilizes 2-AG from DA neurons and inhibits GABA release by activating CB1Rs (Pan *et al.*, 2008a, 2008b; Wang *et al.*, 2015). Prolonged activation of DAD2Rs, induced by enhanced DA levels following cocaine subchronic administration, also leads to a decreased GABA release via cAMP-PKA pathway and in the facilitation of I-LTD induction via cAMP/PKA signaling (Pan *et al.*, 2008a; 2008b). Accordingly, our findings in MAOA^{Neo} ES mice suggest the engagement of a common cAMP-PKA pathway downstream the activation of presynaptic DAD2Rs and CB1Rs in both reducing the probability of GABA release on DA cells and their behavioral readout. Hence, *in vivo* pharmacological blockade of DAD2Rs with sulpiride also prevents cocaine-induced hyperlocomotion, DSI expression and the reduced efficacy of GABA transmission in MAOA*Neo* ES mice. Whether this form of activity-dependent modulation of eCB signaling is caused by enhanced DAD2R-mediated 2-AG release, or decreased termination of 2-AG signaling is elusive yet. In the striatum, for instance, cocaine-induced enhancement of DA levels by activating DAD2Rs also contributes to decreased inhibition of GABA transmission via stimulation of presynaptic CB1Rs (Centonze *et al.*, 2004).
In addition, we cannot exclude the possibility that cocaine stimulates 2-AG synthesis and release through the activation of a mechanism involving the recruitment of sigma-1 receptor with subsequent secretion of extracellular vesicles (Nakamura *et al.*, 2019). Alternatively, one could also speculate that upon subchronic cocaine a physical interaction and heteromer formation between DAD2R and CB1R might occur. In fact, CB1R-DAD2R heterodimers have been found in dendrites and soma of NAc GABA neurons, as well as in terminal regions of glutamatergic afferents (Pickel *et al.*, 2006), where they may modulate the reward and motor responses affected by exposure to substance of abuse. Thus, the interaction between DAD2Rs and CB1Rs might function as a homeostatic mechanism aimed at limiting cocaine-mediated effects. Although we cannot definitely rule this out, one would expect the opposite effect on GABA release because a heterooligomeric receptor complex would shift downstream signaling leading to increased cAMP levels (Kearn *et al.*, 2005).

In conclusion, not only the present thesis supports that MAOA^{Neo} ES mouse model predisposes to AB during the pre-adolescence, but also that cross-sensitizes to psychostimulant effects of cocaine thereby acting as a risk factor for the development of

substance use disorders. MAOA^{Neo} mice might, therefore, meet the criteria for a higher biological sensitivity to stressful environmental stimuli (see Greven *et al.*, 2019 and references therein) as they display enhanced reactivity to the same stimuli. Our results might help to better understand the neuronal mechanisms underlying the pathophysiology of these brain disorders and pave the way to identify pharmacological strategies that could be used to treat AB and drug abuse.

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